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PRENATAL DEXAMETHASONE EXPOSURE AFFECTS MANDIBULAR BONE AND TOOTH DEVELOPMENT IN RATS

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ABSTRACT

Prenatal corticosteroid exposure is associated with important adverse effects on fetuses development. In this study, histomorphometric evaluation of the mandibular bone and mandibular first molar from fetuses exposed to exogenous glucocorticoid in the final period of pregnancy was performed. For this study, six female rats were housed with two male rats for 8 days. The pregnant rats were assigned into two groups (n=3 each): the control group and the dexamethasone group. The dexamethasone received 0.2 mg.kg-1.day-1 water-soluble dexamethasone dose daily diluted in the drinking water from day 14-19 of pregnancy. On the 20th day of gestation, rats were sacrificed, and the fetuses were obtained by cesarean derivation. The weight of each fetus was recorded and euthanized and the head was fixed in 10% phosphate-buffered formalin. Selected samples were evaluated by light microscopy, and the following measurements were recorded: the perimeter of mandibular bone, Meckel's cartilage, and tooth germ; buccolingual length, vertical distance, anteroposterior distance and area of the tooth germ, by the Image J program. There was a statistically significant difference regarding birth weight, mandible perimeter, tooth germ vertical distance, and buccolingual distance; the area of dental germ and germ perimeter were lower in the dexamethasone versus control. There was no statistically significant difference in Meckel's cartilage perimeter and anteroposterior distance of tooth germ between groups. In conclusion, prenatal exposure to excess dexamethasone impairs mandibular bone and tooth development.

KEY-WORDS: *dexamethasone, glucocorticoid, odontogenesis, osteogenesis, pregnancy.*

INTRODUCTION

Glucocorticoids (GC) are a class of steroid hormones that mediate a range of physiological effects in vertebrates, with regulatory roles in development, metabolism, neurobiology, and programmed cell death. Besides the physiological roles, corticosteroids have pharmacological anti-inflammatory and immunosuppressive effects and are among the most prescribed class of drugs in the world.[1]

Numerous compounds with GC activity have been synthesized. Up now, the therapeutic usage of GCs has increased continuously, and they are the standard therapy for diverse disorders, such as asthma, allergy, rheumatism, collagen, vascular and dermatological disorders, inflammatory bowel, and other systemic diseases. Although GCs provide beneficial effects when used for treatments, adverse systemic effects are common.[2]

In adulthood, adverse effects may occur with different prevalence, in different organs, and after distinct durations and dosages of therapy. The side effects may be single or multiple and can vary from cosmetic aspects (e.g., telangiectasia, hypertrichosis) to major concerns, such as diabetes *mellitus*, adrenal atrophy, hypertension, dyslipidemia, and increased risk of infection, among others[2].In children submitted to long-course GC therapy (at least 15 days of treatment) the most reported adverse effects are weight gain, growth retardation, and cushingoid features. Some of the side effects are reversible after the medication is discontinued, while others may be permanent.[3]

Prenatal GC exposure is also associated with important adverse effects. Some medical conditions during pregnancy, such as systemic lupus erythematosus, asthma, and inflammatory bowel disease, are currently treated with long-term GC.[4] Still, in pregnancies with an expected preterm delivery, GC is administered to reduce the risk of neonatal respiratory distress syndrome and mortality.[5] The impact of GC on the newborn depends on the course, dose, and gestational stage at the drug administration.[6] Studies *in vivo* using rats have shown that offspring exposed to dexamethasone in the late period of gestation impaired survival rate,[7] lower body mass,[7, 8] reduced bone mass of long bones,[6,9,10] permanently impaired glucose tolerance and hypertension in adulthood.[8]

Few studies evaluated the effects of GC on mandibular development, and these were limited to mandibular ramus[11] and mandibular ramus and condyle.[12] A more recent study revealed an increased expression of matrix metalloproteinases (MMP) 2 and 9 on the head of the embryonic zebrafish treated with dexamethasone or hydrocortisone and showed abnormal craniofacial phenotype on hydrocortisone-treated fetuses, with decrease lower jaw length.[13]

On the other hand, it is known that teeth from adults treated for a long period with GC present larger predentin, increased formation of secondary dentin, dental pulps with excessive mineralized tissue, and narrowing of the dental pulp chamber.[14,15] Despite that, the effects of GC on tooth germ development remain vague.

