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Effects of gestational and lactational exposure to ethanol on body and bone growth of rat offspring

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ABSTRACT

Introduction: Ethanol is the most widely consumed drug in the world. When this consumption occurs by pregnant women, the harmful effects extend to the fetus. There are few data available on its effects on infants. **Objective:** To evaluate the effects of maternal ethanol consumption during pregnancy (prenatal) as well as during pregnancy and lactation (pre- and post-natal). **Methods:** 12 Wistar rats were divided into ethanol and control groups. Ethanol group received ethanol (4 g/kg/day) via oral gavage from the ninth day of gestation to the 17th day of lactation. The weight and length of the pups were determined at birth and 17 days of age. The length and width of the femur and tibia and the thickness of the epiphysis were measured in the neonates, and the thickness of the articular cartilage, epiphyseal plate, and its zones were measured at 17 days of age. CDC-47 immunoexpression was evaluated and the expressions of aggrecan, collagen type II, and collagen type X were determined by RT-PCR. **Results:** In neonates, rats in the group with prenatal exposure to ethanol were significantly shorter (body length) than the control group. At 17 days of age, the femur of the group with pre-and postnatal exposure to ethanol showed a significantly lower percentage of proliferating chondrocytes, as evidenced by the different CDC-47 immunoexpression between groups. **Conclusion:** Prenatal exposure to ethanol in rats reduced body length at birth while pre- and postnatal exposure to ethanol decreased the proliferation of femoral epiphyseal plate chondrocytes.

Keywords: fetal alcohol spectrum disorder; ethanol; lactation; osteogenesis; prenatal care.

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INTRODUCTION

Bone formation and growth from intrauterine life to puberty occur through endochondral and intramembranous ossification. Endochondral ossification occurs in most bones of the skeleton, including long bones, and begins with the formation of a cartilage template that is gradually replaced by bone[1]. This template is formed by chondrocytes and the extracellular matrix (ECM) components such as aggrecan, type II collagen, and type X collagen, which interact with each other and induce the expression of factors that coordinate the ossification process[2]. Chondrocytes in the template must proliferate,

undergo hypertrophy, and activate the expression of factors crucial for ossification, whereas ECM components support the cells and express factors that prepare the site for the transition from cartilage to bone[3].

After birth, the epiphyseal plate and articular cartilage are formed from remnants of the cartilaginous template. These regions are responsible for longitudinal bone growth during postnatal life. The mechanisms that regulate cellular and molecular events in bone development are complex and interconnected[3]. Any disturbances in these regulatory mechanisms potentially cause growth alterations. Ethanol is one such toxic agent known to affect fetal growth regulation[4].

Ethanol is the most consumed drug worldwide[5]. When consumed by pregnant women, the harmful effects extend to the fetus, as ethanol freely passes through the placenta and reaches fetal blood circulation at concentrations like those found in maternal blood[6]. The condition in which the fetus meets ethanol ingested by the mother is known as prenatal ethanol exposure (PEE). Several associated growth alterations vary significantly in intensity and manifest in different organs[7]. In long bones, PEE is associated with shorter stature[8], shorter bones[9], and alterations in the thickness of epiphyseal plate zones[4]. In addition, PEE predisposes individuals to osteoarthritis in adulthood[10]. Various changes associated with PEE may be related to the action of individual genetic factors that predispose some individuals to the harmful effects of ethanol[11]. Identifying sensitive genes and putative susceptibility markers may be a strategy for preventing future injuries in predisposed individuals[8], considering that no existing therapy can reverse the PEE-induced damage[12].

The effects of ethanol have been studied in the prenatal period[4,7,8], however, many structures, such as bones, are still under development in breastfed individuals. Therefore, they are still vulnerable to external agents that may interfere with growth[13]. We hypothesize that ethanol can be potentially harmful to pups during lactation, as it can be transferred from maternal blood to milk[14-16].

Thus, the objective of this work was to evaluate the effects of maternal ethanol consumption during pregnancy (prenatal) as well as during pregnancy and lactation (pre- and postnatal).

METHODS

The experiment was approved by the Ethics Committee on Animal Use of the *Universidade Federal do Espírito Santo* (registration number 30/2018).

Mating and ethanol administration

Seventeen two-month-old Wistar rats (5 males and 12 females) were used. The rats were maintained in a 12-h light/dark cycle, and the room temperature was maintained at 22±2 °C. All females underwent vaginal cytology to determine the estrous cycle phase[17] and identify the ideal moment for copulation. The presence of sperm in vaginal cytology was determined on day zero of pregnancy (G0).

From G0 onwards, the rats were divided into two groups of six animals each: a group with ethanol consumption from the ninth day of gestation (G9) to the 17th of lactation (ethanol group, n=6), and without ethanol consumption during pregnancy and lactation (control group, n=6). The ethanol consumption group received 4 g ethanol/kg/day, and the alcohol solution was prepared at a concentration of 40% v/v (Ethanol P.A. [Merck/Germany] diluted in distilled water). The volume administered was adjusted weekly according to the weight of the animals[9], and administration was performed by an orogastric tube (gavage) two hours after the start of the light cycle[18]. Animals in the control group received an equal volume of distilled water and experienced comparable stress of manipulation and gavage. Analyses were performed on samples from pups collected at birth and on the 17th postnatal day.

