The Cortical Matrix of Femur in Adult-Aged Wistar Rats is Critically Affected by Maternal Malnutrition During Lactation Period

La Matriz Cortical del Fémur en Ratas Wistar de Edad Adulta se ve Críticamente Afectada por la Malnutrición Materna Durante el Período de Lactancia

Marcio Antonio Babinski¹; Marcio Vinicius Viana Andrade¹; Mario Augusto Sereno Fernandes¹; Jorge Gilberto Cipriano Mendes¹; Monique da Silva Dias Babinski²; Lucas Alves Sarmento Pires² & Vinicius Schott Gameiro²


SUMMARY: Osteoporosis is associated with poor dietary habits. Malnutrition is characterized as a deficit on the intake of necessary nutrients that are essential for optimal health maintenance. It is known that malnutrition during the lactation period can affect the offspring. The present study aims to evaluate the chronic effects caused by maternal energy-protein restriction during lactation period in the offspring. At parturition, Wistar rat dams were divided in three groups: (1) Control group (C) - which received a 23 % protein diet without restrictions; (2) Protein-Energy restriction group (PER) - which received a 8 % protein diet; (3) Energy restriction group (ER) - which received a 23 % protein diet in limited amounts, according to the ingestion of the second group. Each group had 12 pups. After weaning, all pups received free access to a 23 % protein diet until 180 days and then were euthanized. Their femur was excised, decalcified, histologically processed and analyzed under a microscope. The measurements of the osteon lacunae on the C, ER and PER groups were, respectively: 2.1 μm, 10.9 μm and 14.7 μm (p<0.05). A poor ingestion of proteins and calories during lactation period provoked critical and permanent changes on the bone matrix of the femur, which simulated osteoporosis.

KEY WORDS: Bone remodeling, Bone matrix, Undernutrition, Osteoporosis, Wistar rats.

INTRODUCTION

Conditions such as osteopenia and osteoporosis are commonly associated with pathological (or fragility) fractures – which possess a high incidence in women and the elderly (Burge et al., 2007; Court-Brown et al., 2014; Willeumier et al., 2017). These diseases cause morphological changes in bony tissue, which in turn leads to loss of its function. Furthermore, both illnesses are associated with inadequate dietary habits (Pignolo et al., 2010; Warensjö Lemming et al., 2017).

Malnutrition (or undernutrition) is defined as a deficit on the intake of necessary nutrients that are essential for optimal health maintenance. It is not only a product of non-ingestion of food, since irregular dietary habits such as excessive fast food ingestion can cause this condition (Ahsan et al., 2017; Kavle et al., 2018). Despite numerous governmental programs and campaigns, there is still a high incidence of pregnant and lactating women that have an inadequate nutrient intake (Black et al., 2013; Haileslassie et al., 2013; Jeong et al., 2017).

Experimental studies that addressed maternal malnutrition during lactation period showed that this condition can inflict significant changes on bone growth and function in their offspring (Brasil et al., 2005; Fernandes et al., 2007; Fernandes et al., 2008; Babinski et al., 2016; Confortim et al., 2017).

Thus, due to these morphological changes on bony tissue caused by maternal malnutrition during the lactation period associated with the fact that pathological (or fragility) fractures are among the most common types of lesions among osteoporotic and osteopenic patients, the study of maternal malnutrition during lactation period is essential (Court-Brown et al.; Babinski et al.).

¹Orthopedics and Traumatology Department, Salgado Filho Municipal Hospital, Rio de Janeiro, Brazil.
²Programa de Pós-Graduação em Ciências Médicas, Universidade Federal Fluminense, Rio de Janeiro, Brazil.
The study presented herein aims to investigate the effects of maternal malnourishment during lactation period by a histological and morphometric analysis of the cortical dimensions of the femur in adult aged Wistar rats.

MATERIAL AND METHOD

This study was approved by the Animal Care and Use Committee of the State University of Rio de Janeiro. All work presented herein were in accordance with the American Physiological Society guidelines (Howard-Jones, 1985; Bayne, 1996).

