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Prenatal Stress Caused by Movement Restriction Induces Changes in the Development of Skull Bone in CF-1 Mice Progeny

El Estrés Prenatal Causado por Restricción de Movimiento Induce Cambios en la Anatomía Ósea de Cráneo en la Progenie de Ratones CF-1

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SUMMARY: Prenatal stress is associated with changes in body weight and size, and with disorders of the skeletal bone development process. However, there is a lack of documentation on the impact of prenatal stress on skull bone anatomy during the gestation period. Therefore, this research focuses on the short-term effects of prenatal stress on the skull bone anatomy of CF-1 mice on the day of birth. Methodology: Gestating females were divided at random into two groups (control and stressed). The experimental group was subjected to the stress of movement restriction during the final week of gestation. Upon birth the body weight of the progeny was evaluated (control group, n=34; stressed group, n=29). They were then cleaned and stained with alizarin red in order to evaluate the length, width and suture spaces of the skull bone anatomy from superior and inferior views. Results: Gestational stress significantly altered the skull bone anatomy ($p<0.05$) of the offspring at birth in comparison with the control group. Conclusion: Prenatal stress alters the skull bone anatomy of the CF-1 mouse at birth.

KEY WORDS: Prenatal stress; Membranous ossification; Alizarin; Skull bone; CF-1 mouse.

INTRODUCTION

The growth and development of the bone system begins during early prenatal stages and is mainly controlled by 2 cell types: osteoblasts, mononucleate cells responsible for the production of the extracellular bone matrix; and osteoclasts, multinucleate cells responsible for bone matrix resorption for reshaping (Raiz & Kream, 1981; Buckwalter *et al*., 1995). In the skull there is a type of ossification called intramembranous ossification, which is characterized by the addition of undifferentiated cells within layers of connective tissue. These undifferentiated cells give rise to osteoblasts, which will create centers of organic bone matrix formation that spread until the entire bone is formed (Pechak *et al.*, 1986; Buckwalter *et al*., 1995).

Stress is an adaptive response present in all mammals and it allows the organism to deal with physical and/or psychic events that threaten the homeostasis of the internal medium (Chrousos & Gold, 1992). This response is controlled by the hypothalamus, hypophysis and its effectors, with substances such as catecholamines or glucocorticoids. The latter substances not only participate in reestablishing the homeostasis of the internal medium but are also fundamental for the maturation of different fetal organs, one of which is bone tissue (Sliwa *et al*., 2010). Abundant clinical evidence has shown that if the activation of the hypothalamus-hypophysis-adrenal (HHA) axis become chronic, it loses its adaptive value, predisposing the individual to several different types of alteration, from psychiatric disorders, such as depression or anxiety disorders (De Kloet *et al*., 2005) to cardiovascular and metabolic diseases (Barker, 1995).

During pregnancy, the activation of the maternal HHA axis together with the subsequent increase in glucocorticoid (GC) levels in response to situations of acute stress do not significantly alter fetal cortisol levels thanks to the action of the enzyme placental 11-beta-hydroxysteroid dehydrogenase type 2 (11b-HSD2), which metabolizes cortisone with an efficiency of close to 80% (Weinstock, 2008). Nevertheless, conditions of chronic stress lead to prolonged activation of the maternal HHA axis, generating hypercortisolemia, a phenomenon

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associated with a perturbation in the functioning of the enzyme barrier of 11b-HSD2, thus exposing the fetus to elevated levels of GCs (Welberg *et al.*, 2005; Mairesse *et al.*, 2007). This abnormal increase in GC levels during gestation has been associated with a decrease in weight and size of offspring at birth (Welberg *et al*., 2005). Furthermore, activation of the maternal HHA axis is accompanied by a sustained increase in catecholamine plasma concentrations, which induce vasoconstriction of placental arteries, reducing blood flow and oxygen supply to the fetus. These events can alter fetal nutrition and may activate the HHA axis of the fetus (Myers, 1975). It has also been observed that maternal GCs stimulate the production of Corticotropin-releasing hormones and the release of cortisol from the fetus's adrenal glands, thus contributing to the chronic state of fetal hypercortisolemia (Buckigham, 2006).

