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Original Article

Maternal malnutrition during lactation reduces skull growth in weaned rat pups: Experimental and morphometric investigation

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Abstract

The purpose of the present study was to evaluate the effects of maternal protein and energy restriction during lactation on the bodyweight and skull dimensions of pups at weaning. At parturition, Wistar rat dams were randomly assigned to the following groups: (i) control group (C), free access to a standard laboratory diet containing 23% protein; (ii) protein–energy-restricted group (PER), free access to an isoenergetic, protein-restricted diet containing 8% protein; and (iii) energy-restricted group (ER), restricted amounts of a standard laboratory diet. The dimensions of excised pup skulls were measured directly using pre-established anatomical points. Morphometrical analysis of the skulls showed that most of the measurements in the ER and PER groups were significantly lower than in the control group, with the greatest reductions occurring in the PER group. These results show that protein and energy restriction during lactation have an important influence on pup skull development.

Key words: growth and development, morphometry, rats, skull, undernutrition.

Introduction

Malnutrition is the most prevalent nutritional disorder among children in developing countries. Based on World Health Organization data, Onis *et al.* (1993) reported that child malnutrition remains a major public health problem worldwide. In rapidly growing organisms, malnutrition in early life is a serious challenge to which the body will try to adjust in order to survive. Protein malnutrition often occurs during gestation, lactation, and the first 2 years of life (Desai *et al.*, 1980). Some authors have shown that an adequate nutritional status of the mother during gestation and lactation is essential for normal growth and development in humans (Barker, 2000) and animals (Passos *et al.*, 2000).

The quantity or quality of nutrition at these critical periods has permanent consequences for later life. One of the mechanisms for adapting to an inadequate supply of nutrients is to slow down the rate of cell division in tissues and organs, and may lead to altered programming of the structure and function of the system (Lucas, 1998). The concept of programming (metabolic imprinting) links physiological changes in adulthood with physiological changes in the gestational or neonatal period (Lucas, 1998). Chronic diseases in adulthood, such as coronary insufficiency and plurimetabolic syndrome (diabetes mellitus, hypertension and obesity), may be programmed in the initial stages of life (Barker, 2000).

Previous studies have shown that maternal undernutrition during lactation causes alterations in the milk composition (Passos *et al.*, 2000), serum hormone concentration in pups at weaning (Cónsole *et al.*, 2001; Teixeira *et al.*, 2002), and female reproductive system (Engelbregt *et al.*, 2000; Faria *et al.*, 2004; Brazil *et al.*, 2005). Interestingly, some of these alterations persist into adulthood (Passos *et al.*, 2002), thereby reinforcing the concept of metabolic imprinting.

Undernutrition has a wide variety of effects on endocrine systems (Cónsole *et al.*, 2001; Teixeira

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et al., 2002) that can reduce bodyweight (Passos *et al.*, 2000; Teixeira *et al.*, 2002; Santos *et al.*, 2004). Indeed, the use of bodyweight as a measure of growth has shown that protein malnutrition produces smaller-sized individuals (Pucciarelli, 1981; Cotheran *et al.*, 1985; Cameron & Eshelman, 1996).

The craniofacial skeleton is one portion of the body that is critically affected by malnutrition (Pucciarelli & Oyhenart, 1987a,b; Miller & German, 1999). Understanding how the mammalian skull develops is necessary for comprehension of the effect of malnutrition. The skull is not a single developing unit, but rather has two distinct regions, the viscerocranium and the neurocranium (Cheverud, 1982; Pucciarelli & Oyhenart, 1987a,b; Miller & German, 1999). The viscerocranium is used during feeding and breathing, and its growth is continuously subject to muscular loading (Cheverud, 1982; Herring, 1993), whereas the neurocranium houses the brain, and its growth is influenced primarily by brain expansion (Young, 1959). The goal of the present study was to examine the effect of maternal protein and energy malnutrition during lactation on the body size and cranial skeleton growth of the pups at weaning.

Materials and methods

Animal care

The study design and experimental protocols were approved by the Animal Care and Use Committee of the State University of Rio de Janeiro, which based its analysis on the Guide for the Care and Use of Laboratory Animals (Bayne, 1996). The experiments described here were done within the general guidelines of the Brazilian College for Animal Experimentation (COBEA).

