EFFECTS OF PRENATAL TREATMENT WITH METHYL AZOXYMETHANOL ACETATE ON GROWTH, DEVELOPMENT, REPRODUCTIVE PERFORMANCE, LEARNING ABILITY AND BEHAVIOR IN THE RAT OFFSPRING.

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Accepted April 8, 1982

Abstract: SD rats were treated intraperitoneally with methylazoxy-methanol acetate (MAM acetate) at 0, 5 and 20 mg/kg dosage levels on day 14 of gestation, and were allowed to deliver spontaneously. Their offspring were examined for learning ability in a water T-maze at 10 weeks of age. Examinations of postnatal growth, development, reproductive performance, morphological alterations of the brain and other aspects of behavior were also carried out.

The offspring of mothers that received 20 mg/kg of MAM acetate showed a slight decrease (less than 10%) of body weights from birth through adulthood, and slight decreases (2-22%) of liver, kidney, spleen and testis weights at 3 weeks of age as compared to the controls. They also showed slight delays in ear pinna separation, lower incisor eruption, eye and vaginal opening, but had normal appearances and reproductive performance. On the other hand, the cerebrum weights of the offspring in the 20 mg/kg group were approximately 50% less than controls. Microscopically, the micrencephalic offspring showed greatly reduced cerebral hemispheres and abnormal cortical cytoarchitecture. No significant differences were noted between the offspring of the 5 mg/kg of MAM acetate group and the control offspring.

In an open-field behavioral tests, the offspring in the 20 mg/kg group showed a decrease in the latent period and increases in the number of sections crossed and rearings. Their spontaneous night activity measured by a wheel cage increased as compared with controls. In the triple T-maze, the micrence-
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Phallic offspring made 1.4-2.8 times as many errors as controls did throughout trial days.

There were no statistically significant differences in the behavioral parameters between the offspring in the 5 mg/kg group and the control offspring. However, 2 animals in the 5 mg/kg group did not solve the maze problem within 10 minutes of the observation period while all controls successfully escaped from the maze.

Key words: Methylazoxymethanol acetate, postnatal development, behavior, rat.

INTRODUCTION

The teratogenic effects of methylazoxymethanol (MAM) were first detected by Spatz et al (1967). It is most remarkable that the cerebral hemispheres are selectively reduced in size in otherwise normal appearing rats by appropriate prenatal treatment with this chemical and its ester, MAM acetate (Spatz and Laqueur, 1968).

Subsequently, several studies have been done to correlate the MAM-induced structural alterations of CNS with their functional consequences. Haddad et al. (1969) reported the inferior maze performance of the MAM-induced micrencephalic rats. Another aspects of behavior such as activity and emotionality have been reported elsewhere (Ciofalo et al., 1971; Rabe and Haddad, 1972 and Seo et al., 1979).

In all of the above studies, the doses of MAM or MAM acetate were so large as to produce diminished brain weight and its macro- and microscopic changes. It has been suggested that some behavioral changes may be more sensitive indicators of teratogenic effects than morphological alterations (Butcher et al., 1975 and Spyker, 1976).

The present study was undertaken to compare the severe and subtle morphological alterations of rat offspring induced by teratogenic (20 mg/kg) and sub-teratogenic (5 mg/kg) doses of MAM acetate with the subsequent behaviors. In addition, postnatal growth, development and reproductive performance of the offspring were also evaluated since there were relatively few studies incorporating these examinations.

MATERIALS AND METHODS

Animals: SPF female rats of Sprague-Dawley strain (Jcl : SD) were purchased from CLEA Japan Inc. and housed in an air-conditioned animal room of our laboratories, where temperature and relative humidity were maintained at 24±2°C and 55±5%, respectively, with automated light-darkness cycles (12-hr intervals). The animals ate rat pellets (CA-1, CLEA Japan Inc.) at will and had free access to fresh tap water.

The females were paired with the males of the same strain (17 hr). Copulation was established the next morning by the presence of vaginal plugs and the day was designated as day 0 of gestation. They were randomly assigned to one control and two treatment groups. On day 20 of gestation, each female was transferred into a plastic box
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cage floored by wood shavings for nesting. They were allowed to deliver spontaneously. Pups delivered were counted, sexed, weighed and inspected for grossly observable abnormalities and then returned to their own mothers. On day 3 postpartum, the litter size was standardized to 8 with an equal sex ratio when possible, using the random sampling number. On day 21 postpartum, the pups were weaned. Up to 3 male or 3 female pups in a litter were housed separately in a wire-meshed cage for subsequent evaluation of physical development and behavior, until approximately 17 weeks of age.

