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A HISTOMORPHOMETRICAL STUDY OF THE EFFECTS OF ETHANOL ON ENAMEL FORMATION IN RAT MANDIBULAR MOLARS DURING PREGNANCY

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ABSTRACT

The consumption of alcohol during pregnancy causes fetal congenital malformations, including craniofacial and orodental defects, as a result of interference with normal embryonic development. In this work, we examined the effects of alcohol on tooth development and enamel formation in rats. Alcohol was administered to female rats in the drinking water starting at a concentration of 1% followed by weekly increases to 5%, 10%, 15%, 20% and 25%. In the seventh week, the rats were mated and continued to receive 25% alcohol until delivery. On postnatal day 5, three offsprings of each mother were killed and their hemimandibules removed, processed and embedded in araldite. Sections 1 μm thick were cut and stained with 1% toluidine blue and histomorphometric analysis of the dental germ and enamel matrix was done. During the postnatal period, the body weights of the offspring from treated dams were significantly smaller than the controls. In addition, the relative volumes of the tooth germ and enamel matrix were always smaller in the offspring of dams treated with alcohol. These results indicated that the ingestion of alcohol during pregnancy interfered with the development of the tooth germ and the secretion of the enamel matrix.

Key words: Enamel formation, ethanol, molar, rat, tooth, tooth germ

INTRODUCTION

The ingestion of high levels of alcohol during pregnancy causes serious birth defects because of the disruption of normal embryonic development, with fetal alcoholic syndrome being the most devastating of these defects [3]. Fetal alcoholic syndrome is characterized mainly by the retardation of pre- and postnatal growth, CNS deficiencies and a particular set of facial anomalies [4].

The effects of alcohol on craniofacial and orodental development include the formation of small teeth, hypoplastic maxilla [4] and enamel, and other dental anomalies [11]. Studies in mice have shown a delay in the eruption of the incisors (S.A. Tomazela-Herndl, personal communication) and reduced dimensions of the cranium and jaw [5,8,9]. Alcohol also causes cellular alterations in the basal epithelial cells of the tooth germ in the bud stage and in the inner enamel epithelium during odontogenesis [1]. The ingestion of 20% alcohol before and during gestation delays cell proliferation in the tooth germ during the bud stage, as well as calcification of the dentin matrix [8]. These studies show that the influence of alcohol during gestation varies according to the drinking pattern, the period and duration of ingestion, and the doses and routes of administration.

Amelogenesis can be affected by various chemical agents, including tetracycline [21] and nicotine [15]. Alcohol also produces ultrastructural changes in secretory ameloblasts of the tooth germ of mini-pig fetuses after intrauterine exposure to this substance, and results in an abnormal secretory function [14].

In this study, we used histomorphology and histomorphometry to assess the development of the enamel matrix and tooth germ of the first mandibular molar in the offspring of female rats treated with teratogenic doses of alcohol during pregnancy.

MATERIAL AND METHODS

Animals

Two-month-old female Wistar rats weighing 150-230 g were housed individually in standard, clear plastic breeding cages. The rats were fed a commercial diet (Purina rat chow) and water *ad libitum* until the beginning of the experiment. After a week of acclimation, the rats were randomly assigned to either the treated group (given ethanol, n=25) or the control group (n = 17). The treated group was given ethanol (Merck & Co., Inc., Whitehouse Station, NJ, USA) added to the drinking water at a

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starting concentration of 1% (v/v). This concentration was subsequently increased at weekly intervals to 5%, 10%, 15%, 20% and 25% in a manner that allowed the rats to become accustomed to and accept the taste of alcohol, thereby avoiding abstinence or a loss of interest in the solution. Control rats received alcohol-free water, and both groups were fed the same solid diet. The treated rats were given alcohol for six weeks before mating (pre-fertilization period). After this period, female rats were mated overnight with non-alcoholic males. The presence of sperm in the vaginal smear, detected on the following morning, was defined as day 0 of pregnancy. Six pregnant females from each group (controls and treated) were used. During gestation, a 25% alcohol solution was given to the treated group while the control group received alcohol-free water (post-fertilization period). All of the rats were weighed weekly and the food and alcohol consumption was measured daily. This study was approved by the institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP).

