Original Research Paper

Prenatal Ethanol Exposure Affects the Proliferation and Differentiation of the Osteoblasts from Newborn Rats

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Abstract: Alcohol exerts teratogenic effects and its consumption during pregnancy may cause various alterations in the fetus, including deficit of bone development. The objective of this study was to evaluate the initial responses, on osteoblasts isolated from newborn rat calvaria, after prenatal ethanol exposure. Nine pregnant rats were divided into three groups according to the diet fed during pregnancy: Rats fed 20% ethanol, Pair-fed and control were the groups. At 3 days of life, newborn rats were euthanized for removal of the calvaria and isolation of osteogenic cells by sequential enzymatic digestion. The cells were cultured for a maximum period of 14 days. The effect of alcohol was investigated by the measurement of cell adhesion, proliferation and viability, total protein content, Alkaline Phosphatase (ALP) activity and bone matrix formation. The results showed the highest proliferation in ETH group on the 3th day and the highest ALP activity and bone matrix formation, in this group, on the 14th day, indicating that prenatal ethanol seems to affect the proliferation early and the ALP activity and bone matrix formation in more advanced periods.

Keywords: Ethanol, Gestation, Osteoblasts, Rat, Cell Culture

Introduction

Maternal alcohol consumption during pregnancy can have important teratogenic effects. The direct outcomes for the fetus and newborn include spontaneous abortion, low birth weight, prematurity, asphyxia and perinatal mortality (Moraes and Reichenheim, 2007; Silva *et al*., 2011) as well as a range of facial and cerebral anomalies, cognitive and growth retardation, neurological and behavioral problems, a group of conditions called "Fetal Alcohol Spectrum Disorders" (FASD) (Barr and Streissguth, 2001).

Some studies *in vivo* have investigated newborn animals that consumed ethanol before and/or during pregnancy in an attempt to elucidate the mechanisms of action of alcohol on bone tissue of the offspring. Among all the changes that alcohol exposure can cause in fetus and newborn, it has observed delayed ossification, body weight loss, reduced length of individual bones and consequent delay in overall bone growth (Day *et al*., 1989; Day *et al*., 2002; Keiver *et al*., 1997; Keiver *et al*., 1996; Keiver and Weinberg, 2004; Lee and Leichter, 1980, 1983; Ramadoss *et al*., 2006; Simpson *et al*., 2005; Weinberg *et al*., 1990).

Other studies *in vitro* have showed the effect of ethanol added directly to the culture medium of different lines of osteoblastic cells (Chavassieux *et al*., 1993; Friday and Howard, 1991; Gong and Wezeman, 2004; Klein *et al*., 1996). However, no study evaluated the initial responses on osteoblasts of newborns rats, after prenatal ethanol exposure. Thus, the objective of this study is to investigate the behavior of osteoblast of newborn rats, with respect to basic functions, after prenatal ethanol exposure.

Materials and Methods

Animals

All animal procedures were in accordance with the guidelines of the Animal Research Ethics Committee of

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the Sao Jose dos Campos Institute of Science and Technology, Univ Estadual Paulista-UNESP (Protocol No.002/2010 -PA/CEP).

Tree-months-old virgin female Wistar rats, weighing approximately 300g, were mated and pregnancy was confirmed by vaginal smear as described by (Kato *et al.*, 1979). After the confirmation of pregnancy, the rats were kept in individual cages and divided into three groups that received the following diets daily for the 21 days of pregnancy: The Ethanol group (ETH) was fed 20% ethanol solution and rodent lab chow *ad libitum*. The Pair-Fed (PF) group received the same amount of calories as the ETH group. For this purpose, the amount of alcohol and rodent lab chow consumed by animals of the ETH group was measured on the day before and the PF group received the equivalent amount of carbohydrate solution and rodent lab chow on the next day. Control (CONT) animals were fed water and rodent lab chow *ad libitum*. The 20% ethanol solution and carbohydrate solution was obtained as previously described by (Marchini *et al.*, 2012). Treatment consisted of oral self-administration of the liquid and solid diets. Rats were maintained in ambient temperature rooms with lights on between 6h to 18h.

The diets were administered for 21 days of gestation. Diet was presented daily and the amount of calories (solid and liquid diet) ingested by animals was measured daily. On the 21st of pregnancy the newborns were born by natural delivery and remained with their mothers until the day of euthanasia. The dams received the diets, liquid and solid, until the day of euthanasia. All newborns were euthanatized at 3 days old for cell isolation.