Method of MAM acetate treatment: MAM acetate (ASH Stevens Inc., Detroit), after being dissolved in physiological saline was given to females intraperitoneally at dosage levels of 5 and 20 mg/kg on day 14 of gestation. The drug was administered in a volume of 5 ml/kg.

Control females received physiological saline in the same manner as the treatment groups.

Observations:

a. Maternal observation: The body weights and physical appearances of mother animals were registered on day 0 of gestation, daily from day 14 of gestation to delivery, and weekly for 3 weeks thereafter. On day 21 postpartum when the pups were weaned, the mother animals were autopsied and their metrial glands were counted.

b. Observation of the offspring:

(1) Mortality and body weight: The viability and general behavior of the offspring were observed throughout the experiment, and their body weights were recorded on days 0 and 3 postpartum and weekly up to 12 weeks of age.

(2) Organ weight and head-to-tail length: At 3 and 12 weeks of age, the offspring which were scheduled to be sacrificed were exsanguinated via the abdominal aorta and examined for visceral abnormalities. Their major organ weights (cerebrum, cerebellum, liver, kidneys, spleen and testes) and head-to-tail lengths were measured.

(3) Postnatal development: Separation of the ear pinna was examined in all pups on days 3 and 7 postpartum, eruption of the lower incisors on days 11 and 14, and eye opening on days 14 and 21. Auditory acuity by Preyer's reflex and postural abnormality by free fall and mid-air righting reflexes were evaluated on day 21 postpartum. Observation was made on testicular descent or vaginal opening at 5 weeks of age. The males and females, which were scheduled to be sacrificed at 3 and 12 weeks of age, were evaluated for olfaction with a 28% ammonia aqueous solution. The vision was evaluated at night with a flashlight at 5 weeks of age.

(4) Reproductive performance: At 13 weeks of age, one male and female from each litter were mated within the same group but avoiding litter mates. The females were examined for the presence of vaginal plugs and spermatozoa in vaginal saline lavages every morning during 14 days of the breeding period. The females were sacrificed on day 20 of gestation and examined for reproductive status. The live fetuses were weighed, sexed and examined for gross abnormalities.

(5) Histological examination of the brain: Light microscopic observation of the cerebrum was made on the offspring which had been given a water T-maze test. They
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were autopsied about one week after the maze test. The cerebrum of one half of the offspring was removed, fixed in 10% formalin, embedded in paraplast, coronally sectioned at 6 micrometer and then stained with Klüver-Barrera's cresyl violet and luxol fast blue. The cerebrum of the other half of the offspring was processed for Golgi analysis using the Ramon-Moliner's Golgi-Cox technique (1970). The cerebrum was immersed in Golgi-Cox solution for 1 month followed by alkalization for about 24 hours, embedded in celloidin and then coronally sectioned at 120 micrometer.

(6) Behavior test:

i) Open-field test: One male and female at 4 weeks of age were randomly selected from each litter and subjected to an open-field test to observe general activities and emotionality. The test apparatus (ANAC-50-II, Okazaki Sangyo Co., Ltd.) had a circular arena 80 cm in diameter with walls 30 cm high. The floor was marked off into 9 sections: one center circle and 8 sections with equivalent shapes by radii. The field was illuminated by a single 150 W lamp suspended about 150 cm above the center of the field. White noise was generated during the test to mask ambient noise. The animal was placed in the center of the field and kept there in a closed box for 30 sec prior to the initiation of each test. Latency (time spent in the center circle after initiation of the test), sections crossed (a crossing defined as placing both forelegs into each section), rearings, groomings, defecations and urination (number of animals urinated) were measured as well as abnormal gait or behavior during a 3-minute observation period. The rubber floor mat was wiped clean after each animal had been tested.