This study conducted a histomorphometry evaluation of the mandibular body and the mandibular first molar tooth germ from fetuses exposed to excess GC in the late period of pregnancy. It is hypothesized that dexamethasone administration in the third period of gestation may induce mandibular bone and tooth germ growth reductions.

METHODOLOGY

All experiments were conducted following the Brazilian National Council for Animal Experimentation Control (CONCEA), and the experimental protocols approved by the local institution.

Six females and 2 males Wistar rats were used in this study. The animals were housed in climate-controlled conditions (21 + 2°C) and a 12-h light-dark cycle (lights on at 0600). The animals had ad libitum access to food (commercial standard chow for rats, Nuvilab CR-1; Nuvital, Brazil) and filtered tap water. Six-week-old nulliparous Wistar rats were acclimatized for a period of 6 weeks. After habituation, the female rats were housed with male rats for 8 days (three females and one male per cage). The presence of spermatozoa in a vaginal lavage indicated day 0 of gestation and this female rat was transferred to a separate cage. Pregnant rats were housed together (three per cage) until 12 days post conception (dpc), when they were housed separately, but remained in audio-visual and olfactory contact with other animals at all times.

The pregnant rats were randomly assigned into two groups (n=3 each): the control group (CTL) (did not receive any treatment) and the dexamethasone group (DEX). The DEX group received 0.2 $mg.kg^{-1}.day^{-1}$ water-soluble dexamethasone dose daily (Decadron®, Aché, Campinas, SP, Brasil) diluted in the drinking

water from day 14 to 19 of pregnancy. The dexamethasone dose was adjusted daily according to the water intake on the previous day and adjusted to the body mass of the current day. The dose of dexamethasone used was reported in a previous publication using rats as an experimental model.[16] The pregnant rats used in this study were the same used in a previously published study[17] that was handled for the following parameters during the pregnancy period: *i)* daily body weight measure between 13th to 20th dpc and *ii)* tail blood sampling at 13th and 20th dpc. No additional intervention was done in these pregnant rats.

In the 20th dpc, the pregnant rats were sacrificed, and the fetuses were obtained by cesarean derivation. The weight of each fetus was recorded. After that, the fetuses were euthanized by decapitation and the head was fixed in a 10% phosphate-buffered formalin solution for at least 24 hours. Those fetuses not used in this study were euthanized and destined for disposal according to the current sanitary rules.

For the Histomorphometric analysis, the mean weight body of the overall fetus was determined and the five fetuses of each group with the body weight closer to the mean value were included in this analysis. No distinction between males and females was applied for this study. The samples were decalcified with 4.13% EDTA, dehydrated, and embedded in paraffin with the coronal plane parallel to the section plane. Longitudinal sections were performed until the dental follicle on the mesial region of the first mandibular tooth germ was localized. Then, serial sections of 3-µm thickness were collected until the dental follicle ended in its distal region. The number of sections obtained was counted and the anteroposterior distance of the tooth germ was determined (number of sections obtained x 3 µm = mesial-distal length). After that, the section corresponding to the center of each germ was selected and stained with hematoxylin-eosin (Figure 1a).

