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PRENATAL ETHANOL EXPOSURE DISRUPTS THE HISTOLOGICAL STAGES OF FETAL BONE DEVELOPMENT

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Abstract

Maternal ethanol intake during pregnancy results in impairments in general growth and skeletal development in the offspring. We have previously shown that ethanol retards skeletal ossification at doses lower than those that affect growth. Moreover skeletal sites vary in their sensitivity to ethanol effects, with more severe effects occurring in bones that undergo a greater proportion of their development in utero. Taken together, these data suggest that ethanol has specific effects on bone development, and that later stages in the ossification process may be particularly affected. Such effects could have important implications for the offspring's long-term bone health, as studies suggest that the intrauterine environment can program the skeleton. The present study examined the histological stages of bone development to determine if prenatal ethanol exposure alters the morphological development of the growth plate in the fetal rat. Rats were fed a liquid diet containing ethanol (Ethanol, E group), or without ethanol (Pair-Fed, PF, or Control, C groups) for 6 weeks: 3 weeks prior to breeding and during 3 weeks of pregnancy. Fetal tibiae were fixed, decalcified and stained for histological analysis on day 21 of gestation. Maternal ethanol intake resulted in a significant decrease in fetal total bone and diaphysis lengths, compared with tibiae from PF and C fetuses. Although the lengths of the epiphyses were not affected, ethanol disrupted the organization of the histological zones within the epiphyses. Prenatal ethanol exposure decreased the length of the resting zone, but increased the length of the hypertrophic zone. Enlargement of the hypertrophic zone is consistent with an effect of ethanol on the later stages of bone development; however ethanol's effect on the resting zone indicate that earlier stages of bone development may also be disrupted. The functional significance of these morphological changes to long-term bone health remains to be determined.

Keywords

prenatal alcohol exposure; alcohol; bone; skeletal development; fetal bone

INTRODUCTION

There is increasing evidence that the intrauterine environment influences the risk of developing chronic diseases (e.g., cardiovascular disease, diabetes and osteoporosis) later in life [2]. This is thought to occur through fetal programming, which refers to the ability of changes in environmental factors (e.g., nutrition, stress, exposure to toxicants) at critical periods during development to permanently alter the structure, physiology or metabolism of the body [2]. One

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such environmental factor is alcohol (ethanol). Exposure to ethanol in utero is known to have serious long-term effects on the offspring and cause a diverse array of health problems, described collectively as Fetal Alcohol Spectrum Disorder (FASD) [4]. Fetal Alcohol Syndrome (FAS), the most severe manifestation of FASD, is characterized by a distinctive craniofacial dysmorphism, central nervous system abnormalities and growth retardation.

Exposure to ethanol in utero also has a number of effects on the developing skeleton, and these effects do not appear to normalize after birth. Although the long-term effects on skeletal health are unknown, exposure to high levels of ethanol in utero can result in permanent short stature [5] and delayed mean bone age in children up to 14 y of age [9]. In animal (rodent) models, prenatal ethanol exposure results in decreased body length and length of individual bones, delayed ossification and decreased skeletal maturity scores [14,15,17 and 20]. These effects persist at least until weaning [20], but effects at later ages have not been investigated. Prenatal ethanol exposure has also been shown to affect functional measures of bone health, as bone strength was altered by maternal ethanol intake in fetal sheep [6]. It is unknown if prenatal ethanol affects bone function in postnatal life.

Although it is difficult to separate effects on general growth from those on skeletal development, we have shown that prenatal ethanol delays fetal skeletal ossification at doses below those that affect body weight and length [32]. In addition, the skeletal elements differed in their sensitivity to ethanol's effects, with more severe effects occurring in the bones that undergo a greater proportion of their development in utero [32]. These data suggest that ethanol's effects on bone are not limited to decreasing longitudinal growth, and that more subtle effects may occur at levels of maternal drinking below those associated with growth retardation. This is important because recent studies suggest that in addition to low birth weight, impaired bone growth and/or mineralization in utero may decrease peak bone mass and increase the risk of osteoporosis later in life [13]. Moreover, the prenatal environment can program bone cells, resulting in altered responses to stimuli [24] as well as alterations in bone composition and morphology [23 and 35] in adulthood. Thus effects of prenatal ethanol exposure on the fetal skeleton may influence the long-term bone health of the offspring.