Cell Isolation and Primary Culture of Osteogenic Cells

Osteogenic cells were isolated by sequential trypsin/collagenase digestion of calvarial bone obtained from newborn (3 days old). Wistar rats as described previously (Nanci *et al.*, 1996). The pool of cell from each experimental group were plated in 24-well polystyrene plates at a density of 20.000 cells/well. The cells were cultured for a maximum period of 14 days in α-Minimum Essential Medium with L-glutamine (Gibco, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Invitrogen), 7 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), 5 µg/mL ascorbic acid (Mallinckrodt Chemicals, Phillipsburg, NJ, UK) and 50 µg/mL gentamicin (Gibco, Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was changed every 3 days. Progression of the cultures was evaluated by phase contrast microscopy.

Cell Adhesion

For evaluation of cell adhesion, the cells were cultured for 4h. The culture medium was removed and the wells were washed three times with Dulbecco's Phosphate Buffered Saline (PBS) (Gibco, Invitrogen) at 37°C to eliminate unattached cells. The cells were then enzymatically detached (1 mM EDTA, 1.3 mg/mL collagenase type II and 0.25% trypsin; Gibco, Invitrogen) and counted in a hemacytometer. Cell adhesion was expressed as the percentage of the initial number of cells (20.000 cells/well).

Cell Proliferation

For evaluation of cell proliferation, the cells were cultured for 1, 3, 7 and 10 days and enzymatically released from the well by 1 mM EDTA, 1.3 mg/mL collagenase type II and 0.25% trypsin (Gibco, Invitrogen). Aliquots of the solutions of each well were incubated for 5 min with the same volume of 0.4% Trypan blue (Gibco, Invitrogen), which stains nonviable cells and cells were counted in a hemacytometer. Cell proliferation was expressed as the number of cells per well.

Total Protein Content

Total protein content was determined using a modification of the method of (Lowry *et al.*, 1951). Briefly, proteins were extracted from each well with 0.1% sodium lauryl sulphate (Sigma-Aldrich) for 30 min and mixed 1:1 with Lowry solution (Sigma-Aldrich) for 20 min at room temperature. The extract was incubated with Folin-Ciocalteau's phenol reagent (Sigma-Aldrich) for 30 min at room temperature. Absorbance was read in a spectrophotometer (Shimadzu Europa GmbH UV 1203, Duisburg, Germany) at 680 nm. Total protein content was calculated using a standard curve of bovine serum albumin and is expressed as µg/mL.

Alkaline Phosphatase Activity (ALP)

ALP activity was assayed in the same lysates as used for the determination of total protein content and was measured as the release of thymolphthalein from thymolphthalein monophosphate using a commercial kit (Labtest Diagnóstica, Belo Horizonte, MG, Brazil). Briefly, 50 µL thymolphthalein monophosphate was mixed with 0.5 mL of 0.3 M diethanolamine buffer, pH 10.1 and left to stand for 2 min at 37°C. The solution was then added to 50 µL of the lysates obtained from each well and the mixture was incubated for 10 min at 37°C. For color development, 2 mL of a solution of 0.09 M Na_2CO_3 and 0.25 M NaOH was added. After 30 min, absorbance was read at 590 nm and ALP activity was calculated from a standard curve using thymolphthalein. The results are expressed as ALP activity normalized for total protein content on days 7, 10 and 14 of culture.

Analysis of the Mineralized Matrix Nodules

For quantitative analysis of mineralized matrix nodules, the cells were cultured for 14 days. After these periods, the test was carried out according to (Gregory *et al.*, 2004) and (Rosa *et al.*, 2009). After

the extraction of the dye, the absorbance was measured by spectrophotometer (Biotek-EL808IU) at 405 nm.

Cell Viability

For assessment of viability, the cells were cultured for 3, 7 and 10 days and then incubated with 100 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (5 mg mL^{-1}) (Sigma-Aldrich), in PBS (Gibco, Invitrogen) for 4 h at 37°C. The medium was aspirated and 1 mL isopropanol acid (0.04 N HCl in isopropanol) (Sigma-Aldrich) was added to each well. The plates were agitated on a plate shaker for 5 min and 200 μL of this solution was transferred to opaque-walled clear-bottom 96-well plates. Optical density was read at 570-650 nm in a plate reader (Biotek EL808IU, Winooski, VT, USA) (De Oliva *et al.*, 2009). The cytotoxicity was expressed as percentage relative to the control group (100%).

