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Growth and Formation of the Tooth Germ in a Rat Model of Fetal Alcohol Syndrome

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Abstract: The purpose of the present study was to elucidate the influence of alcohol ingestion in pregnant rats on the body and teeth of neonatal rats. Twenty two pregnant rats were divided into a normally-housed control group and alcohol-intake group exposed to alcohol during pregnancy, and 223 neonatal rats from these pregnant rats were used for the study. We observed the amount of water intake and the number of pups in mother rats, and body weight, eye-opening, and tooth development (postnatal day 1, 5, 15, and 20) in neonatal rats. The results showed the decreasing number of pups in the alcohol-intake group. Neonatal rats in the alcohol-intake group exhibited a lower body weight, especially on the first neonatal day, with a significant difference within the group. They also showed a delay in the time to eye-opening. Histopathological examination revealed no significant morphological abnormalities in odontogenic cells, but differential growth of the tooth germ and a delay in dental root formation were observed. The present study confirmed the influences of alcohol ingestion in pregnant rats on neonatal rats, including delays in physical development, differential growth of the tooth germ, and tooth eruption time.

Key words: Tooth germ, Rat, Fetal alcohol syndrome

Introduction

The number of women consuming alcohol has been increasing in Japan in recent years1~6). This is considered to be associated with women’s expanding role in society and the increased opportunity for alcohol consumption. However, there are concerns that alcohol consumption during pregnancy may have adverse effects on the fetus7,8). Fetal Alcohol Syndrome (FAS) is a birth defect in children born to mothers who consumed alcohol during pregnancy, and was established by reports between 1968 and 19739,10~13). In recent years, problems only caused due to alcohol consumption by mothers during pregnancy such as Fetal Alcohol Syndrome (FAS), Fetal Alcohol Effects (FAE), and Alcohol Related Birth Defects (ARBD) are comprehensively called as Fetal Alcohol Spectrum Disorders (FASD)9), and studies have been conducted. These studies have revealed that children born to mothers who consumed alcohol during pregnancy develop various defects in body organs14~18). Although many studies have been conducted regarding the influence on body organs19,20), few studies are available concerning the influence on the oral region21~22). The purpose of the present study was to elucidate the influence of alcohol ingestion in pregnant SD rats on physical and tooth development in neonatal rats.

Materials and Methods

Reference23~35) and pilot investigations were conducted to determine the concentration and administration period of ethanol solution administered to mother rats, and we set experimental conditions which have a minimal influence on pregnant rats and under which changes in fetal rats can be observed.

A total of 223 neonatal rats born to 22 pregnant SD rats (Sankyo Lab Service, Tokyo) were used in the study, and divided into two groups: the control group (8 mother rats, 94 fetal rats), and alcohol-intake group (14 mother rats, 129 fetal rats). Stillborn rats were excluded from the study. Each pregnant rat was housed in a separate metal cage in the animal room under controlled conditions (temperature 22~24°C; humidity 40~60%; 12-hour light/dark cycle 6:00~18:00), and was fed a commercial solid diet (MF, Oriental Yeast Co., Ltd.). The study was conducted in the animal laboratory of Tokyo Dental College. All animal studies were performed in conformity to the guidelines for animal experiments of Tokyo Dental College.

1. Experiment using mother rats

1) Alcohol (water) intake

Mother rats in the control group were fed rat chow and tap
water *ad libitum* during and after pregnancy. Ninety four rats born to these mother rats were observed as a control group.

Mother rats in the alcohol-intake group were fed rat chow and tap water *ad libitum* until day 13 of pregnancy, and 25% ethanol solution in the bottle *ad libitum* from day 14 of pregnancy to the delivery date. This date was chosen based upon the fact that formation of the neonatal rat tooth germ starts on average on day 14 of pregnancy\(^{36,39}\). Rat chow was available *ad libitum* after day 14 of pregnancy. One hundred and twenty nine neonatal rats born to these mother rats were observed as an alcohol-intake group. Postnatal rats were normally housed, and the number of live pups and stillbirths were observed.

2) Measurement of water intake

Water intake (Alcohol intake) in mother rats was measured daily from day 15 of pregnancy to the delivery date.

