Effects of Ethyl Alcohol Administration to Rat Dams during the Gestation Period on Learning Behavior and on Levels of Monoamines and Metabolites in Rat Pup Brain after Birth

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Abstract

Pregnant rats were given 0%, 5%, 10% and 20% ethyl alcohol in drinking water during the gestation period.

We evaluated the brain function of pups born of alcohol-administered dams. Learning ability (Sidman avoidance behavior), the amounts of monoamines (noradrenaline, dopamine, serotonin) and metabolites (3,4-dihydroxyphenyl acetic acid [DOPAC], homovanillic acid [HVA] and 5-hydroxyindole acetic acid [5-HIAA]) in whole brain were examined for neurobehavioral and neurochemical effects.

There was no effect on Sidman avoidance behavior in 56-day-old offspring, but alterations of the amounts of monoamines and their metabolites were observed even in 66-day-old offspring as a result of the dams' exposure to ethanol during pregnancy.

Key words: Ethyl alcohol, Operant behavior, Monoamines, Monoamine metabolites, Gestation period

Introduction

The numbers of Japanese women who drink alcoholic beverages and the amounts they imbibe have increased recently. Jones et al. reported that the children born of mothers with alcohol abuse had anomalies and mental retardation, and designated these children as suffering from fetal alcohol syndrome (FAS). Clarren et al. reported that 80% of children born of mothers with chronic alcoholism suffered from mental retardation. Recent epidemiological studies by Tanaka and Yuan et al. reported a high relationship between the habit of drinking and abnormalities in newborn babies. In experiments using animal models, increases in the frequency of anomalies and fetal death, and decreases in body weight and brain weight both pre and postnatal have been reported by Tanaka et al., while Iwase et al. found alteration in the metabolism of neurotransmitters. A decrease in body weight gain and neurobehavioral deficits has also been demonstrated in pups born of alcohol-administered dams by Iwase et al. Disorders of the central nervous system (CNS) in FAS have been noted via neurobehavioral tests, determination of neurotransmitters, and histochemical and biochemical studies in rats. Several alterations have been shown in the earlier postnatal period of pups exposed to high-dose ethanol during their embryonic period in utero. On the other hand, many studies showed that there was either no effect or recovery in experiments using low-dose exposure and after a long period from the end of high-dose exposure. The purpose of the present study is to examine long-lasting effects of CNS dysfunction. For this purpose, pregnant rats were given ethanol in drinking water from gestation days (GD) 7 to 20, and we studied the effect on pups exposed to ethanol in utero by the shock avoidance test on postnatal day (PND) 56, and the contents of monoamines and their metabolites in the whole brain on PND 66.

Materials and Methods

1. Animals

Forty pregnant Wistar rats (CLEA, Tokyo, Japan) were used in this experiment. They were randomly divided into 4 groups (10 rats per group.) The laboratory diet (CE-2, CLEA, Tokyo, Japan) and drinking water without ethanol were administered ad libitum. Litters of 8 pups (male 4, female 4) from each
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Table 1 HPLC-ECD condition

<table>
<thead>
<tr>
<th>Injection volume</th>
<th>Monoamines</th>
<th>Monoamine metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 μl</td>
<td>0.05M-sodium citrate-citric acid (pH4.5) + Tetrahydrofuran (0.9%)</td>
<td>50 μl 0.075M-sodium citrate-citric acid (pH3.6) + Methyl alcohol (10%) + Tetrahydrofuran (1%) + acetic acid (12%)</td>
</tr>
<tr>
<td>Mobile phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05M-sodium citrate-citric acid (pH4.5) + Tetrahydrofuran (0.9%)</td>
<td>0.075M-sodium citrate-citric acid (pH3.6) + Methyl alcohol (10%) + Tetrahydrofuran (1%) + acetic acid (12%)</td>
<td></td>
</tr>
<tr>
<td>Column (precolumn)</td>
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<td></td>
</tr>
<tr>
<td>ODS-resin (4.0 μm × 250mm)</td>
<td>Ultrasphere-ODS (4.6 μm × 200mm)</td>
<td>Ultrasphere-ODS (4.6 μm × 250mm)</td>
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<td>Column Temp</td>
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<td></td>
</tr>
<tr>
<td>Flow rate</td>
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<tr>
<td>0.9 ml/min</td>
<td></td>
<td></td>
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<td>Detector</td>
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<td></td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Reference electrode</td>
<td>Ag/AgCl</td>
<td></td>
</tr>
</tbody>
</table>

2. Ethanol administration

Pregnant rats were given ethanol solution in drinking water from gestation days (GD) 7 to 20. Ethanol solutions (V/V) of 5, 10, or 20% (5%, 10%, 20%-EtOH) were made from laboratory pure ethanol (Wakojunnyakukogyo, Osaka, Japan) and water. Each solution was given ad libitum to three groups of dams; plain water was given to the control group.

