



## Research Article

# Prenatal Alcohol Exposure Alters Osteoblast Gene Expression in Newborn Rats – Screening Study

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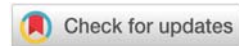
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## Abstract

This study examined the impact of prenatal alcohol exposure on osteoblast gene expression in newborn rats. Sixteen Wistar rats were divided into three groups based on their diet: Alcohol, Pair-fed, and Control. Each group received the specific diet for eight weeks before breeding and during the three weeks of gestation. The newborns were euthanized on the fifth day of life, and their calvaria were processed to isolate primary osteoblasts by sequential enzymatic digestion and then cultured for 10 days. After 10 days of osteogenic cell growth, the cells were processed to extract total RNA. Osteogenesis RT Profiler Rat PCR array was used to evaluate gene expression patterns among all groups. The Control group consumed more solids and liquids than the other groups ( $p < 0.0001$ ). The Control ( $p > 0.005$ ) and Alcohol ( $p > 0.005$ ) groups showed a tendency to weight gain, while the Pair-fed group showed a tendency to lose weight ( $p > 0.005$ ). Out of 84 genes analyzed, 17 showed significant differences between the Alcohol and Pair-fed groups ( $p < 0.05$ ). Of these, some were downregulated, and others upregulated in the Alcohol group compared to the Pair-fed group. In conclusion, prenatal alcohol exposure alters gene expression related to osteogenic differentiation in newborn rats, with a possible consequence on fetal skeletal development.

## Introduction

The teratogenic effects of alcohol are well-documented, and the term “fetal alcohol spectrum disorders (FASD)” has been used to include diverse phenotypes after prenatal alcohol exposure [1-6]. Among all changes that prenatal ethanol exposure causes to the fetus and/or newborns, bone growth

and quality disturbances have been studied in experimental models [7-17]. Additionally, experimental results have demonstrated an effective action of prenatal alcohol exposure on the bone tissue of the fetus and/or newborns, however, the mechanisms in which the alcohol acts on bone tissue of the newborns causing growth delay are still unclear. Under this perspective, our research group studied the effect of

prenatal alcohol exposure on newborns osteoblasts. The first study demonstrated that prenatal alcohol exposure increased osteoblasts proliferation early and increased alkaline phosphatase (Alpl) activity and bone matrix formation in more advanced periods [18]. The second study demonstrated that the prenatal exposure to ethanol induced DNA damage in osteoblasts, as shown by micronucleus formation and a higher percentage of DNA in the comet tail [19]. Taken together, these results suggest that prenatal ethanol consumption has a direct effect on fetal osteoblasts.

Therefore, a gap in the literature regarding how prenatal alcohol exposure affects bone tissue in fetuses/newborns exists. Studies showed that alcohol acts on bone tissue causing various changes such as delayed ossification, body weight loss, reduced individual bone length, and ensuing overall bone growth delay [7,8,10-13,15,20-25]. However, these are consequences of the alcohol action on bone tissue, but the mechanisms by which these changes occur are still unknown. Our previous studies indicated a pathway in which alcohol affects the bone tissue of fetuses/newborns from alcoholic mothers by affecting their offspring osteoblasts [18,19], thus, this study hypothesizes that prenatal alcohol exposure may lead to defects in bone by affecting osteoblast gene expression.

## Materials and methods

### Animals

All animal experiments were approved by the Ethical Committee in Research of São José dos Campos Institute of Science and Technology, UNESP – São Paulo State University (Protocol No. 01/2012-PA/CEP).

A group of sixteen nulliparous female Wistar rats, aged five weeks old, were randomly divided into three groups: i) Alcohol group, the animals were fed a 20% ethanol solution and a solid diet *ad libitum*. The 20% alcohol solution was obtained by dilution of absolute ethanol (ethyl alcohol P.A. ACS-99.5%, Merck, Darmstadt, Germany) in water; ii) Pair-fed group, the animals received an equivalent amount of solid diet and carbohydrate solution as the Alcohol group, and their calorie intake was matched accordingly. To achieve the calorie equivalence, the amount of alcohol and solid diet consumed by the Alcohol group was measured on the previous day, and on the following day, the Pair-fed group was given the same amount of solid diet and carbohydrate solution, as previously described [26,27], along with unrestricted access to water. iii) Control group, the animals had unrestricted access to water and solid food. The intervention involved the voluntary consumption of liquid and solid diets through oral self-administration.

After 8-weeks of dietary control, female rats were mated with Wistar males. The presence of a vaginal plug was used as an indicator of successful mating and designated as gestational day zero. The offspring remained with their mothers, who continued the same dietary regimen until euthanasia, thereby ensuring that the nutritional protocols for each group were maintained throughout the entire experimental period. At five days old, all offspring were euthanized, and their calvariae

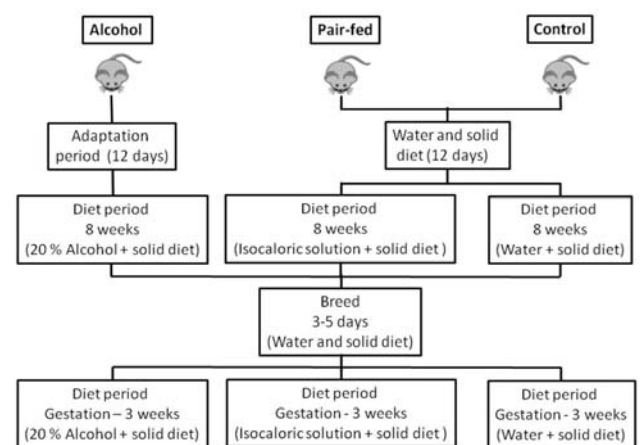


Figure 1: Experimental feeding regimen for rat dams.

were collected for analysis.