ii) Spontaneous motor activity test by a wheel cage method: The spontaneous motor activity of males was measured by the wheel cage method at 8 and 10 weeks of age. The apparatus (Kishimoto Medical Industrial Co., Ltd.) was composed of a wheel cage (30 cm in diameter and 10 cm in width) and a living cage where the animal had free access to food and water. An animal was free to ambulate between both compartments. The animal was transferred from a wire-meshed cage to the wheel cage at approximately 8:00 a.m. on the first day, and then the number of revolutions of the wheel cage was recorded daily for 11 successive days at 6:00 p.m. for daytime activity and 8:00 a.m. for night activity.

iii) Water-filled triple T-maze test: At 10 weeks of age, one male and female in each litter were tested for swimming behavior in a straight channel on the first day, and then for ability to learn to escape from a water-filled triple T-maze following 5 successive days of testing. The straight channel 200 cm long, 15 cm wide and 35 cm high was constructed of a transparent plastic plate and filled with water at 21±1°C to a depth of 25 cm. The animal was placed in the water at one end of the channel and allowed to swim to an exit platform set at the opposite end. The animal was given 3 trials and was examined for the swimming behavior and the elapsed time.

As shown in Fig. 1, the test chamber of the water-filled T-maze developed in our laboratory consisted of 3 parts of gray plastic T-tank 15 cm wide and 35 cm high. The tank was filled with water at 21±1°C to a depth of 25 cm. The platform was placed in
Fig. 1. Top view of the water-filled T-maze apparatus. An animal is placed in the water at the start point (arrow). The goal platform for escape from the water is set at the blind alley 1 or 2. Infrared photocells (dotted line) are fixed on each arm to count the passage of the animal.

one of the blind alleys for the first 3 days, and was then transferred to the other blind alley on days 4 and 5. The animal was given 3 trials per day, and the intertrial rest was 30 sec. The elapsed time and errors were recorded. The errors were calculated as follows:

\[ \text{Errors} = (\text{number of the photocell counts} - 3) \times 1/2 \]

No animal was allowed to remain in the maze for more than 10 minutes in any one trial to prevent exhaustion.

**Statistical analysis:** The results obtained from behavior tests were analyzed by the analysis of variance. The copulation rate and pregnancy rate of the offspring and the number of offspring urinated in the open-field test were analyzed by the Chi-square test. All other parameters were analyzed by the Wilcoxon’s rank sum test on a litter basis.

**RESULTS**

**Maternal observation:** No drug-related physical sign was observed in any group. The average maternal body weight gain during the gestation period in the 20 mg/kg group were significantly lower than that of the control group \((p<0.01)\). In the 5 mg/kg group, no significant difference in maternal body weight gains was shown. The average weight gains during lactation in both treated groups were comparable to those of the control group.

One dam in the 5 mg/kg group did not nurse their pups, and killed all of them by day
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3 postpartum.

Observation of the offspring:

a. Observation at birth: Data obtained from the observation of pups at birth and the number of metrial glands of dams observed at weaning are summarized in Table 1. There was no significant difference between the treatment and control groups in the average number of metrial glands, the average number of pups delivered, sex ratio, death rate and birth rate. No gross malformations were seen in pups from any group.

Table 1. Effect of maternal ip injection of methylazoxymethanol acetate on day 14 of gestation on the developmental state of the newborn rats.

<table>
<thead>
<tr>
<th>Group (mg/kg)</th>
<th>No. females</th>
<th>No. metrial glands (per female)</th>
<th>No. pups delivered</th>
<th>Birth rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>total (per litter)</td>
<td>alive</td>
<td>dead male</td>
</tr>
<tr>
<td>Control</td>
<td>19</td>
<td>247 (13.0)</td>
<td>230 (12.1)</td>
<td>124</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>283 (12.9)</td>
<td>268 (12.2)</td>
<td>143</td>
</tr>
<tr>
<td>20</td>
<td>22</td>
<td>281 (12.8)</td>
<td>262 (11.9)</td>
<td>123</td>
</tr>
</tbody>
</table>

1: The metrial glands were counted on day 21 after delivery or earlier when females were autopsied.
2: Number of dead pups/number of pups delivered x 100.
3: Number of pups delivered/number of metrial glands x 100.
4: Includes one pup whose sex was unknown since a part of body was cannibalized.

b. Observation of pups during the lactation period: The mortality and weaning rates of the offspring are summarized in Table 2. There was no significant difference between the treatment and control groups in the mortalities during the first 3 days after birth and weaning rate.

Table 2. Effect of maternal ip injection of methylazoxymethanol acetate on day 14 of gestation on the survival and weaning rates of rat offspring.