Little is known, however, about the specific effects of ethanol on fetal bone. To date, only one study [25] has examined the effects of prenatal ethanol exposure on the different histological stages of the growth plate of postnatal bone, and no studies have examined prenatal bone. Miralles-Flores and Delgado-Baeza [25] found no effect of ethanol on the tibial growth plate at birth, but did observe a decreased length of the growth plate at postnatal day 15, which resulted from a decrease in the length of the hypertrophic zone. These effects appeared to be independent of ethanol's effects on body weight or tibial length, as they were not abolished by inclusion of these factors as covariates in the analysis [25]. Although this study suggests that ethanol affects specific stages in the process of endochondral ossification, it is unclear if the effects are limited to postnatal development, or could also explain ethanol's observed adverse effects (decreased ossification and growth) on prenatal skeletal development.

The purpose of the present study was to examine the histological stages of bone development to determine if prenatal ethanol exposure alters the morphology of the growth plate in the fetal rat. To maximize the ability to detect potential effects, we chose to use a dose of ethanol (36% ethanol derived calories [EDC]) that consistently results in delayed ossification of fetal bone in the rat model. This dose of ethanol produces blood ethanol levels that are slightly higher than the legal limits for drunk driving (50-100 mg/dL) in Canada and the United States [32], and are well within the range that has been reported in human alcoholics [36]. Fetal Alcohol Spectrum Disorder has a high prevalence, with estimates approximating 1% of live births in the United States [22 and 30]. As the costs to health care associated with osteoporosis are in

the billions of dollars [26 and 27], adverse effects of prenatal ethanol exposure on long-term bone health could represent a significant health care issue.

MATERIALS AND METHODS

Virgin female Sprague-Dawley rats (3-4 months of age, Charles River, St. Constant, Quebec) were weighed (initial body weights 235-286 g, mean 252 g) and assigned to one of three weight-matched groups: Ethanol (E, n = 13), which were fed a liquid diet containing 36% ethanol-derived calories; Pair-Fed (PF, n = 12), which received an isocaloric liquid diet (with the ethanol calories replaced by maltose-dextrin) in amounts equivalent to those consumed by weight-matched rats in the ethanol group; and Control (C, n = 12), which received liquid control diet ad libitum. The diets were obtained from BioServ Inc. (Frenchtown, NJ) and administered for 6 wk: 3 wk prior to breeding, and throughout 21 d of gestation. Rats were maintained in temperature-controlled rooms with lights on between 0600 and 1800 h. Fresh diet was presented daily at 1700 h. Food intake was measured daily and rats were weighed weekly throughout the experiment. Mean time for rats to breed was 3 d (range = 1-7 d) and rats received lab chow ad libitum while in the breeding cages. The appearance of a vaginal plug was considered d1 of gestation. The rats were kept in compliance with the Canadian Council for Animal Care guidelines. Experimental procedures were approved by the University of British Columbia Animal Care Committee.

Peak blood ethanol concentrations (BECs) were determined in a subset of E rats (n = 5) on d10-11 of gestation. Blood samples, obtained by tail nick at 2-4 h after lights out, were centrifuged at 4°C and serum stored at -70°C until analyzed for BEC (kit# 333-B, Sigma Diagnostic, St. Louis, MO).

Fetuses were collected on d21 of gestation (approximately 1.5 d prior to birth) between 0800 and 1200 h. Dams were lightly anesthetized with ether, terminated by decapitation and fetuses euthanized by cervical dislocation. Fetuses were counted and the litter weight determined. Two fetuses from each litter were selected in a blinded fashion (and without reference to sex) and the left tibiae dissected free from soft tissue under a Zeiss dissecting microscope. All fetuses in each litter were not examined because ethanol's effects have been shown to be consistent among fetuses in a litter [11] and previous work in our lab has demonstrated that coefficients of variation for ossification measures among littermates in the E group are similar to those of PF and C groups [32]. The tibia was chosen for analysis as this bone is consistently highly affected by prenatal ethanol exposure [15,17, and 32].