Histological processing

The body weight of each offspring was recorded during the postnatal period and, on the fifth postnatal day, three offspring were superficially anesthetized with ether and killed by cervical dislocation for subsequent removal of their hemimandibles. The tissues were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde for 6 h and then demineralized in 5% EDTA. The hemimandibles were sectioned transversally in the mesial and distal faces of the first mandibular molar (Fig. 1). With this procedure, only the tooth germ of the first mandibular molar remained. Following this, the tissues were dehydrated in a graded series of acetone, infiltrated and embedded in araldite. Parasagittal sections 1 μm thick were obtained from the first mandibular molar in a mesio-distal direction using a Porter Blum MT-2C ultramicrotome and stained with 1% toluidine blue. Using this procedure, the tooth germ was obtained in a similar orientation in all histological sections of the control and treated groups**.** The tooth germ and enamel matrix were evaluated microscopically and histomorphometrically.

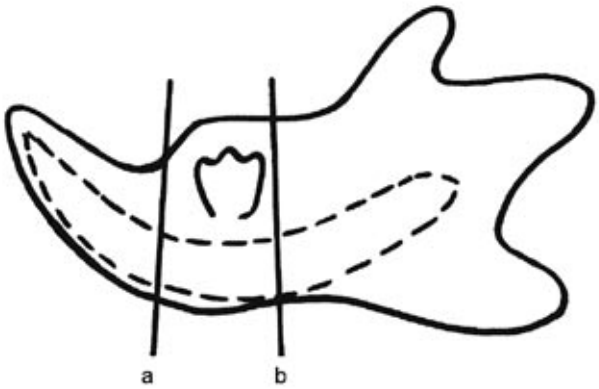

Figure 1. Schematic drawing of the hemimandible with the tooth germ of the first mandibular molar on the fifth post-natal day showing the localization of two transversal sections (**a** and **b**) in the mesial and distal faces, respectively.

Histomorphometry

The histomorphometric analysis consisted of determining the three-dimensional measurements of anatomical structures from bidimensional images of histological sections by geometric and statistical analyses [12]. The parameter used for histomorphometric analysis was the volume density (V_V), which represents the fractional volume occupied by an object in a determined structure.

Two-dimensional profile images of the tooth germ were captured with a light microscope equipped with a video camera connected to a computer. Volume density measurements of the tooth germ and enamel matrix were obtained using an image analyzer, KS 400 (Kontron Electronik, Germany) that elaborated a specific test system consisting of a square lattice of 70 test-points (test area) superimposed over the tooth germ image to allow stereological counting. This system captured all of the tooth germ within the hemimandible. The final magnification used was 40x in which each square measured $4x10^4$ μm^2 (total of 200 $\mu m)^2$. When transformed into mm^3, each point was equivalent to $8x10^{-3}$ mm^3.

Prior to the histomorphometric analysis, the number of observations required to provide an adequate sample size for reliable measurement and subsequent statistical analysis was determined using Chalkey's method [2]. A pilot study was done on some sections to determine the enamel matrix volume density that should be used under our study conditions. With a test system composed of 70 points (tested area), six histological sections per animal, i.e., 420 points corresponding to six tested areas per tooth germ from each rat were needed.

Once the sample size (six serial sections at 10 μm intervals) was established, the V_V of the tooth germ and enamel matrix was determined in 18 treated and control hemimandibles. The volume density of the tooth germ (V_{VG}) and enamel matrix (V_{VE}), expressed in mm^3/mm^3, was estimated by

$$V_{VG} = \frac{P_G}{P} \times 8.10^{-3} mm^3/mm^3 \quad (1)$$

$$V_{VE} = \frac{P_E}{P} \times 8.10^{-3} mm^3/mm^3 \quad (2)$$

where P_G is the number of points coincident upon the tooth germ of each rat, P_E is the number of points coincident upon the enamel matrix from each animal, and P is 6 x 70 (tested area).

