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Morphologic effects of maternal alcohol intake on skull, mandible and tooth of the offspring in mice

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Abstract: Morphologic effects of maternal alcohol intake on skull, mandible and tooth of the offspring in BALB/cJ mice were studied. Adult female mice were given orally 20 % alcohol in drinking water and a diet before and during pregnancy. Maternal blood alcohol levels showed 284.0 ± 1.4 mg/100 ml on gestation day 12.5. Skull and mandible were dissected, weighed and processed for statistical analysis and light microscopic examinations. The results were as follows: 1) The total maternal body weights showed no significant difference during pregnancy between the control and alcohol-treated groups, but the offspring of alcohol-treated females showed weight deficit through the period of gestation and postnatal 28.5 days. 2) The mandible weight of the offspring was less in the alcohol-treated group than the control group through a postnatal period of 28.5 days. 3) The growth of the skull and mandible in the offspring was retarded in the alcohol-treated group through a postnatal period of 28.5 days. Significant statistical differences of the growth were seen in the posterior region of the mandible. 4) Appearance of the tooth germ was delayed on gestation day 14.5. 5) Calcification of the tooth was delayed on postnatal day 1.5. 6) Retardation of the tooth eruption was seen on postnatal day 14.5. It is indicated that the offspring associated with maternal chronic alcoholism showed retardations in their skeletal and dental development, and failed to catch up to the growth of the control animals during a postnatal period of four weeks.

Introduction

Major advances in our understanding of alcohol (ethyl alcohol) on the prenatal and postnatal offspring have been made through clinical¹⁻³⁾ and experimental⁴⁻⁷⁾ studies. The findings which are related to the effects of alcohol during pregnancy and the resulting abnormalities on the offspring from alcoholic mothers have been termed Fetal Alcohol Syndrome (FAS)¹⁾. In the human, FAS has been categorized into three main components: (1) a general somatic growth retardation²⁾, (2) a set of facial appearances noted in the affected offspring³⁾, and (3) microcephaly and mental retardation^{3,4)}.

Alcohol has been given to various laboratory animals by different routes (i.e., intravenous⁵⁾,

by gavage⁶⁾, vapor inhalation⁷⁾, intraperitoneal⁸⁾ and in drinking water⁹⁾). However, the observations reported have not always been in agreement. This may be attributed to the differences of species and strains in their susceptibility to alcohol as well as those in the experimental conditions such as doses⁶⁾, routes⁵⁾, periods¹⁰⁾ and durations¹¹⁾, of alcohol administration.

Little attention has been paid to the observation of dental structures in FAS. In this study, morphologic effects of maternal alcohol intake on the skull, mandible and tooth of the offspring in mice were investigated.

Materials and Methods

Virgin female BALB/cJ mice were obtain-

ed from Tohoku University Animal Center. The laboratory in which animals were retained for the entire experimental period was temperature, humidity and light/dark cycle controlled. The mice were given a commercial diet (F-2, Funabashi Farms, Chiba, Japan) and water. Diet and water were taken ad libitum until the beginning of the experiment.

One hundred and twelve mice were divided into 12 experimental groups (6 animals each) and 10 control groups (4 animals each). The mice were 8 weeks of age at the beginning of the experiment and weighed from 21 and 23 grams. Alcohol solutions in drinking water were given to the experimental groups. The water was replaced by increasing alcohol concentrations in the order 1 %, 5 %, 10 % and 15 % from day 0 to day 12 of the experiment. From day 13 to delivery, the experimental groups were kept on drinking 20 % alcohol and fed on the commercial diet before and during pregnancy. The control groups were also fed on the diet and took water without alcohol ad libitum before and during pregnancy. In all groups, the liquid was given in plastic bottles (150 ml) with a tip to diminish leakage and evaporation. Alcohol intake was measured daily. At 14 and 16 weeks of age, male mice were housed singly and mated daily with female mice between 22 : 30 and 0 : 00. The presence of a vaginal plug was designated as gestation day 0.5.