Six female Wistar rats mated with three male rats (2 females to 1 male). After mating, the six female rats were divided in three distinct groups: the control group (C), which had free access to a standard laboratory diet (in grams per 100g) containing 23 % protein, 68 % carbohydrate, 5 % lipid, 4 % salts and 0.4 % vitamins, and a total of 17,038.7 (kJ/kg) energy; the protein-energy-restricted group (PER), which had free access to an isoenergetic, protein-restricted diet containing 8 % protein; and the energy-restricted group (ER), which was fed with a standard laboratory diet in restricted quantities, which was calculated based on average ingestion of the PER group. This calculi allows comparison between both groups, as previous studies showed that the PER group consumed about 60 % of the amount consumed by the control group, despite the free access to food (Babinski et al.).

Excess pups were removed so that only six pups were kept per dam, since it has been showed to improve the lactation potential of the dam (Fishbeck & Rasmussen, 1987), thus there were 12 pups per group. All pups showed good health condition. The diet was formulated to match the requirements of the American Institute of Nutrition AIN- 93G recommendation for rodent diets (Reeves et al., 1993).

The maternal malnutrition ended at weaning (21 days post-partum) and the pups were housed in groups of three animals per cage and were given unlimited access to the same diet that was used in the C group and ad-libitum water until adult age (day 180). Then, the animals were anesthetized with a lethal thiopental anesthesia (0.15 mL/100 g/body weight), fixed with a 10 % formalin solution (perfused through the left ventricle with buffered saline) and the right femurs of the specimens were excised.

After the excision, they were placed in a 10 % formaldehyde solution for 24 h, then, they were decalcified (EDTA). Histological process was routinely performed in order to include the samples in paraffin blocks. Then, 10 µm thick transversal sections were performed at proximal epiphysis and stained with Hematoxylin/Eosin.

The histological samples were analyzed and quantified according to Nguyen et al. (2017). The microscopic images presented herein had a 400X increase. The areas of the cortical bone that were occupied by lacunae and osteocytes were measured on the three groups. This step was performed with the aid of the Image JÔ software, and the results are presented in micrometers (µm).

Data was reported as mean±standard deviation (SD). Statistical significance of experimental observations was determined by the ANOVA one-way analysis of variance, followed by post-hoc test of Newman-Keuls to compare the three groups (p < 0.05 was considered significant). All statistical analysis was done using GraphPad Prism 6 software (GraphPad Inc., CA, USA).

RESULTS

According to the histological analysis, the control group displayed regular cortical bone tissue and matrix. The osteocytes completely occupied the lacunae and developing Havers’s canals were seen (Fig. 1).

In comparison, the ER group had a less dense and irregular cortical bone, as there were enlarged lacunae with regular osteocytes in its interior. Furthermore, it was also observed irregular lacunae with significant size increase and signs of bone remodeling (Fig. 2).

On the PER group, it was seen that the cortical tissue had an increase in the number of lacunae – which possessed larger areas and no osteocytes (Fig. 3). There were also signs of bone remodeling and bone resorption (Fig. 4). The osteocytes were abundant in larger lacunae, which indicated an increase of cellular death (Fig. 5).

Histomorphometric analysis of the lacunae displayed a mean of 2.1 µm, 10.9 µm and 14.7 µm on the C, ER and PER groups respectively. These differences were statistically significant (p < 0.05). When compared to the C group, the ER and PER measurements were statistically significant. There was also a significant increase of the cortical bone osteon area (µm): ~400 % for the RC group and ~600 % for the PER group.
DISCUSSION

It is known that breastfeeding is essential for the correct development of the newborn (Black et al.). The quality of the milk is subject to the mother’s dietary habits, thus, a good maternal nutrition is essential to provide optimal milk for its offspring (Plecas et al., 2014; Kavle et al.).

Development of the tissues begin during the intrauterine phase, although, after birth, these organs and cells are under the influence of environmental and dietary habits, thus, proper breastfeeding is necessary (Luna et al., 2016).
Hence, the first weeks of life for the newborn are critical. Maternal malnutrition during this timeframe can cause difficulty for the bone tissue to develop in short and long terms – previous experimental studies demonstrated the harmful effects of maternal malnutrition during the lactation period in bone tissue of the offspring. Under these circumstances, the pups which were breastfed by a malnourished mother had an underdevelopment of multiple bones, such as the tibia, mandible, cranium and femur (Fernandes et al., 2007; Fernandes et al., 2008; Degani Junior et al., 2011; Babinski et al.; Luna et al.; Confortim et al.). Moreover, the maternal malnutrition during the gestational period has also been shown to cause detrimental effects on its offspring (Alippi et al., 2002; Lee et al., 2016; Confortim et al.).