These prenatal bone growth and development processes can be influenced by physical factors (Brigton and McCluskey, 1988; Lavine and Grodzinsky, 1987), and nutritional and hormonal factors (Canalis, 1987; Namgung & Tsang, 2003). Of the latter it can be noted that treatments with GCs during gestation have been associated with a fall in fetal linear growth (Holemans *et al*., 2003), decreased bone density (Namgung & Tsang, 2003), and a drop in fetal bone growth (Orzechowski *et al*., 2000; Vos et al., 1995). Similar results were found by Lizana *et al*., (2012) where it was shown that periods of chronic stress as a source of GCs circulating during gestation induce decreases in bone growth in long appendicular bones, though there is still a lack of knowledge on what effects may be caused by an increase in GCs circulating during gestation stages, as a result of prenatal stress, on the anatomy of skull bones.

On the basis of this problem, the objective of this study is to describe the anatomical variations in skull bones as a result of stress due to maternal movement restriction during the final week of gestation in CF-1 mice.

MATERIALS AND METHOD

Animals and laboratory conditions. CF-1 mice were used in the study, 6 females aged 5 to 8 months and 2 breeding males, belonging to the Anatomical Techniques Laboratory of the Pontificia Universidad Católica de Valparaíso (PUCV) in Chile. They were bred and treated under procedures that safeguard bioethical criteria on the handling and care of experiment animals (Sharp, 1998) in accordance with the "Principles of Laboratory Animal Care" (NIH publication N° 86-23, revised 1985), and also in accordance with the directives of the PUCV.

The rodents were kept in plexiglas cages (30cm x 19cm x 13cm) that were cleaned twice a week, under standard laboratory conditions: inverse light-dark cycle of 12 hours (light cycle 20:00 to 8:00) temperature of 18 ± 2° Celsius, with water and food ad libitum (Sharp, 1998).

Upon reaching the minimum weight of 30 grams, the adult females were separated and paired with a breeding male for 5 to 7 days and weighed daily from the start of the pairing process in order to determine the moment of gestation. The females were divided at random into 2 groups: mothers to be stressed during gestation (Sm, n=3) and mothers not subjected to the stress protocol (Cm, n=3). When the offspring were born (control n=34; prenatal stress n=29, irrespective of sex), each was weighed and then euthanized with diethyl ether vapors.

Protocol for movement restriction stress. Each of the gestating females in the Sm group were subjected to a restricted movement stress protocol from day 15 of gestation (G15) until the day before the birth of the offspring by keeping them inside an opaque polyvinyl chloride tube (15 cm in lengthen x 4.5 cm in diameter) for 45 minutes, 3 times a day (09:00; 14:00; 18:00) (Bustamante *et al*., 2010). Only females that were subjected to a minimum of 15 stress sessions were considered in the study, as a minimum for the generation of high levels of corticosterone in the plasma (Buynitsky and Mostofsky, 2009). The females in group Cm were only handled during the process of cleaning their cages.

Procedures for bone cleaning, staining and photography. Once euthanasia had been carried out, the bodies of the offspring were set in formalin at 10% for one week, and were then washed with running water for one hour. Once this process was completed, the bodies were skinned and the internal organs and interscapular fat were extracted; the bone was then macerated with potassium hydroxide (KOH) 2% for two weeks. The staining process was carried out with alizarin red at 0.05% for two days, and the body was finally submerged in a solution of 2% KOH and glycerine 1:1 for one week in order to eliminate any remaining dye. The final samples were preserved in glycerine for a minimum of 3 days for subsequent photographing. Once the staining process was complete the head was separated from the body by a posteroanterior incision, using the cut between the occipital bone and first cervical vertebra as reference points.

The photography process was performed with a trinocular magnifying glass with a zoom feature, model number Q714Z2T (QUIMIS) digital camera with USB connection, model Q712-5megapixeles (QUIMIS), under controlled lighting conditions. The heads were mounted on a glycerine surface and contrasted with a measurement scale.

Measurement procedure. Bone measurements were analyzed using the Image Pro Plus software version 6.0.0.260 (Media Cybernetics, Silver Spring, USA). Thirteen reference points were established on the mouse skulls, distributed over the 2 views (superior and inferior); these were then used to calculate 9 measurements (6 from the superior view and 3 from the inferior; see figure 1). These reference points were placed on the margins between the bones of the different part of the skull for the purposes of measuring the bone dimensions (Fig. 1). Table I shows the linear distance measurement results for each sample.

