Postnatal Behavioral Development in Methylazoxymethanol-induced Microcephalic Rats —A Behavioral Teratology Study—

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Behavioral testings in methylazoxymethanol (MAM)-induced microcephalic rats were conducted. Pregnant Sprague-Dawley rats were treated intraperitoneally with 0, 20 or 40 mg/kg of MAM once a day on day 14 of gestation and were allowed to delivery. Male pups from each litter were examined for open field test at 6 weeks of age and shuttle-box avoidance test at 7 weeks or more of age. In the open field activity of pups, the counts of ambulation and locomoting distance in 40mg/kg group have increased significantly as compared with those in control group. In the shuttle-box avoidance test, the avoidance response rate was dose-dependently high in the session of the 1st day. As to the interaction between the avoidance response rate and sequence of sessions, however, the avoidance response rate in 40mg/kg group was significantly low. Rate of the rats with errors and number of response during the intertrial interval was significantly high in 40mg/kg group. Thus, we could demonstrate functional disturbance in the memory retaining ability *in utero* MAM-exposed rats. — KEY WORDS : behavioral teratology, MAM, open field test, shuttle box avoidance response test

Many reports have been published on behavioral testings to study the effect of specific chemicals and drugs [5, 12, 14, 17] on behavioral development. Behavioral testings, a relatively recent area in reproduction toxicology, are required to develop more reliable methods involving the usage of common positive substances. By this reason we examined the behavioral and morphological disturbances in rats exposed *in utero* to methylazoxymethanol (MAM) which has been known as a potent microcephalia producer [2, 6, 16]. MAM-induced microcephalic rats have been already studied on their behavioral disturbances by means of the open field, rotorod and several maze learning tests [7, 9, 10, 11, 13, 18, 19]. These studies revealed hyperactivity and maze learning deficits in MAM-induced microcephalic rats. However, the relationship between the hyperactivity and the poor avoidance response performance in MAM-

induced microcephalic rats remained to be solved. The present study was conducted in an attempt to characterize the further emotional behavior in MAM-induced microcephalic rats by the open field (OFT) and avoidance response test by two-way shuttle box, and consequently to establish the effectiveness of MAM as a positive control for assessing the behavioral teratology.

Materials and Methods

Animals : Female virgin Sprague-Dawley rats, 10 weeks of age, purchased from Japan SLC Inc. were used. Each female was mated overnight with a mature male of the same strain. Day 0 of gestation was defined by the confirmation of a vaginal plug or sperms in vaginal smears. Pregnant dams were kept in polycarbonated cage (265 (W) × 425 (L) × 150 mm (H)) until weaning. Each offspring was

Fig. 1. Body weight changes in dams *: Significantly different from the control at $p < 0.05$

Table 1. Effects of MAM on delivery index

	MAM (mg/kg)					
	0		20		40	
No. of pregnancy	5		6		6	
Duration of gestation ¹⁾	5	22.2 ± 0.27	6	22.1 ± 0.25	6	22.3 ± 0.29
No. of live offspring ²⁾						
Male	34	6.8 ± 2.2	38	6.3 ± 1.2	31	5.2 ± 1.7
Female	36	7.2 ± 2.2	36	6.0 ± 1.4	25	4.2 ± 2.2
Total	70	14.0 ± 1.6	74	12.3 ± 2.3	56	$9.3 \pm 2.3^*$
No. of stillbirth ²⁾	0		2	0.3 ± 0.8	7	1.2 ± 2.4

1) : Number of complete delivery and duration of gestation in days (mean $\pm$ SD)

2) : Number of total and average offsprings per litter (mean $\pm$ SD) *: Significantly different from the control at $p < 0.05$

Fig. 2. Body weight changes in pups *: Significantly different from the control at $p < 0.05$

individually transferred to a metal cage (760 (W) $\times$ 250 (L) $\times$ 150mm (H)) at 5 weeks of age. Five to six dams were assigned for each group. Only the male pups, randomly culled to 4 or less on day 4 after delivery, were reared by biological mothers. All animals were maintained in a barrier-system sustained animal room with relative humidity at $55 \pm 5\%$, temperature at $23 \pm 1^\circ\text{C}$, and 12-hr lighting (6 : 30-18 : 30 in light).