The experimental feeding regime (Figure 1) was performed according to our previous study [19]. The female rats' weights were assessed twice: at the beginning of the eight-week treatment protocol and after offspring euthanasia. Weight changes were calculated as a percentage using the following formula:

$$\frac{(\text{final weight} - \text{initial weight}) \times 100}{\text{Initial weight}}$$

### Blood alcohol concentration

Blood alcohol concentration in all dams was measured according to the manufacturer's recommendations (EnzyChrom Ethanol Assay Kit, Bioassay Systems, Hayward, CA, USA) as previously described [19]. Peak blood ethanol concentrations were determined in all dams from the Alcohol group on the same day the offspring were euthanized (postnatal day 5). Following euthanasia, the dams were anesthetized for blood collection via cardiac puncture. The anesthetic agents used, along with their respective concentrations, were: xylazine (2.3 g/100 ml AnasedanV R- Vetbrands, Jacarei- Brazil), a sedative and muscle relaxant, and ketamine (1.16 g/10 ml; DopalenV R- Vetbrands, Jacarei- Brazil), a general anesthetic. An anesthetic solution was prepared containing 0.8 ml of xylazine and 0.5 ml of ketamine hydrochloride. Each animal received general anesthesia via intramuscular injection at a dose of 0.1 ml/100 g of body weight. Blood samples were collected between 8:00 and 10:00 a.m., a postprandial period corresponding to the expected peak in blood ethanol concentrations in the dams [28]. Maternal blood was centrifuged at 4°C, and the resulting serum was stored at -80 °C until ethanol concentration analysis, performed using a commercial kit. Blood samples were also collected from control dams to ensure consistent handling across groups and were used as negative controls for ethanol concentration analysis.

### Cell isolation and primary culture of osteogenic cells

Primary osteoblasts were obtained from calvariae as previously described [18,19,29-40]. In brief, calvariae from 5-day-old Wistar rats were dissected and wiped with gauze.

The regions containing cranial sutures were discarded, and the remaining bony tissue was cut into small pieces for digestion with a mixture of enzymes consisting of 0.2% collagenase (Thermo Fisher Scientific, Gibco, Waltham, Massachusetts, EUA) and 0.25% trypsin (Thermo Fisher Scientific, Gibco, Waltham, Massachusetts, EUA). Sequential digestion was carried out at 37°C in an agitating water bath for consecutive periods of 5, 15, and 25 minutes. The digests were centrifuged and resuspended in complete media containing  $\alpha$ -Minimum Essential Medium with L-glutamine (Thermo Fisher Scientific, Gibco, Waltham, Massachusetts, EUA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Gibco, Waltham, Massachusetts, EUA), 7 mM  $\beta$ -glycerophosphate (Sigma/Aldrich, St. Louis, MO, EUA), 5  $\mu$ g/ml ascorbic acid (Mallinckrodt Chemicals, UK), and 50  $\mu$ g/ml gentamicin (Thermo Fisher Scientific, Gibco, Waltham, Massachusetts, EUA). Supernatant from the first digestion was discarded, and the second and third digests were pooled and resuspended in complete media. The pooled material was passed through a 200  $\mu$ m metal screen. An aliquot of the filtrate was stained with trypan blue, and the total cell count and viability were evaluated using a hemocytometer.

Aliquots containing  $1 \times 10^6$  cells from each experimental group were frozen at -80 °C for future use. The cells were thawed and cultured for 10 days in complete media at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The culture medium was replaced every three days, and the advancement of the cultures was assessed using phase contrast microscopy (Inverted Microscope OLYMPUS CK40).

For each experimental group, calvariae were collected from multiple rat pups originating from different dams. Specifically, the Control group consisted of 62 pups from 5 dams, the Pair-fed group comprised 61 pups from 5 dams, and the Alcohol group included 45 pups from 6 dams. Accordingly, the number of calvariae used for primary osteoblast isolation differed across experimental conditions. The samples were then pooled to generate a cell culture, RNA was extracted, and used for gene expression analysis by real-time PCR, following the protocol provided with the QIAGEN PCR array kit in triplicate.

### Real-time PCR

After 10 days of cell culture, RNA was extracted with the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted RNA was quantified (ng/ $\mu$ L) in a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Gibco, Waltham, Massachusetts, EUA). Only RNAs presenting purity between 1.8 and 2.0 were included in this study and treated with DNase. For removal of contaminating DNA, the extracted total RNA was treated with RNase-Free DNase Set (Qiagen, Hilden, Germany), and the RNA was reverse transcribed to complementary DNA (cDNA) using the RT<sup>2</sup> First Strand Kit (Qiagen, Hilden, Germany) according to manufacturer instructions.

Quantitative real-time PCR (qRT-PCR) was used to assess alterations in gene expression, and the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Framingham, MA,

USA) was employed for this purpose using a gene plate "RT2 Profiler Rat Osteogenesis – PARN 026ZA\_12 as instructed by the manufacturer (Qiagen, Hilden, Germany). This plate model is composed of 84 target genes, five housekeeping genes (ACTB –  $\beta$ -actin, HPRT1 – Hypoxanthine phosphoribosyl transferase 1, B2M – Beta-2-microglobulin, LDHA – Lactate dehydrogenase A and RPLP1 – Protein ribosomal greater P1), rat genomic DNA contamination control (RGDC), three controls for reverse transcription (RTC), and three positive controls (PPC) (Table 1 – Supplementary Material).

The conditions of the PCR were an initial cycle at 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C and 30 s at 68 °C, and a final cycle at 72 °C for 10 min. Upon completion of the qRT-PCR, the baseline and CT values were automatically set for each plate, and the same thresholds for all PCR array plates were kept. The data was exported and analyzed by RT2 Profiler PCR Array Data Analysis template v 3.5 (Data Analysis Center | Genetic Analysis Tools | GeneGlobe), where Fold-Change ( $2^{\Delta(-\Delta Ct)}$ ) is the result of normalized gene expression ( $2^{\Delta(-\Delta Ct)}$ ) in the Test Sample divided the normalized gene expression ( $2^{\Delta(-\Delta Ct)}$ ) in the Control Sample [41]. Fold-Regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive or an up-regulation, and the fold-change is equal to the fold-regulation. Fold-change values less than one indicate a negative or down-regulation, and the fold-change is the negative inverse of the fold-change.

### Statistical analysis

The diet data obtained from rats exhibited a non-normal distribution and were analyzed using non-parametric tests (Kruskal-Wallis test followed by Dunn's test). Data obtained from the weight of the rats showed normal distribution and were evaluated using parametric tests (analysis of variance – ANOVA followed by Tukey test).

The qPCR data were analyzed using software available on the company website Qiagen (Data Analysis Center | Genetic Analysis Tools | GeneGlobe). Statistical analysis of gene expression data was performed with the nonpaired Student's t-test and considered statistically significant with  $p$ -values <0.05. Value of the fold-change greater than 2 was defined as increased expression, and the fold-change value of less than 0.5 as decreased gene expression. The analyses and comparisons were performed between the Pair-fed and Control groups, and the Alcohol and Pair-fed groups. These comparisons were made to assess whether the Pair-fed group, our Control group, showed differences in gene expression compared to the Alcohol group.

