Abstract: Previous studies suggest that prenatal alcohol exposure affects fetal bone development, including bone quality. This study evaluated the chemical composition of mandibles from newborn rats after maternal 20% alcohol consumption before and throughout gestation. Nine rats were initially distributed into three groups: an Alcohol group, Pair-fed group, and Control group. The groups were fed prespecified diets for 8 weeks before and the 3 weeks during pregnancy. At age 5 days, eight newborns from each group were euthanized (total, n = 24). Using energy dispersive spectrometry, we evaluated samples of mandibles from newborns to identify changes in bone mineralization, specifically Ca and P concentrations. Ca and P concentrations were lower in the Alcohol group than in the Control and Pair-fed groups (P = 0.003 and P = 0.001, respectively). In summary, alcohol exposure before and throughout gestation reduces mandibular Ca and P concentrations in newborn rats. (J Oral Sci 58, 439-444, 2016)

Keywords: alcohol; gestation; calcium; phosphorous; mandible; rat.

Introduction

Alcohol consumption during pregnancy has been implicated in many brain and facial abnormalities, growth and cognitive delay, and behavioral and neurological complications. Fetal alcohol spectrum disorders is used as a comprehensive term to encompass the variety of phenotypes resulting from prenatal alcohol exposure (1-2).

Considerable evidence indicates that growth delay is one of the most important alterations caused by prenatal alcohol exposure (3-12). Specifically, intrauterine growth delay is partially associated with functional impairment of the placenta during pregnancy (13-14). The placenta acts as an obstacle to protect the fetus from toxic chemicals in the maternal bloodstream, and it is possible that accumulation of such compounds induces alterations in placental cells (15-16). A previous study reported that placental function may be altered by alcohol exposure (17).
Some experimental studies found that animals exposed to alcohol during pregnancy had lower weights at birth, shorter head-tail lengths, shorter bones, less bone resistance, delays in ossification and, consequently, delayed total bone growth (3,4,6-8,10-12). These findings suggest that prenatal alcohol exposure alters fetal bone development and leads to growth delay. Additionally, some studies reported decreased cephalic perimeter, delays in skull growth, and reductions in weight and mandible length (5,18-20), which indicates that bone growth delay attributable to prenatal alcohol exposure is also present in craniofacial bones.

The mechanism by which alcohol causes growth delay is unknown. However, some evidence indicates that growth delay is related to fetal bone quality, because alterations such as ossification delays and decreased bone resistance were present in animals exposed to alcohol during pregnancy (12,13).

Bone is a mineralized tissue composed mainly of calcium (Ca) and phosphorus (P), which together form hydroxyapatite crystals (21). Excess alcohol consumption reduces the quantitative distribution of Ca and P and thus alters the stoichiometric composition of hydroxyapatite (22).

This study examined mandibular calcium and phosphorus concentrations in newborn rats after prenatal alcohol exposure. Because of the known adverse effects of alcohol consumption before and throughout pregnancy on bone quality in newborns, our hypothesis was that alcohol consumption during this period would reduce Ca and P concentrations and the extent of bone mineralization in mandibles of newborn rats.

**Materials and Methods**

**Animals**

Nine virgin female Wistar rats (age, 5 weeks; weight, approximately 120 g) were assigned to one of three groups: (a) an Alcohol group, which received a 20% alcohol solution and *ad libitum* solid diet, (b) a Pair-fed group, which was fed an isocaloric liquid control diet and solid diet with the same number of calories as the diet for the Alcohol group, and (c) a Control group, which received water and an *ad libitum* solid diet. The experimental feeding regime is illustrated in Fig. 1 and is described in detail elsewhere (23). This study was approved by the Ethical Committee on Research at São Paulo State University (UNESP) (protocol no. 04/2012-PA/CEP).

**Determination of blood ethanol concentrations in dams**

After the newborns were euthanized, the dams were anesthetized and cardiac puncture was performed to collect blood samples. Blood samples were then centrifuged at 4°C, and the serum was stored at –80°C until the analysis was performed. Blood ethanol concentration was measured by using a commercial kit (EnzyChrom Ethanol Assay Kit, Bioassay Systems, Hayward, CA, USA). Blood samples were also collected from the Control and Pair-fed rats, to confirm appropriate treatment in these groups (negative controls).

**Newborns**

The dams littered naturally, and the newborns remained with their dams. The dams continued on the experimental diets until their newborns were euthanized by decapitation, on the fifth day of life, and their mandibles collected. At age 5 days, the number of newborns was recorded, and the newborns were weighed and measured. Eight newborns were randomly distributed to each of the three experimental groups, regardless of the total number of animals born alive, and were euthanized by decapitation before mandible collection.