Chemical and treatment : MAM (Aldrich Chemicals Co., Ltd.) was dissolved in physiological saline and intraperitoneally injected to pregnant rats at a dose of 20 or 40mg/kg by single admini at strion on day 14 of gestation. The doses employed were determined referring

Table 2. Brain weight of offspring obtained from mothers injected with MAM

	MAM (mg/kg)		
	0	20	40
No. of offspring	20	20	19
Brain weight (g)	1.97±0.06 (0.60±0.04)	1.64±0.14 (0.50±0.04)**	1.00±0.08 (0.37±0.02)**

Data are expressed as mean±SD. () : Relative weight (brain weight / body weight × 100) ** : Significantly different from the control at $p < 0.01$

Table 3. Open-field behavior of offspring obtained from mothers injected with MAM

	MAM (mg/kg)		
	0	20	40
No. of offspring	20	20	19
Ambulation ¹⁾	74.8±24.0	71.9±38.6	109.8±29.0*
Distance (cm)	1775.7±609.2	1650.6±890.7	2583.8±579.8**
Latency (sec)	2.9±1.9	5.4±5.8	2.8±1.9
Defecation	2.0±1.7	1.8±1.7	1.5±1.4

Data are expressed as mean±SD 1) : Number of sections crossed
* : Significantly different from the control at $P < 0.05$ ** : Significantly different from the control at $P < 0.01$

the study of Haddad *et al.* [9]. The volume and concentration of each dosing solution were adjusted to 0.2 ml/100g body weight. The control rats were given with physiological saline in the same manner with MAM treatment.

Observation and testings : Dams were observed for general sign, duration of gestation, delivery and maternal care. Body weight of dams was weekly measured from day 0 of gestation to 21 days after delivery.

Pups were observed for general sign and their body weight was measured weekly until 7 weeks of age. At 6 weeks of age, open field test (OFT) was conducted between 11 : 00 and 15 : 00 in the day of examination according to the method described elsewhere [1]. The data obtained were analyzed with an image processor with a personal computer (NEC, PC 98 series).

At 7 weeks or more of age, the two-way shuttle-box avoidance test was conducted between 11 : 00 and 15 : 00 in the day of examination once for 3 successive days. As conditioned stimuli (CS), a lighting and a buzzer sound (2.5 kHz, 50 dB) for 10 sec were

applied to each pup. Those which failed to learn the CS, received unconditioned electric stimulus (UCS of 0.5 mA) for 5 sec from the floor grid. The interval between trials was not fixed and varied for 15 to 45 sec. Thirty trials were done as the trial in each day. The avoidance or escape rate was expressed as the percent of avoidance or escape response in 30 trials of the day. The data were analyzed by Kruskal-Wallis nonparametric one way analysis of variance, χ^2 -test, one way analysis of variance and repeated measurement.

Results

Changes in body weight in control and MAM-treated dams and pups are shown in Figs 1 and 2, respectively. The delivery index is also shown in Table 1.

In the MAM treated dams, no abnormal findings were observed in general sign, delivery and maternal care. There were no significant differences in duration of gestation. The increase of body weight was significantly inhibited in 40mg/kg group than in control group on day 21 of gestation ($p < 0.05$) (Fig.