Statistical analysis. The data was analyzed with descriptive statistics and processes using the U Mann-Whitney test for unpaired, non-parametric data, considering the independent variable of prenatal stress. The dependent variables comprised the lengths between the skull bone reference points. A result was considered statistically significant for $p \leq 0.05$. The program Graphpad Prism 5 was used to analyze the results.

RESULTS

Body weight

Gestating females. No significant differences were found in the body weight during the gestation period between the females in the two study groups (data not shown).

Offspring. The offspring of females subjected to stress during gestation (group S) showed an average body weight significantly lower that the offspring of females not subjected to the protocol (group C) on day P0 ($p=0.0037$).

Skull bone anatomy

Superior view. The offspring of group S presented a significantly shorter anteroposterior length for the nasal bone in comparison with those of group C (D1) ($p<0.01$). A similar situation was found for the anteroposterior length of the fron-

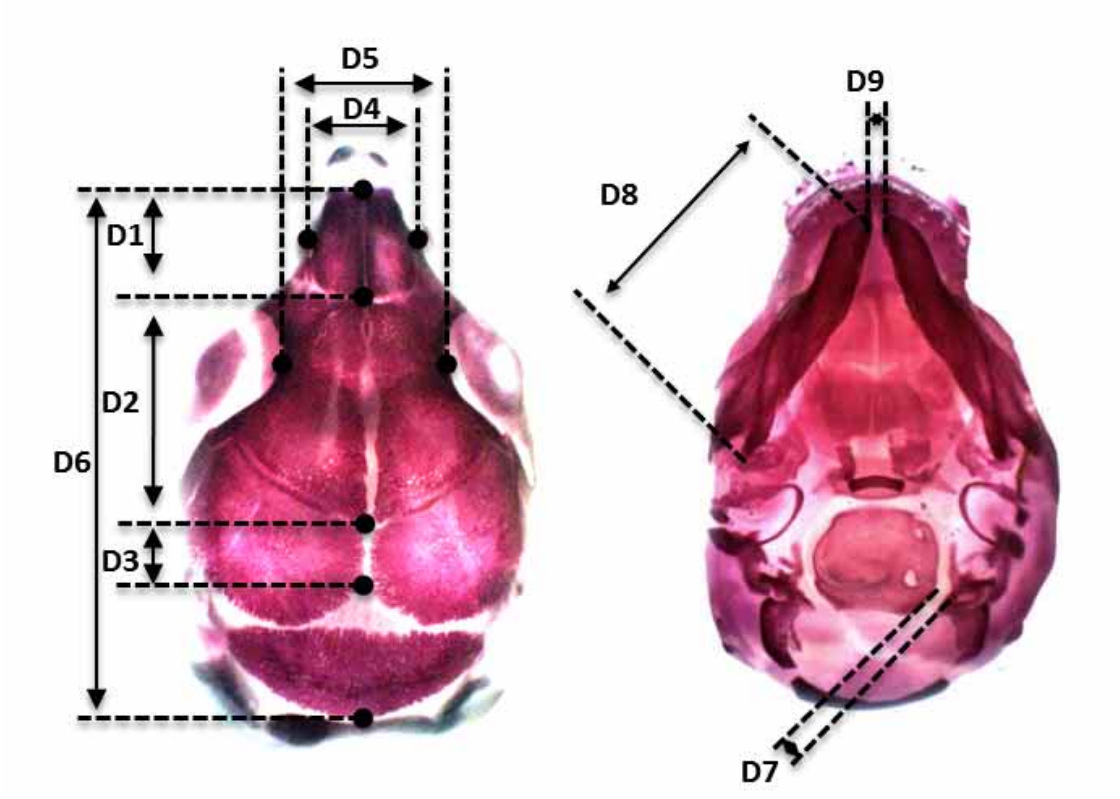

Fig 1. Linear measurement point on the skull of the CF-1 mouse. Linear distance (D). Superior view (A); Nasal Bone Length (D1); Frontal Bone Length (D2) (sagittal margin length of the frontal bone); Parietal Bone Length (D3) (sagittal margin length of the parietal bone); Nasal Bone Width (D4); between right and left orbit (D5) (minimum width); total length (D6) (nasal bone to occipital pole). Inferior view (B); between basi-and ex-occipital bones (D7); anterior end of mandibular (D8) (to posterior end of angular process); intermandibular space (D9).