Measurements of body weight and length, and femur and tibia length and width

Mean measurements of body weight and length, as well as the length and width of the right femur and tibia, were obtained from three pups randomly chosen from each mother. The length of the animal was determined by measuring the distance between the nape and the base of the tail. Measurements of the right femur and tibia were performed with a digital caliper. The length of each bone was measured from the proximal epiphysis surface to the distal epiphysis surface, whereas the width was measured in the mid-diaphysis.

Histological processing and histomorphometric analyses of growth cartilages

The right femur and tibia of one animal from each offspring (n=6) were fixed in 10% buffered formalin and decalcified with EDTA. Subsequently, the bones were longitudinally sectioned, processed using the paraffin embedding technique, subjected to microtomy, and stained using the hematoxylin-eosin (HE) technique. In newborn rats, the growth plate was measured along the entire length of the cartilaginous epiphysis of the distal femur and proximal tibia using the average of three equidistant measurements in a 10x objective.

The hypertrophic zone was measured using an average of 15 equidistant points in a 10x objective. In the distal femur and proximal tibia of 17-day-old rats, the total thickness of the growth plate corresponding to each zone (resting, proliferative, and hypertrophic) was evaluated. Furthermore, the articular cartilage thickness and deep zone were evaluated. Fifteen measurements were performed in three different fields using a 10x objective. All the measurements were performed using a Leica DM500 optical microscope equipped with a Leica ICC50 camera.

Immunohistochemical analyses

Histological sections of the distal femur and proximal tibia of one animal from each offspring (n=6) were used for immunohistochemical analyses. In neonatal rats, immunostaining analysis was performed in three distinct fields in the central epiphysis region. In 17-day-old rats, three distinct fields of the proliferative zone were evaluated.

Anti-CDC47 antibody (47DC14; Neomarkers, Fremont, CA/USA; 1:100 dilution for rat bones at birth and 1:50 for rat bones at 17 days of age) was used with streptavidin-biotin-peroxidase (Dako, St Louis, MO/USA). Antigen retrieval was performed by heating samples in a water bath at 98 °C. Histological sections were incubated in a humid chamber to block endogenous peroxidase and nonspecific proteins (Dako, St Louis, MO/USA) and incubated for 12 h with the primary antibody. Incubation was then performed with a secondary antibody, followed by streptavidin-peroxidase. Diaminobenzidine was used as chromogen (Dako, St Louis, MO/USA).

Histological sections were counterstained with Harris hematoxylin. Negative control was obtained by omitting the primary antibody, and histological sections of the rat spleen were used as positive controls. In samples from neonatal rats, 200 cells were counted in three different fields of the central region of the cartilaginous epiphysis using a 40x objective. Stained cells were classified as CDC47 positive, and the values were converted to percentages. In samples from rats at 17 days of age, the analysis was performed by counting 100 cells in three different fields of the proliferative zone in a 10x objective. Cells that showed staining were classified as CDC47-positive, and the values were converted to percentages.

Expression of gene transcripts by RT-qPCR in cartilage

The expression quantification of aggrecan, collagen type II, and collagen type X was performed by quantitative reverse transcription-polymerase chain reaction (RT-qPCR) in fragments of the articular cartilage of the left femur of newborn and 17-day-old rats. For this analysis, the articular cartilage of two animals from each offspring was collected and mixed to make one sample (n=6). The cartilage was placed in TRIzol™ (Invitrogen, Carlsbad, CA/USA), frozen in liquid nitrogen for 2 h, and then stored in a freezer at −80 °C. Extraction of mRNA was performed using TRIzol™ according to the manufacturer's instructions. RNA concentration was determined by using a spectrophotometer. For cDNA synthesis, a commercial SuperScript® III First-Strand Synthesis SuperMix kit (Invitrogen, Carlsbad, CA/USA) was used according to the manufacturer's instructions. Subsequently, a commercial Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Carlsbad, CA/USA) was used according to the manufacturer's instructions. The primers were designed based on the Rattus norvegicus mRNA sequence: GAPDH (forward: TTCTTGTGCAGTGCCAGCC, reverse: GTAACCAGGCGTCCGATACG), aggrecan (forward: CACACGCTACACACTGGACT, reverse: TCACACTGGTGGAAGCCATC), collagen type II (forward: GTTCACGTACACTGCCCTGA, reverse: AAGGCGTGAGGTCTTCTGTG) collagen type X (forward: GAAACAGGTGTCTGACTTAC, reverse: TACTTCCAGTGGAATAGAAG). Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. The results obtained for each group were quantitatively compared after normalization to the *Rattus norvegicus* GAPDH expression.

Statistical analysis

The study design was completely randomized, and the mean and standard deviation were determined for each variable. Means were compared using the student's t-test. All data were analyzed using the GraphPad Prism computational package. Differences were considered statistically significant at $p<0.05$.