## Results

### Dam diet and weight

The values of solid diet, liquid diet, and the changes in the weight (percentage) were shown in Table 1 (changes in body weight were calculated based on measurements obtained at the beginning of the eight-week treatment protocol and

**Table 1:** Analysis comparing solid diet consumption and liquid diet consumption (Kruskal-Wallis test) and changes in the weight (%) (ANOVA test).

Groups	Control	Pair-fed	Alcohol
<i>Solid diet (g/day/rat)</i>			
Average	50.85 <sup>ab</sup>	28.79 <sup>a</sup>	28.02 <sup>b</sup>
Standard deviation	11.25	9.13	6.77
Sample size (n) <sup>*</sup>	5	5	6
<i>Liquid Diet (ml/day/rat)</i>			
Average	91.67 <sup>ab</sup>	34.6 <sup>ac</sup>	52.63 <sup>bc</sup>
Standard deviation	18.74	11.95	15.41
Sample size (n) <sup>*</sup>	5	5	6
<i>Weight (% gain)</i>			
Average	49.89 % <sup>e</sup>	-1.67 <sup>de</sup>	29.93 % <sup>d</sup>
Standard deviation	19.27	7.46	15.21
Sample size (n) <sup>*</sup>	5	5	6

<sup>a</sup> Control vs. Pair-fed ( $p < 0.0001$ )

<sup>b</sup> Control vs Alcohol ( $p < 0.0001$ )

<sup>c</sup> Alcohol vs. Pair-fed ( $p < 0.0001$ )

<sup>d</sup> Alcohol vs. Pair-fed ( $p = 0.0099$ )

<sup>e</sup> Control vs. Pair-fed ( $p = 0.0003$ )

\* Sample size (n) – considering the number of animals that were pregnant in each group.

after offspring euthanasia). The Control group consumed a higher amount of solid diet when compared to the pair-fed and Alcohol groups ( $50.85 \pm 11.25$  g) ( $p < 0.0001$ ). The Pair-fed group ingested all the solid diet offered to them. The food consumption between Pair-fed and Alcohol was equivalent ( $28.8 \pm 9.13$  g and  $28.02 \pm 6.77$  g, respectively) (Table 1).

Concerning the liquid diet, although the Pair-fed group received a carbohydrate solution, which is equivalent to an alcohol solution in calories, the rats did not ingest all the solution available to them. The Alcohol group consumed an average of  $52.63 \pm 15.41$  mL of alcohol solution, and the Pair-fed group ingested  $34.6 \pm 11.95$  mL of carbohydrate solution ( $p < 0.0001$ ). In the liquid diet, the Control group also showed higher consumption ( $91.67 \pm 18.74$  mL) than the alcohol and Pair-fed groups ( $p < 0.0001$ ) (Table 1).

Weight gain was observed in both the control and Alcohol groups ( $49.89 \pm 19.27$  % and  $29.93 \pm 15.21$  %, respectively). However, the Pair-fed group showed weight loss ( $-1.67$  %  $\pm 7.46$  g) differing significantly from the Alcohol and Control groups ( $p = 0.0099$  and  $p = 0.0003$ , respectively).

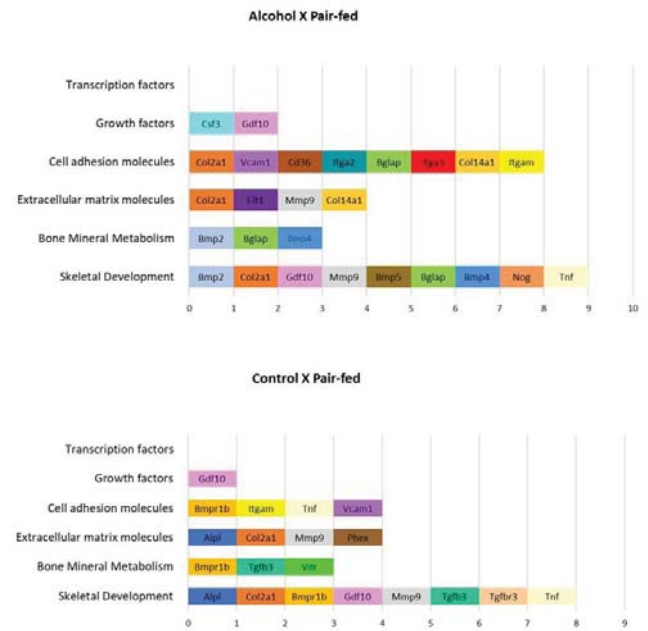
### Determination of blood ethanol concentrations in dams

As previously described [19] the Alcohol group had an average blood ethanol concentration of  $1280 \pm 730$  mg/dL, while the Pair-fed and Control groups showed negative alcohol concentration in the blood.

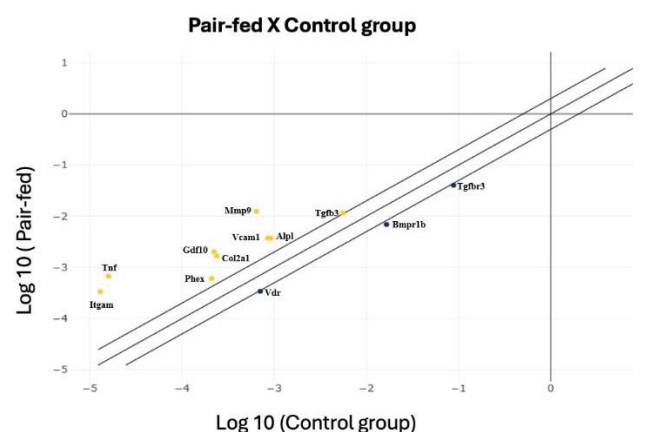
### qRT-PCR

The threshold cycle (Ct) values predominantly fell below 25 or were observed between 25 and 30 across all experimental groups, thereby confirming the reliability of this study. Beta-2 microglobulin (B2m) was selected as a normalizing reference. The bone gene set allows the separation of genes by category according to the biological events of osteogenesis: skeletal development, bone mineral metabolism, extracellular matrix molecules (ECM), cell adhesion molecules, growth factors, and transcription factors (Qiagen company catalog provides this

separation of genes by category according to the biological events of osteogenesis, **Table 2 – Supplementary Material**). A first general analysis showed expression variation in 23 bone genes that fall into 3 main categories: Skeletal development (38,2%), Cell adhesion molecules (23,5%), and Bone mineral metabolism (20,5%). Extracellular matrix molecules correspond to 11.7% of the modulated genes. Growth and transcription factors correspond to 2.9 % each. Few genes are related to more than one category. A diagram was provided to categorize the biological events associated with the genes that exhibited differential expression (Figure 2).