Tibiae were fixed in formalin for 3 d, decalcified in 10% w/v ethylenediaminetetraacetic acid (EDTA, pH 7.4) for 5 d, rinsed in four 1 h washes of 70% ethanol, and embedded in paraffin. Care was taken to ensure that all bones were oriented in the same direction during embedding to minimize differences in the angles at which the bones were sectioned, and a single investigator (M.E.S.) processed all bones to maintain consistency. Tibiae were sectioned (5 µm thickness) longitudinally through the entire bone and sections were stained with hematoxylin and eosin for histological examination. The longest section of each tibia was selected for analysis.

Photomicrographs (Retiga EX Digital CCD monochrome camera) were taken under light microscopy (Axioscop 2 Microscope) at 31.5X magnification for assessment of total bone and diaphyseal length, diaphyseal width, and distal epiphyseal length, and at 63X magnification for assessment of the lengths and areas of the different chondrocyte zones in the proximal epiphysis. Photomicrographs were imported into Adobe Photoshop and the parameters to be measured were delineated based on cell morphology. Figure 1 shows the boundaries used for the morphometric analyses. Figure 1A illustrates the boundaries for the lengths of the total

bone, diaphysis and distal epiphysis, and the width of the diaphysis. Total bone length and diaphyseal length were measured centrally along the long axis of the bone, from end to end for total bone length, and from the distal to the proximal epiphyses for diaphyseal length. The length of the distal epiphysis was determined from the mean of five measurements made parallel to the long axis of the bone, taken at equal intervals across the width of the bone, from the end of the epiphysis to the bottom of the hypertrophic zone. The width of the diaphysis was measured perpendicularly to the long axis of the bone at its longitudinal midpoint.

Figure 1B shows the boundaries for the measurements of the lengths and areas of the resting, proliferative, and hypertrophic zones of the proximal epiphysis. Criteria for delineation of the different histological zones were based on Howell & Dean [12] and Miralles-Flores and Delgado-Baeza [25]. Specifically, the resting zone was defined as the top of the epiphysis to the top of the proliferative zone, the proliferative zone was defined as the first flattened chondrocyte to the top of the hypertrophic zone, and the hypertrophic zone was defined as the first rounded hypertrophic chondrocyte to the last rounded hypertrophic chondrocyte. The length of each zone was determined from the mean of five measurements made parallel to the long axis of the bone, taken at equal intervals across the width of the bone. The total length of the proximal epiphysis was calculated from the sum of the lengths of the resting, proliferative and hypertrophic zones. The area of each zone was also determined using the boundaries delineated as described above (shown in Figure 1B). All parameters were measured using Image J software [29]. The value for each litter represents the mean of two fetuses and the n per group represents the number of litters.

A single investigator, blinded to treatment group, performed all measurements. To determine intra- and inter-rater reliability of the measures, measurements of total bone length, bone width and diaphysis length (n = 9) and resting, proliferative and hypertrophic zone length (n = 22) were performed on a subset of the tibiae by the same investigator at a later date, and by a different investigator, respectively.

Group (diet) differences were determined for all measures, except maternal food intake, by one-way ANOVAs, followed by Newman-Keuls post-hoc tests. Maternal food intake was assessed using a two-way repeated measures ANOVA, with factors of time (repeated measure; wk 1, 2, 3, 4, 5, and 6 of feeding) and diet (E, PF and C), followed by Newman-Keuls post-hoc tests. Level of significance was $p < 0.05$. Values presented are the mean $\pm$ SE. Intra- and inter-rater reliability were assessed using the intraclass correlation coefficient (ICC), models 2 and 3, respectively [28 and 31]. Values for ICC greater than 0.75 were considered to represent high reliability [31].