RESULTS

All the females used in the experiment were pregnant. The gestational period ranged from 20–22 days. No significant difference was observed in the weight of female mothers between the groups (data not shown).

Body weight and length and bone length and width

No significant difference was observed in the weight of the animals at birth and 17 days of age between the groups (Table 1). Neonates in the ethanol group were significantly shorter than those in the control group. However, no significant difference in body length was observed at 17 days of age between the groups (Figure 1). Additionally, no significant differences were observed between the groups regarding the length and width of the femur and tibia of neonates and 17-day-old rats (Figure 2).

Table 1: Mean and standard deviation of weight and body length of rats at birth (newborn) and 17 days of age in the control and ethanol groups. *p<0.05.

	Newborn (n=6)		17 days (n=6)	
	Control	Ethanol	Control	Ethanol
Weight (g)	6.12 ±0.97	5.75 ±0.51	33.88 ±4.61	32.71 ±2.73
Body length (mm)	34.35 ±1.79	31.9 ±1.83*	65.31 ±4.65	64.9 ±3.02

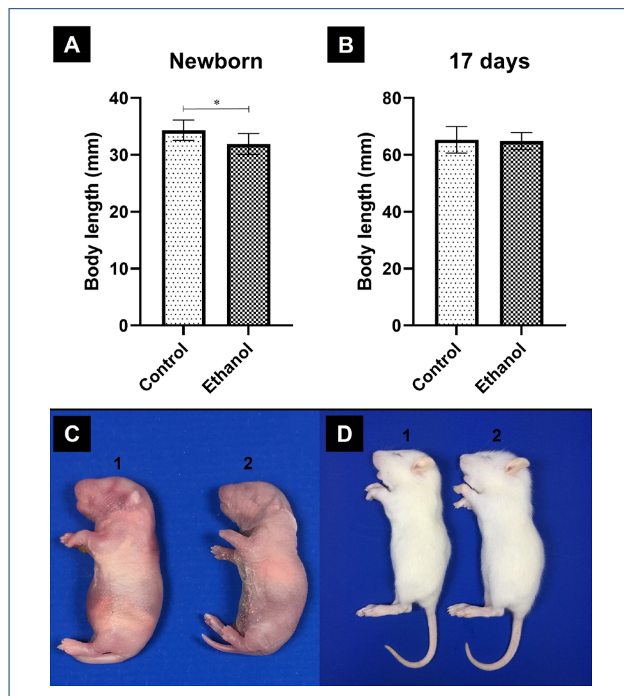

Figure 1: Body length assessment of rats at birth and 17 days of life. A) Mean ± standard deviation of body length (mm) of animals at birth in the control and ethanol groups (n=6). B) Mean ± standard deviation of body length (mm) of animals at 17 days of age in the control and ethanol groups (n=6). C) Rat pups at birth illustrate size differences between animals. C-1: Pup from the control group. C-2: Pup from the ethanol group. D) Rat pups at 17 days of age illustrating similarity in body size between animals. D-1: Pup from the control group. D-2: Pup from the ethanol group. *Statistical difference ($p<0.05$).

Histomorphometry and cartilage proliferative index

Regardless of the bone, the thickness of the cartilaginous epiphysis of the neonate, as well as the epiphyseal plate of the 17-day-old rat and its zones, did not differ significantly between groups (data not shown).

The proliferation rate of tibial and femoral chondrocytes, assessed by CDC47 immunoexpression, did not differ significantly between the groups in neonatal rats (Figure 3). However, in 17-day-old animals, the proliferative zone of the femoral epiphyseal plate of rats exposed to ethanol presented significantly less proliferation, meaning a lower percentage of CDC47-positive cells than that in control (Figure 4).

Expression of aggrecan, type II collagen, and type X collagen in cartilage

At birth and 17 days of age, no significant differences in the gene expression of Aggrecan, type II collagen, and type X collagen transcripts from the femoral articular cartilage were observed between the groups (data not shown).

DISCUSSION

Prenatal exposure to ethanol in rats causes intrauterine growth restriction and reduces body length at birth. Notably, the size of the pups at weaning was equal between the groups, although exposure to ethanol persisted in the postnatal period. The effects of ethanol on the tibia and femur were different. Compared to the control, prenatal and postnatal exposure to ethanol decreased the proliferation of chondrocytes from the epiphyseal plate solely in the femur without reducing the bone length in the same period.

The choice of ethanol administration using oral gavage was made based on the pattern of consumption observed in humans, which shows a growing percentage of binge alcohol consumption when copious amounts of ethanol are ingested in a brief period of time[19]. The administered dose was 4 g/kg ethanol, which generated a mean concentration of 87 mM in maternal blood circulation. This concentration can be easily achieved by individuals who consume moderate amounts of alcoholic beverages. As a result of maternal consumption, ethanol is found at an average concentration of 58 mM in fetal circulation[20].