**Figure 2:** Diagram categorizing the biological events associated with the genes that showed differential expression between Alcohol and Pair-fed and Pair-fed and Control groups. The genes were separated according to the biological events of osteogenesis.



**Figure 3:** Scatter Plot in Log 10 showing the normalized expression of the genes on the PCR Array between Pair-fed and Control groups. The center diagonal line indicates unchanged gene expression, while the outer diagonal lines show the selected fold regulation threshold. Genes with data points beyond the outer lines in the upper and lower corners are up-regulated or down-regulated, respectively. Note significant ( $p < 0.05$ ) downregulation (blue dots) of the Bmpr1b (-2.16), Tgfb3 (-1.39), and Vdr (-3.47) genes and up-regulation (yellow dots) of Alpl (-2.43), Col2a1 (-2.78), Gdf10 (-2.69), Itgam (-3.48), Mmp9 (-1.91), Phex (-3.22), Tgfb3 (-1.94), Tnf (-3.47) and Vcam1 (-2.43) genes.



Chrd	Chordin	-1,47	0,019	2,65	0,299
Col10a1	Collagen, type X, alpha 1	-1,03	0,792	2,66	0,689
Col14a1	Collagen, type XIV, alpha 1	<b>-2,26</b>	0,005*	3,25	0,141
Col1a1	Collagen, type I, alpha 1	1,39	0,003	1,34	0,654
Col1a2	Collagen, type I, alpha 2	1,12	0,044	1,52	0,302
Col2a1	Collagen, type II, alpha 1	<b>-3,19</b>	0,006*	<b>6,93</b>	0,003*
Col3a1	Collagen, type III, alpha 1	-1,2	0,005	1,3	0,398
Col4a1	Collagen, type IV, alpha 1	-1,11	0,237	-1,13	0,511
Col5a1	Collagen, type V, alpha 1	1,08	0,361	1,54	0,558
Col6a1	Collagen, type VI, alpha 1	-1,01	0,871	1,37	0,746
Comp	Cartilage oligomeric matrix protein	1,85	0,001	1,96	0,431
Csf1	Colony stimulating factor 1 (macrophage)	1,29	0,139	1,15	0,972
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	-1,47	0,023	2,4	0,093
Csf3	Colony stimulating factor 3 (granulocyte)	<b>4,14</b>	0,001*	1,22	0,826
Ctsk	Cathepsin K	1,29	0,024	1,4	0,565
Dlx5	Distal less homeobox 5	1,15	0,359	1,22	0,364
Egf	Epidermal growth factor	-1,37	0,012	1,86	0,046
Fgf1	Fibroblast growth factor 1	1,31	0,781	2,48	0,531
Fgf2	Fibroblast growth factor 2	-1,41	0,031	-2,07	0,149
Fgfr1	Fibroblast growth factor receptor 1	1,73	0,030	-1,39	0,321
Fgfr2	Fibroblast growth factor receptor 2	1,31	0,255	1,44	0,176
Flt1	Fms-related tyrosine kinase 1	<b>-3,6</b>	0,011*	1,13	0,630
Fn1	Fibronectin 1	1,94	0,002	-1,19	0,519
Gdf10	Growth differentiation factor 10	<b>-5,99</b>	0,001*	<b>9,09</b>	0,001*
Gli1	GLI family zinc finger 1	-1,13	0,418	1,72	0,029
Icam1	Intercellular adhesion molecule 1	1,42	0,023	1,11	0,827
Igf1	Insulin-like growth factor 1	-1,36	0,006	1,93	0,008
Igf1r	Insulin-like growth factor 1 receptor	1,71	0,008	-1,67	0,075
lhh	Indian hedgehog	-1,01	0,967	1,55	0,030
Itga2	Integrin, alpha 2	<b>-3,41</b>	0,001*	1,12	0,439
Itga3	Integrin, alpha 3	<b>2,19</b>	0,001*	-1,54	0,114
Itgam	Integrin, alpha M	<b>-27,3</b>	0,001*	<b>25,68</b>	0,001*
Itgav	Integrin, alpha V	1,48	0,003	-1,65	0,001
Itgb1	Integrin, beta 1	1,28	0,063	-1,2	0,220
Mmp10	Matrix metalloproteinase 10	-1,55	0,001	-4,44	0,179
Mmp2	Matrix metalloproteinase 2	1,31	0,111	-1,4	0,070
Mmp8	Matrix metalloproteinase 8	-5,21	0,087	3,56	0,107
Mmp9	Matrix metalloproteinase 9	<b>-8,61</b>	0,002*	<b>19,43</b>	0,002*
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	1,5	0,042	-1,26	0,234
Nog	Noggin	<b>-11,08</b>	0,005*	2,45	0,078
Pdgfra	Platelet-derived growth factor alpha polypeptide	1,37	0,016	-1,4	0,162
Phex	Phosphate regulating endopeptidase homolog, X-linked	-1,52	0,000	<b>2,89</b>	0,013*
Runx2	Runt-related transcription factor 2	1,44	0,039	-1,11	0,649
Serpinh1	Serine (or cysteine) peptidase inhibitor, clade H, member 1	1,26	0,041	1,32	0,577
Smad1	SMAD family member 1	-1,9	0,201	-1,32	0,133
Smad2	SMAD family member 2	-1,19	0,145	-1,09	0,547
Smad3	SMAD family member 3	1,08	0,549	-1,11	0,373
Smad4	SMAD family member 4	1,18	0,279	-1,82	0,026
Smad5	SMAD family member 5	1,02	0,917	-1,32	0,021
Sost	Sclerostosis	-1,01	0,967	-1,1	0,611
Sox9	SRY-box containing gene 9	1,77	0,028	-1,79	0,016
Sp7	Sp7 transcription factor	1,57	0,200	1,05	0,903
Spp1	Secreted phosphoprotein 1	1,19	0,012	-1,14	0,047
Tgfb1	Transforming growth factor, beta 1	1,58	0,044	-1,24	0,400
Tgfb2	Transforming growth factor, beta 2	-1,72	0,031	1,84	0,031
Tgfb3	Transforming growth factor, beta 3	-1,37	0,076	<b>2,08</b>	0,035*
Tgfb1	Transforming growth factor, beta receptor 1	1,3	0,031	-1,52	0,037
Tgfb2	Transforming growth factor, beta receptor II	-1	0,884	1,06	0,883
Tgfb3	Transforming growth factor, beta receptor III	1,24	0,209	<b>-2,19</b>	0,002*
Tnf	Tumor necrosis factor (TNF superfamily, member 2)	<b>-55,12</b>	0,003*	<b>42,19</b>	0,003*
Tnfsf11	Tumor necrosis factor (ligand) superfamily, member 11	1,01	0,838	1,15	0,172
Twist1	Twist homolog 1 (Drosophila)	1,44	0,006	-2,14	0,074
Vcam1	Vascular cell adhesion molecule 1	<b>-5,11</b>	0,003*	<b>4,38</b>	0,004*
Vdr	Vitamin D (1,25-dihydroxyvitamin D3) receptor	1,95	0,014	<b>-2,07</b>	0,008*
Vegfa	Vascular endothelial growth factor A	-1,01	0,857	-1,13	0,566
Vegfb	Vascular endothelial growth factor B	-1	0,897	-1,55	0,099