From gestation day 7.5, pregnant females were weighed every other day. On gestation day 12.5, a blood sample was taken from the retro-orbital sinus and collected in heparinized capillary tubes. Blood alcohol levels were determined by gas-liquid chromatography according to the method of Hessel and Modglin¹²⁾.

On gestation days 14.5, 16.5 and 18.5, pregnant females (28 control, 32 experimental) were anaesthetized with chloroform and killed by decapitation. The number and location of live, dead and resorbed fetuses were recorded. Live fetuses were observed under a dissecting microscope to examine then for gross physical abnormalities. Heads were fixed in Bouin's solution for 24 hours and then stored in 70 % alcohol. When pregnant fe-

Fig. 1 Landmarks and measurements of skull and mandible.

Skull: Pa-T (height), Na-Oc (total length), Ad-Ad' (articulation width). Mandible: Me-Me' (height to the bottom of angular process), Cd-Cd' (condylar height), Co-Co' (coronoid process height), I-Cd (mandible length), Me-Cd (condylar length), Me-Co (coronoid process length).

males (12 control, 40 experimental) gave birth, weights were recorded. Each offspring was weighed daily thereafter. On postnatal days 1.5, 3.5, 7.5, 14.5, 21.5 and 28.5, skulls and mandibles were removed, weighed and fixed in 4 % paraformaldehyde and 1 % glutaraldehyde. Skulls and mandibles were placed directly on X-ray film for obtaining the dorsal and ventral radiographs. Linear measurements of the skull and the mandible were made on the radiographs at 1/1 magnification. (Fig. 1)

Mandibles from postnatal day 3.5 to 28.5 mice were demineralized in 4 % EDTA solution and embedded in paraffin. Sagittal and frontal sections, 4 μ m thick, were obtained and stained with haematoxylin and eosin (H. E.) and by the Von Kossa's method.

The statistical significance of the difference of the means between the control and experimental groups was examined by Welch-t-test.

Results

The experimental mice consumed daily 480 mg of alcohol (3.1 ml/20 % alcohol)/animal on average. Maternal blood alcohol level

Table 1 Frequency of resorption in pregnant females

	Control	Experimental
Pregnant females (P)	28	32
Total implants (T)	304	224
Fetuses (F)	197	89
Litter size (L)	7.0	2.8
% Resorption (R)	35.2	60.3

$L = F/P, R = 100 \times (T - F)/T$

showed 284.0 ± 1.4 mg/100 ml/ on gestation day 12.5. A prolonged gestational period of 22 days was presented in 8 (25 %) of 32 alcohol-treated mothers (vs. 21 days in the control animals). There was an effect of alcohol intake on the litter size (number of fetuses/one pregnant female). The frequency of fetal resorption was higher in the experimental group than in the control group (Table 1).

Body Weight :

Weights of mother, fetus and offspring (Fig. 2)-No significant differences were found in the maternal body weight between the control and the experimental groups during the four weeks of the prefertilization period (Fig. 2A). Maternal body weights showed significant differences between the two groups during the gestation period (Fig. 2A). Significant differences were found in the fetal body weight between the control and experimental groups on gestation days 14.5, 16.5 and 18.5 (Fig. 2B). Significant differences were found in the body weight of the offsprings during

A : Maternal body weight before and during pregnancy.

B: Fetal body weight during a gestational period.

C : Offsprings body weight.
NS (not significant), * $p < 0.05$, ** $p < 0.01$,
*** $p < 0.001$ (Welch-t-test).

Results are expressed as the mean $\pm$ S.D..

Fig. 2 Body weight.

the four weeks of the postnatal period (Fig. 2C). On postnatal day 28.5, the reduction percentage was 14.4.

Mandible weight (Fig. 3)-Significant differences were found on postnatal days 14.5, 21.5 and 28.5. An increase in the difference was shown from postnatal day 14.5 to 21.5. A decrease in the difference was shown from postnatal day 21.5 to 28.5. On postnatal day 21.5, the reduction percentage was 30.8.