2. Experiment using neonatal rats

1) Body weight measurement

Body weight in neonatal rats was measured on neonatal day 1, 5, 15, and 20. Before the measurement, neonatal rats were euthanized using diethyl ether (Wako Pure Chemical Industries, Ltd.).

2) Eye opening assessment

Eye opening was assessed on neonatal day 15 according to the criteria of Grawiler J, and Leist, K.T.\(^{37}\), and Adams, J. and Buelke-Sam J.\(^{38}\).

3) Assessment of tooth eruption

The lower left first molar was observed both in the alcohol-intake and control groups. Considering that tooth eruption begins on average on neonatal day 19\(^{36,39}\), we compared the degree of tooth eruption on neonatal day 20 based on the criteria below.

0: Unerupted
1: Occlusal surface partially erupted
2: Occlusal surface fully erupted, but below the occlusal plane
3: Fully erupted to the occlusal plane

4) Tooth development

The tooth germ of the lower left first molar and growth after eruption were observed. Before the removal of the test tooth and jawbone, rats were euthanized using diethyl ether (Wako Pure Chemical Industries, Ltd.) on neonatal day 1, 5, 15, and 20, followed by saw-cutting of the jawbone including the test tooth, and fixation with 4% paraformaldehyde for three days at 4°C. Samples were rinsed with 0.1M cacodylate buffer after fixation, and decalcified with 10% EDTA-2Na for 4 weeks at room temperature. After the completion of decalcification, samples were rinsed with distilled water, dehydrated with a graded ethanol series, and embedded in paraffin. The automated tissue processor (VPM-1500, Tissue Tek) was used throughout these procedures. After embedding, samples were serially sectioned (7 µ m), and stained with hematoxylin-eosin (H-E). The section containing the tooth in parallel with the tooth axis, and crossing the buccolingual midline (center of the tooth crown) was chosen for observation. It is observed tooth crown on neonatal day 1, 5. The tooth crown was completed on neonatal day 15, 20, I observed root, because that growth is in the way.

The data obtained in this way were statistically analyzed and evaluated by means of Student’s t-tests.

Results

1. The number of live pups and stillbirths

The average number of pups in the control group was 11.75 (n=8), with a maximum of 17 and a minimum of 8. The gestation period was always 21 days excluding one period of 22 days. There was no stillbirth or maternal death.

The average number of pups in the alcohol-intake group was 9.43 (n=14), with a maximum of 17 and a minimum of 1. The number of pups was smaller than that in the control group, and significant individual variation was observed. The gestation period was mostly 22 days. Rats born to 8 out of 22 mother rats were stillborn (36.36%). Most of the stillborn rats (embryos) were in their early developmental stage, and few individuals could be confirmed. The precise number of stillbirths remained unknown due to the large variations in growth. There was no maternal death.

The number of pups in test rats is shown in Fig. 1.

2. Water intake by mother rats

The average water intake of mother rats in the control group was 42.03 ml/day ± 16.47 (n=8). Water intake did not change significantly until day 19 of pregnancy, but showed decreases on day 20 and 21 of pregnancy. This is considered to be due to a decreased number of mother rats who consume water in preparation for birth. The measurement value on day 22 was excluded from analysis since all births were completed on day 21 excluding one mother rat in the control group.

The average alcohol intake of mother rats in the alcohol-intake group was 22.06 ml/day ± 16.47 (n=14). Alcohol intake did not change significantly throughout the alcohol administration period. There was a tendency whereby water intake decreased before and during delivery, and increased significantly after delivery, resulting in marked individual variability. Therefore, the measurement value on day 22 was excluded from analysis. The time course of water intake in test rats is shown in Table 1 and Fig. 2.

3. Body weight of newborn rats

Body weight differences between rats born on the same day in the control group are shown in Table 3 and Fig. 3.

A moderate inter-individual variability was observed on postnatal day 1 and 20, but other days did not show any discernible difference.