3. Shock avoidance test

Two male litters that were randomly selected from each dam were used for this test on PND 56. Female litters were not used in order to avoid the influence of menstruation. The schedule of the shock avoidance test followed the Sidman avoidance schedule: shock-shock interval 5s; response-shock interval 30s; shock intensity 100V; and 3mA for 0.5s. All avoidance tests were performed for 60 min a day for 10 days. Because no warm-up effect was evident in the latter half of each test, we analyzed the data of the latter half to evaluate the learning ability. The data were expressed as avoidance rates: A={(S-G)/S} x 100 (%), where A is the avoidance rate, S is the total number of electric stimuli when no response occurred, and G is the total number of shocks given to the rat.

4. Determination of the levels of monoamines and their metabolites

All male pups were decapitated, and the whole brain except the olfactory bulb was removed. These brain samples were immediately frozen and stored at -80°C until determination. We homogenized each brain sample with 3,4-dihydroxybenzylamine, 3,4-dihydroxyphenylpropionic acid as the internal standard for monoamines (noradrenaline [NA], dopamine [DA], 5-hydroxytryptamine [5-HT]) and their metabolites (3, 4-dihydroxyphenyl acetic acid [DOPAC], homovanillic acid [HVA] and 5-hydroxyindole acetic acid [5-HIAA]). After that we added n-butanol and n-heptane to homogenized brain tissue and extracted monoamines from the aqueous layer, and their metabolites from the organic layer according to the method of Shibanoki et al. We measured levels of monoamines and their metabolites using liquid chromatography (L-5000, Yanagimoto Co.) with an ECD detector (VMD-501, Yanagimoto Co.) under the conditions shown in Table 1.

5. Statistics

We calculated mean values and standard deviations of avoidance rates and levels of monoamines and their metabolites for each group. Differences between two groups were tested by Student’s t test and Aspin-Welch’s t test. Statistical analyses of simple regression lines or trends between the concentration of ethanol administered and the levels of monoamines, the levels of their metabolites and the ratio reflecting the activation of turnover, were performed.

Results

1. Examination of surface

Macroscopic anomalies, abnormal behavior and motor disturbances such as convulsion, hemiparalysis, and ataxia were not found until PND 66.

2. Water intake

During ethanol administration to dams, the volume of water intake decreased with increased concentrations of ethanol solution. We estimated the mean volume of pure ethanol intake from the mean water intake for each group (see Table 2). The higher the concentration of ethanol solution administered, the higher the intake of pure ethanol by dams. But the ratio of the mean volume of pure ethanol intake from one group to another was less than the ratio of the mean water intake between corresponding groups. No significant differences in water intake between two groups were found after the end of ethanol administration.

3. Body weight

The mean body weight of dams administered 20%-EtOH showed a significant decrease (6-11.5%) compared with controls.

Table 2 Intake of drinking water and EtOH administration of each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intake volume of drinking water (ml/kg/day)</th>
<th>Dose of ethyl alcohol administration (g/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.0 ± 3.7</td>
<td>-</td>
</tr>
<tr>
<td>5%-EtOH</td>
<td>25.3 ± 3.4</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>10%-EtOH</td>
<td>21.6 ± 3.0</td>
<td>5.7 ± 0.8</td>
</tr>
<tr>
<td>20%-EtOH</td>
<td>15.9 ± 2.2</td>
<td>8.4 ± 1.2</td>
</tr>
</tbody>
</table>
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from PND 9 through 20. The mean body weight of the group administered 10% EtOH was significantly lower (5.5%) than that of controls at PND 19 and 20.

The mean body weight of pups from dams administered 20% EtOH was 10% lower until PND 7, 9% lower at PND 28, and 7.7% lower at PND 35 than in controls. No significant differences in mean body weight between other groups and controls were found.