Alcohol group corresponds to  $1280 \pm 730$  mg/dL blood ethanol concentration. The maternal blood ethanol concentration is rarely known in human studies and is often not measured in animal studies, making it difficult to compare among studies. The measured values in the present study are considered a high ethanol dosage because even the lower measured values (550 mg/dL blood ethanol concentration) are still considered a high ethanol dosage [14,51–53]. In this way, the prenatal alcohol exposure was able to damage the DNA of osteoblasts, suggesting a direct action of alcohol on these cells and consequently on bone formation [19]. As expected, the same prenatal alcohol exposure was also able to modulate osteoblast gene expression. Taken together, these findings indicate that alcohol changes the bone tissue by affecting the osteoblasts.

Osteoblasts and osteoclasts are the pivotal cells involved in bone turnover: they are responsible for bone formation and bone resorption, respectively. In particular, osteoblasts arise from the osteogenic differentiation of mesenchymal stem cells through a process regulated in different steps. Osteocytes, other important skeletal cells that derive from mature osteoblasts and are surrounded by secreted extracellular matrix, regulate osteoblast and osteoclast activity and, consequently, maintain bone homeostasis. Alterations of mesenchymal stem cells may occur during commitment or differentiation towards the osteogenic lineage, causing demineralization or bone loss in different pathological settings [54].

Within the array of genes participating in osteogenic differentiation, certain ones are already recognized for their specific functions in this intricate process. For this reason, some genes are more evaluated in studies on bone formation, like osteopontin (Spp1), alkaline phosphatase (Alpl), and osteocalcin (Bglap), which are the most investigated osteogenic-related genes [55,56]. In the present study, 84 genes related to osteogenesis were evaluated in osteoblasts differentiated *in vitro* (primary pre-osteoblasts obtained from the calvaria of fetuses from the three groups). Variations in gene expression were evaluated by real-time PCR and clustered in categories: skeletal development, bone mineral metabolism, extracellular matrix molecules (ECM), cell adhesion molecules, growth factors, and transcription factors. The results showed that alcohol alters the genes expression in practically all biological events related to osteogenesis. Among the 17 genes that showed significant changes in gene expression between the Alcohol group compared to the Pair-fed group, 12 genes presented downregulation. The Bmp5, Col2a1, Gdf10, Mmp9, Nog, and Tnf have been observed in skeletal development. The Cd36, Itga2, Itgam, and Vcam1 genes were found in association with cell adhesion molecules. The Col14a1, Col2a1, and Flt1 genes have been related to extracellular matrix molecules. In contrast, the Bglap, Bmp2, Bmp4, Csf3, and Itga3 genes were upregulated in the Alcohol group compared to the Pair-fed group. Changes in Bglap, Bmp2, and Bmp4 gene expression are related to skeletal development and bone mineral metabolism. The Csf3 is a growth factor, and the Bglap and Itga3 genes are usually involved with cell adhesion, according to the manufacturer's description of the plate model chosen (Rat Osteogenesis (PARN 026ZA\_12/ Figure 2).

Osteocalcin, also referred to as bone gamma-carboxyglutamate (gla) protein, is a non-collagenous protein primarily found in bone tissue and is notably up-regulated during the later stages of cellular differentiation. This stage coincides with the onset of mineralization, suggesting that osteocalcin may play a part in the regulation of matrix mineralization [57]. In a prior investigation by our research team [18], the impact of alcohol was explored through assessments of cell adhesion, proliferation, viability, total protein content, Alkaline Phosphatase activity, and bone matrix formation. The findings indicated that prenatal ethanol exposure enhances cell differentiation, leading to increased alkaline phosphatase production and mineralized bone matrix formation on the 14th and 21st days of cultivation. The current study reveals an overexpression of the osteocalcin gene (Bglap) in the Alcohol group. Taken together, these outcomes suggest that the upregulation of this gene may be associated with heightened matrix mineralization, as observed in our prior work [18]. This overexpression of the Bglap gene likely contributes to accelerated mineralization noted in our previous investigation. Studies have shown that animals exposed to prenatal alcohol exhibit smaller bones or reduced stature [7,8,11–15,23,24,43]. One potential mechanism behind this phenomenon could be the acceleration of mineralization due to alcohol-induced overexpression of Bglap, as demonstrated in this study, resulting in faster bone mineralization and, consequently, premature cessation of bone growth.

Numerous cytokines and growth factors play pivotal roles in bone formation, with BMPs (bone morphogenetic proteins) particularly central to skeletogenesis. BMPs contribute to mesenchyme condensation, skeleton morphogenesis, growth plate development, osteoblast differentiation, and the regulation of postnatal bone and cartilage maintenance [58,59]. Among the 14 known BMPs, BMP-2, 4, 5, 6, 7, and 9 exhibit potent osteogenic activity [58]. Our results demonstrated prenatal alcohol exposure alters the expression of BMPs, with Bmp5 showing reduced expression while Bmp2 and Bmp4 are overexpressed. This suggests that alcohol directly affects osteoblasts, influencing their differentiation process. In addition, BMP-2 is particularly significant as it significantly enhances Bglap expression, with short-term BMP-2 expression being crucial for inducing irreversible bone formation [58]. Considering these findings and the results of Bglap and BMP-2 expression in our study, it can be inferred that prenatal alcohol exposure upregulates these genes, promoting bone formation. This supports the hypothesis of alcohol's mechanism in bone formation and its role in accelerating bone mineralization, potentially contributing to the shorter stature observed in descendants of alcohol consumers.