<table>
<thead>
<tr>
<th>Group (mg/kg)</th>
<th>No. live pups at birth</th>
<th>No. pups died by day 3 postpartum (%)</th>
<th>No. live pups after reduction</th>
<th>No. pups died from day 3 to 21 postpartum (%)</th>
<th>No. pups weaned (weaning rate %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>226(19)</td>
<td>3 (1.3)</td>
<td>151(19)</td>
<td>0</td>
<td>151 (100)</td>
</tr>
<tr>
<td>5</td>
<td>266(22)</td>
<td>16 (6.0)</td>
<td>168(21)</td>
<td>0</td>
<td>168 (100)</td>
</tr>
<tr>
<td>20</td>
<td>257(22)</td>
<td>4 (1.6)</td>
<td>174(22)</td>
<td>4</td>
<td>170 (97.7)</td>
</tr>
</tbody>
</table>

1: Number of dead pups/number of live pups at birth x 100.
2: Litter sizes were standardized to 8 or less pups at day 3 postpartum.
3: Number of dead pups/number of pups after reduction x 100.
4: Number of weanlings/number of pups after reduction x 100.
5: Numbers in parentheses represent litters.
6: Twelve pups from one litter died because the mother did not nurse them.
7: Three male and one female pups from one litter died during day 7-11 postpartum.
Fig. 2. Effect of maternal i.p. injection of methylazoxymethanol acetate on day 14 of gestation on the body weight growth of the offspring.

\*or\*\*; Significant at p < 0.05 or p < 0.01 by the Wilcoxon's Rank Sum Test (two-sided).

c. Body weight: Average body weights of male and female offspring from birth to 12 weeks of age are shown in Fig. 2. The average body weights in the 20 mg/kg group were lower than those of the control group and a significant difference was shown from 3 to 12 weeks of age in male and from 3 to 10 weeks of age in females (p < 0.05 or p < 0.01). The average body weights of males and females in the 5 mg/kg group were comparable to controls.

d. Organ weight and head-to-tail length: The major organ weights and head-to-tail lengths of male and female offspring at 3 and 12 weeks of age are shown in Tables 3, 4, 5 and 6.

i) Brain: The appearance of the brain of 3-week-old males in each group is shown in Fig. 3. The reduction of the cerebrum was remarkable in the 20 mg/kg group. The absolute cerebrum weights of males and females at 3 weeks of age were decreased by 47.4% and 49.1% of controls, respectively. The relative weights of males and females at this age were also severely decreased as compared with controls (males: 39.7% and females: 47.5%). Similar decreased cerebrum weights were observed in males and females at 12 weeks of age (absolute weights of males: 47.6%; females: 49.3%, relative weights of males: 41.2%; females: 46.4%).

The cerebellum weights in the 20 mg/kg group were slightly greater than controls at 3 and 12 weeks of age (absolute weights: 3.4–8.5%, relative weights: 10.5–18.2%).

In the 5 mg/kg group, both cerebrum and cerebellum weights of males and females
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Table 3. Organ weight and head-to-tail length of male offspring at 3 weeks of age (average ± S.D.)

<table>
<thead>
<tr>
<th>Group (mg/kg)</th>
<th>No. offspring (No. litters)</th>
<th>Body weight (g)</th>
<th>Organ weight (g)</th>
<th>Head-to-tail length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>brain cerebrum</td>
<td>cerebellum liver kidneys spleen testes</td>
</tr>
<tr>
<td>Control</td>
<td>20 (16)</td>
<td>53.8±4.17</td>
<td>1.12±0.04 0.29±0.02 2.31±0.26 0.61±0.07 0.27±0.04 0.24±0.03 20.8±0.69</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>22 (19)</td>
<td>54.2±3.58</td>
<td>1.18±0.05 0.29±0.01 2.34±0.18 0.61±0.05 0.27±0.04 0.25±0.02 21.3±0.82</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>25 (19)</td>
<td>48.4±5.89** 0.60±0.06** 0.30±0.02 1.98±0.32** 0.53±0.06** 0.21±0.05** 0.22±0.03* 20.5±1.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* or **: Significant at p<0.05 or p<0.01 by the Wilcoxon's Rank Sum Test (two sided).

Table 4. Organ weight and head-to-tail length of female offspring at 3 weeks of age (average ± S.D.)