Animals and experimental model

Wistar rats obtained from Biomedical Center, State University of Rio de Janeiro were housed at 25 ± 1°C and on a 12 h light–dark cycle (lights on from 07.00 hours to 19.00 hours) throughout the experiment. Six 3-month-old, virgin female rats were housed with three male rats at a proportion of 2:1 on individual cage. After mating each female was placed in an individual cage and had a normal pregnancy, receiving food and water ad libitum until delivery. The number of pups born was similar, six per pregnant rat, totalling 12 per group. All pups were in good health and there was no statistical difference in bodyweight or linear growth.

Pregnant Wistar rats were randomly separated at delivery into three groups (two per group): (i) control group (C), free access to a standard laboratory diet (in grams per 100 g) containing 23% protein, 68% carbohydrate, 5% lipid, 4% salts and 0.4% vitamins, 17 038.7 total energy (kJ/kg); (ii) protein–energy-restricted group (PER), free access to an isoenergetic, protein-restricted diet containing 8% protein; and (iii) energy-restricted group (ER), standard laboratory diet in restricted quantities that were calculated based on the mean ingestion of the PER group. We have previously shown that the PER group consumes approximately 60% of the amount consumed by the control group, despite having free access to food (Passos *et al.*, 2000). Hence, the ER and PER groups ingested essentially the same amount of food.

The protein-restricted diet was prepared in the laboratory at State University of Rio de Janeiro (Table 1) using the control diet with replacement of part of its protein content with cornstarch. The amount of the latter was calculated to replace the same energy content of the control diet. Vitamin and mineral mixtures were formulated to equal those found in the control diet and to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets (Reeves *et al.*, 1993). To evaluate the nutritional state, the food consumption and bodyweight (Fig. 1) were monitored throughout the experiment. Within 24 h of birth, excess pups were removed so that only six pups were kept per dam, because it has been shown that this procedure maximizes lactation performance (Fischbeck & Rasmussen, 1987). Malnutrition was started at birth, which was defined as

Table 1. Diet composition

Ingredients (g/kg)	Control‡	PER§
Total protein†	230.0	80.0
Corn starch	676.0	826.0
Soybean oil	50.0	50.0
Vitamin mixture¶	4.0	4.0
Mineral mixture¶	40.0	40.0
Macronutrient composition (%)		
Protein	23.0	8.0
Carbohydrate	66.0	81.0
Fat	11.0	11.0
Total energy (kJ/kg)	17 038.7	17 038.7

†Principal protein resources were soybean wheat, steak, fish and amino acids.

‡Standard diet for rats (Nuvilab-Nuvital, Curitiba, Paraná, Brazil).

§The PER diet was prepared in the laboratory at State University of Rio de Janeiro by replacing part of the protein content of the control diet with cornstarch. The amount of the latter was calculated to replace the same energy content of the control diet.

¶Vitamin and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets (Reeves *et al.*, 1993).

PER, protein–energy restriction.

day 0 of lactation (d0), and was ended at weaning (d21). To evaluate the nutritional state, the food consumption and bodyweight of the pups were monitored throughout the experiment. At weaning six rats were anesthetized with thiopental anesthesia (0.1 mL/100 g bodyweight) and perfused through the left ventricle with buffered saline followed by formalin solution.

Morphometric parameters

After perfusion the skulls were excised, dissected, weighed and fixed in 4% formalin in 0.1 mol/L phosphate buffer (pH 7.4) by immersion for 24 h at room temperature prior to being measured. The skull width, length and height were measured as defined in Table 2 and illustrated in Fig. 2. All of the measurements were made to the nearest 0.01 mm using callipers. The anatomical terminology was based on Greene (1963) as adapted for veterinary anatomy (Schaller, 1999).