4. Shock avoidance test

Figure 1 shows the avoidance rate during the latter half of the test. Each group achieved high avoidance rates of about 70% at the first trial, and accomplished avoidance rates of about 90% at the 10th trial. No significant differences in avoidance rates between ethanol-administered groups and the control group were found.

5. Brain weight

The whole-brain weights, minus the olfactory bulb, of pups on PND 66 were as follows; control: 1799 ± 55mg, 5%-EtOH administered group: 1766 ± 55mg, 10%-EtOH administered group: 1783 ± 68mg, and 20%-EtOH administered group: 1764 ± 66mg. No significant differences in mean brain weight between ethanol-administered groups and the control group were found.

6. Brain levels of monoamines and their metabolites

Figure 2 shows the brain levels of noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (5-HT), 3,4-dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), a metabolite of DA, and 5-hydroxyindole acetic acid (5-HIAA), a metabolite of 5-HT. Statistical analyses of simple regression lines between the concentration of administered ethanol and the levels of monoamines and their metabolites were performed. As a result, significant correlations between the levels of NA for pups and the concentration of ethanol administered to dams were found. There was a positive correlation coefficient of 0.404 (p<0.001) between the level of DA and the concentration of ethanol, and a correlation coefficient of 0.712 (p<0.001) between the level of 5-HT and the concentration of ethanol. In the case of metabolites, we obtained a negative correlation coefficient of -0.505 (p<0.001) between the level of DOPAC and the concentration of ethanol, and a correlation coefficient of -0.559 (p<0.001) between the level of HVA and the concentration of ethanol, and a correlation coefficient of -0.607 (p<0.001) between the level of...
5-HIAA and the concentration of ethanol. If ethanol administration to dams did not affect the synthesis of monoamines for pups, the synthesis of monoamines could be considered constant, and the ratio of the metabolite level to the level of monoamine could be assumed to reflect the activation of turnover for metabolism. These ratios are shown in Figure 3. There was a significant difference in the DOPAC/DA ratio between the 5%-EtOH-administered group and the control, and a significant difference in the HVA/DA, 5-HIAA/5-HT ratios between the 5- and 10%-EtOH-administered groups and the control. Moreover, we analyzed simple regression lines between the concentration of administered ethanol and the levels of these ratios. We obtained a negative correlation coefficient of -0.589 (p<0.001) between the DOPAC/DA ratio and the concentration of ethanol, a correlation coefficient of -0.62 (p<0.001) between the HVA/DA ratio and the concentration of ethanol, and a correlation coefficient of -0.74 (p<0.001) between the 5-HIAA/5-HT ratio and the concentration of ethanol.

Discussion

In the shock avoidance test, no significant differences in the avoidance rates between ethanol-administered groups and the control group were found. Riley et al. reported that gave liquid diets containing 8, 19 and 32% of total calories as ethanol to pregnant rats from GD 6 to 16, and carried out passive avoidance tests for offspring of ethanol administered dams on PND 18. Dams consumed an average (±SE) of 3.57 (±0.2) g/kg/day of pure ethanol in the 8% group, 8.49 (±0.33) g/kg/day in the 19% group and 14.3 (±0.56) g/kg/day in the 32% group. Learning deficits were found in the 32% group but were not found in the 8 and 19% groups. Riley et al. also reported that they gave liquid diets containing 17 and 35% of total calories as ethanol to pregnant rats from GD 5 to 20, and the T-maze test to offspring of ethanol administered dams on PND 20 to 21. In this experiment dams consumed an average (±SE) of 6.66 (±0.17) g/kg/day of pure ethanol in the 17% group, and 12.96 (±0.37) g/kg/day in the 35% group. A decrease in learning ability was found in the 35% group, but not in the 17% group. In our experiment dams consumed an average (±SD) of 3.3 (±0.4) g/kg/day of pure ethanol in the 5%-EtOH administered group, 5.7 (±0.8) g/kg/day in the 10%-EtOH administered group and 8.4 (±1.2) g/kg/day in the 20%-EtOH administered group. The maximum consumption of pure ethanol by dams in our experiment was lower than those of Riley et al. Their experiments and our investigation showed no learning deficits at low ethanol exposure.