tal bone in the horizontal plane (D2) ($p<0.001$). On the other hand, the length of the parietal bone shows no significant differences between the two groups (D3). Evaluating the length from the anterior end of the nasal bone to the posterior end of the occipital bone it was found that the offspring in the control group showed significantly longer measurements than those of group S (D6) ($p<0.05$). For lateromedial lengths in the horizontal plane from the superior view it was found that for the group S offspring the width of the nasal bone was significantly smaller that for the offspring of group C (D4) ($p<0.001$). With regard to the width of the frontal bone it was found that the group C offspring showed a significantly longer width than the offspring of group S (D5) ($p<0.01$) (Table I and Fig. 2).

Inferior view. The length from the anterior end of the jaw to the posterior end of the angular process was evaluated, showing that the measurements for the offspring of group S were significantly shorter than for group C (D8) ($p<0.001$), while the width between the ends of the intermandibular sutures and the occipital zone was seen to be significantly higher for group S than for group C (D7 and D9) ($p<0.05$) (Table I and Fig. 2).

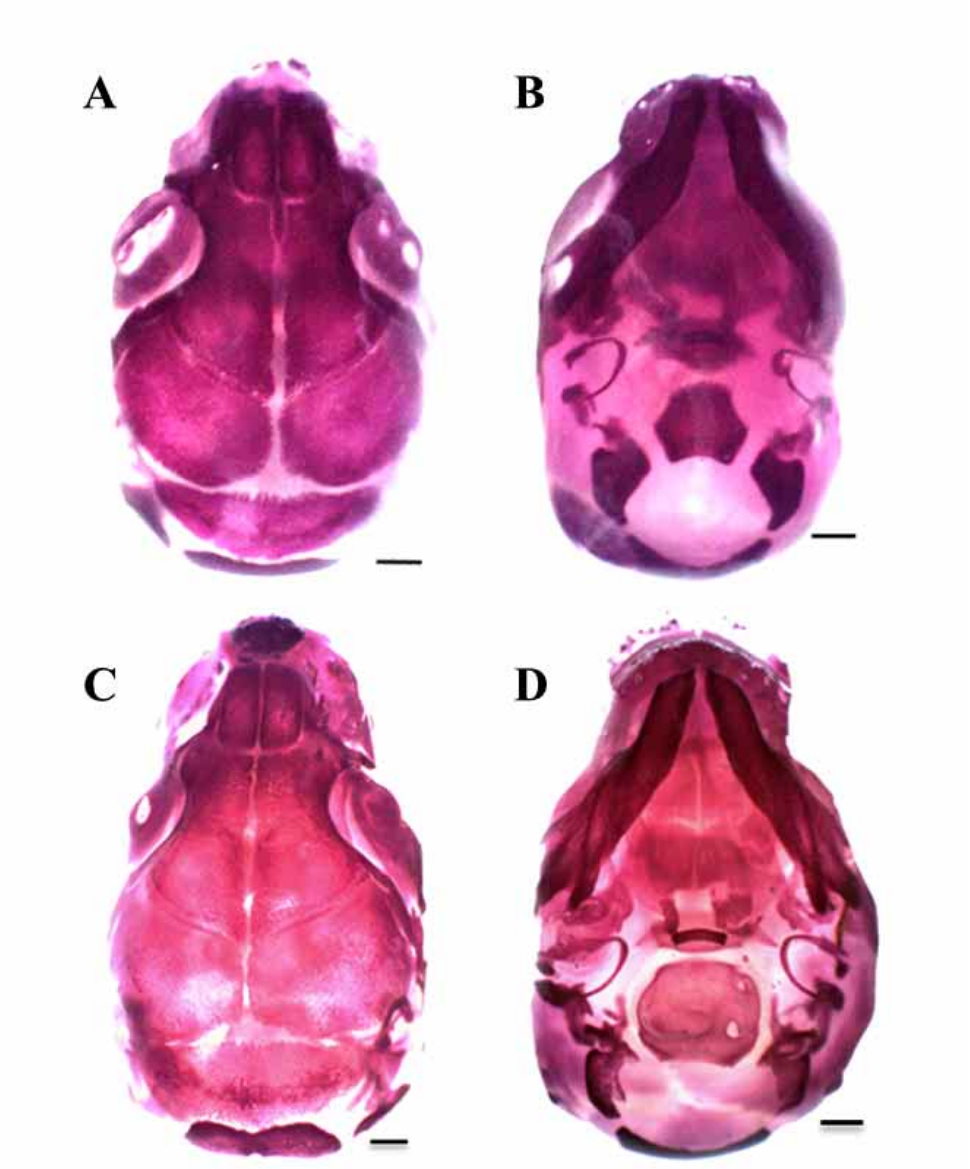

Fig 2. Cleaned CF-1 mouse skulls subsequently stained with Alizarine red. Specimens subjected to prenatal stress due to movement restriction (A-B). Superior view (A); inferior view (B). Control specimens (C-D). Superior view (C); inferior view (D). All specimens are from day 1 after birth. Scale bars indicate 0.5 cm.

Table I. Linear measurements from CF-1 mouse skulls.

Superior view	D1	D2	D3	D4	D5	D6
Stress	1.93±0.028	4.12±0.034	1.95±0.038	1.95±0.013	4.91±0.026	9.63±0.053
Control	2.08±0.035†	4.31±0.032‡	1.92±0.031	2.04±0.017‡	5.03±0.043†	9.76±0.055*
Inferior view	D7	D8	D9			
Stress	0.357±0.012*	5.82±0.061	0.40±0.015†			
Control	0.335±0.009	6.15±0.048‡	0.33 ±0.009			

D: distance (mm); mean±SD; (*) p<0.05; (†) p<0.01; (‡)p<0.001

DISCUSSION

The objective of this study was to describe anatomical variations in skull bones as a result of stress due to maternal movement restriction during the final week of gestation in CF-1 mice.

With regard to the body weight of the females during gestation, as no significant differences between the two groups were found, it can be stated that the stress treatment during the gestation period did not have a significant effect on general maternal nutrition, which is very important in studies of this type, as this allows us to discard the factor of maternal nutrition as a possible cause of any alterations observed.