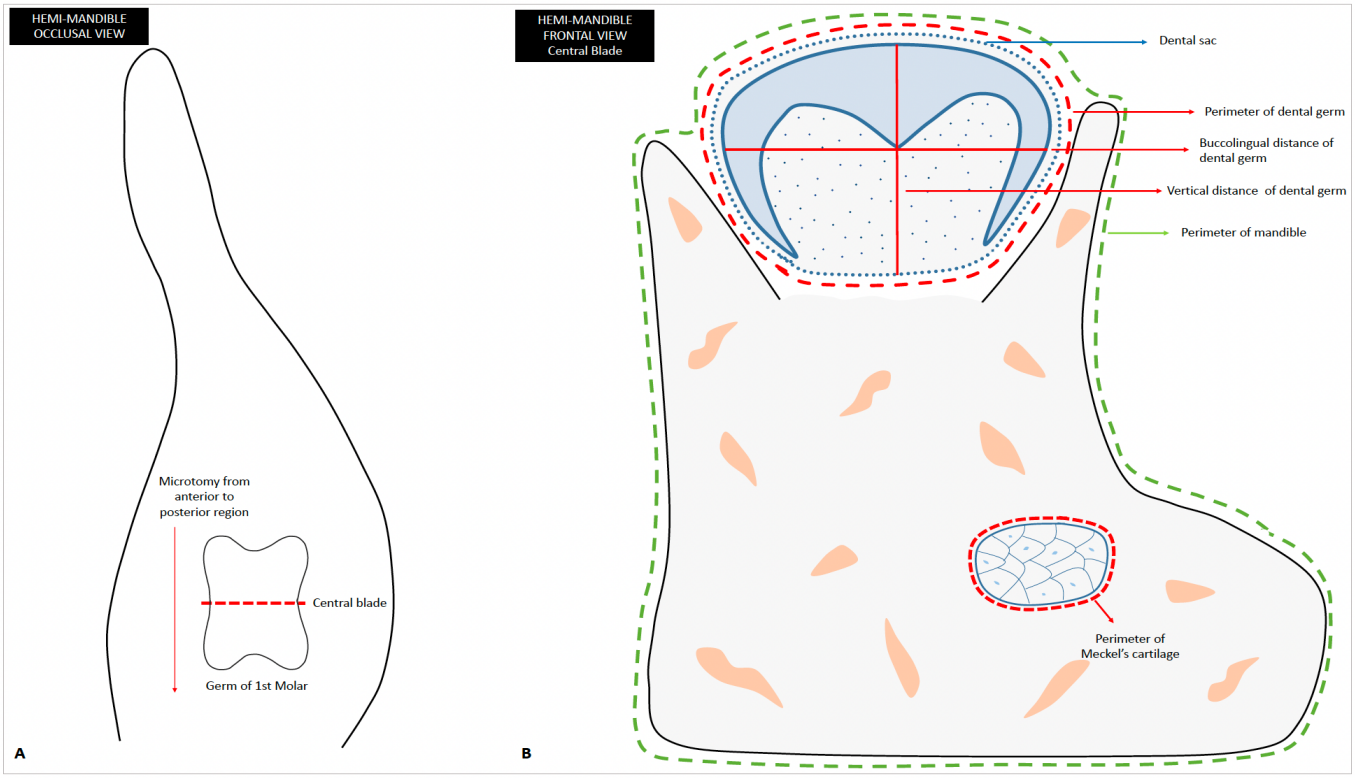

Figure 1: (A) Occlusal view of mandibular bone. The dashed line indicates the central blade collected from the center of 1st mandibular bone. (B) Frontal view of mandibular bone. Dashed lines indicate measurement of perimeter of the mandible, Meckel's cartilage, and dental germ; red lines indicate linear measurements of dental germ.

Images were captured using an Olympus® Bx41 microscope (Olympus, SP, Brazil) with a 3.3-pixel camera and Q-capture Pro 5.1 software (Q-imaging, BC, Canada). Analysis of the images was conducted using NIH Image J program. It measured the perimeter of the mandibular bone surrounding the tooth germ and the perimeter of Meckel's cartilage in the central slide selected. Also, it was measured in the tooth germ: perimeter, buccolingual length, vertical distance, and per unit value (P.U) (Figure 1b).

The P.U is a system used to express quantities as fractions of a defined base unit quantity, and it was applied in this study to estimate the area of dental germ from the data obtained. The product of three dimensions (height x anteroposterior distance x buccolingual length) was determined for each sample and named as "area". The sample with a higher value of the area was considered the base value (base value = 1 P.U). Each of the remaining samples had its area value divided by base value, revealing the fraction:

PU = (area) / (base area)

Data were tabulated on Excel 2016 (Microsoft Office 2016; Microsoft, Redmond, United States of America) and analyzed using SPSS Statistics 25 (SPSS Inc., IBM). Normality tests were conducted to assess the normal distribution of data. Based on normality test results, the parametric unpaired *t*-test and non-parametric Mann-Whitney test were used for between-group comparisons. Statistical significance was set at α=0.05.

Table 1 – Association of birth weight, Meckel's cartilage, mandible and tooth germ development with dexamethasone treatment

		Dexamethasone Treatment	No Treatment	p
Birth weight (gr)	Mean (SD)	2.4 (0.2)	3.9 (0.08)	**< 0.001[a]**
	Median	2.603	3.915	
Meckel's Cartilage Perimeter (µm)	Mean (SD)	523.2 (32.3)	521.4 (75.5)	0.963[a]
	Median	529	559	
Mandible Perimeter (µm)	Mean (SD)	2. 563 (186.2)	3.422,8 (205.3)	**< 0.001[a]**
	Median	2.585	3.363	
TOOTH GERM				
Vertical distance (µm)	Mean (SD)	285 (44.2)	408 (25.0)	**0.001[a]**
	Median	298	489	
Buccolingual distance (µm)	Mean (SD)	405.4 (59.3)	490 (31.4)	**0.03[a]**
	Median	375	489	
Anteroposterior distance (µm)	Mean (SD)	1.125,6 (92.9)	1.199,6 (144.1)	0.465[b]
	Median	1.179	1.131	
Area of dental germ (µm)	Mean (SD)	0.4 (0.1)	0.7 (0.1)	**0.005[a]**
	Median	0.4	0.7	
Germ Perimeter (µm)	Mean (SD)	1.233,4 (126.3)	1.530,8 (59.9)	**0.001[a]**
	Median	1.246	1535	