Table 4. Learning ability of offspring obtained from mothers injected with MAM

		MAM (mg/kg)		
		0	20	40
No. of offspring		20	20	19
Avoidance rate (%)	day 1	28.5±18.0	43.2±22.8	55.1±19.2*
	day 2	59.7±19.9	64.9±22.4	59.8±25.1
	day 3	72.5±15.4	68.7±22.5	66.5±22.2
Escape rate (%)	day 1	69.7±17.4	53.3±21.8	35.3±16.7
	day 2	40.0±19.4	33.7±20.6	35.5±18.4
	day 3	27.5±15.4	28.4±19.0	27.4±16.3
No. of errors	day 1	0.6±1.0	1.0±1.7	2.9±6.5
	day 2	0.1±0.3	0.4±1.0	1.4±3.4
	day 3	0.0±0.0	0.9±3.0	1.8±6.4*
Avoidance latency (sec)	day 1	4.4±1.3	3.3±1.4	3.3±0.8
	day 2	4.3±0.9	3.8±1.4	3.4±1.0
	day 3	4.5±0.8	4.3±1.0	4.1±1.2
Escape latency (sec)	day 1	1.4±0.3	1.6±0.6	1.5±0.4
	day 2	1.0±0.2	1.0±0.3	1.3±0.5
	day 3	0.7±0.2	0.7±0.2	0.9±0.4
No. of response during the ITI	day 1	10.5±10.2	8.2±8.9	25.2±12.7*
	day 2	10.8±10.7	11.8±10.6	20.1±11.4*
	day 3	11.2±10.3	14.9±16.0	20.9±17.9

Data were expressed as mean±SD.
the control at $p<0.05$

* : Significantly different from

Fig. 3. Avoidance response rate in pups * : Significantly different from the control at $p<0.05$

1.). After the delivery, the body weight in 40 mg/kg group tended to be lower than that in the control. The number of surviving pups at birth in 40 mg/kg group was significantly smaller than that in the control ($p<0.05$).

The body weight of pups in 40 mg/kg group was significantly lower than that in control group through the entire examination period ($p<0.05$) (Fig. 2). As to the general sign in weaning pups of 40 mg/kg group, a startle response to the contact stimulus and an gallop-like abnormal gait were noticed.

In the MAM-treated groups, as shown in Table 2, the brain weight was dose-dependently less than in the control group and the difference in the ratio the brain to body weight was significant ($p<0.01$).

Table 3 shows the results of the open-field test. The counts of ambulation and locomotion distance in the 40 mg/kg group have increased significantly as compared with those in control group (ambulation : $\chi^2=2.91$; $p<0.05$, distance $\chi^2=3.05$; $p<0.01$). The latency to leave

the starting point in 20 mg/kg group prolonged slightly, although there were no significant differences in the latency and defecation between the control and treated groups.

As shown in Table 4, the avoidance response rate was dose-dependently high in the session of the 1st day and significant in 40 mg/kg group ($F=7.90$, $df=2/57$, $p<0.05$). As to the interaction between the avoidance response rate and sequence of sessions, however, the avoidance response rate in 40 mg/kg group was significantly low ($F=5.93$, $df=4\text{ e}/114\text{ e}$, $p<0.05$); namely the increase of avoidance response rate was not recorded in 40 mg/kg group in the 2nd and 3rd sessions (Fig. 3).

The number of error was increased in dose-dependent manner through 3 days sessions. In fact, no animals in control group failed in the 3rd session, whereas 4 out of 19 in 40 mg/kg group failed during the same session. Rate of the rats with errors was significantly high in 40 mg/kg group ($\chi^2=4.692$, $df=1$, $p<0.05$).

As to the latency for avoidance and escape there were no significant differences between control and treated groups.

The number of avoidance response during the intertrial interval (ITI) in 1st and 2nd sessions was significantly high in 40 mg/kg group (1st day; $F=13.87$, $df=2/57$, $p<0.05$; 2nd day; $F=3.98$, $df=2/57$, $p<0.05$).