**X-ray spectrometry**

Mandibular Ca and P concentrations were measured by energy dispersive spectrometry (EDS; Bruker AXS Inc., Madison, WI, USA; Institute of Science and Technology, São José dos Campos [UNESP]). After euthanasia, the mandibles were immersed in 10% formalin solution for
The bones were then prepared for EDS analysis by dehydrating all specimens in progressively higher concentrations of alcohol (50%, 60%, 70%, 80%, 90%, and 100%). During the dehydration process the specimens were immersed in each of the aforementioned solutions for 2 h. After each immersion, specimens were washed for 30 min in running water before being added to the next solution. The specimens were immersed in the final concentration (100%) for 24 h, thus ending the process. After that, the specimens were stored at 4°C. This procedure is required in order to decrease the noise during scanning electron microscopic analysis of specimens, because of the low electric conductivity of the specimen. To standardize sectioning, specimens were placed inside a PVC ring-shaped matrix (dimensions: diameter = 1,905 cm, height = 10 mm). Chemically activated acrylic resin (RAAQ; Jet, Artigos Odontológicos Clássico; São Paulo, SP, Brazil) was poured into the matrix, up to a height of 2 mm. The specimens were then placed onto this base. To ensure proper positioning, measurements were taken so that the center of the specimen matched that of the matrix. The specimens were then glued with white glue (Tenaz-Pritt Henkel Ltd., São Paulo, SP, Brazil) onto the acrylic resin base surface, after which additional acrylic resin was poured on the specimen. The specimens were cut axially with the aid of a precision cutting device (Isomet Buehler; Lake Bluff, IL, USA) and then sand-blasted with the aid of an automatic polishing machine (EcoMet 250; Buehler ITW Company, Lake Bluff, IL, USA) with 600-grit silicon carbide paper. The specimens were then inspected by a scanning electron microscope (Inspect S50; FEI Worldwide Corporate Headquarters, Hillsboro, OR, USA) and EDS analysis (Bruker AXS Inc., Madison, WI, USA) with the aid of software Espirit 4.1 (Bruker AXS Inc.). Using EDS, we quantified Ca and P concentrations by utilizing the mapping function of the software at ×5,073 magnification. The software automatically selected the size of a reading area corresponding to approximately 2,025 µm² (Fig. 2). Five reading areas, at predetermined sites, were selected for each specimen, as shown in Fig. 3.

**Statistical analysis**

Because spectrophotometric and dietary data were non-normally distributed, nonparametric tests (Kruskal-Wallis and Mann-Whitney tests) were used. Data for fetal body weight were normally distributed; thus, parametric tests were used (ANOV A and Tukey test). There was no statistical adjustment of the samples. A $P$ value of less than 0.05 was considered to indicate statistical significance.

**Results**

**Dam diet**

The consumption of the liquid and solid diets was compared by using Kruskal-Wallis and Mann-Whitney tests (Table 1). Consumption of the solid diet was significantly greater in the Control group (19.1 ± 4.2 g) than in the Alcohol and Pair-fed groups ($P < 0.001$ for both comparisons). Solid diet consumption did not significantly differ between the Pair-fed and Alcohol groups (11.9 ± 3.9 g vs 11.8 ± 2.9 g). Consumption of the liquid diet was significantly greater in the Control group (19.1 ± 4.2 g) than in the Alcohol (27.1 ± 6.6 mL) and Pair-fed groups (17.5 ± 8.3 mL) ($P < 0.001$ for both comparisons).

**Alcohol consumption**

The mean quantity of 20% alcohol solution ingested was 25.4 mL/day/rat in the Alcohol group, which corresponds to a mean of 51.28% of dietary calories from alcohol (Table 2).
Blood ethanol concentrations in dams
The mean blood ethanol concentration in the Alcohol group was 1533 ± 455.7 mg/dL. As expected, the Pair-fed and Control groups had undetectable concentrations of alcohol in blood.

Weight of newborns
The weights of newborns were compared by using ANOVA and the Tukey test (Table 3). The Control group was significantly heavier (12.33 ± 1.01 g) than the Alcohol and Pair-fed groups (P < 0.0001 for both comparisons). The difference between the Alcohol and Pair-fed groups was not significant (P = 0.875).

Ca and P concentrations
Some specimens were lost during sample preparation, which resulted in a difference in the numbers of specimens in the experimental groups (Table 4). After EDS, mean Ca and P concentrations were calculated, and the data were analyzed by using the Kruskal-Wallis and Mann-Whitney tests. Ca and P concentrations in atomic percentage (at%) were significantly lower in the Alcohol group (2.34 ± 0.90 and 1.03 ± 0.41 at%, respectively) than in the other groups. Additionally, neither Ca nor P concentration significantly differed between the Pair-fed and Control groups (P = 0.668 and P = 0.617, respectively).