Moreover, they reported that a decrease of learning ability was found early at PND 18, but not at PND 41 to 53. This indicated that the effect of ethanol exposure disappeared with passage of a long period of time after the end of exposure. Another reason why no effect on learning was found in our experiment was that the test was performed on PND 56.

To study the mechanism of neurobehavioral deficits occurring in children born of alcohol-abusing mothers, we investigated learning behavior and neurotransmitters of mature offspring born of dams administrated ethanol during pregnancy. Driscoll et al. compared the effects of gestational alcohol exposure in humans to the effects in a rat model. From their report, it was inferred that the absolute amount of alcohol consumed by alcohol-abusing mothers during pregnancy was 0.3 - 4.1 g/kg/week. The absolute alcohol amount of 3.3 ± 0.4 g/kg/day that the 5%-EtOH groups ingested in our experiment was much greater than this value in humans. Moreover, Driscoll et al. reported that the blood alcohol level when rats ingested 10 - 14 g/kg/day was over 100 mg/dl, and that if women consumed the same volume in one hour as alcoholic mothers who had borne children with significantly decreased IQs consumed in one day, their blood alcohol levels would be around 100 mg/dl. From corresponding blood alcohol levels in humans and rats, Driscoll et al. also concluded that the effect on the fetus when dams ingested 10 - 14 g/kg/day had the same effect on the fetus as in mothers who had abused alcohol in pregnancy. In our experiment, the maximum amount of absolute alcohol consumed by rats was 8.4 ± 1.2 g/kg/day. This intake corresponded to less than that of alcoholic mothers of children with low IQs.

One factor involved in the variation in the levels of monoamines and their metabolites in the brain has been thought to be stress, such as an electrical shock in avoidance tests. Imori and Bliss et al. reported that the level of NA in the rat brain decreased with foot-shock stress. Bliss et al. also reported that the influence of shock, even on the monoamine with the slowest metabolic rate, disappeared after 16 hours. Because rats were decapitated more than 24 hours after the end of the shock avoidance test in our experiment, electric shock did not influence levels of monoamines in the brain. Another factor varying the level of 5-HT is malnutrition. Malnutrition causes a decrease of the level of 5-HT in the brain due to a decrease of tryptophan from which

<table>
<thead>
<tr>
<th>DA region</th>
<th>observed day (postnatal day)</th>
<th>alteration in content</th>
<th>metabolism</th>
<th>reference</th>
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<tbody>
<tr>
<td>whole brain</td>
<td>5, 10</td>
<td>⇔</td>
<td>Detering, Druse</td>
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<tr>
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<td>1</td>
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<td>↑</td>
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<tr>
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<td>↓</td>
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<td>cerebrum</td>
<td>5, 8, 10</td>
<td>↑</td>
<td>↑</td>
<td>Iwase</td>
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<tr>
<td>cortex</td>
<td>19, 35</td>
<td>↓</td>
<td>↑</td>
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<table>
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<th>5-HT region</th>
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<td>↑</td>
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<tr>
<td>whole brain</td>
<td>21</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
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<tr>
<td>cerebrum</td>
<td>1</td>
<td>↑</td>
<td>↑</td>
<td>Iwase</td>
</tr>
<tr>
<td>hypothalamus &amp; septal</td>
<td>18</td>
<td>↓</td>
<td>↑</td>
<td>Detering</td>
</tr>
</tbody>
</table>

Table 3 Effects of the gestational exposure to ethanol on monoamines. (⇑ : increase without significance, ↑ : significant increase, ↓ : significant decrease)
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5-HT is biosynthesized. In our experiment there was no influence of malnutrition because the level of 5-HT in the EtOH-administered groups was higher than the level in controls.