[a]unpaired *t*-test; [b]Mann-Whitney Test

RESULTS

Of the 3 rats of DEX, two had 12 fetuses each, and one had 13 fetuses, while on the CTL one rat had 14, one had 11, and one had 9 fetuses.

Histological evaluation of Meckel's cartilage, mandibular bone, and mandibular first molar revealed that both groups were at the same stage of development. The mandibular bone is under expansion and ossification, involving all the dental germ, except in its superior region, where remains the dental lamina connected to the oral epithelium. The Meckel's cartilage is present under the dental germ and in a medial position, near to the tongue, in all animals, with no difference.

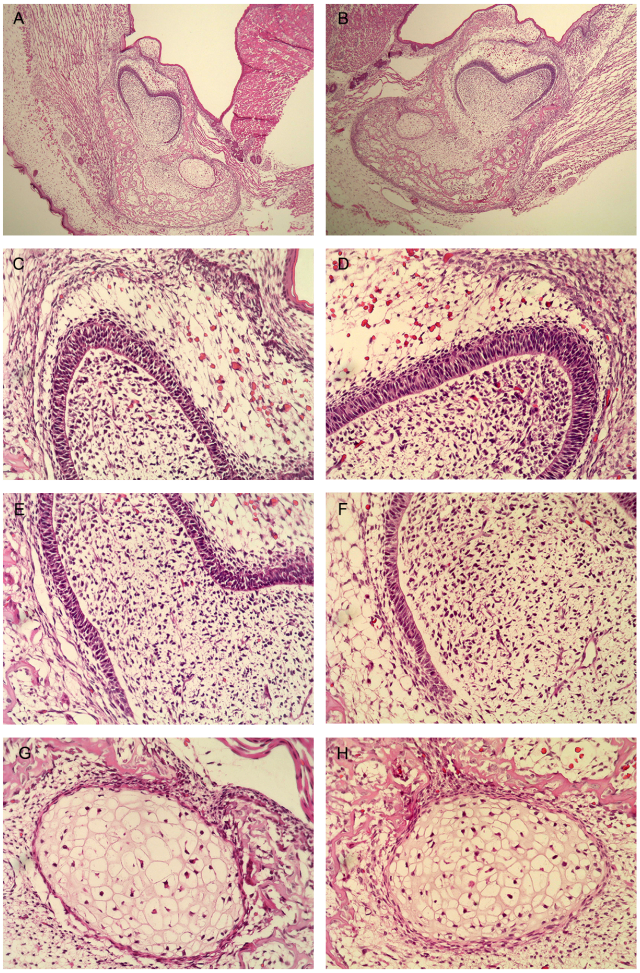

Figure 2: Images of some histologic slides analyzed. (a, b) mandible and dental germ (40X). (c, d) Outer enamel epithelium (400X). (e,f) cervical loop (400X). (g,h) Meckel's Cartilage (400X). a, c, e, g: DEX; b, d, f, h: CTL.

The tooth germ of the mandibular first molar of all animals was at the bell stage of development. The enamel organ presents the inner epithelium, on the cusp region, with columnar cells. Above these is the stratum intermedium and stellate reticulum; below it the dental papilla periphery cells are condensate. The extremity of the inner epithelium is forming the cervical loop. The dental follicle involves all dental germs. The dental lamina is still present, connected to the oral epithelium. Figure 2 shows histological sections from the mandible and dental germ of CG and DG animals.

Histomorphometric evaluation, on the other hand, revealed reduced fetus weight ($p<0.001$); mandible perimeter ($p<0.001$); tooth germ vertical distance ($p<0.001$) and buccolingual distance ($p=0.03$); area of dental germ ($p=0.005$); and germ perimeter ($p=0.001$). There was no statistically significant difference in Meckel's cartilage perimeter ($p=0.963$) and anteroposterior distance of tooth germ ($p=0.465$). The results are detailed in Table 1.