<table>
<thead>
<tr>
<th>Group (mg/kg)</th>
<th>No. offspring (No. litters)</th>
<th>Body weight (g)</th>
<th>Organ weight (g)</th>
<th>Head-to-tail length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>brain cerebrum</td>
<td>cerebellum liver kidneys spleen</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15 (14)</td>
<td>48.6±8.16</td>
<td>1.06±0.06 0.27±0.02 2.10±0.46 0.56±0.12 0.25±0.07 19.9±1.29</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>21 (18)</td>
<td>52.2±4.23</td>
<td>1.06±0.04 0.28±0.02 2.29±0.27 0.62±0.06 0.27±0.05 20.9±0.88*</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>20 (17)</td>
<td>46.9±6.44</td>
<td>0.54±0.05 0.29±0.02* 1.94±0.36 0.55±0.08 0.22±0.06 20.2±1.13</td>
<td></td>
</tr>
</tbody>
</table>

Relative weight (absolute weight/body weight ×100)

* or **: Significant at p<0.05 or p<0.01 by the Wilcoxon's Rank Sum Test (two sided).
Table 5. Organ weight and head-to-tail length of male offspring at 12 weeks of age (average ± S. D.)

<table>
<thead>
<tr>
<th>Group (mg/kg)</th>
<th>No. offspring (No. litters)</th>
<th>Body weight (g)</th>
<th>Organ weight (g)</th>
<th>Head-to-tail length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19 (19)</td>
<td>423.4±32.17</td>
<td>1.45±0.06</td>
<td>18.49±1.89</td>
</tr>
<tr>
<td>5</td>
<td>21 (21)</td>
<td>405.6±25.08</td>
<td>1.42±0.05</td>
<td>17.90±1.86</td>
</tr>
<tr>
<td>20</td>
<td>21 (21)</td>
<td>391.4±36.38**</td>
<td>0.76±0.08**</td>
<td>17.69±2.32</td>
</tr>
</tbody>
</table>

**; Significant at p<0.01 by the Wilcoxon's Rank Sum Test (two sided).

Table 6. Organ weight and head-to-tail length of female offspring at 12 weeks of age (average ± S. D.)

<table>
<thead>
<tr>
<th>Group (mg/kg)</th>
<th>No. offspring (No. litters)</th>
<th>Body weight (g)</th>
<th>Organ weight (g)</th>
<th>Head-to-tail length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18 (18)</td>
<td>243.2±15.00</td>
<td>1.36±0.06</td>
<td>9.32±0.82</td>
</tr>
<tr>
<td>5</td>
<td>21 (21)</td>
<td>244.0±19.80</td>
<td>1.32±0.07</td>
<td>9.53±0.96</td>
</tr>
<tr>
<td>20</td>
<td>19 (19)</td>
<td>230.5±19.06**</td>
<td>0.69±0.10**</td>
<td>9.47±1.21</td>
</tr>
</tbody>
</table>

Relative weight (absolute weight/body weight × 100)

| Control       | 18 (18)                     | -               | 0.56±0.04        | 3.84±0.35              | 0.75±0.06 0.21±0.03 - |
| 5             | 21 (21)                     | -               | 0.54±0.03        | 3.91±0.24              | 0.78±0.07 0.22±0.04 - |
| 20            | 19 (19)                     | -               | 0.30±0.04**      | 4.10±0.32*             | 0.75±0.05 0.23±0.02* - |

* or **; Significant at p<0.05 or p<0.01 by the Wilcoxon's Rank Sum Test (two sided).

were comparable to controls at any age.

ii) Other major organ weight and head-to-tail length: In the 20 mg/kg group, the absolute liver, kidney, spleen and testis weights and the relative liver and spleen weights

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of 3 week-old males were significantly lower than controls (p<0.05 or p<0.01). The relative testis weight of 12 week-old males and relative liver and spleen weights of 12 week-old females were significantly greater than controls (p<0.05). In the 5 mg/kg group, the absolute and relative weights of major organs in males and females were comparable to the corresponding control values at both 3 and 12 weeks of age.

The head-to-tail lengths of males and females in the treatment groups were comparable to controls at any age except for a significant increase in 3 week-old females in the 5 mg/kg group.

e. Postnatal development: The ear pinna separation, eruption of lower incisors, eye and vaginal openings tended to be retarded in the treatment groups, especially in the 20 mg/kg group. However, there was no statistically significant difference in any developmental sign between the treatment and control groups, and all the retarded offspring in the treatment and control groups showed normal developmental signs when examined again several days later.

