Maternal undernutrition during lactation leads to reduction in skull size and thickness of adult-aged Wistar rats

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Abstract

Introduction: It is known that the quality and quantity of milk is directly related to the dietary habits of the mother. Despite that, the rates of maternal malnutrition during lactation are increasing in several countries; thus, observing its effects on the offspring is relevant. The present study aims to verify the effects of maternal malnutrition during breastfeeding on the skulls of adult-aged Wistar rats.

Material and methods: Thirty-six newborn rats were divided in three groups: the control group, in which the mother received a regular commercial diet containing 23% protein in unlimited amounts; the protein-energy restriction group, in which the dam received a commercial diet containing 8% protein in unlimited amounts; the energy restricted group, in which the dam received a commercial diet containing 23% of protein in limited amounts. After weaning, all rats received the same diet as the control group until 180 days of age. Then, the rats were euthanized, and their crania were excised and measured in radiographic images. Afterwards, their skull was decalcified with nitric acid (5%) and histological samples were obtained and the thickness of the diploe was verified. Descriptive statistics and ANOVA followed by the Newman-Keuls test were performed for comparison purposes.

Results: It was observed that the skull from the protein-energy restriction and energy-restriction groups was smaller and thinner than that of the control group in several parameters.

Conclusions: Maternal malnutrition during the lactation period caused long-term effects in skull morphology of Wistar rats. These effects could not be reversed after regulation of the diet.

Key words: maternal malnutrition, skull, Wistar rat, histology, morphometrics, breastfeeding.

Introduction

Malnutrition is characterized as poor ingestion of essential nutrients that are necessary for health maintenance. This is caused either by famine or poor dietary habits [1, 2].

Breastfeeding is essential for the correct development of the newborn and it brings benefits to the mother as well. However, proper dietary
habits are essential for both the pregnant and lactating mother [3, 4].

Despite that, maternal malnutrition rates are still fairly high among the general population. Hence, it is still a public health issue in developing or underdeveloped countries [5–7].

An inadequate amount of micronutrients is one of the main causes of maternal malnutrition in low income countries – essentially due to the lack of resources [5]. On the other hand, cultural factors and myths regarding the mother’s diet during the lactation period are another cause of nutrient restriction [6].

Several studies have proposed that maternal malnutrition during pregnancy [8], the lactation period [9–11] or during both timeframes [12–14] can cause several developmental changes in multiple tissues of the offspring.

Some of these studies evaluated the central nervous system (CNS) and behavioral changes [13, 14], while other studies observed changes in muscle tissue [12] and bony tissue [10, 11, 15], although regarding bones, most of these studies performed morphometric evaluations of the studied bones (e.g., femur, cranium). In addition, there are a few studies that addressed the long-term effects of bone growth on offspring whose mothers were malnourished during the lactation period [10, 11, 16].

It is known that the development of the cranium is essential for the consolidation of the CNS. This relation is mutual, since diseases that affect the CNS often impact skull growth [17].

Thus, the study of maternal malnutrition during the lactation period and its effects on cranium development is relevant. The present study aims to analyze the skull of adult aged Wistar rats whose mothers were malnourished during the breastfeeding period.

Material and methods

The study presented herein was approved by the Animal Care and Use Committee of the State University of Rio de Janeiro (CEUA/036/2010), which based its analysis on the Guide for the Care and Use of Laboratory Animals [18].

Furthermore, this experiment complies with the ARRIVE guidelines and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

Six healthy adult female Wistar rats gave birth to a significant number of healthy pups. After birth, the progenitors were divided into three groups with two dams each: a protein-energy restricted group (PER), which received in unlimited amounts a commercial diet containing 8% protein; an energy restricted group (ER), which received a commercial diet containing 23% protein in limited amounts, according to the amount ingested by the PER group; and a control group (C), in which the progenitors received unlimited amounts of a commercial diet with 23% protein.