Growth of Skull, Mandible and Tooth :

The differences in the growth of skulls and mandibles between the two groups were mac-

Fig. 3 Mandible weight in postnatal offsprings. ** $p < 0.01$.

Fig. 4 Radiographs of skulls and mandibles on postnatal day 14.5. Control group (C). Experimental group (E).

roscopically and radiographically appreciable from postnatal day 14.5 (Fig. 4).

Linear measurements of skulls (Fig. 5)-The mean values of the measurements for each postnatal day showed that the height, length and width of the skull increased gradually from postnatal day 14.5 to 28.5. Reduction of the growth was seen in the experimental groups. Significant differences were found in the total length (Na-Oc) on postnatal days 14.5, 21.5 and 28.5. Significant differences were found in the height (Pa-T) on postnatal days 14.5 and 21.5, but not on postnatal day 28.5. No significant differences were found in the articulation width (Ad-Ad') on postnatal days 14.5, 21.5 and 28.5.

Fig. 5 Linear measurements of skulls in postnatal offsprings.

Length (Na-Oc), Width (Ad-Ad'), Height (Pa-T).

NS (not significant), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Linear measurements of mandibles (Fig. 6)-The mean values of the measurements for each postnatal day showed that the height and length of the mandible increased gradually from postnatal day 14.5 to 28.5. Reduction of the growth was seen in the experimental groups. Significant differences were found in the condylar height (Cd-Cd') on postnatal days 14.5, 21.5 and 28.5. On postnatal day 14.5, significant differences were found in all lengths and heights of the mandibles except the height to the bottom of angular process (Me-Me'). On postnatal day 21.5, no significant differences were found in all lengths and heights of the mandibles except Cd-Cd'. On postnatal day 28.5, significant differences were found in all lengths and heights of the mandibles except the mandible length (I-Cd). Decreases in the differences between the control and experimental groups during a period from postnatal day 14.5 to 21.5, and increases in the differences from postnatal day 21.5 to 28.5 were shown in the condylar length (Me-Cd), the coronoid process length (M-Co), the coronoid process height (Co-Co') and Me-Me'. Decreases in the differences from post-

Fig. 6 Linear measurements of mandibles in postnatal offspring.

Length (I-Cd, Me-Cd, Me-Co), Height (Co-Co', Cd-Cd', Me-Me').

NS (not significant), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

natal days 14.5 and 28.5 were shown in Cd-Cd' and I-Cd.

Tooth germ (Fig. 7)-Appearance of the tooth germ was histologically shown on gesta-

tion day 14.5 in the control group. The oral epithelium proliferated to form the bud at an early developmental stage of the tooth germ of the mandibular first molar. A layer of tall columnar cells appeared adjacent to the future dental papilla (Fig. 7a). The bud of the mandibular first molar showed an early developmental stage on gestation day 14.5 in the experimental group. Thick cuboidal epithelial cells proliferating downward from the dental lamina were present with mitotic figures (Fig. 7b).

Calcification of the tooth (Fig. 8)-Odontoblasts in the most advanced region of the cusps of the mandibular first molar histologically showed the beginning of dentin formation on postnatal day 1.5 in the control group. A continuous layer of calcification was formed adjacent to the dentinoenamel junction as revealed by the Von Kossa's method (Fig. 8a). Dentin matrix in the mandibular first molar on postnatal day 1.5 in the experimental group did not show any sign of calcification (Fig. 8b). Calcification of dentin matrix showed no differences at a light microscopic level between the control and experimental groups from postnatal day 3.5 to 28.5.

Tooth eruption (Fig. 9)-Light microscopic observations on longitudinal and frontal sections of the mandibular first molar showed the prefunctional eruptive phase on postnatal day 14.5 in the control group. The cusps of the crown approached to the oral mucosa, and the lingual cusp was evidently exposed to the oral cavity. The connective tissue between

a) Control: An early developmental stage with high columnar cells.

b) Experimental: An early developmental stage with thickened cuboidal cells.