Ethanol was administered from the ninth day of gestation, which correlates to the fourth week of human gestation when the fetal bone is developed, and limb development begins[11]. Pups were collected at birth and at 17 days of age to represent the effects of exclusive PEE (at birth) and PEE associated with ethanol transfer through lactation (17 days of life). In an experimental model, it was proven that puppies that feed on milk from females that consume alcoholic solutions have ethanol in their blood, thus demonstrating that breastfeeding constitutes a form of exposure of puppies to ethanol[21]. The spontaneous weaning of rats occurs at the end of the third week of life[13]. However, samples were collected on the 17th day to obtain animals fed exclusively on milk since pups begin to ingest solid food from the 18th day[22].

The analysis began with the measurement of body weight and length. At birth, the animals had similar body weights. However, the body length of rats at birth was significantly shorter in the PEE group. One of the problems associated with ethanol consumption is inadequate nutrition[23]. Alcoholics often fail to eat properly when drinking alcoholic beverages and thus experience weight loss and malnutrition, which can impair fetal growth in pregnant women[24]. In addition, the energy provided by ethanol interferes with nutrient absorption[23].

In the present study, no difference in weight was observed in the mothers (data not shown). In other experiments conducted by our team, no interference of maternal consumption of ethanol on the weight gain of the mothers was observed[25,26]. Thus, females who consumed ethanol did not present a malnutrition condition that could affect fetal development. Therefore, the data points to a shorter stature associated with intrauterine exposure to ethanol, without the direct involvement of nutritional factors. Children and adolescents with PEE are smaller than average[8], thus demonstrating that humans are susceptible to a clinical manifestation of PEE like that found in rats.

		Newborn (n=6)		17 days (n=6)	
		Control	Ethanol	Control	Ethanol
Femur	Length (mm)	6.91 ±0.41	6.81 ±0.43	15.45 ±0.7	15.51 ±0.43
	Width (mm)	0.95 ±0.04	0.96 ±0.06	2.1 ±0.11	2.07 ±0.07
Tibia	Length (mm)	7.7 ±0.45	7.46 ±0.84	19.4 ±0.81	19.53 ±0.78
	Width (mm)	0.8 ±0.05	0.78 ±0.05	1.57 ±0.06	1.52 ±0.08

Figure 2: Macroscopic analysis of long bones of rats at birth (newborn) and 17 days of age. A) Mean ± standard deviation of femoral and tibial length and width of rats at birth (newborn) and 17 days of age in the control and ethanol groups. B) Bone samples from newborn rats. C) Bone samples from rats at 17 days of age. No significant difference were observed between the groups regarding the length and width of the femur and tibia ($p \geq 0.05$).

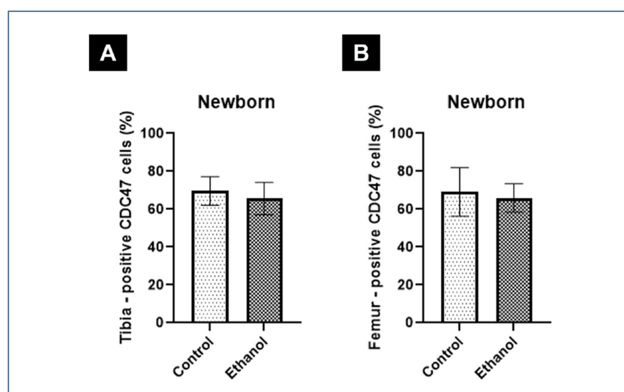

Figure 3: Percentage of anti-CDC47 immunostaining-positive cells in the tibia (A) and femur (B) of neonatal rats (n=6).

Pups with PEE presented a shorter body length at birth; however, at 17 days of age, the animals overcame the initially shorter stature despite continuous exposure to ethanol in the postnatal period. While some studies report that growth impairments extend throughout life[8,20], others indicate that accelerated growth may occur after birth, compensating for the delay in intrauterine development[27]. In a study conducted in mice[28], it was demonstrated that PEE between days 0.5 and 8.5 of gestation has different effects on pup weight, depending on measurement time. Fetuses with PEE collected in G16 did not show growth restriction, having the same weight as the control animals. However, on the 21st day of life, mice with PEE were lighter than the animals in the control group. This trend was maintained until the 5th day