Regarding the body weight of the progeny evaluated at P0, it was found that group S showed a significantly lower weight than the control group. This result is in agreement with the study by Lordi *et al*., (2000), who evaluated the offspring of females stressed during the last trimester of gestation. These results can be explained by two mechanisms associated with the effect of GCs and catecholamines on the developing fetus: the first states that the GCs cause an increase in circulating glucose, an alteration of insulin receptors and an increase in lipolysis, leading to lower weight at birth in comparison with control animals (Ain *et al*., 2005). Another mechanism, mediated by catecholamines, is proposed by Lordi *et al*., (2000), who postulate that a stressing agent increases plasmatic catecholamines, leading to vasoconstriction of the umbilical blood vessels, decreasing the flow of nutrients to the fetus, thus affecting its general growth and development.

The principal finding of the present study was identification of variation in the skull bone anatomy due to a chronic stress process during gestation. Upon evaluation of the skull bone anatomy it can be seen that most skull bone lengths and widths measured in the study are lower in the animals subjected to stress during gestation, in comparison with the control group. This suggests that the effect of prenatal stress reduced growth and development of skull bones, a result which may be explained by the possible increase in GCs circulating in the fetus during the final trimester of gestation, produced by the stress applied to the gestating females. It has been reported that increase in GCs during growth periods can negatively affect the amount and functionality of osteoblasts and osteocytes (Hardy & Cooper, 2009). It has also been shown that GCs such as dexamethasone in in vitro skull osteoblast cultures decrease the expression of cell differentiation factors, altering their function during the ossification and knitting process of different sutures (Coussense *et al*., 2008) and thus possibly having a direct effect on skull ossification. A second mechanism that may alter this process results from the increase in catecholamines during the gestational stress period, which may lead to alterations in fetal blood flow and subsequent alterations in the availability of nutrients necessary for bone growth processes (Lordi *et al.*, 2000). The anteroposterior length of the parietal bone showed no significant differences with the control group. This may be explained by the anatomy of this zone, which is curved in the sagittal plane, and therefore, when measuring the 2 dimensions from above, the depth is lost and possible differences in bone growth are attenuated. Regarding the sutures from the inferior view of the skull, in both the occipital zone and the mandible zone for the offspring subjected to stress during gestation an increase can be seen in the separation between the borders of the sutures, which implies a slower suture knitting process and this may be directly related to the finding for skull bone growth. One possible explanation for this change, in addition to the aforementioned mechanisms, is the increase in circulating GCs. This may alter the function of key molecules in the suture knitting process (Ignelzi *et al.*, 2003), such as the growth factor of fibroblasts (Wang *et al*., 2013), thus altering the skull suture knitting and ossification process. It is important to note