The Growth differentiation factor 10 (Gdf10), also referred to as bone morphogenetic protein 3B (Bmp3b), belongs to the transforming growth factor beta (TGF- $\beta$ ) superfamily [58,60]. Gdf10 is strongly expressed in developing skeletal structures in embryos and in bones, the brain (especially the cerebellum), the aorta, and adipose tissues in adult rodents [61,62]. Moreover, Gdf10 plays a role in inhibiting osteoblast differentiation [63] and is crucial for head formation in *Xenopus* embryos [64]. In

our study, the alcohol-exposed group demonstrated reduced expression of the *Gdf10* gene, supporting the hypothesis of alcohol's direct impact on osteoblasts and bone development. Furthermore, we can infer its action, particularly in skull bone formation, given the essential role of this gene in head development.

In the present study, cells from animals exposed to prenatal alcohol consumption exhibited reduced expression of two genes associated with the extracellular matrix (ECM) structure, *Col2a1* and *Col14a1*. Type II collagen (*Col2a1*) is a key component of the cartilage matrix. Along with other proteins and proteoglycans, *Col2a1* forms complex extracellular scaffolds that support mechanical forces, maintain physiological homeostasis, and provide anchoring sites for chondrocytes, extracellular matrix molecules, and growth factors [65]. Beyond its structural role, *Col2a1* serves as an important extracellular signaling molecule, regulating chondrocyte proliferation, metabolism, and differentiation [66]. The degradation and reduction of *Col2a1* are considered characteristic pathological markers [67–69]. Type- XIV collagen (*Col14a1*) is often present in areas of high mechanical stress [70–74].

Previous research [74] revealed that mice lacking *Col14a1* are viable. However, detailed examinations of their skin and tendons showed defects in fibril growth and fiber assembly during embryonic development. Due to altered fibrillogenesis, the fiber structure was compromised, leading to significantly reduced biomechanical functions in these tissues [74]. This suggests that *Col14a1* is crucial for ECM assembly and tissue function in tendons and skin. This way, our findings suggest that prenatal alcohol exposure also impacts the bone ECM.

In the current study, the expression of six genes associated with cell adhesion—*Itga2*, *Itgam*, *Itga3*, *Cd36*, *Vcam-1*, and *Bglap*—was found to be altered. Cell adhesion is essential for all multicellular organisms, enabling interaction and coordination within cell populations.

Integrins, a key class of cell adhesion receptors, play a vital role in “maintaining the integrity of the cytoskeletal-extracellular matrix linkage.” This concept was first established in the 1970s and 1980s through the research of Erkki Ruoslahti and Richard O. Hynes, who are recognized as the pioneers of integrin research [75]. Integrins consist of an alpha ( $\alpha$ ) subunit and a beta ( $\beta$ ) subunit, each with a specific affinity for different ECM components. The integrin family comprises 24 known members, which play active roles in regulating cellular growth, differentiation, and apoptosis. Each integrin heterodimer performs distinct functions in specific contexts, although some functions partially overlap with those of other integrin family members [76]. Our results revealed that the *Itga2* and *Itgam* genes were underexpressed, while the *Itga3* gene was overexpressed in the group exposed to alcohol during pregnancy. This corroborates our findings that prenatal alcohol exposure also affects the bone ECM.

*Cd36* is a glycoprotein embedded within cell membranes and is found in a variety of immune and non-immune cells, including bone cells [77–79]. It interacts with various

external ligands, implicating this receptor in diverse biological processes. While the involvement of *Cd36* in various physiological functions is well-documented, its specific role in osteoblasts remains to be fully understood. Kervokova et al. (2013) investigated *Cd36*'s impact on bone metabolism and osteoblast activities, revealing that mice lacking *Cd36* exhibited an osteopenic phenotype in trabecular bone. *In vitro* studies on bone marrow-derived mesenchymal stem cells and osteoblasts from *Cd36*-deficient mice showed decreased cell viability and expansion. Additionally, key osteoblastic transcription factors like *Runx2* and *Osterix*, along with osteocalcin and bone sialoprotein expression, were downregulated in *Cd36*-deficient cells. These findings suggest that *Cd36* plays a crucial role in bone metabolism, ensuring proper bone formation. In our current investigation, we observed reduced expression of the *Cd36* gene in the Alcohol group. This outcome underscores the impact of prenatal alcohol exposure on osteoblasts, potentially disrupting normal bone formation processes.

Vascular cell adhesion molecule 1 (*Vcam-1*) was initially recognized as a glycoprotein on the surface of endothelial cells in 1989. This glycoprotein is alternatively referred to as *CD106* and can be triggered for expression. *Vcam-1* expression is activated by pro-inflammatory cytokines, including *Tnf $\alpha$* , and also by ROS, oxidized low-density lipoprotein, high glucose concentration, toll-like receptor agonists, and shear stress. Under high levels of inflammation and chronic conditions in some diseases, *Vcam-1* is also expressed on the surface of other cells, including tissue macrophages, dendritic cells, bone marrow fibroblasts, myoblasts, oocytes, Kupffer cells, Sertoli cells, and cancer cells [80]. In this study, the *Vcam-1* gene exhibited reduced expression following prenatal alcohol exposure, likely due to the underexpression of the *Tnf* gene, which activates this protein.

Tumor necrosis factor alpha (*Tnf $\alpha$* ) functions as a pro-inflammatory cytokine, prompting the expression of various inflammatory molecules, including other cytokines and cell adhesion molecules [80]. While *Tnf*'s impact on bone tissue, particularly in bone resorption, is established [81], its effect on the differentiation of mesenchymal stromal cells into osteoblasts remains contentious. Some research indicates *Tnf* inhibits this differentiation, while others suggest it can stimulate osteogenic differentiation by increasing levels of *Runx2*, *Osterix*, osteocalcin, *Bmp-2*, and alkaline phosphatase [81]. The conflicting roles of *Tnf* in mesenchymal stromal cell osteogenic differentiation likely stem from differences in cellular stages, *Tnf* concentration, and exposure duration. In our current investigation, we noted a decrease in the expression of the *Tnf* gene and an increase in the expression of the osteocalcin gene. This observation further supports the previously observed trend of positive regulation identified in other studies.