The dams’ malnourishment started on day 0 of the experiment (immediately post-partum) throughout the lactation period (21 days). Six male pups were assigned to each dam, in order to maximize breastfeeding potential [10], totaling 12 pups per group.

After weaning, the pups were randomly placed in cages in groups of three. All pups received the same commercial diet (23% of protein) until adult age (180th day). Then, the rats were euthanized with a lethal dose of thiopental and their cranium was excised and fixed in a 10% formalin solution.

The skulls were weighed and X-rays were obtained for measurement purposes (Figure 1). Morphometric parameters were previously defined by Fernandes et al. [9] (Table I).

Afterwards, the skulls were routinely decalcified in a solution of 5% nitric acid and histological samples were obtained (hematoxylin/eosin stain was used). The thickness of the diploe was measured in four different anatomical landmarks (nasion, vertex, bregma and inion) with the Image J software. Five random measurements were performed in five random fields (40× magnification) in all skulls and expressed as millimeters (mm) (Figure 2). The lacunae were examined in five random fields of the cortical bone (100× magnification) from each region and expressed as percentage (ratio of empty lacuna number to the total lacuna count) and their

Figure 1. X-ray of the rat’s skull depicting the morphometric parameters. Lateral view and superior view

Figure 2. Measurement of the diploe thickness (40× H&E)
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Mean area was measured (expressed in µm) and analyzed.

**Statistical analysis**

Statistical analysis was performed with the aid of the IBM SPSS statistical software. The data presented here are reported as mean ± standard deviation (SD). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test to compare the three groups (p < 0.05 was considered significant).

**Results**

**Macroscopic analysis**

The mean weight of the crania was 9 g, 8 g and 7 g in the C, ER and PER groups, respectively. These values were not statistically significant (p > 0.05).

The H1, H2, H3, H4 and H5 values were significantly reduced in the PER group in comparison to the C group (p < 0.05). However, when the C and ER or the PER and ER groups were compared, most of these values did not have a statistical significant difference. This also was observed in the W1, W2, W3, L3, L4 and L7 measurements.

These measurements are covered in detail in Table II and Figure 3.

**Microscopic analysis**

It was observed on the histological images that the PER and ER group had more empty lacunae on the cortical bone than the C group (Figure 4). Rates of empty lacuna on the C, ER and PER groups were 7.65 ±2.23%, 8.36 ±3.67%, and 10.41 ±2.91% (p > 0.05), respectively. Histomorphometric analysis of the lacunae showed

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**Table I. Parameters used in the morphometric analysis according to Fernandes et al. (2008)**

| Parameter | Definition |
|-----------|------------|
| Height 1 (H1) | 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 (H2) | 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 (H3) | Maximum height of the orbital cavity = distance between the right upper and lower walls of the orbit – level of the infraorbital fissure |
| Height 4 (H4) | 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 (H5) | 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 (L1) | Maximum length of the neurocranium (rectangular measurement) = distance between the external occipital protuberance and the alveolar margin of the incisive bone |
| Length 2 (L2) | Maximum length of the dorsoventral neurocranium (linear measurement) = distance between the external occipital protuberance and the alveolar margin of the incisive bone |
| Length 3 (L3) | 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 (L4) | Maximum length of the nasal bone = anterior tip of nasal bone – suture between the nasal and frontal bone in the median plane |
| Length 5 (L5) | 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 (L6) | 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 (L7) | Maximum length of the orbital cavity = distance between the most ventral aspect of the right infraorbital and supraorbital margin |
| Width 1 (W1) | 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 (W2) | 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 (W3) | 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 (W4) | Distance between the tympanic bulla = anteromedial edge of the right tympanic bulla – anteromedial edge of the left tympanic bulla |
Table II. Morphometric results of the skull in the adult-aged rats (mean ± SD of 12 pups per group)