Statistical Analysis

The results are representative of experiments performed with three distinct primary cultures prepared from the offspring of the animals that composed the groups (n=18 wells per parameter). These results are reported as the mean ± standard deviation and a *p* value ≤0.05 was considered to be statistically significant. Alcohol consumption was analyzed by Kruskal-Wallis and Dunn's tests. Cell adhesion was compared by Student t-test and Mann-Whitney test. Cell proliferation, ALP activity, total protein content and mineralized matrix nodules were analyzed by Kruskal-Wallis and Mann-Whitney tests. Lastly, cell viabiltity was analyzed by Z test.

Results

Dam Diet

The values of solid and liquid diet are compared and described in Tables 1 and 2 (Kruskal-Wallis and Dunn's tests). By analyzing the solid diet, CONT group showed a statistically greater consumption than ETH and PF groups (p=<0.0001 and p=<0.0001, respectively) and ETH group was similar to PF group. According to the liquid diet, CONT group again showed a statistically greater consumption than ETH and PF groups (p=0.0004 and p=<0.0001, respectively). However the ETH group showed higher values than PF group (p=0.0006).

Table 1. Analysis comparing solid diet and liquid diet from dams during pregnancy (Kruskal-Wallis test)

Groups	ETH	PF	CONT
Solid diet (g/day/rat)			
Average	12.1	12.0	19.6
Stardard deviation	1.4	1.8	1.5
p value	<0.0001[a]	<0.0001[a]	<0.0001[a]
Liquid Diet (ml/day/rat)			
Average	28.2	13.1	41.0
Stardard deviation	2.6	6.4	6.6
p value	<0.0001[a]	<0.0001[a]	<0.0001[a]

[a]Value considered statistically significant

Table 2. Statistical *p* values comparing solid diet and liquid diet from dams during pregnancy (Dunn's test)

Groups	ETH	CONT
Solid diet		
CONT	<0.0001[a]	
PF	>0,9999	<0.0001[a]
Liquid diet		
CONT	0.0004[a]	
PF	0.0006[a]	<0.0001[a]

[a]Statistically significant difference

Table 3. Analysis of alcohol consumption

Alcohol consumption	
Average intake of alcohol solution 20% (mL/day/rat)	28.2 mL
Average intake of absolute alcohol (mL/day/rat)	5.6 mL
Average intake of absolute alcohol (g/day/rat)[a]	4.5 g
Feed intake (g/day/rat)	12.1 g
Feed intake (Kcal/day/rat)[b]	28.8 Kcal
Alcohol consumption (Kcal/day/rat)[c]	31.9 Kcal
% Calorie diet from alcohol (average per day/rat)	52.4 %

[a]Considering that 1 mL of alcohol is equivalent to 0.790 g
[b]Considering that 1 g of commercial food (Labina-Purina®, Paulinia, Brazil) has 2.386 Kcal
[c]Considering that 1 g of alcohol is equivalent to 7.1 Kcal

Analysis of Alcohol Consumption

The average amount of 20% alcohol solution consumed daily per animal was 28.2 ml. With this data it was able to calculate the amount of alcohol consumed in other units of measurement (Table 3). The percentage of calories from the alcohol diet was also calculated. The results showed that on average 52.4% of dietary calories came from alcohol consumption.

Cell Adhesion

On the 4th day, a significant difference in the cell adhesion test was observed between the CONT and PF groups (p=0.016). The cell adhesion was higher in the CONT group (Fig. 1). The cell adhesion in ETH group was similar to CONT and PF groups.

Cell Proliferation

The cell proliferation test showed a significant difference between the groups at 3, 7 and 10 days. On the 3th day, the ETH group presented higher cell proliferation than PF group (p=0.005), which showed higher values than CONT group (p=0.026). On the 7th day ETH group was similar to PF group and both showed higher values than CONT group (p=0.008 and p=0.048). On the 10th day, the ETH group presented higher cell proliferation than CONT group (p=0.004). However, ETH group was similar to PF group, which was similar to control group. These results can be observed in Fig. 2.

Total Protein Content

Total protein content showed a significant difference between the groups only at 7 e 14 days. On the 7th day, ETH group was similar to PF group and both presented lower values than CONT group (p=0.016 and p=0.005). On the 14th day ETH group was similar to PF group again

and both showed higher total protein content than CONT group (p=0.001). These results can be observed in Fig. 3.