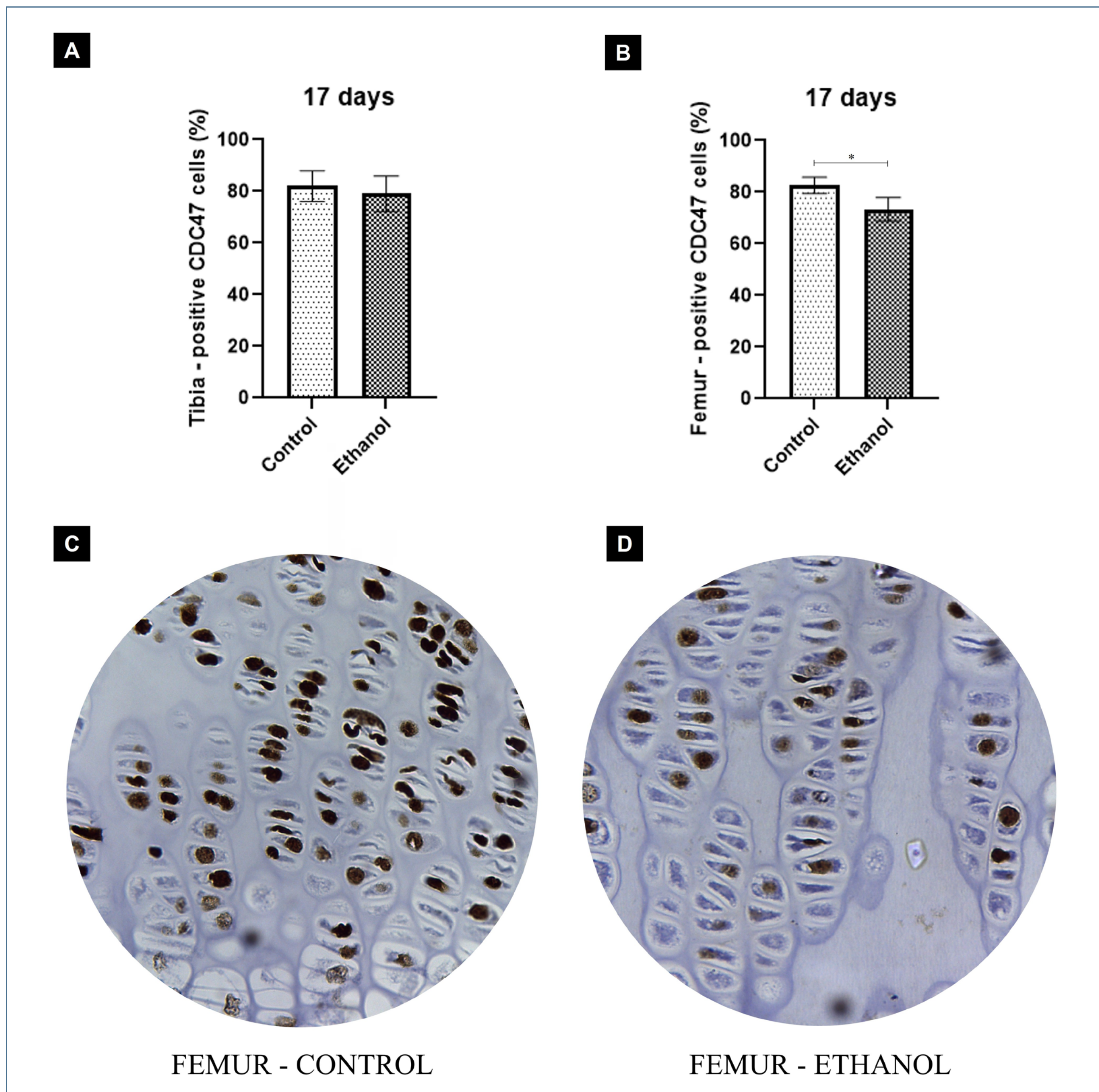

Figure 4: Anti-CDC47 antibody immunostaining in 17-day-old rats. A) Percentage of cells with positive immunostaining in the tibia (n=6). B) Percentage of cells with positive immunostaining in the femur (n=6). *Statistical difference (p<0.05). C) Femur from the control group showing all nuclei with positive immunostaining in the proliferation zone of the epiphyseal plate. Counterstained with Harris hematoxylin. D) Femur from the ethanol group demonstrating decreased immunostaining-positive cells in the proliferation zone of the epiphyseal plate. Counterstained with Harris hematoxylin.

of life, indicating that PEE in the first week of gestation results in postnatal growth restriction. However, from the sixth week onwards, the weight of the animals becomes statistically similar, indicating compensatory growth. A compensatory mechanism was not identified in our study; however, it did not seem to be related to differences in maternal fitness between groups or litter size. One of the possible mechanisms involved in postnatal compensatory growth is a systemic increase in the concentration of insulin-like growth factor (IGF). IGF-II is related to prenatal growth; after birth, there is a predominance conversion from IGF-II to IGF-I. However, children with PEE have low levels of IGF-II soon after birth, and these levels increase after 1 year of age, which contradicts expectations. The authors believe that if there is adequate postnatal nutrition, the organism can activate compensatory growth mechanisms[27].

PEE can induce various morphological changes in growth cartilages according to various methodologies. In a model of spontaneous consumption of an ethanol-containing liquid diet,

a thickness increase of the tibial hypertrophic zone and reduction of the resting zone was observed in rat fetuses collected at the end of the gestational period, i.e., on the 21st day of gestation[4]. Four-week-old females from offspring with PEE between days G9 and G20 via maternal gavage showed a reduction in the femoral epiphyseal plate thickness[29]. Additionally, adult rats from offspring with PEE between days G11 and G20 had a reduced number of chondrocytes in the articular cartilage superficial zones of the femur and tibia[30]. In our study, the ethanol exposure started in G9 through gavage did not impose significant changes in bone length and histomorphometry of growth cartilages in neonates and pups at weaning. Similarly, a study performed on the tibia of rats with gavage-induced PEE presented no changes in bone length when ethanol was administered between days G1 and G19[31].