In humans, maternal malnutrition during pregnancy has been shown to be associated with the late onset of diseases such as osteoporosis (Hyde et al., 2017). Thus, the metabolic programming theory has been gaining recent attention (Dennison et al., 2010; Barker, 2012).

Osteoporosis disrupts the normal arrangement of the bone, as is characterized as low bone mass and micro-architectural deterioration of bone tissue, thus, an osteoporotic bone is more susceptible to fractures and more prone to be damaged during orthopedic procedures (Dennison et al.; Court-Brown et al.; Babinski et al.).

According to the analysis presented herein, there were several alterations on bone matrix of the ER and PER groups. This might indicate that rats that are breastfed by malnourished mothers may develop a disturbance of bone metabolism in the future, in which bone resorption is increased (predominance of osteoclasts) and bone consolidation is decreased. This fact led us to believe that our experiment simulated osteoporosis type II, which could not be reversed with nutritional reposition.

There is a lack of experimental studies regarding the chronic effects of maternal malnutrition during lactation period (e.g. rats that received regular diet after being fed by a malnourished mother). A study performed by Babinski et al. showed that the femur was significantly affected by malnutrition during lactation period, and the morphological effects remained until the adult life of the animals, even after receiving unlimited amounts of a diet with protein and energy after weaning. Furthermore, their study also presented significant alterations of the head and anatomical neck diameters, together with alterations of the cervical-diaphyseal angle in rats from ER and PER groups, in comparison to the control group.

The present study demonstrated that catch-up growth is not possible in the offspring that is subject to maternal malnutrition during lactation period. This is in accordance with several studies (Cesani et al., 2003; Babinski et al.), although there are studies that indicate otherwise (Jones et al., 2011), thus, there is still no consensus on this matter.

**CONCLUSIONS**

In summary, maternal protein-energy malnutrition during lactation provokes critical and irreversible changes on bone matrix of the femur in rat offspring. Diet regulation did not reversed the effects caused by the maternal malnutrition, thus the metabolic programming theory should be further expanded to the lactation period as well.

Our results showed that the experimental model was viable in order to reproduce previous results (Fernandes et al., 2007; Fernandes et al., 2008; Degani Junior et al.; Babinski et al.) Furthermore, the present study reiterates the importance of a protein-energy adequate diet to the breastfeeding mother during gestational and lactation periods.

**RESUMEN:** La osteoporosis se asocia con malos hábitos alimenticios. La desnutrición se caracteriza como un déficit en la ingesta de los nutrientes necesarios que son esenciales para un mantenimiento óptimo de la salud. Se sabe que la malnutrición durante el período de lactancia puede afectar a la descendencia. El presente estudio tiene como objetivo evaluar los efectos crónicos causados por la restricción energética-proteína materna durante el período de lactancia en la descendencia. En el parto, las hijas de rata Wistar se dividieron en tres grupos: (1) grupo control (C) - que recibió una dieta con 23 % de proteína sin restricciones; (2) Grupo restricción de energía de proteína (PER) - que recibió una dieta con 8 % de proteína; (3) Grupo restricción de energía (ER) - que recibió una dieta de 23 % de proteína en cantidades limitadas, de acuerdo con la ingestión del segundo grupo. Cada grupo tenía 12 crías. Después del destete, todas las crías recibieron acceso libre a una dieta con 23 % de proteína hasta 180 días y luego fueron sacrificadas. Su fémur fue extirpado, descalcificado, procesado histológicamente y analizado bajo un microscopio. Las mediciones de las lagunas de osteón en los grupos C, ER y PER fueron, respectivamente: 2,1 mm, 10,9 mm y 14,7 mm (p <0,05). Una mala ingesta de proteínas y calorías durante el período de lactancia provocó cambios críticos y permanentes en la matriz ósea del fémur, que simulaba osteoporosis.

**PALABRAS CLAVE:** remodelación ósea; Matriz ósea; Desnutrición; Osteoporosis; Ratas Wistar.