RESULTS

There were no significant differences in the number of fetuses per litter among the diet groups (data not shown). Fetal body weights were significantly affected by diet (ANOVA $p < 0.001$). Maternal ethanol intake resulted in significantly lower mean fetal body weights (2.64 ± 0.05 g) compared with PF (2.99 ± 0.07 g; $p = 0.001$) and C (3.10 ± 0.07 g; $p < 0.001$) fetuses. The body weights of PF and C fetuses were not significantly different from one another.

Visual examination of the tibia under 31.5x magnification showed no obvious abnormalities in the ethanol-exposed, compared with PF and C, fetuses (Figure 2A), other than a decrease in total bone length. Intra- and inter-rater reliability was high (> 0.75) for all parameters assessed, with ICC values ranging from 0.76 to 0.99.

The effect of ethanol on total length of the fetal tibia is shown in Figure 2B. Tibial length was significantly affected by diet (ANOVA $p < 0.001$), and ethanol exposure resulted in significantly

shorter tibiae compared with PF and C fetuses ($p < 0.001$). There was no significant difference in tibial length between PF and C fetuses.

The shortening of the tibia by ethanol exposure appeared to be the result of a shorter diaphysis (ANOVA $p = 0.009$), with E fetuses ($1488 \pm 47 \mu\text{m}$) having significantly shorter diaphyses compared with PF ($1689 \pm 50 \mu\text{m}$, $p = 0.017$) and C ($1672 \pm 49 \mu\text{m}$, $p = 0.012$) fetuses, which did not differ from each other. When expressed relative to total tibial length, the differences among the diet groups did not reach significance (E: $36.1 \pm 0.9\%$, PF: $38.6 \pm 0.91\%$, C: $38.4 \pm 0.88\%$; ANOVA $p = 0.108$).

In contrast with ethanol's effect on the diaphysis, the lengths of the proximal or distal epiphyses were not significantly affected by ethanol, when expressed as an absolute measure (proximal: E: $1317 \pm 19 \mu\text{m}$, PF: $1337 \pm 21 \mu\text{m}$, C: $1358 \pm 27 \mu\text{m}$; distal: E: $1253 \pm 18 \mu\text{m}$, PF: $1298 \pm 15 \mu\text{m}$, C: $1284 \pm 20 \mu\text{m}$) or relative to total tibial length (proximal: E: $32.2 \pm 0.5\%$, PF: $30.6 \pm 0.5\%$, C: $31.2 \pm 0.5\%$; distal: E: $30.5 \pm 0.5\%$, PF: $29.7 \pm 0.4\%$, C: $29.6 \pm 0.6\%$). There was also no effect of diet on the width of the diaphysis (E: $492 \pm 15 \mu\text{m}$, PF: $518 \pm 12 \mu\text{m}$, C: $526 \pm 12 \mu\text{m}$).

While the total length of the epiphyses was not affected by ethanol, the organization of the different zones within the epiphyses was disrupted (Figure 3). In the proximal epiphysis, significant diet effects were found on the lengths of both the resting (ANOVA $p = 0.015$) and hypertrophic (ANOVA $p = 0.017$) zones (Figure 3A). Fetuses exposed to ethanol had a shorter resting zone compared with PF ($p = 0.027$) and C ($p = 0.019$) fetuses, and a longer hypertrophic zone than PF fetuses ($p = 0.016$) (Figure 3A). The increase in hypertrophic zone length in E compared with C fetuses approached significance ($p = 0.055$). No significant differences in zone lengths were found between PF and C fetuses. When expressed relative to total tibial length (Figure 3B), the effect of diet on the hypertrophic zone was still apparent (ANOVA $p < 0.001$), with the length in E fetuses being longer than that of PF ($p < 0.001$) and C ($p = 0.004$) fetuses. The differences in the resting zone length between E vs. PF and C fetuses disappeared, however, indicating that the decrease in resting zone length was proportional to the decrease in total tibial length. There was no significant effect of ethanol exposure on the proliferative zone length, expressed either as an absolute measure or relative to total tibial length.