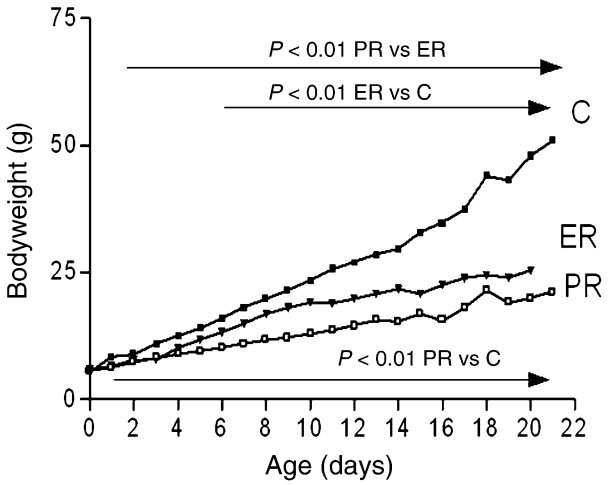

Figure 1. Bodyweight gain of pups in the control (C), protein-restricted diet (PER) and energy-restricted diet (ER) groups up to 21 days of age. Group C: free access to water and a diet containing 23% protein; group PER: free access to water and a diet containing 8% protein; group ER: free access to water and limited access to a commercial diet containing 23% protein, which corresponded to the same amount ingested in the previous day by rats in group PER. The results are the mean ± SD of 12 pups per group.

Statistical analysis

The data are reported as mean ± SD. Statistical significance of experimental observations was determined using one-way analysis of variance followed by Newman–Keuls test to compare the three experimental groups. The level of significance was set at $P < 0.05$. All statistical analysis was done using GraphPad Prism 4 statistical software (GraphPad, XXX, CA, USA).

Results

Figure 1 shows the bodyweight gain of pups in the three groups. The pups of dams fed a protein-restricted diet during lactation had a lower weight gain than the control group throughout the study (up to 21 days of age; $P < 0.01$), with the difference between these two groups being approximately 58%. The pups in group ER had a lower weight gain (approx. 46% less) than the controls from day 6 onwards ($P < 0.01$). The PER group had a lower weight gain than the ER group from day 2 until the end of the study ($P < 0.01$).

The cranial morphometric measurements are shown in Table 3. The values for heights 1, 2 and 5 in the ER ($P < 0.05$) and PER ($P < 0.001$) groups were significantly smaller than those of the control group, with the difference being greater in the PER group. In contrast, the values for heights 3 and 4 were lower than the controls only in the PER group

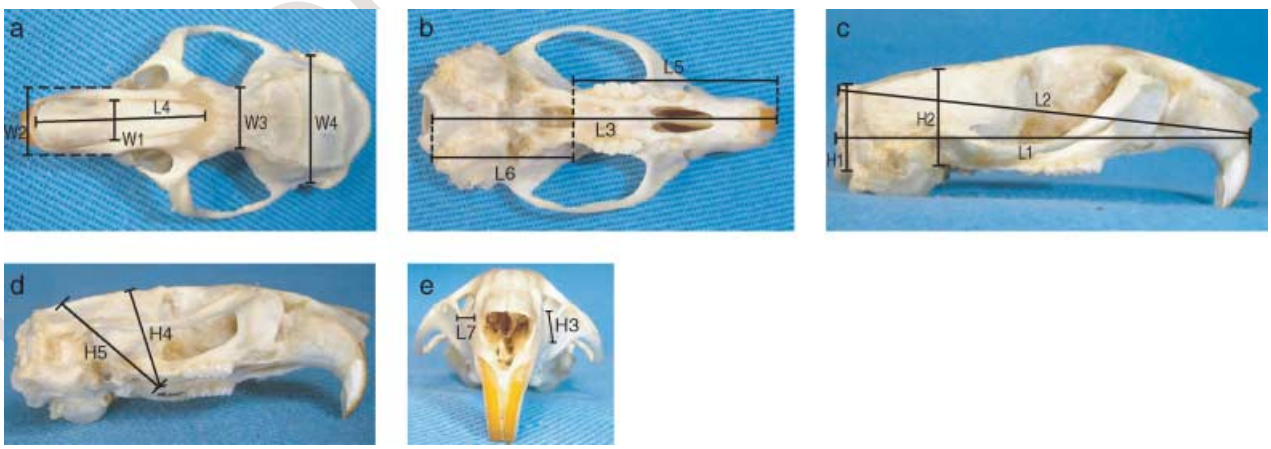

Figure 2. Rat skull showing the measurements used in the morphometric analysis. Definitions of acronyms are given in Table 2. (a) Dorsal view; (b) ventral view; (c) lateral view; (d) ventrolateral view; (e) frontal view.