Statistical analysis

The results were expressed as the mean ± SD. The normality of the data was tested using Shapiro-Wilk's W

test [16] followed by Student's *t* test to compare the results for the control and treated groups. Values of $p \leq 0.05$ indicated significance [19].

RESULTS

During pregnancy, the average volume of alcohol ingested by the treated group was 24.78 g/kg/day (34.04 ml of 25% alcohol per rat). The initial weight (281 ± 15.94 vs 246 ± 9.70) and the ended weight of pregnant females rats (368 ± 22.53 vs 288 ± 15.71) were significantly reduced in treated group, despite maintaining a healthy appearance; the average weight gain of the controls was approximately 50% greater than that of the treated rats (Table 1). The gestational period of the treated mothers was longer than that of the control rats, and the pups of treated dams weighed 21% less than the control pups at birth (4.77 ± 0.23 g vs 6.04 ± 0.82 g) and on the fifth postnatal day (7.56 ± 0.47 g vs 9.58 ± 1.57 g).

Histomorphologic analysis in the control group revealed normal structural characteristics of the tooth germ in the appositional stage (Fig. 2). Ameloblasts appeared as tall, columnar epithelial cells, with elongated basal nuclei, and vacuoles were occasionally seen in the supranuclear region of these secretory cells. The apical cytoplasm contained dense, round granules of different sizes. The stratum intermedium showed a single layer of cells with large, round nuclei (Fig. 3A) and, in the alcohol-treated group, there was a reduction in the thickness of the enamel matrix (Fig. 3B). This was confirmed by histomorphometric analysis, which showed that the volume density of the enamel matrix and the tooth germ was significantly lower in the offspring of treated dams compared to control dams (~50% and ~12% lower, respectively) (Fig. 4).

Table 1. Gestational period and weight gain in female rats, number of offspring per dam, and weight of litters at birth and on post-natal day 5.

Parameter	Control (n = 6)	Treated (n = 6)
Gestational time (days)	21	22 ± 0.3 *
Weight gain (g)	87 ± 13	42 ± 9.4 *
Total number of offspring/ female rat	14.5 ± 1.5	7.3 ± 2.7 *
Offspring weight (g) at birth	6.0 ± 0.8	4.8 ± 0.2 *
Offspring weight (g) five days after birth	9.6 ± 1.6	7.6 ± 0.5 *

The values are the mean ± SD. *$p \leq 0.05$ compared to the controls.

DISCUSSION

Various methods for studying the effects of alcohol consumption have been reported in the literature and differ in the species, ethanol concentration, experimental groups, duration of ethanol administration and periods of exposure used [1,8,9]. In the present study, oral administration was used because it is more similar to human exposure. During pregnancy, the average volume of alcohol ingested by the treated group was 24.78 g/kg/day (34.04 ml of 25% alcohol per rat). In most studies on gestational alcoholism, this concentration is sufficient to produce teratogenic effects in the offspring [1,8-10,17].

As show here, exposure to ethanol before and during gestation affected the gestational period, litter size (number of offspring/dam) and body weight of the offspring of alcoholic dams, a finding that agreed with other studies [8-10]. Alterations in maternal nutrition represent an important source of interactive factors in fetal alcoholic syndrome in most animal models. However, studies in rodents have shown that ethanol directly affects the development of embryonic tissue and results in a low birth weight, even when the nutritional status of the dams is maintained to allow normal weight gain during pregnancy [9,20]. Our findings are consistent with other studies [5,7] which have shown that alcoholic dams lose body weight during pregnancy and have a smaller number of offspring compared to isocalorically fed dams. These results suggest that alcohol directly affects capacity to bear offspring, regardless of the nutritional status.