Discussion
Alcohol intake during pregnancy is related to bone tissue changes in newborns (3,4,6-8,10-12,24). Decreased bone resistance and delays in ossification indicate that the bone quality of fetuses is altered by alcohol consumption. Additionally, studies reported that prenatal alcohol exposure results in craniofacial skeletal abnormalities (5,18-20,24). These findings led to our hypothesis, namely, that maternal alcohol consumption would adversely affect mandibular bone mineralization in newborns and decrease Ca and P concentrations and formation of hydroxyapatite crystals. This hypothesis was confirmed: newborns in the Alcohol group had significantly lower mandibular Ca and P concentrations.

In our study, the dams ingested an average of 25.4 mL per day of a 20% ethanol solution, which corresponded to a mean of 51.28% of total dietary calories. A proportion of 35% to 40% of dietary calories from alcohol is considered a high dose. Such doses have been used in previous studies to achieve high blood alcohol concentrations.
concentrations (11,25-27). High maternal blood alcohol concentrations were confirmed in this study by measuring alcohol concentrations in blood. The mean blood ethanol concentration in the dams in the Alcohol group was 1533 ± 455.7 mg/dL, which is much higher than the legal limits for drunk driving (50-100 mg/dL) in the United States and Canada (28). Furthermore, the threshold required in order to reduce skeleton ossification and fetal body length and weight is slightly higher than the limits for drunk driving. Therefore, this threshold should be considered as equivalent to heavy drinking (28,29).

Alcohol has a high energy content, which leads to a sense of fullness and provides a high percentage of calories in the total diet, thus reducing the usual amount of solid nutrients ingested (30,31). This explains why the animals in the Alcohol group ingested less solid food as compared with the Control group, which was expected. The Pair-fed group was used as a control for nutritional intake, as the same amount of solid food was ingested by the Alcohol group and Pair-fed group. Liquid diet consumption differed between the Pair-fed and Alcohol groups; however, there was no difference in Ca and P concentrations between the Pair-fed and Control groups, which indicates that the diet did not affect the results.

Previous studies reported that prenatal alcohol exposure reduced skeletal ossification in newborn rats, although the severity of the effect varied (4,6-8). The severity of the adverse effects of prenatal alcohol exposure increases as the dose ingested by dams during pregnancy rises; however, the effects on the developing skeleton are evident even when the dose is moderate (11).

A previous study found that prenatal ethanol intake had significant effects on fetal Ca homeostasis, which caused a reduction in fetal blood Ca levels and subsequent fetal hypocalcemia after 6 weeks of maternal alcohol exposure. Blood alcohol and blood Ca concentrations were inversely related in newborns. The authors of the study noted that although the severity of the effects of ethanol was weakly associated with the duration of maternal ethanol intake, the association with relative exposure of the fetus to ethanol was stronger (10).

In this study, the duration of maternal alcohol consumption was 11 weeks. Previous studies used shorter exposure periods: 6 and 3 weeks (10) and 5 weeks (7). Fetal blood alcohol concentration was not evaluated; however, maternal blood alcohol concentration was measured and confirmed a high ethanol concentration in the blood of dams. Because ethanol crosses the placenta, alcohol was probably transported to the fetus. Fetal peak blood ethanol concentration is strongly correlated with maternal peak blood ethanol concentration (32).

The prolonged maternal alcohol consumption (8 weeks before and 3 weeks during pregnancy) and high maternal blood alcohol concentrations observed in this study significantly decreased Ca concentration in fetal mandibles, perhaps because of systemic hypocalcemia, as previously reported (10).

Fetal body weight was lower in the Alcohol and Pair-fed groups. This weight decrease was not directly caused by ethanol ingestion; instead, it was probably the result of nutritional changes. A prenatal intake of 36% ethanol-derived calories reduced fetal body weight in previous studies (10,11). Some studies found that low birth weight and deficient fetal bone development resulted in lower peak bone mass and an increased risk of future osteoporosis (33-36).

Previous studies reported that prenatal alcohol exposure causes delays in fetal skeletal development (10) and force reduction in fetal bones (12) and that it reduces craniofacial bone development after birth (20). Our results were consistent with these past reports. We believe that bone deficiencies are related to alterations in hydroxyapatite composition, which are caused by decreases in Ca and P concentrations. Future studies should investigate whether and how reductions in mandibular Ca and P are related to systemic hypocalcemia.

Acknowledgments

This study was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo – The State of Sao Paulo Research Foundation) (grant number 2012/10643-3) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazilian Federal Agency for Support and Evaluation of Post-graduate Education), Brazil.

References

6. Weinberg J, D’Alquen G, Bezio S (1990) Interactive effects...
of ethanol intake and maternal nutritional status on skeletal development of fetal rats. Alcohol 7, 383-388.