A significant inter-individual variability was observed on the first postnatal day in the alcohol-intake group, but the difference
The number of live birth progressively decreased over time (Table 2, Fig. 3). Comparison between the two groups showed a lower body weight in the experimental group than in the control group. However, no difference was observed on postnatal day 15, suggesting that rats in the alcohol-intake group also grew rapidly under normal conditions.

4. Eye-opening (postnatal day 15)

In the control group, eye-opening was observed in 8 out of rats (53.33%). In the alcohol-intake group, eye-opening was observed in 4 out of 13 rats (30.77%).

5. Lower first molar tooth eruption on postnatal day 20

The tooth eruption status of the lower first molar on postnatal day 20 in the control and alcohol-intake groups is shown in Table 3.

In the control group, the occlusal surface was confirmed in all individuals, but did not reach the occlusal plane. In the alcohol-intake group, 67% showed a partially erupted occlusal surface, and 33% showed a fully erupted occlusal surface, but not reaching the occlusal plane. The tooth eruption delayed in the experimental group. (p<0.01)

4. Tooth development

The sagittal section crossing the buccolingual midline (center of the tooth crown) of the lower left first molar was chosen for observation.

1) Postnatal day 1

In the control group, the developmental stage of the tooth germ was the late bell stage (after the initiation of calcification) (Fig. 4). Columnar ameloblasts and odontoblasts were observed, and enamel and dentin formation had started (Fig. 5). The width of...
Figure 4. Postnatal day 1 in the control group. The developmental stage of the tooth germ was the late bell stage. Bar 500µm

Figure 5. Postnatal day 1 in the control group. Columnar ameloblasts and odontoblasts were observed. Bar 50µm

Figure 6. Postnatal day 1 in alcohol-intake group. The developmental stage of the tooth germ was the early bell stage. Bar 500µm

Figure 7. Postnatal day 1 in alcohol-intake group. Columnar ameloblasts and odontoblasts were observed, but enamel formation had not started. Bar 50µm

Figure 8. Postnatal day 5 in the control group. Tooth crown formation had been proceeding, but root formation had yet to commence. Bar 500µm

Figure 9. Postnatal day 5 in the control group. The thickness of enamel and dentin increased significantly. Bar 50µm

Figure 10. Postnatal day 5 in alcohol-intake group. Tooth crown formation had been proceeding, but the tooth germ height was lower than in the control group. Bar 500µm

Figure 11. Postnatal day 5 in alcohol-intake group. The overall level of formation was smaller than in the control group. Bar 50µm
Figure 12. Postnatal day 15 in the control group. Tooth crown morphogenesis had nearly completed, and enamel formation was complete. Bar 500µm

Figure 13. Postnatal day 15 in the control group. Root formation had started, but was incomplete. Bar 0.5µm

Figure 14. Postnatal day 15 in alcohol-intake group. Tooth crown morphogenesis had nearly completed, and enamel formation was complete. Bar 500µm

Figure 15. Postnatal day 15 in alcohol-intake group. Root formation had started, but was incomplete. Bar 0.5µm

Figure 16. Postnatal day 20 in the control group. Tooth crown morphogenesis was complete, and tooth eruption was observed. Bar 500µm

Figure 17. Postnatal day 20 in the control group. The opening of the apical formation could not be identified due to apical formation migration in the buccolingual direction as the root grew. Bar 0.5µm

Figure 18. Postnatal day 20 in alcohol-intake group. Tooth crown morphogenesis was complete, and tooth eruption was observed. Bar 500µm

Figure 19. Postnatal day 20 in alcohol-intake group. Most of the dental roots were short
the tooth germ (maximum mesiodistal contour) was 2.3 ± 0.14 mm (n=8).

In the alcohol-intake group, the developmental stage of the tooth germ was the early bell stage (pre-calcification period). Columnar ameloblasts and odontoblasts were observed, but enamel formation had not started in most individuals (Fig.6). Although enamel formation was observed in a few individuals, it was still in a very early stage. The formation of a thin dentin layer was observed, but the level of formation was smaller than that in the control group (Fig. 7). Tooth germ width (maximum mesiodistal contour) was 1.9 ± 0.16 mm (n=8) It was smaller than control group. (p<0.01)

2) Postnatal day 5

In the control group, tooth crown formation had been proceeding, but root formation had yet to commence (Fig. 8). Columnar ameloblasts and odontoblasts were observed, and the thickness of enamel and dentin increased significantly (Fig. 9). Tooth germ width (maximum mesiodistal contour) was 3.0 ± 0.07 mm (n=5).