that stress is a process that can induce alterations in several different ways, many of which have not been fully proven in bone growth. The results of this study are similar to those obtained by Minagi *et al*., (2009), who applied a movement restriction stress protocol during the second trimester of gestation, observing alterations in skull ossification, particularly in the occipital zone.

It may therefore be concluded that stress due to movement restriction induces alterations in the skull anatomy of progeny at birth, which indicates that stress during gestation is a factor that affects the intramembranous growth and ossification process of skull bones, though the ways in which these alterations are produced is still unknown.

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RESUMEN: El estrés prenatal se ha asociado con alteraciones en el peso y tamaño corporal, además de trastornos en el proceso de osificación del esqueleto en desarrollo. Sin embargo, existen escasos antecedentes acerca del impacto del estrés prenatal sobre la anatomía ósea craneal durante el periodo de gestación. Por lo tanto, la presente investigación estudió los efectos a corto plazo del estrés prenatal sobre la anatomía ósea craneal del ratón CF-1 en el día de nacimiento. Las hembras gestantes fueron divididas aleatoriamente en dos grupos (control y estresado), el grupo experimental fue sometido a estrés por restricción de movimiento durante la última semana de gestación. Al nacimiento se evaluó el peso corporal de la progenie (grupo control n:34; grupo estresado n:29), para posteriormente diafanizar y teñir con alizarina roja, evaluando dimensiones longitudinales, anchos y espacios suturales de la anatomía de los huesos de cráneo por la vista superior e inferior. El estrés gestacional alteró significativamente la anatomía de los huesos de cabeza ósea ($p<0,05$) de las crías en el momento del nacimiento con respecto a los controles. El estrés prenatal altera la anatomía de los huesos craneales del ratón CF-1 evaluado al nacer.

PALABRA CLAVE: Estrés prenatal; Osificación membranosa; Alizarina roja; Cabeza ósea; Ratón CF-1.

REFERENCES

Barker, D. J. Intrauterine programming of adult disease. *Mol. Med. Today., 1*:418-23, 1995.

Brighton, C. T. & McCluskey, W. P. Response of cultured bone cells to a capacitively coupled electric field: inhibition of cAMP response to parathyroid hormone. *J. Orthop. Res., 6*: 567-71, 1998

Buckigham, J. C. Glucocorticoids: exemplars of multitasking. *Brit. J. Pharmacol., 147*:258-268, 2006.

Buckwalter, J. A.; Glimcher, M. J.; Cooper, R. R.; Recke, R. Bone biology. *J. Bone Joint. Surg. Am., 77*:1256-75, 1995.

Bustamante, C.; Bilbao, P.; Contreras, W.; Martínez, M.; Mendoza, A.; Reyes, A.; Pascual, R. Effects of prenatal stress and exercise on dentate granule cells maturation and spatial memory in adolescent mice. *Int. J. Dev. Neurosci., 28*:605-9, 2010.

Canalis, E. Effect of growth factors on bone cell replication and differentiation. *Clin. Orthop. Relat. R., 193*:246-63, 1985.

Chrousos, G. & Gold, P. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. *JAMA., 267*:1244-52, 1992

Coussens, A.; Hughes, I.; Morris, P.; Powell, B. & Anderson P. *In Vitro* Differentiation of Human Calvarial Suture Derived Cells With and Without Dexamethasone Does Not Induce *In Vivo*-Like Expression. *J. Cell. Physiol., 218*:183-91, 2009.

De Kloet, E. R.; Joels, M. & Holsboer, F. Stress and the brain: from adaptation to disease. *Nat. Rev. Neurosci., 6*:463-75, 2005.

Hardy, R. & Cooper, M. S. Bone loss in inflammatory disorders. *J. Endocrinol., 201*:309-20, 2009.