Alkaline Phosphatase Activity (ALP)

The ALP test showed a significant difference between the groups at 7, 10 and 14 days. On the 7th and 10th day ETH group was similar to PF group and both (p=0.001; p=0.001; p=0.003 and p=0.001) presented higher values than CONT group. On the 14th ETH group showed higher ALP than PF and CONT groups (p=0.011 and p=0.001). In this same period, PF group presented higher values than CONT group (p= 0.001). These results can be observed in Fig. 4.

Analysis of the Mineralized Matrix Nodules

The analysis of mineralized matrix nodules showed significant difference between ETH groups to PF (p= 0.005) and CONT group (p<0.001). ETH group presented higher values than PF and control groups. These results can be observed in Fig. 5.

Cell Viability

The data obtained in the MTT test showed that the mean of cell viability in ETH group was above 100%.

When ETH and PF groups were compared to CONT group (100%), only ETH group showed a significant difference from CONT group, on the 3th day (p<0.001). In this period, ETH group presented the highest amount of viable cells (Fig. 6).

When ETH and PF groups were compared between them, there was significant difference (p=0.002). On the 3th day, ETH group was different from all periods from PF group (p<0.001). On the 7th day, ETH group was different from PF group on the 3th day (p<0.001).
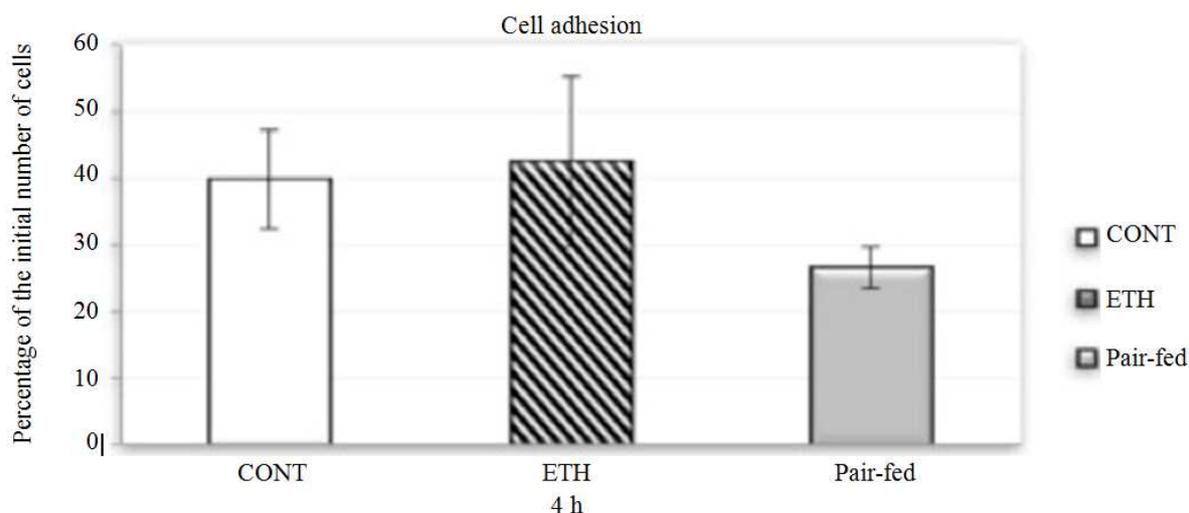

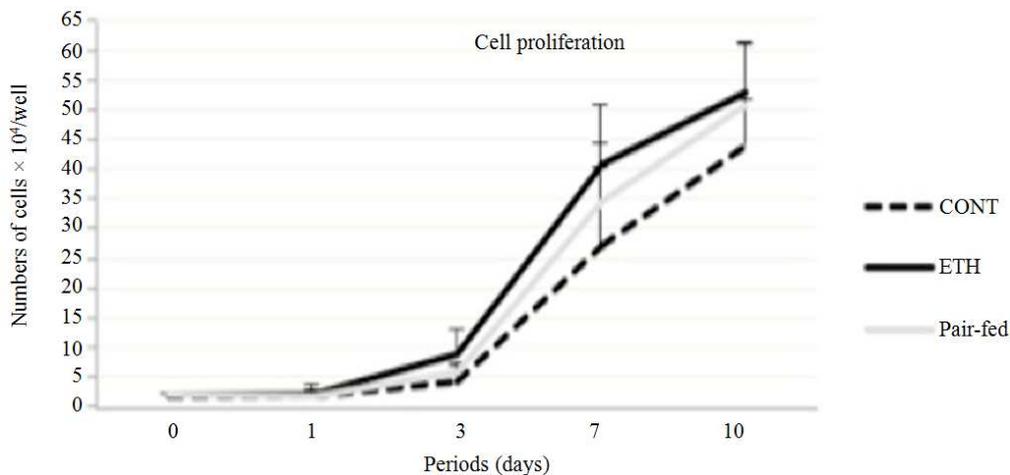Fig. 1. Graph showing the mean ± standard deviation percentage of cell adhesion after 4 h