*Flt1*, also known as Fms Related Receptor Tyrosine Kinase 1, is a protein-coding gene that is alternatively referred to as *Vegfr1* (Vascular Endothelial Growth Factor Receptor 1). It encodes a member of the vascular endothelial growth factor receptor (VEGFR) family. Expression of this protein is observed

across various non-endothelial cell types, including vascular smooth muscle cells and macrophages [82]. While the primary focus of research on Flt1 has centered around angiogenesis, studies have also noted its expression in other cell types, such as osteoblasts and osteoclasts [83-86]. Accumulating evidence suggests potential implications of the VEGF/Flt-1 system in bone formation [87,88]. Recently, Xu and colleagues [89] demonstrated that Sod3 and its downstream gene, Flt1, influence osteogenic and adipogenic differentiation through the PI3K/AKT and MAPK pathways, ultimately impacting bone mass. In the current study, the alcohol-exposed group exhibited reduced expression of the Flt1 gene, underscoring the effect of prenatal alcohol exposure on osteogenic differentiation.

The Csf3 gene encodes granulocyte colony-stimulating factor (GCSF), a cytokine that functions as a hematopoietic growth factor governing the survival, growth, and specialization of granulocyte precursors and neutrophil activity [90]. Despite lacking a direct physiological role in bone regulation, GCSF exhibits pharmacological impact on the skeleton *in vivo* [91]. The mechanisms by which pharmacological GCSF acts on the osteoblast lineage or osteoclasts have not been fully resolved; however, it is clear that the pharmacological effects of GCSF on the skeleton result from indirect action on the osteoblast lineage, as the GCSF receptor is not expressed by osteoblasts or osteocytes [92,93]. In the current study, the cells exposed to alcohol during development displayed heightened expression of the Csf3 gene; this finding indicates that alcohol exerts an indirect influence on osteoblasts as well.

Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that play a crucial role in the regeneration of the ECM by degrading its components. These proteins target and cleave structural elements of the ECM, such as collagen and gelatin, facilitating its breakdown and subsequent regeneration [94]. Under both physiological and pathological conditions, bone cells actively express MMPs, which are believed to be vital for the viability and functionality of osteoclasts, osteoblasts, and osteocytes. Additionally, these MMPs are crucial for the formation and development of chondrocytes, all of which are influenced by bone ECM [94]. Given the dynamic nature of bone tissue and the necessity for various enzymes to degrade the organic components of the bone matrix, the actions

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integrin heterodimer performs distinct functions in specific contexts, although some functions partially overlap with those of other integrin family members [76]. Our results revealed that the Itga2 and Itgam genes were underexpressed, while the Itga3 gene was overexpressed in the group exposed to alcohol during pregnancy. This corroborates our findings that prenatal alcohol exposure also affects the bone ECM.

CD36 is a glycoprotein embedded within cell membranes and is found in a variety of immune and non-immune cells, including bone cells [77-79]. It interacts with various external ligands, implicating this receptor in diverse biological processes. While the involvement of Cd36 in various physiological functions is well-documented, its specific role in osteoblasts remains to be fully understood. Kervokova et al. (2013) investigated Cd36's impact on bone metabolism and osteoblast activities, revealing that mice lacking Cd36 exhibited an osteopenic phenotype in trabecular bone. *In vitro* studies on bone marrow-derived mesenchymal stem cells and osteoblasts from Cd36-deficient mice showed decreased cell viability and expansion. Additionally, key osteoblastic transcription factors like Runx2 and Osterix, along with osteocalcin and bone sialoprotein expression, were downregulated in Cd36-deficient cells. These findings suggest that Cd36 plays a crucial role in bone metabolism, ensuring proper bone formation. In our current investigation, we observed reduced expression of the Cd36 gene in the Alcohol group. This outcome underscores the impact of prenatal alcohol exposure on osteoblasts, potentially disrupting normal bone formation processes.

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Tumor necrosis factor alpha (Tnf $\alpha$ ) functions as a pro-inflammatory cytokine, prompting the expression of various inflammatory molecules, including other cytokines and cell adhesion molecules [80]. While Tnf's impact on bone tissue, particularly in bone resorption, is established [81], its effect on the differentiation of mesenchymal stromal cells into osteoblasts remains contentious. Some research indicates Tnf inhibits this differentiation, while others suggest it can stimulate osteogenic differentiation by increasing levels of Runx2, Osterix, osteocalcin, Bmp-2, and alkaline phosphatase [81]. The conflicting roles of Tnf in mesenchymal stromal cell osteogenic differentiation likely stem from differences in cellular stages, Tnf concentration, and exposure duration. In our current investigation, we noted a decrease in the

expression of *Tnf* gene and an increase in the expression of the osteocalcin gene. This observation further supports the previously observed trend of positive regulation identified in other studies.

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The *Csf3* gene encodes granulocyte colony-stimulating factor (GCSF), a cytokine that functions as a hematopoietic growth factor governing the survival, growth, and specialization of granulocyte precursors and neutrophil activity [90]. Despite lacking a direct physiological role in bone regulation, GCSF exhibits pharmacological impact on the skeleton *in vivo* [91]. The mechanisms by which pharmacological GCSF acts on the osteoblast lineage or osteoclasts have not been fully resolved, however, it is clear that the pharmacological effects of GCSF on the skeleton result from indirect action on the osteoblast lineage, as the GCSF receptor is not expressed by osteoblasts or osteocytes [92,93]. In the current study, the cells exposed to alcohol during development displayed heightened expression of the *Csf3* gene; this finding indicates that alcohol exerts an indirect influence on osteoblasts as well.

Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that play a crucial role in the regeneration of the ECM by degrading its components. These proteins target and cleave structural elements of the ECM, such as collagen and gelatin, facilitating its breakdown and subsequent regeneration [94]. Under both physiological and pathological conditions, bone cells actively express MMPs, which are believed to be vital for the viability and functionality of osteoclasts, osteoblasts, and osteocytes. Additionally, these MMPs are crucial for the formation and development of chondrocytes, all of which are influenced by bone ECM [94]. Given the dynamic nature of bone tissue and the necessity for various enzymes to degrade the organic components of the bone matrix, the actions of MMPs and their inhibitors hold significant physiological importance, in this manner, can state that MMPs are crucial mediators of bone physiology [94]. The literature indicates that the expression of matrix metalloproteinase-9 (MMP-9) varies as a child grows, and this enzyme is expressed in trophoblasts and osteoclasts during early development, suggesting its role in bone implantation and resorption [94]. Furthermore, the

absence of certain molecules, including MMP-9, during skeletal development lead to significant defects in long bone plates, impeding normal bone production [94]. In the present study, alcohol caused in the under expression of the *Mmp-9* gene. This finding provides further compelling evidence that alcohol impacts the ECM, thereby influencing the bone development of offspring whose mothers consumed alcohol during pregnancy.

*Noggin*, also referred to as *Nog* and encoded by the *Nog* gene, is a secreted homodimeric glycoprotein involved in the development of various body tissues, including nerve tissue, muscles, and bones [95]. In bone tissue, *Nog* regulates bone morphogenetic protein signaling by preventing these proteins from binding to cell receptors, thereby inhibiting signal transduction. By this mechanism, it modulates various signaling pathways during cartilage and bone formation in human development [95]. This gene is considered very important for bone development, as mutations in the *Nog* gene result in alterations in bone morphogenetic protein signaling, leading to different types of dysplasias [96-99]. Furthermore, some studies suggest *Nog* as a potential candidate for craniofacial disorders [100-102]. Our results showed that prenatal alcohol exposure led to reduced expression of the *Nog* gene, which may have influenced the expression of the *Bmp2*, *Bmp4*, and *Bmp5* genes, as changes in their expression were observed in the alcohol-exposed group. Additionally, literature suggests that the *Nog* gene plays a role in head formation; thus, the under expression of this gene in our study indicates that the cranial bone changes observed in previous studies may be related to this gene alteration [16,21,22,24].

It is well known that alcohol has low nutritional value, however its metabolism by the liver provides energy to the body (7.1 kcal/g), leading to a sense of satiety. Thus, nutritionally important foods are replaced by alcohol, which commonly leads to malnutrition due to the lower intake of nutrients [103,104]. In agreement, in our study, there was a significant decrease in solid food intake by the animals of the Alcohol group when compared to the Control group. Considering that the Alcohol group ingested fewer nutrients, the presence of the Pair-fed group was important to control this variable. Regarding nutrient deficiency, mimicked by the Pair-fed group, our results showed alterations in the gene expression of 12 genes evaluated. The *Bmpr1b*, *Tgfb3* and *Vdr* genes were downregulated in Pair-fed group compared to Control group. These genes have been observed in cell adhesion molecules (*Bmpr1b*), skeletal development (*Bmpr1b* and *Tgfb3*) and bone mineral metabolism (*Bmpr1b* and *Vdr*). On the other hand, the *Alpl*, *Col2a1*, *Gdf10*, *Itgam*, *Mmp9*, *Phex*, *Tgfb3*, *Tnf* and *Vcam1* genes were upregulated in the Pair-fed group compared to the Control group. The *Alpl*, *Col2a1*, *Gdf10*, *Mmp9*, *Tgfb3* and *Tnf* genes are important for skeletal development, while the *Itgam* and *Vcam1* genes have been associated with cell adhesion and *Phex* gene have been associated bone mineral metabolism (Figure 2).

In general, our findings suggest that this direct action on osteoblast gene expression may be part of the mechanism involved in arrested development and impaired skeletal function

due to prenatal alcohol exposure. The mechanisms underlying alcohol's impact on bone formation remain unclear. Examining gene expression linked to osteogenic differentiation holds the potential to unravel these mechanisms in bone development, given that osteoblasts and ECM are two pivotal elements in the process [105].

Although the methodology adopted for the treatment of the animals in our study is well described and previously performed by other authors [14,16,18,19,27,51], some limitations should be pointed out.

Firstly, the liquid diet of isocaloric group (Pair-fed group) was not strictly controlled, although nutritional pairing was accomplished regarding the solid food. Moreover, the extent of the pups' exposure to alcohol during the breastfeeding period was not assessed. Despite these limitations, this kind of methodology have been well accepted in the scientific environment [14,16,18,19,26,27,51].

Another limitation that should be highlighted is the absence of individual sample evaluation for each animal. Since we have evaluated the cells jointly and not individually, we could not check the individual gene expression or difference between males and females. However, our findings represent a screening for selection of some genes that should be investigated individually in male and female newborns using the same model where the cells are extracted from the calvariae of newborns while still in the pre-osteoblastic mesenchymal cell stage. In our model, the cells are allowed to differentiate into osteoblasts during *in vitro* cultivation, without the addition of alcohol to the culture medium. This approach proved to be suitable to assess the influence of alcohol on gene expression during fetal development, demonstrating a direct effect of alcohol on bone development. Future *in vitro* and *in vivo* studies should also explore osteoclast behavior to assess the full effect of maternal ingestion on bone turnover of the newborns.

In summary, our study evaluated the gene expression of osteoblasts from the calvaria of newborn rats whose mothers received alcohol prior to breeding and during the three weeks of gestation. A suitable alcohol level could be detected in the blood of these mothers. As expected, the animals from the Alcohol group exhibited a tendency to weight gain, even with lower ingestion of food. The poor nutritional intake of the Alcohol group was compensated with the insertion of the Pair-feed group which received lower nutrients. After the treatment of the mothers, pre-osteoblast of the newborns was extracted from the calvaria and differentiated into primary osteoblasts that were posteriorly evaluated regarding osteogenic genes. Altered expressions were detected in 17 genes of which some were downregulated and others upregulated in the Alcohol group.

## Conclusion

This study demonstrate that ethanol directly affects fetal osteoblasts by altering gene expression related to osteogenic differentiation in newborn rats, including Itgam, Nog, Tnf,

and Mmp-9. Such alterations may impair overall fetal skeletal development. This study highlights the potential repercussions of maternal alcohol consumption on the skeletal health of offspring, paving the way for further investigations into its long-term effects.

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## Conflict of interest statement

The authors report no conflicts of interest related to this study.

## Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Ethics approval statement

This study was approved by the ethics committee of São José dos Campos Institute of Science and Technology, UNESP – São Paulo State University (Protocol No. 01/2012-PA/CEP).

(Supplementary Material)

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