Figure 4 shows the effect of ethanol on the areas of the different histological zones. There was a significant effect of diet on the absolute area of the resting (ANOVA $p = 0.019$) and proliferative (ANOVA $p = 0.032$) zones (Figure 4A). The resting zone area of E tibiae was significantly smaller ($p = 0.016$) than that of PF tibiae, but the difference between E and C did not reach significance ($p = 0.078$). Similarly, the area of the proliferative zone was significantly smaller in E compared with PF ($p = 0.025$) tibiae, whereas there was no significant difference between E and C. When expressed relative to total tibial length (Figure 4B), the differences disappeared. There were no significant differences in the areas of any of the zones between PF and C fetuses. Despite the decrease in tibial length, the area of the hypertrophic zone was not decreased in E fetuses, and there were no significant differences among absolute or relative hypertrophic zones areas. The differences among the areas of the zones were generally consistent with the differences in zone lengths. The apparent difference in ethanol's effect on the area (significant) vs length (not significant) of the proliferative zone is likely due to the fact that the area measure reflects the combination of length and width measures, both of which alone showed small (and insignificant) decreases with ethanol exposure.

Maternal ethanol intake throughout gestation in the E group averaged $11.3 \pm 0.2 \text{ g/kg}$ body weight per day, and mean peak BEC on day 10-11 of gestation was $114 \pm 16 \text{ mg/dL}$. Maternal food intake over the 6 wk of feeding (3 wk prior to pregnancy and 3 wk during gestation), averaged weekly, ranged from 0.196 to 0.297 g food/kg body weight/day and was significantly

affected by diet (ANOVA $p < 0.001$) and time (ANOVA $p < 0.001$), with a significant diet by time interaction (ANOVA $p < 0.001$). Overall, there was no significant difference in the food intake of E and PF dams, which both consumed less than C dams (p 's < 0.001). The post-hoc test of the individual groups over time showed no significant differences between the E and PF groups for any individual week, indicating that differences between E and PF groups are the result of ethanol intake and not differences in caloric or nutrient intake. Although E and PF dams ate less than C dams prior to and throughout the first week of gestation (weeks 1-4), food intake was not significantly different between PF and C dams during week 5, or among E, PF and C dams for week 6. Maternal weight gain during gestation varied with diet (ANOVA $p < 0.001$), and was not significantly different between E (84 ± 5 g) and PF (77 ± 4 g) dams, while the C dams gained significantly more weight during gestation (115 ± 6 g) than both the E and PF dams (p 's < 0.001).

DISCUSSION

This study examined the effects of prenatal ethanol exposure on the different histological stages of endochondral bone formation as a first step in determining the specific effects of ethanol on fetal bone development. Consistent with previous studies [14,15,17,20,25,32, and 37], prenatal ethanol exposure resulted in decreased fetal body weight and tibial length in the rat. The decreased tibial length appeared mainly to reflect a shorter diaphysis, as there was no significant effect of ethanol on the total length of the epiphyses. However, although the length of the epiphyses was not affected, the cellular organization of the zones within the epiphyses was altered by prenatal ethanol exposure. Ethanol decreased the length of the resting zone (proportional to the decrease in total tibial length), but this decrease was offset by an increase in the length of the hypertrophic zone. These data suggest that ethanol affects bone development at multiple stages. Moreover, ethanol's effects on the hypertrophic zone are consistent with the hypothesis that ethanol has specific effects on the later stages of bone development [32].