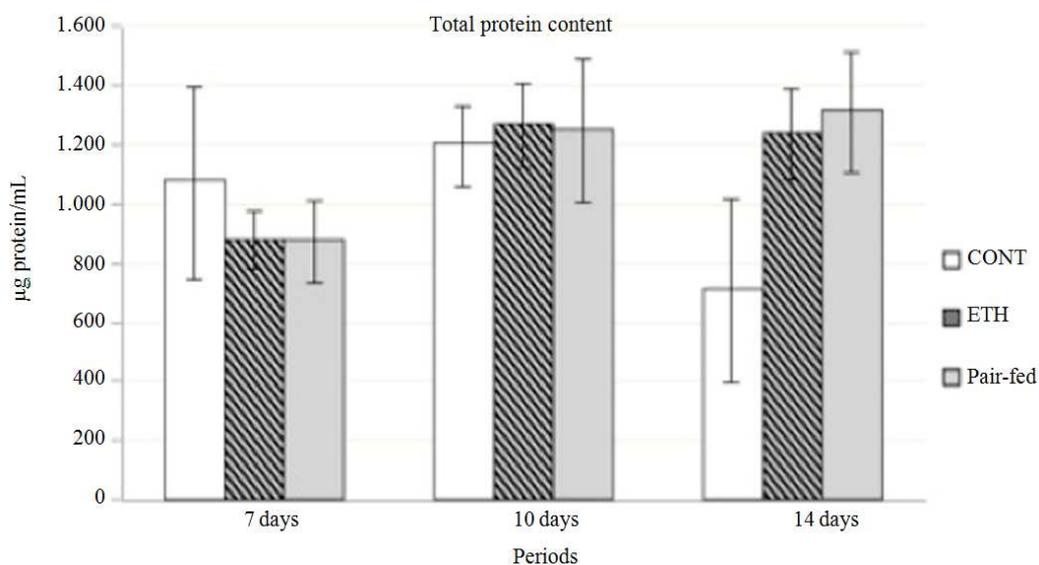Fig. 2. Graph showing the mean ± standard deviation of number of cells after 1, 3, 7 and 10 days

Fig. 3. Showing the mean ± standard deviation of total protein content in µg/mL, after 7, 10 and 14 days
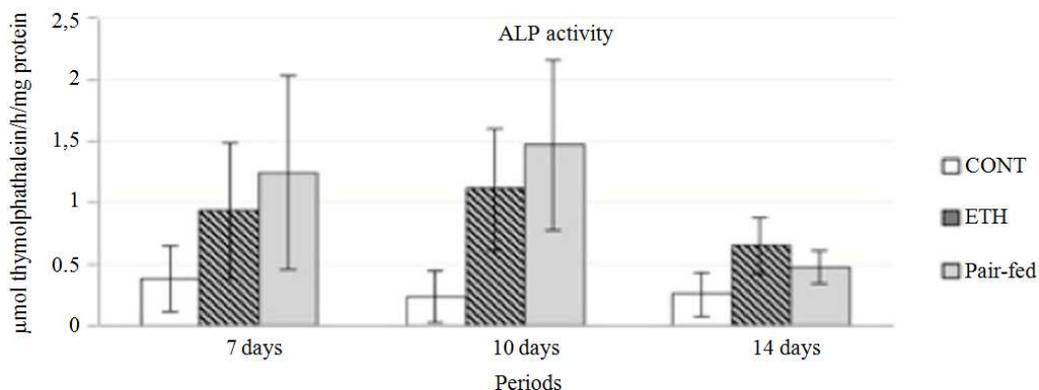