Table 2. Parameters used in the morphometric analysis

Parameter	Definition
Height 1	Maximum height of the neurocranium (occipital level of the braincase) = distance between the uppermost tip of the external occipital crest and the level of the occipital foramen (border)
Height 2	Maximum height of the neurocranium (parietal level of the braincase) = distance between the anteromedial edge of the right tympanic bulla and the most dorsoventral surface of the skull
Height 3	Maximum height of the orbital cavity = distance between the right upper and lower walls of the orbit – level of the infraorbital fissure
Height 4	Maximum height of the neurocranium (fronto-parietal level of the braincase) = distance between the posterior nasal spine (posterior palatine extremity) and the union point of the coronal and sagittal sutures
Height 5	Maximum height of the neurocranium (parieto-occipital level of the braincase) = distance between the posterior nasal spine (posterior palatine extremity) and the union of the lambdoid and sagittal sutures
Length 1	Maximum length of the neurocranium (rectangular measurement) = distance between the external occipital protuberance and the alveolar margin of the incisive bone
Length 2	Maximum length of the dorsoventral neurocranium (linear measurement) = distance between the external occipital protuberance and the alveolar margin of the incisive bone
Length 3	Maximum length of the basal neurocranium (linear measurement) = distance between the most ventral aspect of the foramen occipital and the alveolar margin of the incisive bone in the median plane
Length 4	Maximum length of the nasal bone = anterior tip of nasal bone – suture between the nasal and frontal bone in the median plane
Length 5	Maximum length of the palatine bone = distance between the posterior nasal spine (posterior palatine extremity) and the alveolar margin of the incisive bone in the median plane
Length 6	Maximum length of the sphenoid bone = distance between the most ventral aspect of the foramen magnum and the posterior nasal spine (posterior palatine extremity) in the median plane
Length 7	Maximum length of the orbital cavity = distance between the most ventral aspect of the right infraorbital and supraorbital margin
Width 1	Nasal width = distance between the right margin of the nasomaxillary suture (level of the medial infraorbital border) – the left margin of the nasomaxillary suture
Width 2	Premaxillary width = distance between the rightmost lateral aspect of the premaxillary, medial infraorbital border – the leftmost lateral aspect of the premaxillary, medial infraorbital border
Width 3	Frontal width = distance between the rightmost constricted region of the frontal (temporal line, level of the zygomatic–malar process suture) – the leftmost constricted region of the frontal
Width 4	Distance between the tympanic bulla = anteromedial edge of the right tympanic bulla – anteromedial edge of the left tympanic bulla

($P < 0.001$). The measurements for heights 3, 4 and 5 were significantly lower in the PER group compared to the ER group ($P < 0.05$).

All of the measurements for length (1–7) were significantly lower in the two malnourished groups (ER and PER) compared to the control group ($P < 0.05$), except for lengths 4 and 5 (ER vs C; $P > 0.05$). Only the parameter length 4 was significantly lower in the ER group compared to the PER group ($P < 0.05$); there was no difference between these two groups in the other lengths.

All of the measurements for width (1–4) were significantly lower in groups PER ($P < 0.001$) and ER ($P < 0.05$) compared to the control group; there was no significant difference between groups ER and PER ($P > 0.05$; Table 3).

Discussion

The development of the craniofacial skeleton is critically affected by malnutrition, and several studies have examined the effect of nutritional deficiencies on bone growth during gestation (Pucciarelli & Oyhenart, 1987b), lactation (Pucciarelli & Oyhenart, 1987a; Miller & German, 1999), gestation and lactation (Toews & Lee, 1975), and the postweaning period (Riesenfeld, 1967; Riesenfeld, 1973; Pucciarelli, 1981). Different forms of retarded cranial growth have been reported, depending on the type of malnutrition and/or its intensity, as well as the period in which the stress was applied. Additionally, growth of the craniofacial components in rats may be influenced by sex, breed or strain, and nutritional status (Pucciarelli, 1981; Pucciarelli & Oyhenart, 1987a). Because there is no consensus regarding the morphometric parameters that should be analyzed, in the present study we used some parameters adapted from Miller and German (1999) and Pucciarelli and Oyhenart (1987a).