| Parameter | Groups          | P-value | C vs. ER | C vs. PER | ER vs. PER |
|-----------|-----------------|---------|----------|-----------|------------|
| Height 1  | C 12.49 ±0.42   | ER 12.17 ±0.29 | PER 11.29 ±0.38 | > 0.05 | < 0.05 | < 0.05 |
| Height 2  | C 14.93 ±0.59   | ER 14.70 ±0.28 | PER 14.29 ±0.38 | > 0.05 | < 0.05 | < 0.05 |
| Height 3  | C 4.97 ±0.32    | ER 4.58 ±0.22 | PER 4.49 ±0.25 | < 0.05 | < 0.05 | > 0.05 |
| Height 4  | C 13.49 ±0.37   | ER 13.29 ±0.34 | PER 13.02 ±0.60 | > 0.05 | < 0.05 | < 0.05 |
| Height 5  | C 18.03 ±0.58   | ER 17.70 ±0.40 | PER 17.10 ±0.70 | > 0.05 | < 0.05 | > 0.05 |
| Length 1  | C 48.06 ±1.37   | ER 47.52 ±1.05 | PER 47.43 ±0.99 | > 0.05 | > 0.05 | < 0.05 |
| Length 2  | C 47.81 ±1.45   | ER 47.17 ±1.00 | PER 47.14 ±0.95 | > 0.05 | > 0.05 | < 0.05 |
| Length 3  | C 45.79 ±1.37   | ER 44.91 ±1.03 | PER 44.74 ±1.06 | > 0.05 | < 0.05 | < 0.05 |
| Length 4  | C 19.64 ±0.71   | ER 19.04 ±0.83 | PER 18.83 ±0.46 | > 0.05 | < 0.05 | > 0.05 |
| Length 5  | C 26.92 ±0.58   | ER 26.59 ±0.61 | PER 26.47 ±0.52 | > 0.05 | < 0.05 | > 0.05 |
| Length 6  | C 27.04 ±0.81   | ER 26.72 ±0.59 | PER 26.73 ±0.54 | > 0.05 | > 0.05 | > 0.05 |
| Length 7  | C 6.25 ±0.31    | ER 5.85 ±0.35 | PER 5.59 ±0.22 | > 0.05 | > 0.05 | < 0.05 |
| Width 1   | C 4.48 ±0.19    | ER 4.38 ±0.22 | PER 4.17 ±0.22 | > 0.05 | < 0.05 | > 0.05 |
| Width 2   | C 8.03 ±0.23    | ER 7.69 ±0.16 | PER 7.61 ±0.13 | < 0.05 | < 0.05 | > 0.05 |
| Width 3   | C 7.40 ±0.19    | ER 7.25 ±0.21 | PER 7.09 ±0.22 | > 0.05 | < 0.05 | > 0.05 |
| Width 4   | C 17.06 ±0.41   | ER 17.16 ±0.25 | PER 17.00 ±0.34 | > 0.05 | > 0.05 | > 0.05 |

C – control group, ER – energy restricted group, PER – protein-energy restricted group. P < 0.05 is considered significant.

Figure 3. Graphic showing the macroscopic morphometric results (mean and standard deviation) of the three groups. H – height, L – length, W – width
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Regarding skull thickness, there was a statistically significant difference of the vertex between the three groups \((p < 0.05)\). In addition, there were statistically significant differences between the C and PER groups regarding the bregma, nasion and vertex regions \((p < 0.05)\). Detailed measurements of each region can be observed in Table III and Figure 5.

**Discussion**

The breastfeeding period is essential for the correct development of the newborn as it benefits multiple systems of the organism. It is known that the mother’s diet directly affects the quality and quantity of the produced milk. Despite that, there are significant rates of maternal malnutrition in developing and developed countries \([1, 2, 4–6, 19]\).

The effects of maternal malnutrition during pregnancy have been intensively studied in the literature. These studies have proven the metabolic imprinting (or fetal programming) theory, in which the effects caused during the intra-uterine life may induce metabolic disturbances in later periods \([19–24]\).

However, recent studies have shown that the lactation period is also a critical window for inducing developmental changes in several tissues. Thus, metabolic imprinting may occur up until the end of the breastfeeding period \([7, 9–12, 14–16, 25]\).