To verify the proliferative activity of chondrocytes, immunohistochemical analysis was performed for CDC-47, a protein present in the nucleus of proliferating cells[32]. At birth, no difference was observed between groups; however, at 17 days, the number of CDC-47 positive chondrocytes was reduced in the proliferative zone of the femoral epiphyseal plate of treated rats. This result proves that ethanol inhibits chondrocyte proliferation, and this effect does not affect all bones equally, as no changes were found in the tibia. To the best of our knowledge, this is the first study to evaluate in vivo the effect of ethanol on chondrocyte proliferation in rat offspring exposed to ethanol from the gestational period to the end of the lactation period. However, several studies have evaluated the proliferation of other cell types, such as central nervous system cells. A study reported that ethanol inhibits the proliferation of glial cells[33], whereas another work observed a reduction in the proliferation of cells in the hippocampus[34]. In bone tissue, human osteoblasts cultured in a medium containing ethanol showed a reduction in cell proliferation. The authors suggested that the reduction in osteoblast proliferation caused by ethanol may be one of the mechanisms involved in osteopenia observed in alcoholics[35].

The growth rate of the cartilage epiphyseal plate is related to the ability of chondrocytes to proliferate and the hypertrophy of terminal chondrocytes. The cells have a defined and predetermined proliferative capacity. Thus, the initial accelerated bone growth slows down as the chondrocytes replicate because the cells enter a physiological process known as senescence[36]. Therefore, conditions that inhibit chondrocyte proliferation are believed to slow the growth plate senescence, resulting in temporary inhibition of bone growth that may be followed by faster-than-expected compensatory growth[37]. The femur showed no significant difference in thickness and length between groups, although the chondrocyte proliferation rate differed between the groups. One reason for this is that signs of reduced bone growth take some time to appear when the rate of chondrocyte proliferation is impaired. Hence, the reflexes on femoral length of lower proliferative activity of the femoral chondrocytes of 17-day-old animals would have been observed at a later stage. This reduction in proliferative activity appeared to be delayed since it didn´t caused reduction in either the bone length or the thickness of the proliferation zone. Notably, the effects of ethanol on the lactation period represent the sum of what was administered from the ninth day of pregnancy until the moment the consequences appeared. The lactation period in rats is short; however, this is a critical issue that must be considered for women.

To identify molecules critical for endochondral ossification that could be affected by ethanol, expression levels of aggrecan, collagen type II, and collagen type X gene transcripts were evaluated. Aggrecan is a structural proteoglycan that mediates cell-cell interactions and interactions between cells and the extracellular matrix. It forms a gel that lubricates the joint and, together with type II collagen, confers the cartilaginous resistance properties[2]. Chondrocyte maturation in the epiphyseal plate leads to a reduction in the production of type II collagen and an increase in type X collagen as the cells mature. This altered collagen expression is vital because it prepares the tissue for the final steps that precede mineralization[38]. Changes in the expression and synthesis of these components can result in several types of damage, ranging from impaired chondrocyte survival to chondrodysplasia[2]. In our previous study using cultures of articular chondrocytes from neonatal rats, the addition of ethanol to the culture medium reduced the expression of aggrecan without altering the expression of type II collagen[39]. However, in the present study, no changes were found in the expression of any of the three evaluated components of the extracellular matrix, indicating that these are not the preferred targets of ethanol in vivo. When extrapolating to an in vivo situation, the direct mechanisms that result in the changes in vitro may be suppressed by several factors simultaneously affected by ethanol throughout the organism[40]. Another possibility is that the clinical manifestations observed in individuals with PEE occur due to alterations in later transcription stages, such as protein synthesis, which was not evaluated in this study.

The results from this study collectively suggest that prenatal exposure to ethanol in rats reduces body length at birth and that this parameter is equal between groups at weaning, despite remaining postnatal exposure to ethanol. Pre- and postnatal exposures to ethanol decrease the proliferation of femoral epiphyseal plate chondrocytes compared to that in the control.

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REFERENCES

1. Grigoriou E, Trocle A, Dormans JP. The growth plate: embryologic origin, structure, and function. In: Polin RA, Abman SH, Rowitch DH, Benitz WE, Fox WW. Fetal and neonatal physiology. 5th ed. V. 2; Philadelphia: Elsevier; 2017; p. 1421-9.e2.

2. Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M. Endochondral ossification: How cartilage is converted into bone in the developing skeleton. Int J Biochem Cell Biol. 2008;40(1):46-62. https://doi.org/10.1016/j.biocel.2007.06.009

3. Ono N, Kronenberg HM. Developmental biology of musculoskeletal tissues for tissue engineers. In: Stoddart MJ, Craft AM, Pattappa G, Gardner OFW. Developmental biology and musculoskeletal tissue engineering: principles and applications. London: Academic Press; 2018; p. 1-24.