In the alcohol-intake group, tooth crown formation had been proceeding, but the tooth germ height was lower than in the control group, and root formation had yet to commence (Fig. 10). Columnar ameloblasts and odontoblasts were observed. Although enamel and dentin were formed (Fig. 11), the overall level of formation was smaller than that in the control group. Tooth germ width (maximum mesiodistal contour) was 2.8 ± 0.07 mm (n=5). It was smaller than control group. (p<0.01)

3) Postnatal day 15

In the control group, tooth crown morphogenesis had nearly completed, and enamel formation was complete. Although ameloblasts of the enamel–free area in the occlusal head decreased in height and flattened, the tooth remained unerupted (Fig. 12). Root formation had started, but was incomplete, with a short root and wide apical foramen (Fig. 13). The tooth crown width was about 3 mm, the same width as that on neonatal day 5. In the alcohol-intake group, tooth crown morphogenesis was nearly complete, and enamel formation was complete. Although ameloblasts in the occlusal head decreased in height, the tooth...
remained unerupted (Fig. 14). Root formation had started, but was incomplete, with a short root and wide apical foramen (Fig. 15). The tooth crown width was about 2.8 mm, the same width as that on neonatal day 5.

**4) Postnatal day 20**

In the control group, tooth crown morphogenesis was complete, and tooth eruption was observed (Fig. 16). The section used for the observation was parallel to the tooth axis and crossed the buccolingual midline. The opening of the apical foramen could not be identified due to apical foramen migration in the buccolingual direction as the root grew (Fig. 17).

In the alcohol-intake group, tooth crown morphogenesis was complete, and tooth eruption was observed (Fig. 18). Most of the dental roots were shorter than in the control group, and apical foramen were confirmed in some cases (Fig. 19).

**Discussion**

Although some experimental studies on FAS have been performed by K. Sulik et al., Ashwell KW et al., Sasaki et al., and Nakamura et al., alcohol was administered intraperitoneally in most of these studies, with few studies using oral administration. This is considered to be due to a significant difference in water (alcohol) intake under the ad libitum feeding condition, and the difference in alcohol sensitivity among test rats, which make it difficult to obtain clear results. In the present study, 25% ethanol was administered orally ad libitum, and problems shown above were observed. We shall discuss the following items first, and then the overall influence of alcohol.

**1. The number of pups, the stillbirth ratio, and the volume of water intake**

There was a difference in the average number of pups between the control group (11.75) and the alcohol-intake group (9.43). Pups born to 8 out of 22 mother rats (36.36%) were stillbirths. Since alcohol was administered orally in the present study, a significant difference was observed in water intake levels in mother rats, but the influence on fetal rats is unclear. Considering that the number of pups showed an increase in variation instead of an overall decrease in the alcohol-intake group compared to the control group, it is speculated that the influence of alcohol was greater than that of water intake. A liposoluble substance with a molecular weight under 600 can easily cross the placenta, suggesting that alcohol may influence the apoptosis balance, suggesting that alcohol may prevent fetal development, resulting in a decrease in the number of pups or an increase in stillbirths. Furthermore, considering the significant difference in the number of pups, it is considered that there is a wide variety in the sensitivity to alcohol between mother rats.

**2. Body weight of neonatal rats, and eye-opening period**

It is widely known that growth impairment is a leading symptom of FAS. Intrauterine growth impairment is believed to be present in more than 80% of FAS, and the results of the present study also showed a lower body weight in neonatal rats in the alcohol-intake group, as reported in previous studies. There is also a report that fetal growth impairment cannot be corrected by catch-up growth. The results of the present study also showed that growth in the alcohol-intake group did not catch up with the control group up until postnatal day 5, as reported in previous studies. The difference in body weight was not observed on neonatal day 15, and was minimal on neonatal day 20. This suggested that the delay in neonatal growth may catch up after a certain period of time.