All males in each group showed normal descent of testes at 5 weeks of age. Auditory acuity and righting reflexes of offspring at 3 weeks of age were all normal in the treatment and control groups. With respect to olfaction tested at 3 and 12 weeks of age, avoidance response to ammonia was observed in all offspring of each group. In the vision test carried out at 5 weeks of age, all offspring in each group positively responded to the light.

f. Reproductive performance: There were no significant differences between the treatment and control groups in the copulation rate, pregnancy rate, the numbers of implantation sites, resorptions and live fetuses, sex ratio and average fetal body

Fig. 3 Brains of 3 week-old male rat offspring whose mothers received saline (left), 5 mg/kg (center) or 20 mg/kg (right) of methylazoxymethanol acetate on day 14 of gestation. Note severe retardation on prosencephalic development in the offspring treated with 20 mg/kg of methylazoxymethanol acetate. The brain of the offspring treated with 5 mg/kg of methylazoxymethanol acetate is similar to the controls.

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weights. With respect to gross malformations, one fetus in the 20 mg/kg group had exencephaly associated with open eyelids.

g. Histological examination of the brain: The principal feature of the brain of the offspring in the 20 mg/kg group was small cerebral hemispheres (Fig. 3). Microscopically, the cerebral cortex of the animals in the 20 mg/kg group was strikingly reduced in thickness compared with controls and had abnormal cortical cytoarchitecture. The small granular cells which are contained in layer II–IV of the control cortex were not recognized as discrete layers in the thinned cortex. Immediately beneath the molecular layer (layer I) were medium and large sized neurons which resembled the normal occupants of layer V, although this middle layer appeared more densely packed with smaller granular cells. Beneath this group of cells and contiguous to white matter was a layer smaller and more uniform granular cells which resembled normal layer VI (Figs. 4A–C).

Fig. 4 Dorsolateral cerebral cortices of 11 week-old rat offspring whose mothers received saline (A and D) 5 mg/kg (B and E) and 20 mg/kg (C and F) of methylnitroazoxymethanol acetate on day 14 of gestation. In the offspring whose mothers received 20 mg/kg of methylnitroazoxymethanol acetate, the outer laminae are deficient (C) and apical dendrites of the pyramidal cells are shortened, bifurcated from the base of the perikaryon and oriented in all directions without any order (F). The pyramidal cells of the offspring whose mothers received saline or 5 mg/kg of methylnitroazoxymethanol acetate have apical dendrites oriented towards the surface in an orderly fashion (D and E). A–C: cresyl violet and luxol fast blue staining, D–F: Golgi-Cox staining. (50×)
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Fig. 5 Frontal sections through hippocampus of 11 week-old male rats whose mothers received saline (A and D), 5 mg/kg (B and E) and 20 mg/kg (C and F) of methylazoxymethanol acetate on day 14 of gestation. Note defects in the pyramidal cell layer of Ammon's horn with a gap and displacement of neurons (arrow) into adjacent layers (C), and irregularly oriented dendrites of the pyramidal cells (F). The hippocampal formation of the offspring in the 5 mg/kg group is normal (B and E). A-C: cresyl violet and luxol fast blue staining, D-F: Golgi-Cox staining. (50×)

Analysis of the Golgi-Cox stainings revealed that the pyramidal cells in the thinned cortex had a reduced branching pattern and shortened apical dendrites. The pyramidal cells in some thinned cortices had apical dendrites which bifurcated from the base of the perikaryon and oriented in all directions without organization, whereas the pyramidal cells of the control cortex had apical dendrites oriented towards the surface in an orderly fashion (Figs. 4D–F).

Moreover, clusters of ectopic neurons appeared in the hippocampus of some animals in the 20 mg/kg group. The ectopic cells were located in the stratum radiatum and stratum oriens (Fig. 5C). Irregularly oriented dendrites of the pyramidal neurons in the 20 mg/kg group were observed by analysis of the Golgi-Cox stain (Fig. 5F). There was no sex difference in the above histologic findings of the brain in the 20 mg/kg group.