Many researchers have reported effects of ingested EtOH on the level of monoamines in the brain of the adult rat after acute or chronic alcohol ingestion, and during abstinence. The reviews by Kuriyama and Ohkuma, and Aboudonia reported that the synthesis of monoamines is intensified and the levels of monoamines in the brain are unchanged or increased, while turnover of the monoamine metabolism tends to increase and the level of metabolites is increased by acute or chronic alcohol ingestion. The alteration of monoamines in the brains of pups from ethanol administered dams in our experiment was different from the alteration in ethanol-ingested adult rats. The results that have been reported for the alteration of monoamines in the brains of pups exposed to ethanol in utero are shown in Table 3 (refer also to the survey by Norton et al.). Each of these results shows a prolonged effect on the level of monoamines after birth. Our results showed that in the pup brain on PND 66, the levels of NA, DA, 5-HT increased, and conversely the levels of DOPAC, HVA, 5-HIAA decreased as the concentration of ethanol administered to dams increased. This result is partially consistent with past results. Iwase gave 10%-EtOH to pregnant mice and investigated the levels of monoamines in the pup brain on PND 1, 5, 8 and 30. In his report the level of NA in the EtOH group was higher on PND 1, the level of DA was significantly higher on PND 8 and tended to be high on the other PNDs, and the level of 5-HT was significantly higher on all PNDs than the level in controls. Our results are consistent with the results obtained by Iwase as well as with the report of Rawat et al. that the levels of NA and 5-HT increased. A dose-effect relationship was observed between the concentration of administered ethanol and the levels of monoamines and their metabolites.

It was shown that the turnover rate of DA was similar to that of 5-HT from the similar correlation coefficients for the DOPAC/DA ratio and 5-HIAA/5-HT ratio. The reason for this result is believed to be as follows. There are common enzymes, monoamine oxidase (MAO) and aldehyde dehydrogenase (ALDH), in the metabolic pathways of both DA and 5-HT (Fig. 4). MAO is localized on the outer membrane of mitochondria while ALDH is mostly localized in the mitochondria. Iwase also studied the activities of MAO, and reported that in the 10% EtOH group the activities of both MAO-A for which the substrates were NA, DA and 5-HT, and MAO-B for which the substrate was DA, were lower than those in controls on PND 5. However, after PND 30 there was no significant difference between the EtOH group and controls. On the other hand, Ledig et al. gave 20%-EtOH to female rats for one month before mating and analyzed the activities of cytosolic alcohol dehydrogenase (ADH) and mitochondrial ALDH in the pup brain. They reported that the activity of ADH tended to be lower and the activity of ALDH was significantly lower at 8 weeks. At 24 weeks both enzyme activities were significantly lower than in controls. Although their administration period was different from ours, there is a possibility that ethanol exposure during pregnancy also induces prolonged decreases in the activities of ADH and mitochondrial ALDH in the pup brain after birth, causing a decrease in the turnover rate of the monoamine metabolism.

Several hypotheses for why ethanol causes FAS, especially

Fig. 4-1 Pathway of the metabolism of noradrenaline.

Fig. 4-2 Pathway of the metabolism of dopamine.

Fig. 4-3 Pathway of the metabolism of serotonin.
CNS dysfunction have been proposed. Indirect effects of ethanol and acetaldehyde are zinc deficiency in mothers during pregnancy, dysfunction of placental transport of nutrients, and fetal hypoxia as a result of placental hypoxia. Reduced DNA and protein synthesis, and consequently a decreased rate of cell division in the embryo, are considered to be direct effects. Structural alterations of neurons and synapses caused by these effects have been shown in the cerebral cortex, hippocampus and cerebellum, and these structural alterations are assumed to cause CNS dysfunction.

We need to consider the cause of CNS dysfunction not only from the structural point of view, but also from a functional view regarding the decreased activities of ADH and ALDH in the brain and consequent alterations of the monoamine metabolism. Moreover, we need to study the relationship between alterations in the monoamine metabolism and learning and behavior that do not involve shock avoidance.

Newborns are affected not only by ethanol through the placenta but are exposed through mother’s milk. Alcoholic mothers often drink not only during pregnancy but also during lactation. Therefore experiments designed with ethanol administration to dams during late pregnancy and to pups during lactation may be better models for reflecting the real situation.

**Conclusion**

In this experiment rat dams were administered ethanol in drinking water ad libitum during pregnancy. For pups from these dams we analyzed the postnatal body weight change, the Sidman shock avoidance test, brain weight and level of monoamines and metabolites in the brain. There was no effect on body weight change, the Sidman shock avoidance test or brain weight. However, alterations in the levels of monoamines and metabolites were shown in the groups administered 5%, 10%, 20% EtOH even on PND 66. Moreover, dose-effect relationships were observed between the concentration of administered ethanol and the levels of monoamines and their metabolites.

**References**