The present study showed that the skull in the PER group was significantly smaller than in C or ER groups. Moreover, the diploe was significantly thinner in the PER group in two anatomical landmarks. Thus, there was observed a negative impact in skull growth caused by maternal protein-energy restriction during the lactation period.

Despite the methodological differences, the results presented herein are similar to the studies performed by Ramirez Rozzi et al. \([26]\) and Luna et al. \([15]\), as there were significant alterations in the crania size of the malnourished groups.

Catch-up growth is considered as the compensatory growth during the recovery window after growth restriction. It can be complete (type A) when the animal reaches normal adult size, or incomplete (type B) when it fails to reach the regular growth curve. The concept of catch-up growth was well explained by Boersma and Wit \([27]\).

Much is known about catch-up growth; however, there is uncertainty whether catch-up growth is possible or not as there is a mosaic of different

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Histological findings of the lacunae of the control (A), energy restricted (B) and protein-energy restriction (C) groups. A higher number of empty lacunae (black arrowheads) can be observed in the protein-energy restriction group (C). 200×/H&E
results in the literature, especially when both periods (pregnancy and breastfeeding) are considered separately [10, 11, 25, 28].

In the present study, catch-up growth is considered as the period in which the skull growth of the rats from the PER and ER groups would take to reach the skull growth of the C group. A previous study performed by Fernandes et al. [9] observed similar results to ours, albeit in young rats. When their results are compared to the ones presented herein, it is clear that catch-up growth was not possible since these changes in skull growth continued even after regular dietary intake of the adult aged rats.

Furthermore, a slightly higher percentage of empty lacunae could be observed in the ER and PER groups. Although they were not statistically significant, these results may indicate that there is indeed an injury of bony tissue caused by maternal malnutrition during the lactation phase.

The study of the lacunae which surrounds the osteocyte is used to measure pathological changes on bony tissue caused by osteoporosis; for instance, thus, it can be a predictor of abnormalities within the bone [29, 30].

Moreover, conditions such as premature synostosis can cause impairment of CNS development; in addition, diseases of the CNS (such as Zika) can impact skull growth as well since their ontogenetic process is closely related and mutually dependent [17].

In this fashion, our results might indicate that the changes in skull growth are associated with neurological alterations of the PER groups observed in the studies performed by Belluscio et al. [13] and Natt et al. [14].

**Table III.** Diploe thickness of the three groups (mean ± SD of 12 pups per group)

| Thickness [mm] | Groups | P-value |
|----------------|--------|---------|
|                | C      | ER      | PER    | C vs. ER | C vs. PER | ER vs. PER |
| Bregma         | 0.30 ±0.03 | 0.29 ±0.04 | 0.27 ±0.02 | > 0.05 | < 0.05 | < 0.05 |
| Inion          | 0.37 ±0.01 | 0.37 ±0.04 | 0.35 ±0.04 | > 0.05 | > 0.05 | > 0.05 |
| Nasion         | 0.34 ±0.04 | 0.32 ±0.03 | 0.32 ±0.01 | < 0.05 | < 0.05 | > 0.05 |
| Vertex         | 0.26 ±0.03 | 0.24 ±0.02 | 0.21 ±0.05 | < 0.05 | < 0.05 | < 0.05 |

C – control group, ER – energy restricted group, PER – protein-energy restricted group. P < 0.05 is considered significant.

**Figure 5.** Graphic showing the histomorphometric results (mean and standard deviation) of the bregma (A), inion (B), nasion (C), and vertex (D) of the three groups.
In conclusion, the study presented herein revealed that there were several changes in the skull and the diploe of rats whose dams were malnourished during the breastfeeding period. The results of this study also showed that these changes could not be reversed even after regular dietary intake between the weaning period and the adult age, thus concluding that catch-up growth was not possible.

Conflict of interest
The authors declare no conflict of interest.

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