Fig. 7 Photomicrographs of the tooth bud of the mandibular first molar on gestation day 14.5. (H.E. $\times 200$)

a) Control : Calcification of dentin (an arrow).

b) Experimental : Lack of calcification (an arrow).

Fig. 8 Photomicrographs of longitudinal sections in the most advanced area of the mid-cusp of the mandibular first molar on postnatal day 1.5. Ameloblasts (A). Odontoblasts (O). (Von Kossa's method $\times 200$)

a) Control : Eruptive phase with the lingual cusp emerged into the oral cavity.

b) Experimental : Maturation phase with normal shape. Alveolar bone (an arrow) overlying the anterior part of the eruptive pathway is seen.

Fig. 9 Photomicrographs of longitudinal sections of the mandibular first molar on postnatal day 14.5. (H.E. $\times 150$)

the dental epithelium and the oral epithelium disappeared. Occlusal surfaces were occasionally covered with the degenerative reduced dental epithelium (Fig. 9a). In the experimental group, the mandibular first molar was surrounded by the fibrous connective tissue. The overlying bone was seen at the surface of the dental epithelia toward which the tooth erupts. There was a close connection between the inner and outer dental epithelia covering the entire enamel surface. These histological features were observed in 10 (66 %) of 15 experimental offsprings (Fig. 9b).

Discussion

Blood alcohol levels are important data for

the extrapolation from animal to human. In most cases, animals must be given much higher doses of alcohol to obtain an effect that might occur in human¹³. Animals could metabolize many drugs much faster than human¹³. The mouse and rat, for example, could metabolize alcohol at rates of 300 and 550 mg/kg/hr respectively, whereas in human 100 mg/kg/hr¹³. Maternal blood alcohol levels of 74 to 98 mg/100 ml had no effect on fetuses in rats^{14,15}. Moderate amounts of alcohol intake during pregnancy did not seem to affect fetuses in the human^{16,17}, but preceding and maternal high levels of alcohol intake, particularly at an early stage of pregnancy caused a prenatal effect such as fetal death¹⁸⁻²⁰.

The prolongation of the gestational period in pregnant alcoholic animals has also been reported¹⁹⁾. A high embryonic resorption rate obtained in this study suggests that even a moderate alcohol intake (blood alcohol level 284 mg/100 ml) during periods of pre-fertilization and pregnancy might have adverse effects on gestation in mice as generally found in other experimental animals, and the human^{3,19,21,22)}.

In most animal models, it has been postulated that maternal nutritional alterations would be a primary interactive factor in FAS^{6,23,24)}. Furthermore there has been a report that the degree of FAS was dependent upon the state of the maternal liver and not on amounts of alcohol intake²⁵⁾. On the other hand, alcoholic mothers showed decreases in their body weight and in the number of offsprings, compared with isocaloric fed control mothers^{19,26,27)}. These data reflect the direct effects of alcohol, regardless of the nutritional factors^{18,26,27)}. The detrimental effect on the fetal weight was potentiated in the offspring of alcohol-treated mothers. Alcoholic mothers weighed less than the controls at the time of pregnancy. Lower body weight of mothers at the impregnation time might result in a risk to the offsprings with lower body and mandible weights at birth.

Considerable evidences have shown that alcohol inhibited the brain development and was exerted on the skull growth and expansion^{28,29)}. The reduction of skull length may be a manifestation of disorganized neural and mesenchymal growth²⁸⁾. Linear measurements of skulls in this study may suggest the retardation of active growth at cranial sutural sites. Growth of the human brain begins shortly after organogenesis reaches a maximum rate around birth and continues through the first few years of life^{30,31)}. Maxillary and mandibular hypoplasia have been reported in severe human and animal FAS cases.^{1,14,25,28)} Linear measurements of mandibles in this study show that the initial target of alcohol was the posterior region of the mandible. This may be explained by the teratogenic action of alcohol on the mitotic activity of the condyle playing an important role in the mandibular growth. Blood alco-