The eye-opening period is a growth index, and eye-opening occurs on average on postnatal day 12. However, previous studies indicated that eye-opening is delayed in rats with FAS. In the present study, we examined the eye-opening status on postnatal day 15. Eye-opening was observed in 4 out of 13 rats (30.77%) in the alcohol-intake group, and 8 out of 15 rats (53.33%) in the control group. The eye-opening ratio was low in the control group, and was lower in the alcohol group, suggesting the influence of alcohol (ethanol) on eye-opening.

These results suggested that the delay in body growth was overcome in the early stage, but the influence on the development of the nervous system remained and adversely affected the sensory organs and intelligence.

**3. Eruption of the lower first molar**

The lower first molar in rats erupts on postnatal day 19 on average. However, previous studies have indicated a delay in tooth eruption in rats with FAS. In the present study, we observed the tooth eruption status of the lower first molar on postnatal day 20. The alcohol-intake group showed 6 cases (67%) of partially erupted occlusal surfaces (level 1), and 3 cases (33%) of fully erupted occlusal surfaces, but below the occlusal plane (level 2). The control group showed 15 cases (100%) of fully erupted occlusal surfaces, but below the occlusal plane (level 2). These results showed that alcohol administration to pregnant rats may cause a delay in tooth eruption in their pups.

**4. Tooth growth**

Enamel formation generally starts on neonatal day 1. However, the alcohol-intake group showed no enamel formation, little dentin formation, and a relatively small tooth germ, suggesting a delay in the differential growth of the tooth germ.
No significant morphological abnormality was observed in ameloblasts and odontoblasts, and there was no influence on differentiation of the inner enamel epithelium in the enamel-free area of the crown. These results indicate that alcohol intake has no significant influence on cell formation despite a few days delay in tooth crown development in the alcohol-intake group. A significant difference was observed in root formation on postnatal day 20. This is considered to be caused by the delay in tooth germ differentiation, especially the delay in the stretching of Hertwig's epithelial sheath, resulting in delayed tooth eruption. The influence of alcohol intake by mothers on fetuses has been investigated as fetal alcohol spectrum disorder, and various disorders developing in fetal organs have been elucidated.

The present study revealed an influence of alcohol intake on neonatal growth in rats. There was a difference in growth on postnatal day 1. The present study focused on tooth growth, and no local disorders in the tooth germ, hard tissue dysplasia, and morphological changes of cells involved in tooth formation were observed. However, the delay in physical growth affected differential growth of the tooth germ, and the delay in the initiation of calcification, and tooth germ growth. Since we only observed rats younger than postnatal day 20, subsequent growth remains unclear. However, it is likely that the influence of alcohol intake on fetal rats persists over a long period of time. To know influence in detail of alcohol, we need investigation of liver function. We observation of tooth germ of the rat which there was little of the number of the live birth is necessary.

Conclusions

We investigated the influence of alcohol administered to pregnant rats on the physical growth of pups, especially tooth development, to elucidate its effects from a growth perspective, and the following conclusions were obtained. The alcohol-intake group showed a lower number of pups and a higher stillbirth rate than the control group. Differences in body weight were found between the control and alcohol-intake groups on postnatal day 1 and 5, and the alcohol-intake group showed a lower body weight. However, the body weight discrepancy between groups narrowed as they grow, suggesting that growth in newborns catches up after a certain period of time. The eye-opening ratio on postnatal day 15 was lower in the alcohol-intake group, which was considered to be due to the influence of alcohol. The eruption of the lower first molar was delayed in the alcohol-intake group. In tooth germ formation, no significant morphological abnormalities were observed in cells involved in tooth formation, but a delay in the differential growth of the tooth germ was observed in the alcohol-intake group. The delay reached a maximum on postnatal day 1. The growth gap was reduced as they grew, but still remained on postnatal day 20.

These results confirmed that alcohol intake by pregnant rats can cause a delay in neonatal growth. Although alcohol intake is considered to have no significant influence on tooth germ formation, a delay in the differential growth of the tooth germ and delay in root formation accompanying the delay in body growth were observed.

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