Craniofacial underdevelopment was evident in weaned rats whose mothers were fed PER or ER diets during lactation (Table 3), and those changes

Table 3. Morphometric analysis of skull growth in rat pups at weaning

Parameter (mm)	C	ER	PER	*P* C vs ER	*P* C vs PER	*P* ER vs PER
Height 1	5.4 ± 0.3	4.9 ± 0.3	4.8 ± 0.3	<0.05	<0.001	>0.05
Height 2	9.5 ± 0.2	8.9 ± 0.5	8.7 ± 0.5	>0.05	<0.001	>0.05
Height 3	2.3 ± 0.5	2.0 ± 0.1	1.8 ± 0.1	>0.05	<0.001	<0.05
Height 4	9.3 ± 0.2	9.2 ± 0.1	8.9 ± 0.2	>0.05	<0.001	<0.05
Height 5	12.3 ± 0.4	11.2 ± 0.4	9.0 ± 0.3	<0.05	<0.001	<0.05
Length 1	32.2 ± 1.0	30.5 ± 1.1	30.0 ± 0.9	<0.05	<0.001	>0.05
Length 2	32.6 ± 0.9	30.3 ± 1.2	29.6 ± 1.0	<0.05	<0.001	>0.05
Length 3	28.3 ± 0.6	25.5 ± 0.8	25.0 ± 1.2	<0.01	<0.001	>0.05
Length 4	9.3 ± 0.8	8.9 ± 0.7	7.6 ± 0.8	>0.05	<0.001	<0.05
Length 5	16.2 ± 0.7	16.0 ± 0.5	15.1 ± 0.8	>0.05	<0.01	>0.05
Length 6	10.7 ± 0.4	9.8 ± 0.5	9.5 ± 0.3	<0.05	<0.001	>0.05
Length 7	2.8 ± 0.8	2.1 ± 0.1	1.9 ± 0.2	<0.05	<0.001	>0.05
Width 1	3.6 ± 0.1	3.2 ± 0.07	3.0 ± 0.07	<0.05	<0.001	>0.05
Width 2	5.2 ± 0.1	5.0 ± 0.09	4.9 ± 0.1	<0.05	<0.001	>0.05
Width 3	5.9 ± 0.2	5.6 ± 0.1	5.4 ± 0.1	<0.01	<0.001	>0.05
Width 4	15.0 ± 0.3	14.5 ± 0.3	14.2 ± 0.3	<0.01	<0.001	>0.05

Mean ± SD of 12 pups per group.
C, control group; ER, energy-restricted group; PER, protein–energy-restricted group.

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were accompanied by quantitative alterations in the bodyweight. These findings confirm previous observations (Ramos *et al*., 1997; Passos *et al*., 2000; Teixeira *et al*., 2002) that undernutrition (ER and PER groups) leads to a lower weight gain from the first day of lactation onwards (Fig. 1).

The deficiency in bodyweight gain seen in malnourished offspring could result from a reduction or absence of growth hormone (GH) because food deprivation reduces the number of GH secretory cells, as shown by immunostaining of hypothalamic sections for GH-releasing hormone (GHRH) and quantification of the mRNA levels for GHRH and GH (Brogan *et al*., 1997; Cónsole *et al*., 2001). Morphometric and ultrastructural analysis of hypophyseal cells from adult monkeys fed a protein-restricted diet containing 10% protein have shown a decline in the number of somatotrophic, lactotrophic, gonadotrophic and tireotrophic cells. The volumetric density and frequency distribution of these cells were also significantly lower (Herbert, 1980a,b; Heindel *et al*., 1988; Cónsole *et al*., 2001).

Leptin, a circulating hormone secreted by adipose cells that controls the amount of food ingested and energy expenditure, plays a role key in the homeostasis of bodyweight (Rosenbaum & Leibel, 1998). Energy restriction during lactation causes a drastic reduction in the plasma leptin levels of offspring until weaning (Léonhardt *et al*., 2003). Consequently, low levels of leptin could alter the normal functioning of the hypothalamic–hypophyseal (GH) target organ (bone) axis.

Another hypothesis for the retardation in bone development seen in PER and ER rats may be related to inadequate maturation of the hypothalamic–hypophyseal (GH)-target organ (bone) axis in the offspring as a result of maternal malnutrition. In the present case low hormonal stimulation may be insufficient to stimulate normal development of the craniofacial bones.