The brain in the 5 mg/kg group were histologically normal (Figs. 4-5).

h. Behavior test:

i) Open-field test: The results obtained from the open-field test are shown in Table 7. In the latent period in males and females, there was no statistically significant difference between the treatment and control groups, but the period tended to be decreased in a dose-related manner. The numbers of sections crossed and rearing times for males and females tended to increase in a dose-related manner, and there was
significant difference in both parameters in females in the 20 mg/kg group compared with controls (sections crossed: $F=7.5$; $df=2/60$; $p<0.01$, rearing times: $F=6.7$; $df=2/60$; $p<0.01$). The number of urinated females in the 20 mg/kg group was significantly fewer than controls ($p<0.05$). There was no significant difference in the number of groomings and defecations between the treatment and control groups.

ii) Spontaneous motor activity test by a wheel cage method: There were no significant differences in the number of daytime revolutions between the treatment and control groups. The average daily number of night revolutions in the 8 and 10 week-old male offspring for 11 days are shown in Fig. 6. The daily number of revolutions varied

![Fig. 6](image)

Fig. 6 Spontaneous motor activity of male offspring, whose mothers were injected with methylaazoxymethanol acetate on day 14 of gestation, in the night by a wheel cage method.

Analysis of variance revealed that the offspring in the 20 mg/kg group was significantly more active than the controls ($p<0.01$)
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considerably from animal to animal during the 11 day experimental period. Accordingly, a square root transformation was done on the data prior to an analysis of variance.

At 8 weeks of age, the number of night revolutions increased in all groups as the days of the observation proceeded. The trend of increase was more distinct in the 20 mg/kg group than that of the 5 mg/kg or control group. An analysis of variance revealed significant treatment effect (Group; F=9.3, df=2/27, p<0.01) and time effect (Day; F=4.8, df=10/297, p<0.01) on the average number of night revolutions. Group comparisons showed that animals in the 20 mg/kg group, but not in the 5 mg/kg group, were significantly more active than controls (p<0.01). At 10 weeks of age, similar results were obtained from the wheel activity test (Group: F=13.1, df=2/253, p<0.01; Day: F=7.9, df=10/253, p<0.01) without significant differences between results obtained at 8 and 10 weeks of age.

iii) Water-filled triple T-maze test: There was no significant difference in the swimming speed in the straight channel between the treatment and control groups. The swimming behavior of animals was all normal in each group.

The total errors and elapsed time of 3 trials on each day in the triple T-maze test are shown in Fig. 7. There was no significant sex difference and the results from both sexes were combined. The number of errors in all groups gradually decreased from day

Fig. 7 Learning behavior of 10 week-old rat offspring whose mothers were injected with methylazoxymethanol acetate on day 14 of gestation. Each animal was given 3 trials a day in the water-filled triple T-maze, and the goal platform was transferred to other side on day 4 of examination. Analysis of variance revealed that the offspring in the 20 mg/kg group made significantly more errors than the controls (p<0.01).
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1 to 3. When the goal platform was transferred to the other location (day 4), the number of errors was markedly increased but rapidly decreased on the next day.

Two males in the 5 mg/kg group and 3 males and 2 females in the 20 mg/kg group failed to reach the goal platform at various trials within the 10-minute time limit. These animals were guided to the platform and given an error score of 50.5 for males and 67.5 for females (one more than the maximum error score for any animal solving the maze in less than 10 minutes).

An analysis of variance performed on errors revealed significant group effect (F = 40.3; df = 2/590; p < 0.01) and day effect (F = 14.2; df = 4/590; p < 0.01). Comparisons showed that both males and females in the 20 mg/kg group, but not in the 5 mg/kg group, made significantly more errors than controls (p < 0.01).

The elapsed time in the water-filled triple T-maze test corresponded with the number of errors. There were significant group effect (F = 22.4; df = 2/590; p < 0.01) and day effect (F = 9.8; df = 4/590; p < 0.01) on the elapsed time. A significant difference in the elapsed time was also noted in the 20 mg/kg group compared to controls (p < 0.01).

DISCUSSION

MAM is known to be a mutagenic, carcinogenic and teratogenic alkylating agent (Smith, 1966; Laqueur and Spatz, 1968 and Spatz et al., 1967). Spatz and Laqueur (1968) first discovered that prenatal exposure to MAM can produce micrencephaly in the rat. There are many reports on structural and functional deficits of the micrencephalic rat induced by MAM or MAM acetate (Haddad et al., 1969; Rabe and Haddad, 1972 and Johnston and Coyle, 1979).