The effect of prenatal ethanol exposure on resting zone length suggests that ethanol may have effects on early stages of long bone development, which may contribute to the decrease in total bone length seen in this and other studies with high levels of ethanol exposure. Development of the fetal skeleton begins with the condensation of mesenchymal cells in the embryo, which subsequently differentiate into chondrocytes and form the cartilage anlagen of the skeleton. The initial size of a condensation is a reflection of the growth potential of the bones that will result from that condensation [10]. Thus, it is possible that ethanol exposure reduces the initial size of the mesenchymal cell condensation, resulting in a smaller anlagen, and this reduction in size is reflected in the smaller resting zone in the ethanol-exposed fetus. Ethanol has been shown to enhance the differentiation of mesenchymal cells into chondrocytes in chick limb buds *in vitro* [19], but whether this precocious chondrogenesis results in a smaller cartilage anlagen or influences the fate of the developing limb in some other way has not been investigated.

Previous work in our lab demonstrated that the ossification (proportion of bone that is mineralized) of some bones in the fetal rat is decreased at levels of ethanol below those required to decrease body weight and length [32]. Moreover, ethanol's effects varied by bone, with the sensitivity to ethanol being greatest in those bones that start to ossify earlier in gestation [32] and thus undergo more of their development *in utero* [34]. These data led us to hypothesize that ethanol disrupts the later stages of bone development, such as cartilage mineralization or resorption, chondrocyte apoptosis, angiogenesis or osteoblast function. The increased length of the hypertrophic zone seen in the ethanol fetuses, combined with the lack of a significant effect of ethanol on the length of the proliferative zone, support this hypothesis. For example, hypertrophic zone enlargement has been shown to occur with disruptions in later stages of bone

development, such as angiogenesis or resorption of calcified cartilage, and is often associated with a decrease in diaphysis and total bone length [33 and 39].

The only other study to histologically examine the effects of prenatal ethanol exposure on developing bone is that of Miralles-Flores and Delgado-Baeza [25]. In that study, dams were fed ethanol for 30 d prior to and during gestation, and the tibia of offspring examined at birth or postnatal d15. Ethanol exposure resulted in decreased tibial length at both ages, but only affected the organization of the growth plate at postnatal d15. Ethanol decreased the length of the hypertrophic zone, which resulted in a decrease in total growth plate length. The length of the resting zone was not measured. The differences between our results and those of Miralles-Flores and Delgado-Baeza [25] may be due to differences in the ages of the rats (gestation day 21 vs. at birth vs. postnatal day 15), as the regulation of bone development differs between the pre- and postnatal periods. Thus, it is possible that the effect of prenatal ethanol exposure on the expression of local growth factors and/or systemic hormones that affect bone development differs during the prenatal and postnatal periods. Alternatively, it is possible that differences in level of ethanol exposure could account for the differences between the studies. It is common in many labs to remove dams from ethanol-containing diets for 1-2 d prior to birth as ethanol can cause complications during parturition. It is unknown if this was the case in the Miralles-Flores and Delgado-Baeza [25] study, but if so, the removal of ethanol during this period could have allowed the bone to partially recover, resulting in no significant effects on the growth plates of those pups at birth.

The mechanisms by which ethanol affects fetal bone development are unknown. Ethanol crosses the placenta [8] and so may have direct effects on bone development, or may act indirectly through effects on fetal/maternal hormones or nutrition. Ethanol has been shown to inhibit osteoblast proliferation and function in vitro [18] and thus prenatal ethanol exposure may affect fetal bone development through direct effects on osteoblasts. Moreover, chronic ethanol exposure in the adult results in increased total bone and bone marrow fat content in both humans and animals [7 and 38], and ethanol has been shown to inhibit osteogenic differentiation [7] and increase adipogenesis [38] in mesenchymal stem cells. Interestingly, preliminary data indicate that the tibia of fetal guinea pigs prenatally exposed to ethanol also has increased fat content (Keiver and Brien, unpublished data), thereby suggesting that ethanol also alters the balance between adipogenesis and osteogenesis in the fetus. In addition, ethanol intake is known to affect number of maternal/fetal systems, including the glucocorticoid [40], insulin-like growth factor [3] and calcium-regulating [1,15,16, and 17] systems, all of which could potentially affect bone development.