4. Snow ME, Keiver K. Prenatal ethanol exposure disrupts the histological stages of fetal bone development. Bone. 2007;41(2):181-7. https://doi.org/10.1016/j.bone.2007.04.182

5. Lovely CB. Animal models of gene–alcohol interactions. Birth Defects Res. 2019;112(4):367-79. https://doi.org/10.1002/bdr2.1623

6. Popova S, Charness ME, Burd L, Crawford A, Hoyme HE, Mukherjee RAS, et al. Fetal alcohol spectrum disorders. Nat Rev Dis Primers. 2023;9:11. https://doi.org/10.1038/s41572-023-00420-x

7. Dörrie N, Föcker M, Freunscht I, Hebebrand J. Fetal alcohol spectrum disorders. Eur Child Adolesc Psychiatry. 2014;23(10):863-75. https://doi.org/10.1007/s00787-014-0571-6

8. Day NL, Leech SL, Richardson GA, Cornelius MD, Robles N, Larkby C. Prenatal alcohol exposure predicts continued deficits in offspring size at 14 years of age. Alcohol Clin Exp Res. 2002;26(10):1584-91. https://doi.org/10.1097/01.ALC.0000034036.75248.D9

9. Pan Z, Zhang X, Shangguan Y, Hu H, Chen L, Wang H. Suppressed osteoclast differentiation at the chondro-osseous junction mediates endochondral ossification retardation in long bones of Wistar fetal rats with prenatal ethanol exposure. Toxicol Appl Pharmacol. 2016;305:234-41. https://doi.org/10.1016/j.taap.2016.06.021

10. Ni Q, Wang L, Wu Y, Shen L, Qin J, Liu Y, et al. Prenatal ethanol exposure induces the osteoarthritis-like phenotype in female adult offspring rats with a post-weaning high-fat diet and its intrauterine programming mechanisms of cholesterol metabolism. Toxicol Lett. 2015;238(2):117-25. http://dx.doi.org/10.1016/j.toxlet.2015.07.017

11. Fish EW, Murdaugh LB, Sulik KK, Williams KP, Parnell SE. Genetic vulnerabilities to prenatal alcohol exposure: Limb defects in sonic hedgehog and GLI2 heterozygous mice. Birth Defects Res. 2017;109(11):860-65. https://doi.org/10.1002/bdr2.1026

12. Ramadoss J, Magness RR. Vascular effects of maternal alcohol consumption. Am J Physiol Hear Circ Physiol. 2012;303(4):H414-21. https://doi.org/10.1152/ajpheart.00127.2012

13. Barbosa MMB, Aguiar Júnior FCA, Maia CS, Tenório FCAM, Oliveira LS, Medeiros JP. Aspectos morfológicos do estômago de ratos submetidos ao desmame precoce. Brazilian J Heal Ver. 2020;3(5):13876-89. https://doi.org/10.34119/bjhrv3n5-200

14. Kesäniemi YA. Ethanol and Acetaldehyde in the Milk and Peripheral Blood of Lactating Women After Ethanol Administration. J Obstet Gynaecol Br Commonw. 1974;81(1):84-6.

15. Carmo MGT, Nascimento CMO, Martin A, Herrera E. Ethanol intake during lactation impairs milk production in rats and affects growth and metabolism of suckling pups. Alcohol. 1999;18(1):71-6. https://doi.org/10.1016/s0741-8329(98)00070-6

16. Giglia RC. Alcohol and lactation: An updated systematic review. Nutr Diet. 2010;67(4):237-43. https://doi.org/10.1111/j.1747-0080.2010.01469.x

17. Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: Some helpful considerations. Brazilian J Biol. 2002;62(4A):609-14. https://doi.org/10.1590/S1519-69842002000400008

18. Walker BM, Ehlers CL. Age-related differences in the blood alcohol levels of Wistar rats. Pharmacol Biochem Behav. 2009;91(4):560-5. https://doi.org/10.1016/j.pbb.2008.09.017

19. Jones SA, Lueras JM, Nagel BJ. Effects of binge drinking on the developing brain. Alcohol Res. 2018;39(1):87-96.

20. Shen L, Liu Z, Gong J, Zhang L, Wang L, Magdalou J, et al. Prenatal ethanol exposure programs an increased susceptibility to non-alcoholic fatty liver disease in female adult offspring rats. Toxicol Appl Pharmacol. 2014;274(2):263-73. http://dx.doi.org/10.1016/j.taap.2013.11.009

21. Sengupta P. The laboratory rat: Relating its age with humans. Int J Prev Med. 2013;4(6):624-30.

22. Vilaró S, Viñas O, Remesar X, Herrera E. Effects of Chronic Ethanol Consumption on Lactational Performance in Rat: Mammary Gland and Milk Composition and Pups' Growth and Metabolism. Pharmacol Biochem Behav. 1987;27(2):333-9. https://doi.org/10.1016/0091-3057(87)90577-6

23. Barve S, Chen SY, Kirpich I, Watson WH, McClain C. Development, prevention, and treatment of alcohol-induced organ injury: the role of nutrition. Alcohol Res. 2017;38(2):289-302.