hol can directly affect the placenta and has adverse effects on the calcium balance³²⁾ and ossification. Alcohol passing through the placenta has teratogenic effects on organ growth and enzymatic effects on electrolyte metabolism³³⁾. However, the subsequent effects on calcium balance in the offspring have not been reported, except for some reports on transient hypocalcemia in children with human FAS¹⁾. Alcohol may relate to the fetal development³³⁾ even in the absence of the obvious developmental or physical malformations. The subtle effects may last into adulthood with hypoplastic characteristics of FAS¹⁾. Observations of dental tissues had showed alterations of tooth size and faulty enamel formation in human FAS³⁴⁾.

In vitro binding of Epidermal Growth Factor (EGF) to mouse and human odontogenic tissues has been studied at various developmental stages³⁵⁻³⁷⁾. Receptors for this growth factor might be inhibited by alcohol^{38,39)}. Recent studies on the binding of EGF to various odontogenic tissues suggest that this growth factor may have a significant role in the development of tooth organs and the differentiation of mesenchymal cells. Retardation of the growth of tooth organs may be due to the inhibition of EGF by alcohol.

The basic architecture of the tooth is established during embryogenesis. It is regulated by sequential epithelio-mesenchymal interactions, especially at an early developmental stage of the tooth before calcification of dentin begins. Delayed calcification of dentin might not be caused by derangements and disruptions of developing tissues, but rather be a result of reduction of cells followed by delayed functions⁴⁰⁾. A recent ultrastructural study on tooth germs from mini-pig fetuses in alcohol-treated mothers has reported that mitochondria of secretory ameloblasts showed abnormal shapes and deposition of paracrystalline material in the matrix⁴¹⁾.

The mechanisms of tooth eruption seem to be multifactorial processes⁴²⁾. The rate of tooth eruption is abnormal in several pathological conditions and syndromes (i.e., cleidocranial dysplasia, Down syndrome⁴³⁾ and osteopetrotic animals⁴⁴⁾). Retardation of tooth eruption has been shown in offsprings of ma-

caque monkeys whose mothers were exposed to alcohol⁴⁵⁾. In the apical tissues of developing tooth root, EGF uptake with heavy labeling of vessel walls and dental mesenchymal cells has recently been shown by Thesleff⁴²⁾. One of the in vivo effects of EGF was the precocious incisor eruption in newborn mice⁴⁶⁾. Mechanisms of tooth eruption and those responsible for the tooth precocity by EGF injection are not fully understood yet.

This study showed that alcohol affected the general growth of the fetus and offspring of mice, although a postnatal experimental period was not long enough to demonstrate the alterations in the adulthood. The mecha-

nisms of action of alcohol could be due to a variety of processes, including the interactions at membrane levels of developing cells, and to the alterations in growth factors and cellular metabolism needed for normal growth.

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抄録 : BALB/cJ マウス母獣のアルコール摂取が仔の頭蓋, 下顎, 歯の形成に及ぼす影響について, 形態計測・病理組織学的に観察した。実験群には妊娠前, 妊娠中に20%のアルコールを, 正常群には水を自由摂取させた。母獣の血中アルコール濃度は妊娠12.5日で $284.0 \pm 1.4 \text{ mg/100ml}$ を示した。その結果, 1) 母獣の体重に有意差はなかったが, 仔の体重の軽量化がみられた。2) 出生後28.5日に至る仔の下顎骨重量の軽量化がみられた。3) 出生後14.5, 21.5, 28.5日の下顎骨には発育遅延がみられ, 下顎骨後方の成長に明らかな有意差がみられた。4) 歯胚の形成は出生後14.5日で差異がみられた。5) 歯の石灰化は出生後1.5日で差異がみられた。6) 歯の萌出遅延は出生後14.5日でみられた。以上より, 母獣のアルコール中毒は, 仔の頭蓋, 下顎, 歯の形成過程に影響し, 出生後4週ではその回復はみられないことが判明した。

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