Ethanol's effects on the morphology of the growth plate in the present study were fairly subtle. The mechanism(s) underlying these effects will likely be difficult to elucidate, as ethanol affects multiple maternal/fetal systems that could alter bone development and may act by more than one mechanism. Moreover, the complexity of the regulatory cascades involved in bone development will make it challenging to pinpoint the mechanisms underlying ethanol's effects. Therefore it may be important to first perform studies to determine the functional significance of these effects. Previous work has shown that ethanol's effects on skeletal development do not resolve after birth but extend into postnatal life in both humans [9] and animals [21 and 25]. Although the implications of the effects of prenatal ethanol exposure on the long-term bone health of the offspring are unknown, studies have shown that changes in the prenatal environment may program bone cells during fetal life and result in long-term effects. For example, maternal protein restriction [23] and the administration of IL-1 [35] or a synthetic estrogen [24] to pregnant dams results in alterations in skeletal morphology, composition and cellular responsiveness that are apparent in the offspring in adulthood. Whether the subtle effects on the epiphysis seen in the current study will result in significant functional effects postnatally is an important area for investigation.

In conclusion, this study demonstrated that prenatal ethanol exposure resulted in a decrease in fetal bone length, which appeared to be due to a shorter diaphysis and a decrease in the length of the resting zone. Importantly, ethanol resulted in subtle changes in the organization of the epiphysis, specifically an increase in the length of the hypertrophic zone. The effects on the hypertrophic zone support the hypothesis that ethanol affects the later stages of bone development, such as those that occur during or after cartilage mineralization, while the effect on the resting zone indicates that there may also be effects on the earlier stages of the process. Further research is needed to determine the significance of these effects for long-term bone health and the mechanisms that underlie them.

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Figure 1. Morphometric parameters analyzed in fetal rat tibia stained with hematoxylin and eosin. (A) Total bone length, width, diaphyseal length and distal epiphyseal plate length were measured under 31.5X magnification. (B) The boundaries of the resting (RZ), proliferative (PZ) and hypertrophic (HZ) zones were delineated based on cell morphology under 63X magnification. The length of each zone was determined from the mean of five measurements per zone. (RZ, dotted line; PZ, dashed line; HZ, solid line). Scale bar in A and B represent 400 μm and 200 μm , respectively.

Figure 2.

Total length of the fetal tibia. (A) Tibiae from ethanol (E), pair-fed (PF) or ad lib control (C) fetuses at 31.5X magnification. There were no obvious differences, other than total bone length, among the tibia at this magnification. (B) The effect of maternal ethanol intake (E), pair-feeding (PF) or ad lib control (C) diet on the total length of the fetal tibia. Values are mean $\pm$ SE. Values for n are given in the bars. Groups sharing the same letter are significantly different by Newman-Keuls post hoc test: ^a, ^b $p < 0.001$.

Figure 3.

Resting, proliferative and hypertrophic zone lengths in the proximal epiphysis. The effects of maternal ethanol intake (E), pair-feeding (PF) or ad lib control (C) diet on (A) absolute length of the resting, proliferative and hypertrophic zones in the proximal epiphysis and (B) length of the resting, proliferative and hypertrophic zones in the proximal epiphysis relative to total tibial length. Values are mean $\pm$ SE. Values for n are given in the bars. Groups sharing the same letter are significantly different by Newman-Keuls post hoc test: (A) ^a $p = 0.027$, ^b $p = 0.019$, ^c $p = 0.016$, ^d $p = 0.055$ (B) ^a $p < 0.001$, ^b $p = 0.004$

Figure 4.

Area of the resting, proliferative and hypertrophic zones in the proximal epiphysis. The effects of maternal ethanol intake (E), pair-feeding (PF) or ad lib control (C) diet on (A) absolute area of the resting, proliferative and hypertrophic zones in the proximal epiphysis and (B) area of the resting, proliferative and hypertrophic zones in the proximal epiphysis relative to total tibial length. Values are mean $\pm$ SE. Values for n are given in the bars. Groups sharing the same letter are significantly different by Newman-Keuls post hoc test: (A) ^a $p = 0.016$, ^b $p = 0.024$.