Fig. 4. Showing the mean ± standard deviation of alkaline phosphatase activity in thymolphthalein micromol/h/mg protein/mL, after 7, 10 and 14 days
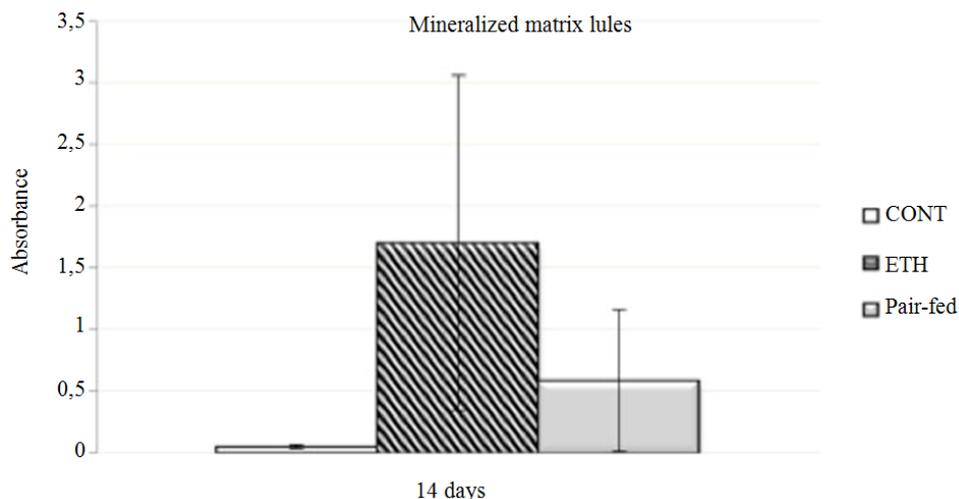
Fig. 5. Showing mean ± standard deviation of absorbance obtained with analysis of the mineralized matrix nodules after 14 days
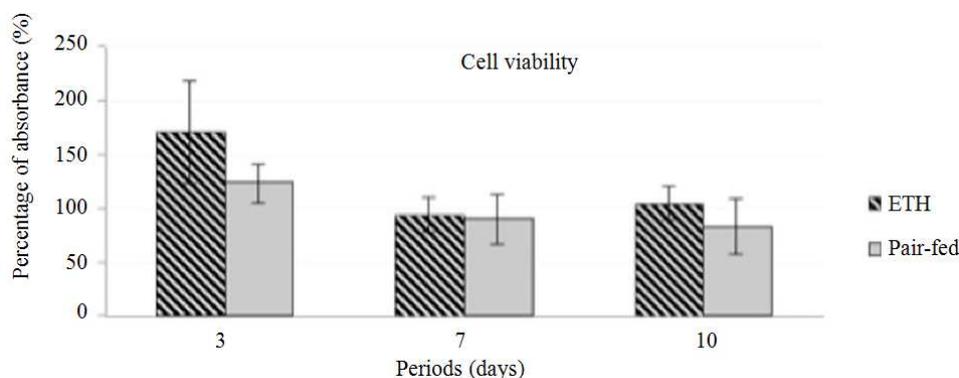

Fig. 6. Graph showing the mean ± standard deviation percentage of absorbance, obtained with MTT assay, after 3, 7 and 10 days

Discussion

Studies have indicated that prenatal ethanol exposure affects the skeletal development of the fetus and/or newborn (Day *et al*., 1989; Day *et al*., 2002; Keiver and Weinberg, 2004; Lee and Leichter, 1980, 1983; Ramadoss *et al*., 2006; Simpson *et al*., 2005; Weinberg *et al*., 1990) but the mechanisms whereby this occurs are still unclear. In order to investigate these mechanisms, this study was the first that evaluated the initial responses on osteoblasts of newborns rats, after prenatal ethanol exposure.

The average volume of alcohol solution ingested by ETH group was 28.2 (ml/day/rat), which corresponded to an average of 52.4% of total calories came from alcohol. This data suggest that the amount of alcohol consumed by the animals of this study was sufficient for producing alterations in skeletal development of newborns rats, once in other studies 36% of total calories of the rats came from alcohol and this amount was

sufficient to decreased fetal body weight, skeletal ossification concentrations and length of bone (Keiver and Weinberg, 2003, 2004; Simpson *et al*., 2005).

In addition, the average of cell viability in this study was above 100% for ETH group. This finding indicates that prenatal ethanol exposure, did not damage the cell viability on osteoblasts of newborns rats.