24. Young JK, Giesbrecht HE, Eskin MN, Aliani M, Suh M. Nutrition implications for fetal alcohol spectrum disorder. Adv Nutr. 2014;5(6):675-92. https://doi.org/10.3945/an.113.004846

25. Cardoso KMMC, Gomes LA, Reis AMS, Silva CMO, Tamiasso NV, Serakides R, et al. Phenotype and synthesis activity of joint chondrocytes extracted from newborn rats with prenatal ethanol exposure. Hum Exp Toxicol. 2021;40(12 Suppl):S414-22. https://doi.org/10.1177/09603271211045949

26. Gomes LA, Cardoso KMMC, Reis AMS, Melo FG, Serakides R, Ocarino NM. Effect of ethanol consumption during pregnancy and lactation on bone histomorphometry and in vitro osteogenic differentiation of bone marrow mesenchymal stem cells in maternal rats. Alcohol. 2021; 95:51-64. https://doi.org/10.1016/j.alcohol.2021.07.001

27. Aros S, Mills JL, Iñiguez G, Avila A, Conley MR, Troendle J, et al. Effects of prenatal ethanol exposure on postnatal growth and the insulin-like growth factor axis. Horm Res Paediatr. 2011;75(3):166-73. https://doi.org/10.1159/000319706

28. Kaminen-Ahola N, Ahola A, Maga M, Mallitt KA, Fahey P, Cox TC, et al. Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. PLoS Genet. 2010;6(1):e1000811. https://doi.org/10.1371/journal.pgen.1000811

29. Wu Z, Pan Z, Wen Y, Xiao H, Shangguan Y, Wang H, et al. Egr1/p300/ACE signal mediates postnatal osteopenia in female rat offspring induced by prenatal ethanol exposure. Food Chem Toxicol. 2020;136:111083. https://doi.org/10.1016/j.fct.2019.111083

30. Ni Q, Tan Y, Zhang X, Luo H, Deng Y, Magdalou J, et al. Prenatal ethanol exposure increases osteoarthritis susceptibility in female rat offspring by programming a low-functioning IGF-1 signaling pathway. Sci Rep. 2015;5:14711. https://doi.org/10.1038/srep14711

31. Perry V. Effects of prenatal alcohol exposure on 3-week-old Sprague-Dawley rat proximal tibia: an immunohistochemical and three-dimensional micro-computed tomography X-ray investigation. [Dissertation]. [Johannesburg]: University of the Witwatersrand, 2018; p. 49-80.

32. Rojiani M V, Siemann DW, Rojiani AM. Cell proliferation index determination by immunohistochemical detection of hCDC47 protein. Appl Immunohistochem Mol Morphol. 2010;18(3):278-82. https://doi.org/10.1097/PAI.0b013e3181c6c949

33. Guizzetti M, Catlin M, Costa LG. Effects of ethanol on glial cell proliferation: relevance to the fetal alcohol syndrome. Front Biosci. 1997;2(5):93-8. https://doi.org/10.2741/a230

34. Redila VA, Olson AK, Swann SE, Mohades G, Webber AJ, Weinberg J, et al. Hippocampal cell proliferation is reduced following prenatal ethanol exposure but can be rescued with voluntary exercise. Hippocampus. 2006;16(3):305-11. https://doi.org/10.1002/hipo.20164

35. Friday KE, Howard GA. Ethanol inhibits human bone cell proliferation and function in vitro. Metabolism. 1991;40(6):562-5. https://doi.org/10.1016/0026-0495(91)90044-w

36. Lui JC, Jee YH, Garrison P, Iben JR, Yue S, Ad M, et al. Differential aging of growth plate cartilage underlies differences in bone length and thus helps determine skeletal proportions. PLoS Biol. 2018;16(7):e2005263. https://doi.org/10.1371/journal.pbio.2005263

37. Gafni RI, Weise M, Robrecht DT, Meyers JL, Barnes KM, De-Levi S, et al. Catch-up growth is associated with delayed senescence of the growth plate in rabbits. Pediatr Res. 2001;50(5):618-23. https://doi.org/10.1203/00006450-200111000-00014

38. Staines KA, Pollard AS, McGonnell IM, Farquharson C, Pitsillides AA. Cartilage to bone transitions in health and disease. J Endocrinol. 2013;219(1):R1-12. https://doi.org/10.1530/JOE-13-0276

39. Tamiasso NV, Silva CMO, Reis AMS, Ocarino NM, Serakides R. Ethanol alters phenotype and synthesis activity of rat neonatal articular chondrocytes grown in 2- and 3-dimensional culture. Cartilage. 2019;13(12 Suppl):839S-46. https://doi.org/10.1177/1947603519870862

40. Maran A, Zhang M, Spelsberg TC, Turner RT. The dose-response effects of ethanol on the human fetal osteoblastic cell line. J Bone Miner Res. 2001;16(2):270-6. https://doi.org/10.1359/jbmr.2001.16.2.270