The loss of bodyweight and osseous tissue in the ER and PER groups may be caused by a reduction in the rate of metabolism. Part of this decline results from a reduced energy intake and a consequent decrease in the thermal effect of food, while part is attributable to the reduced size of the mass available for metabolization. However, whether there is also a metabolic adaptation, defined here as a reduction in the metabolic rate that is disproportional to the decreased size of the respiring mass, is a subject of continued debate. In their investigation of the biology of semistarvation, Keys *et al*. (1950) defined metabolic adaptation as 'a useful adjustment to altered circumstances' (Heilbronn & Ravussin, 2003).

The present results agree with reports showing that undernutrition during lactation delays offspring growth (Engelbregt *et al*., 2000; Delemarre *et al*., 2002) and craniofacial development (Pucciarelli & Oyhenart, 1987a; Miller & German, 1999).

The development of the neurocranium depends on the growth/development of the brain (Young, 1959; Rozzi *et al*., 2005) and nutritional state. Based on the present findings of an attenuated neurocranial

development in the ER and PER groups (as shown by the measurements for heights 1, 2, 4 and 5, lengths 1–3 and width 3), we suggest that the brain was proportionally underdeveloped in the treated groups. Similarly, the present analysis of the viscerocranium (height 3, lengths 4–7 and widths 1 and 2) suggested that the development of the respiratory and suction functions of the treated groups was lower than that of the control group. The present findings support the studies of Cheverud (1982) and Herring (1993), who showed that the viscerocranium is used during suckling and breathing, and that its growth is continuously subject to muscular loading. We believe that in the present study, the nutritional influence was stronger than the biomechanical influence. The present results agree with Rozzi *et al.* (2005), who showed that environmental stress during development resulted in transitional growth perturbations.

The effects of a nutritional deficit on skull growth and craniofacial dimensions in the rat are not uniform, but depend on the period in which the deficit occurs (Widdowson *et al.*, 1964; Riesenfeld, 1967; Toews & Lee, 1975; Pucciarelli, 1980; Pucciarelli, 1981; Pucciarelli *et al.*, 1990). According to Miller and German (1999), the viscerocranium must grow faster than the neurocranium and is more susceptible to epigenetic factors such as dietary protein levels than is the neurocranium (Pucciarelli, 1980; Pucciarelli, 1981; Miller & German, 1999). Additionally, craniofacial growth generally follows the same pattern in mammals, with growth of the viscerocranium contributing more than growth of the neurocranium to the changes seen postnatally (Enlow, 1966; Michejda *et al.*, 1979; Pucciarelli, 1981; Sirianni *et al.*, 1982; Miller & German, 1999). The present data show that craniofacial (viscerocranium and neurocranium) growth did not follow the same pattern as postnatal growth in the two malnourished groups. Rozzi *et al.* (2005) reported that the lower postnatal neurocranial growth seen in mammals resulted from earlier development of the brain compared to the other structures (Sirianni *et al.*, 1982; Hartwig, 1995).

Cheverud (1982, 1995) suggested that environmental integration is strong in functionally or developmentally integrated traits when the neurocranium and viscerocranium are treated as two different units, whereas genetic integration is stronger than environmental and phenotypic integration when the skull is considered as a whole. This differential growth rate probably reflects the functional demands of the viscerocranium and the application of muscular forces to the facial skull on aging (Lightfoot & German, 1998; Jones *et al.*, 2007). Evidence from the present study supports the idea that the functional demands of the viscerocranium are greater after birth and that, to reach functional adult proportions, growth in this area occurs at a higher rate. Hence, there was an increased chance of being affected by an epigenetic factor such as dietary protein level (Miller & German, 1999).

Widdowson *et al.* (1964) stated that the effects of protein–energy malnutrition are apparently dependent on the time at which this malnutrition occurs. Hence, undernutrition during fetal or neonatal life determines the extent to which there will be recovery in growth (McCance & Widdowson, 1962; Chow & Lee, 1964; Alippi *et al.*, 2002). Malnutrition may occur in any phase of growth, that is, gestation, suckling, weaning or later periods, and the specific effects associated with each period may or may not be similar and/or reversible (Miller & German, 1999; Alippi *et al.*, 2002).

Conclusion

Maternal nutritional state during lactation can affect the development of the craniofacial skeleton. Morphometric analysis of the skull demonstrated a significant reduction in most of the parameters of the two treated groups, specially the PER group, when compared to the controls. This attenuated growth involved both the neurocranium and viscerocranium, and was probably more affected by the maternal nutritional status than by biomechanical stress on the suction and respiratory functions.

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