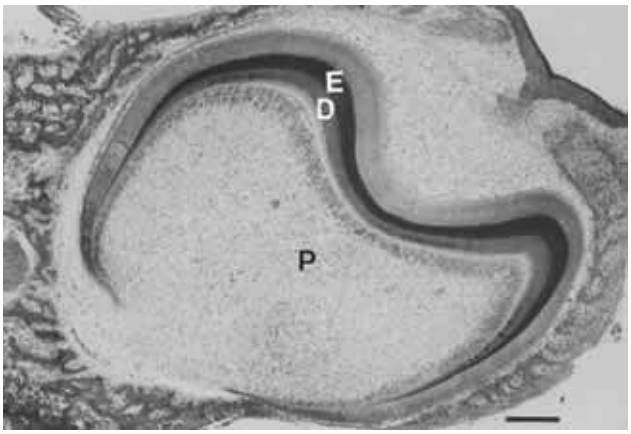

Figure 2. Photomicrograph of the first mandibular molar on the fifth post-natal day showing the structural characteristics of the tooth germ in the appositional stage. **E** – enamel, **D** – dentin, **P** – pulp. Toluidine blue staining. Bar = 169 μm.

In the present study, pair-fed control rats were not used because there is no evidence of morphological differences in the mandibular size [7,9] and tooth development [8,10] of offspring from *ad libitum* and pair-fed control dams.

The chronic ingestion of ethanol during gestation delays the development and differentiation of embryonic tissues and organs, and leads to an overall reduction in animal growth [7,13,17]. In agreement with this, alterations in tooth size and faulty enamel formation have been reported in human fetal alcoholic syndrome [1,4,8,10]. In the present study, histomorphometric analysis showed that the tooth germ volume at the appositional stage was significantly smaller in litters from treated dams compared to the controls, indicating that alcohol interfered with the process of differentiation and the formation of the tissues in this phase. This finding agrees with Hernandez-Guerrero *et al.* [10], who also observed that the tooth germs of offspring from ethanol-treated mice were morphometrically smaller and that the maternal ingestion of alcohol reduced the expression of epidermal growth factor (EGF) in mouse molar dental follicles, which contributed to the reduction in tooth germ size. Campos and Duranza [1] reported cellular alterations in the tooth germ of mouse molars in the bud and cap stages; the most important alterations were found in the inner enamel epithelium of the tooth germ. Since these cells will differentiate into ameloblasts and begin producing enamel, these modifications could explain the reduction in the enamel matrix seen in our experiments. Guerrero [8] reported retardation in the tooth bud formation in ethanol-treated mice. In these animals, the mandibular bud of the first molar consisted of thick cuboidal epithelial cells, whereas in the controls the tooth germ in bud stage consisted of cylindrical cells at the periphery and polygonal cells in the central area.