In the present study, the cerebral hemispheres were selectively reduced in size by prenatal treatment with MAM acetate at 20 mg/kg. The histological findings of the cerebrum from the micrencephalic rat were comparable to those of Johnston and Coyle (1979) and Singh (1977 and 1980). Since MAM acetate has the property of methylating nucleic acids in the fetal rat brain (Nagata and Matsumoto, 1969) and has a cytotoxic effect on the neuroblast (Spatz and Laqueur, 1968), it is reasonable that the above regions, where cells are actively proliferating, were selectively interfered with MAM acetate. Although an increase in cerebellum weights of the micrencephalic rat was observed in the present study, we have no ready explanation for this result.

Whereas cerebrum weights of the micrencephalic rats were severely reduced by approximately 50%, their body weight loss was less than 10%. The micrencephalic rats also showed slight delays in onset of ear pinna separation, lower incisor eruption, eye and vaginal opening. Delays in these developmental signs seemed to be associated with growth retardation. In addition, the micrencephalic rats had normal visual, olfactory and auditory functions, righting reflex, swimming behavior and reproductive performance.

The above results of the present study suggested that the micrencephalic rats
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induced by the prenatal treatment of MAM acetate provided a model that can be used to study behavior, especially learning ability of animals with morphological changes of the CNS, since effects of the generalized growth retardation on the behavior were minimal.

The micrencephalic rats induced by MAM acetate were reported to show an increase in adult activity as measured by a photocell system (Rabe and Haddad, 1972) and open-field test (Seo et al., 1979). However, Ciolfalo et al. (1971) found a non-significant increase in the open-field test in the micrencephalic rats. Our observation is in accord with studies of Rabe and Haddad and Seo et al. The micrencephalic rats showed hyperactivity in both open-field test and wheel cage method. Since it is postulated that the hippocampus has some kind of inhibitory function on behavior (Kimble, 1968), the hyperactivity seen in the micrencephalic rats may be considered to be related to hippocampal damage produced by the prenatal treatment of MAM acetate.

In the present study, the micrencephalic rats were given the open-field test at 4 weeks of age and showed a decrease in the latent period and increases in the number of sections crossed and rearings. Seo et al. (1979) also described similar results in the micrencephalic rats of the same strain at 8–9 weeks of age. These findings indicated that the functional changes of the micrencephalic rats were permanent as their structural changes persisted into adulthood.

Rabe and Haddad (1972) reported that the micrencephalic rats induced by MAM acetate were deficient on spatial reversal and maze-type problem-solving tasks, but were not impaired in their performance on commonly used operant conditioning schedules or in acquiring a discrimination learning set. Ciolfalo et al. (1971) also reported mild deficits in motor and Y-maze performance in the micrencephalic rats. In the present study, the micrencephalic rats showed learning deficits in the water-filled triple T-maze, which was developed in our laboratory.

Although average errors of the 5 mg/kg group were comparable to controls in the water-filled triple T-maze, 2 rats in the 5 mg/kg group failed to reach the goal platform at various trials. Similar severe learning deficit was observed in neither the present nor the past control rats but was in 5 rats in the 20 mg/kg group. Although obvious morphological changes were not detected in rats in the 5 mg/kg group, the severe learning deficit found in 2 rats in this group may suggest that some behavioral changes are sensitive indicators of teratogenic effects than morphological alterations (Butcher et al., 1975 and Spyker, 1975). However, there were large variations in the water-filled triple T-maze as well as open-field test and activity test by a wheel cage compared with anatomical measures.

The water-filled triple T-maze is considered to be a useful apparatus to study the learning ability of rats in behavioral teratology from the following points of view. First, water mazes have been used in numerous teratological studies, and have several advantages over dry maze. Since no reward but escape is present, there is no need for preceding training by food deprivation. Second, swimming may require less motor
coordination than land locomotion, and thus be less affected by motor impairment (Brunner and Altman, 1973). Third, cost-efficiency is one of the critical points for a method to be suitable for general screening purpose in the behavioral teratology (Buelke-Sam and Kimmel, 1979), and the water-filled triple T-maze is relatively inexpensive in time and money.

LITERATURE CITED


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