Due to possible nutritional changes generated by the consumption of alcohol, an isocaloric group was used to establish a nutritional pairing. Thereby, the alterations caused by alcohol, only have considered relevant when EHT group showed a significant difference from PF and CONT groups.

Our results showed that prenatal ethanol exposure improved the proliferation in early stages (on the 3th day) and the ALP activity and bone matrix formation in more advanced periods, at 14 and 21 days, respectively.

The viability was analyzed by MTT assay. Bromide (3-(4,5-dimethylthiazol-2il)-2,5-diphenyl tetrazolium (MTT) test is an assay that shows not only

the number of cells, but also the level of its metabolic activity because it is based on the activity of enzymes, such as succicil dehydrogenase, which is present in viable cells (Mosmann, 1983).

The cells of ETH group showed higher levels of proliferation than PF and control groups on the 3th day. Moreover, the greatest number of viable cells was seen during this period in ETH group. These results indicate that during this period the ETH group presented higher number of cells than other groups and these cells were viable.

This stimulatory effect in the proliferation of osteoblasts was also observed on cells removed from the femur of rats submitted to chronic alcohol consumption in a previous study (Rosa *et al.*, 2008). On the other hand, some studies have demonstrated an antiproliferative effect of alcohol on osteoblastic cells (Chavassieux *et al.*, 1993; Friday and Howard, 1991; Klein *et al.*, 1996). However, these studies have not evaluated the performance of alcohol systemically because alcohol was added directly to the culture medium of different lines of osteoblastic cells.

The total protein content is associated with the capacity of synthesis of the cells and have been considered a parameter to evaluate the osteogenesis *in vitro* (Beloti and Rosa, 2005; Rosa and Beloti, 2005). Besides, the production of enzyme alkaline phosphatase is also an indicator of bone formation (Mödder and Khosla, 2008) because this enzyme captures phosphate ions during the process of osteogenesis, being essential to mineralization (Delgado-Calle *et al.*, 2011). When mineralized matrix of bone tissue is observed, the presence of cell differentiation can be confirmed (Hoemann *et al.*, 2009).

Based on the results of this study, prenatal ethanol exposure improves the cell differentiation, promoting an increase in the production of alkaline phosphatase and mineralized bone matrix, on the 14th and 21st, respectively. These events are directly correlated, according to previous studies (Beloti and Rosa, 2005; Rosa and Beloti, 2005).

A previous study (Klein *et al.*, 1996) verified an alteration in the ALP synthesis in the ETH group, similar to our study that showed highest levels of ALP in the ETH group in the last period. On the other hand, the reduction in ALP activity in the groups submitted to ethanol was observed in other study that did not evaluate the performance of alcohol systemically (Friday and Howard, 1991). Probably, our findings may provide more accurately support in relation to effects of chronic ethanol consumption on osteoblastic cell differentiation, because our evaluation was systemic.

Rosa *et al.* (2008) observed a decrease in the mineralized bone matrix when administered alcohol systemically in rats and did ETH exposure of cell cultures. However this author evaluated the stems cells of the rats which were exposed to alcohol, different of our study that showed the responses on osteoblasts of newborns rats, after prenatal ethanol exposure.

Other studies involving more complex cellular mechanisms are needed to clarify why the alcohol enhances the proliferation and differentiation of osteoblasts in some periods and what the relationship of these results with *in vivo* findings showing that alcohol impairs the ossification.

Conclusion

Our study was the first that realized prenatal ethanol exposure and verified initial responses on osteoblasts of newborns rats. The ethanol affected the proliferation of these cells early and the ALP activity and bone matrix formation in more advanced periods.

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Author's Contributions

Isabel Chaves Silva Carvalho: Development of research idea, care/treatment of animals, running the experiments, analysis of whole data, writing draft of whole manuscript and final approval of manuscript.

Dennia Perez de Andrade, Noala Vicensoto Moreira Milhan and Evelyn Luzia de Souza Santos: Care / treatment of animals, running the experiments, reading draft, correcting and final approval of manuscript.

Cristina Pacheco Soares: Compiling and analysis of whole data, reading draft, correcting and final approval of manuscript.

Rosilene Fernandes da Rocha: Project was conceived, planned and coordinated, reading draft, correcting and final approval of manuscript.

Luana Marotta Reis de Vasconcellos: Supervising research work, reading draft, correcting and final approval of manuscript.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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