DISCUSSION

It is estimated that 1% to 3% of adults worldwide take long-term GC, which 20% of patients use for more than 6 months and 5% for over 5 years.[16] GC influences whole-body homeostasis and has an important impact on the skeletal system. In adults under continuous or excessive GC treatment, the most common skeletal disorder is GC-induced osteoporosis (up to 50% in patients using GC longer than 6 months),[17] while children have longitudinal growth impairment[18] and fetuses exposed to GC during pregnancy have growth restriction.[10,13,19]

GC acts on a molecular basis and the cellular response is generally mediated by the GC receptor (GR), which has multiple domains involved in DNA binding, ligand binding, and transcriptional regulation. Rapid non-genomic effects of GR also occur; however, genomic control is the main action of GC in stress response and therapy[17]. Depending on the

physiological state and specific cell type, the GR induces cell proliferation, apoptosis, and differentiation, commonly through gene transcription control, which could be positively or negatively regulated.[18]

The longitudinal growth restriction in children exposed to GC is due to the direct effects of GC on the chondrocytes of the growth plate. *In vitro* and *in vivo* studies have shown that dexamethasone has an inhibitory effect on chondrocyte proliferation, but regarding cellular differentiation, while *in vitro* studies have shown differentiation promotion, *in vivo* studies showed commitment.[18]

Hillegass et al. (2008)[13] showed aberrant craniofacial cartilage development in zebrafish embryos exposed to dexamethasone (smaller ceratohyal cartilage length) and hydrocortisone (smaller ceratohyal cartilage length and lower jaw length) and related this to the impairment of the anterior growth and migration of the pharyngeal cartilages. Besides that, there were higher expression levels of MMP2 and MMP-9 on the head, which was related to the degradation of extracellular matrix and proper cellular migration and tissue organization during embryogenesis.[13]

In our study, there were no differences between Meckel's cartilage perimeter measurement in CTL and DEX groups. Since in this study Meckel's cartilage was evaluated in a single slide, it was not possible to determine its longitudinal length, which might reveal any difference between groups. The primordium of Meckel's cartilage is distinguishable at E13.5, but it became differentiated after E15.5. Chondrocytes express GR Nr3c1 at E15.5 and from E17.5 to E18.5 when the cartilage begins to degenerate.[20] Because dexamethasone was administered onward E14 and the GR Nr3c1 is expressed later, is likely that the signaling to Meckel's cartilage development was no disturbed and this could be the reason we did not find any difference between the groups.

GC also affects bone tissue and is essential in the development and maintenance of bone mass. Physiological levels of GCs are essential in osteoblast differentiation and maturation, through Wnt Signaling, as demonstrated by Zhou et al. (2008) in calvarial osteoblasts derived from GR-deficient mice[21]. The authors also showed that transgenic mice with disrupted GC signaling in osteoblasts present delayed cranial bone development with larger bone sutures, hypoplastic bones, reduced extent of mineralized bone and smaller overall size of the head.[22]

On the other hand, pharmacological doses of GCs affect bone homeostasis, which occurs in two phases: an early rapid phase of bone loss due to excessive bone resorption and a second, by bone formation, hampered.[18] GC at pharmacological levels increases the expression of RANKL, which is related to differentiation and osteoclast activation period, and downregulates the expression of osteoprotegerin (OPG), the decoy receptor of RANKL, promoting an increase of bone resorption.[23]

The impairment of bone formation results from lower osteoblasts proliferation, differentiation, function, and survival.[18] O'Brien et al. (2004)[24] revealed that in transgenic mice with blocked GC action on osteoblasts and osteocytes, there was normal bone development and turnover as compared with wild-type animals, but when an excess of GC was administered there were increased osteoblast and osteocyte apoptosis in wild-type mice. Likewise, osteoblasts, osteoid area, and bone formation rate were significantly higher in GC-treated transgenic mice.[24]

Cheng et al. (2017)[25] demonstrated that chicken embryos exposed to dexamethasone had reduced maxillary formation (part or full absence of the bone) and related this with the harmed Neural Crest Cells (NCCs) generation and differentiation. In this study, the expression of HNK1 (a marker for migratory NCCs), PAX7 (a marker for premigratory and migratory NCCs), and Ap-2a (a marker for cranial NCCs) were lower in

dexamethasone-treated compared to non-treated animals, and the co-expression of these markers with apoptosis markers were higher. Overall, embryos treated with dexamethasone showed skull developmental delay, lower body weight, and increased mortality.[25]