Holemans, K.; Aerts, L. & Van Assche, F. A. Fetal growth restriction and consequences for the offspring in animal models. *J. Soc. Gynecol. Invest., 10*:392-9, 2003.

Ignelzi, M. A. Jr.; Wang, W. & Young, A. T. Fibroblast growth factors lead to increased Msx2 expression and fusion in calvarial sutures. *J. Bone. Miner. Res., 18*:751-9, 2003.

Lavine, M. D. & Grodzinsky, A. J. Current concepts review. Electrical stimulation of repair of bone. *J. Bone and Joint Surg., 69-A*:626-30, 1987.

Lizana, P.; Henríquez, R. & Muñoz, P. Prenatal Stress Caused by Movement Restriction Induces Changes in the Appendicular Osseous Development of CF-1 Mice Progeny. *Int. J. Morphol., 30*:1132-8, 2012.

Lordi, B.; Patin, V.; Protais, P.; Mellier, D. & Caston, J. Chronic stress in pregnant rats: effects on growth rate anxiety and memory capabilities of the offspring. *Int J. Psychophysiol., 37*:195-205, 2000.

Mairesse, J.; Lesage, J.; Breton, C.; Bréant, B.; Hahn, T.; Darnaudéry, M.; Dickson, SL.; Seckl, J.; Blondeau, B.; Vieau, D.; Maccari, S. & Viltart, O. Maternal stress alters endocrine function of the feto-placental unit in rats. *Am. J. Physiol. Endocrinol. Metab., 292*:1526-533, 2007.

Minagi, T.; Izumi, Y.; Chatani, F. & Matsumoto, K. Restraint-induced maternal stress and alteration of ossification in mouse fetuses. *Congenit. Anom. (Kyoto)., 49*:108-12, 2009.

Myers, R. E. Maternal psychological stress and fetal asphyxia: a study in the monkey. *Am. J. Obstet. Gynecol., 122*:47-59, 1995.

Namgung, R. & Tsang, R. C. Bone in the pregnant mother and newborn at birth. *Clin. Chim. Acta., 333*:1-11, 2003.

Orzechowski, A.; Ostaszewski, P.; Brodnicka, A.; Wilczak, J.; Balasin´ ska, B.; Jank, M.; Grzekowska, K.; Ploszaj, T.; Olczak , J. & Mro´wczyn´ ska, A. Excess of glucocorticoids impairs whole-body antioxidant status in young rats Relation to the effect of dexamethasone in soleus muscle and spleen. *Horm. Metab. Res., 32*:174-80, 2000.

Pechak, D.G.; Kujawa, M. J. & Caplan, A.I. Morphological and histochemical events during first bone formation in embryonic chick limbs. *Bone., 7*:441-458, 1986.

Raiz, L. G. & Kream, B. E. Hormonal control of skeletal growth. *Ann. Rev. Physiol., 43*:225-38, 1981.

Sharp, P.; La Regina, M. The Laboratory Rat. *CRC. Press. LLC., 2*:33-51, 1988.

Sliwa. E.; Dobrowolski, P. & Piersiak, T. Bone development of suckling piglets after prenatal, neonatal or perinatal treatment whit dexamethsone. *J. Anim. Physiol. An., 94*:293-306, 2010.

Vos, P.; Saladin, R.; Auwerx, J. & Staels, B. Induction of ob gene expression by corticosteroids is accompanied by body weight loss and reduced food intake. *J. Biol. Chem., 270*:15958–61, 1995.

Wang, C.; Dawes, L. J.; Liu, Y.; Wen, L.; Lovicu, F. J. & McAvoy, J. W. Dexamethasone influences FGF-induced responses in lens epithelial explants and promotes the posterior capsule coverage that is a feature of glucocorticoid-induced cataract. *Exp. Eye. Res., 111*:79-87, 2013.

Weinstock, M. The long-term behavioural consequences of prenatal stress. *Neurosci. Biobehav. Rev., 32*:1073-86, 2008.

Welberg, L. A.; Thrivikraman, K.V. & Plotsky, P. M. Chronic maternal stress inhibits the capacity to up-regulate placental 11-hydroxysteroid dehydrogenase type 2 activity. *J. Endocrinol., 186*:7–12, 2005.

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