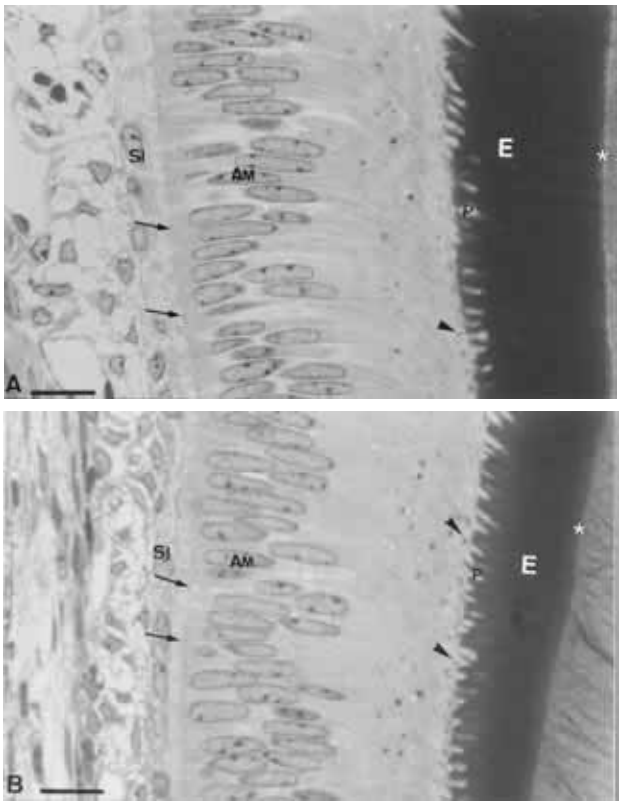

Figure 3. Photomicrograph from the lingual vertent region of the tooth germ. **A** – control group, **B** – treated group. **AM** – ameloblasts, **E** – enamel, **P** – ameloblastic processes, **SI** – stratum intermedium, **arrowhead** – granules in the apical cytoplasm, **arrows** – terminal bars and (*) dentino-enamel junction. Toluidine blue staining. Bar = 16 μm.

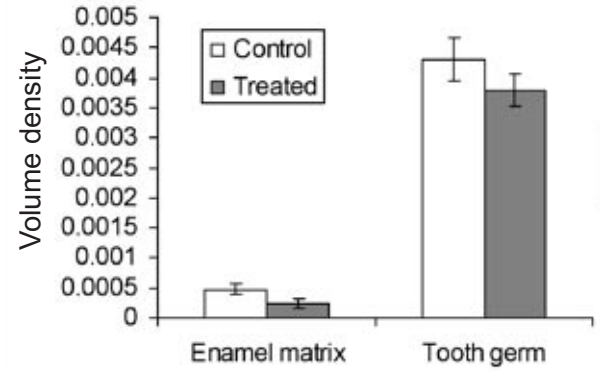

Figure 4. Mean values ± SD of the volume density (mm^3/mm^3) of the enamel matrix and tooth germ of offspring on the fifth post-natal day in the control and treated groups.

The mechanism by which alcohol affects the tooth germ is not yet fully understood. Alcohol may affect a variety of processes, including interaction with the membranes of developing cells and alterations in the growth factors and cellular metabolism needed for normal growth. Alcohol is a potent teratogen that affects the proliferation, migration and differentiation of neural crest cells, thereby retarding the development of cellular structures that are dependent on morphogenesis [17]. The cells of the neural crest differentiate in several directions during embryonic development and give rise to various structures, including most of the embryonic connective tissue of the facial area that contributes to the development of the teeth [18].

Since ethanol inhibits EGF receptors [6], it is possible that EGF may be prevented from exerting its function during ameloblastic differentiation,

hence delaying cellular activity and reducing deposition of the enamel matrix. This suggestion agrees with Guerrero [8] who reported that the delayed calcification of dentin at postnatal day 1.5 in alcohol-treated animals resulted in reduced cellular activity and delayed functions because of the effects of alcohol in the early stages of embryonic development. An ultrastructural study of tooth germs in mini-pig fetuses from alcohol-treated dams showed that the mitochondria of secretory ameloblasts had an abnormal shape, with the deposition of paracrystalline material in the matrix and the abnormal deposition of stippled intercellular material. These changes suggested that the administration of ethanol during pregnancy influenced the secretory function of the ameloblasts and, hence, enamel formation [14].

The results of the present study, and those of other investigations [13,16], have shown that the tissue responses to the ingestion of alcohol during pregnancy are not uniform. Hence, we observed that the reduction in volume density was more pronounced in the enamel matrix than in the tooth germ as a whole, which may indicate that ameloblasts were more susceptible to ethanol than other cells during the period analyzed. In support of this conclusion, Phillips and Krueger [13] stated that cells with intense metabolic activity show a specific response when exposed to alcohol.

In conclusion, alcohol intake during pregnancy reduced the development of the tooth germ and enamel matrix formation, with a greater effect on the latter. The immunohistochemical analysis of the tooth germ of offspring from alcoholic and control rats currently in progress should contribute to our understanding of the cellular effects of maternal alcohol intake on enamel formation.

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