Although the histologic evaluation of our study did not show any disturbance in mandibular bone tissue among animals, the histomorphometric evaluation exposed a significantly smaller mandible in DEX animals. Our results suggest that the development of mandibular bone, which is growing during the fetal and postnatal phases, was restricted by medication. Furthermore, our study also demonstrated lower birth weight in dexamethasone-treated animals.[25]

With regard to tooth development, it seems that similarly to bone tissue, physiological levels of GC are necessary for tooth development. Wang et al. (2019) showed that betamethasone stimulates the proliferation of DPSCs and up-regulates the expression of alkaline phosphatase, dentin sialophosphoprotein (DSPP), osteocalcin in both DPSCs and SHEDs.[26] In this way, Ritchie et al. (2004) showed that dexamethasone up-regulates the expression of DSPP and collagen type I expression on rat tooth organ cultures and suggested that the use of dexamethasone increased extracellular matrix synthesis and mineralization in the pulp.[15]

The results of our study pointed to a significantly smaller dental germ on GC-treated animals. Duarte et al. (2014).[20] showed in the mouse that at E13.5 the molar is at the early bud stage GR Nr3c1 is expressed on dental lamina; at E15.5 the molar is in cap stage and GR Nr3c1 is expressed in the enamel organ stellate reticulum, dental lamina and the surrounding mesenchyme; at E.17.5 the molar is in bell stage and GR Nr3c1 is expressed inner enamel epithelium, outer enamel epithelium, stratum intermedium, dental lamina, and dental papilla.[20]

In transgenic mice with over-expression of GR Nr3c1, delayed tooth formation, microdontia, and oligodontia of the third, and less commonly the second molar were reported. When wild animals were exposed to dexamethasone, it was observed delay in the differentiation of internal epithelial cells and dental papilla cells to ameloblasts and odontoblasts.[27]

During odontogenesis, an important expansion occurs in the enamel organ and stellate reticulum during E14.5 to E16.5 period.[20] Since these tissues express GR and are sensitive to GC, the smaller size of tooth germ seen in DEX animals of our study may be a result of the dexamethasone administered from E14 to E19. The histological evaluation of our study showed that all tooth germs evaluated were at the bell stage, with no difference in cell differentiation among CTL and DEX animals.

The mechanisms of physiological and iatrogenic effects of GC in mandibular bone and tooth germ remain elusive. In the future, advanced molecular biological analyses such as gene expression profiles and genome-wide screens will help to identify the genes that mediate the actions of GC and may contribute to controlling adverse effects.

CONCLUSION

We concluded that therapeutic doses of dexamethasone influence mandibular bone and tooth development emphasizing the importance of considering GC exposure during pregnancy to as minimal as possible.

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RESUMO

A exposição pré-natal aos corticosteroides é associada a efeitos adversos importantes no desenvolvimento do feto. Neste estudo foi realizada análise histomorfométrica do osso mandibular e primeiro molar inferior de fetos expostos a glicocorticóide exógeno no período final da gravidez. Para este estudo, seis ratos fêmeas foram alojadas com dois ratos machos durante 8 dias. As ratas grávidas foram divididas em dois grupos (n=3 cada): grupo controle e grupo dexametasona. A dexametasona recebeu 0,2 mg.kg-1.dia-1 dose diária de dexametasona solúvel diluída na água de beber do 14º ao 19º dia de gestação. No 20º dia de gestação, as ratas foram sacrificadas e os fetos coletados. O peso de cada feto foi registrado e, em seguida, foram eutanasiados e a cabeça fixada em formalina tamponada com fosfato a 10%. As amostras selecionadas foram avaliadas por microscopia de luz, sendo registradas as seguintes medidas: perímetro do osso mandibular, cartilagem de Meckel e germe dentário; comprimento vestíbulo-lingual, distância vertical, distância anteroposterior e área do germe dentário, pelo programa Image J. Houve diferença estatística significativa com relação ao peso ao nascer, perímetro mandibular, distância vertical do germe dentário e distância vestíbulo-lingual; a área do germe dental e o perímetro do germe foram menores na dexametasona. Não houve diferença estatística significativa no perímetro da cartilagem de Meckel e na distância anteroposterior do germe dentário entre os grupos. Em conclusão, a exposição pré-natal a dexametasona influencia o desenvolvimento ósseo e dentário da mandíbula.

PALAVRAS-CHAVE: dexametasona, glicocorticoide, odontogênese, osteogênese e gravidez

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