Discussion

The results obtained from OFT in the present study indicated a significant increase in the ambulation in the MAM-induced microcephalic rats, which was in consistent with the report by Haddad *et al.* [19]. The increase of ambulation in OFT has generally been regarded as an expression of emotional disturbance in the behavioral test using experimental animals. In the lower dose (20 mg/kg) group in the present study, no increase in the ambulation was recorded. The offsprings of this group were microcephalic, though to a lesser degree, as indicated in the brain weight data. Thus the OFT may have some difficulties with the detection of slight emotional disturbance.

In the shuttle box avoidance test, the mean avoidance response rate and number of response during the ITI were significantly high in 40 mg/kg group. It seems that these findings may have related to the increase of

ambulation and locomotion distance in OFT due to the increase of meaningless spontaneous motor activity in the microcephalic rats exposed *in utero* with MAM. The ratio of the number of animals with higher avoidance response rate to the number of animals which exhibited more response during ITI in session 1 of each group was examined. It was apparently high in the treated groups (42.9% in control group versus 83.3% in 20 mg/kg group, 88.9% in 40 mg/kg group), and significant in 40 mg/kg group ($\chi^2=3.88$, $df=1$, $p<0.05$). As a result, it is obvious that the individuals in control group which showed a high response count during ITI did not necessarily exhibit a high avoidance response rate. On the contrary, the individuals with high spontaneous motor activities in the MAM-treated groups tended to show high avoidance response rate. Therefore, it seems that the avoidance responses counted in the MAM-treated groups contained both real response due to conditioned stimulus and "make-believe response" originated from exciting reaction against the buzzer sound and lighting. These assumptions would be encouraged by the fact that the interaction between the rate of avoidance response and sequence of sessions in the MAM-treated groups was significantly low. There is no doubt about functional disturbance in the memory retaining ability *in utero* MAM-exposed rats.

Alcohol and X ray irradiation have been known to produce hyperactivity in offsprings by *in utero* exposure [3, 8]. Bond and Digiusto [4] and Tamaki [15], who studied learning ability of offsprings exposed *in utero* with these agents in shuttle box avoidance test, found a decrease in avoidance response ability after alcohol treatment and an increase of that after X ray irradiation. In the present study, the authors found the restlessness as revealed by OFT and avoidance response test in MAM-induced microcephalic rats, similar to the case of microcephalic rats produced by *in utero* X ray irradiation. Different from the findings on MAM-induced microcephalia, however, Tamaki [15] reported that the rate of avoidance in *utero* X ray-irradiated microcephalic rats through 2 days sessions was significantly higher in the 2nd session than in the 1st session. Accordingly, it seemed that the entity of the functional disturbance in terms of learning ability in experimental microcephala-

ly is not an uniform one, but different each other according to the cause of the microcephalic condition.

It may be concluded that MAM is a potent teratogen which produces morphological as well as behavioral abnormalities and useful as a positive control in practical behavioral teratology because of its reproducibility in the toxicity. In addition, such combination of two or more methods as described in the present paper was considered desirable to obtain much information.

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メチルアゾキシメタノール誘発小頭症
ラットにおける生後の行動発達
——行動奇形学的研究——

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Methylazoxymethanol (MAM) 誘発小頭症ラットを用いた行動学的な検討を行った。MAMは Sprague-Dawley 系ラットの妊娠14日に0, 20および40 mg/kgの投与量で腹腔内へ単回投与された。母体を自然分娩させて得られた雄の出産児は、6週齢でオープンフィールドテストに、7週齢以降でシャトルボックスによる条件回避学習試験に供した。オープンフィールドテストにおいて、40mg/kg群の通過区画数および移動距離が対照

群に比して有意に増加した。条件回避学習試験において、試行1日目の回避率が用量依存的に高値を示した。しかしながら、回避率と検査日の交互作用でみると、40mg/kg群の回避率は有意に低い結果となった。また、40mg/kg群ではエラーしたラットおよび試行間間隔中の移動回数に増加が認められた。以上のように、MAMの胎生期暴露は出産児の記憶保持能力に障害をもたらすことが示唆された。