Biological Trace Element Research
https://doi.org/10.1007/s12011-018-1499-3

Effects of Nutritional Deficiency of Boron on the Bones of the Appendicular Skeleton of Mice

Alejandro A. Gorustovich[1] · Forrest H. Nielsen[2]

Received: 17 August 2018 / Accepted: 28 August 2018

Abstract
Scientific evidence has shown the nutritional importance of boron (B) in the remodeling and repair of cancellous bone tissue. However, the effects of the nutritional deficiency of B on the cortical bone tissue of the appendicular skeleton have not yet been described. Thus, a study was performed to histomorphometrically evaluate the density of osteocyte lacunae of cortical bone of mouse femora under conditions of nutritional deficiency of B and to analyze the effects of the deficiency on the biomechanical properties of mouse tibiae. Weaning, 21-day-old male Swiss mice were assigned to the following two groups: controls (B+; n = 10) and experimental (B−; n = 10). Control mice were fed a basal diet containing 3 mg B/kg, whereas experimental mice were fed a B-deficient diet containing 0.07 mg B/kg for 9 weeks. The histological and histomorphometric evaluations of the mice fed a B-deficient diet showed a decrease in the density of osteocyte lacunae in the femoral cortical bone tissue and the evaluation of biomechanical properties showed lower bone rigidity in the tibia.

Keywords Boron · Cortical bone · Osteocyte lacunae · Histomorphometry · Biomechanical properties

Introduction

Bone tissue is a specialized form of dense connective tissue composed of cells and a mineralized extracellular matrix [1]. The cellular composition of the adult skeleton is composed of osteocytes (90–95%), osteoblasts (5%), and osteoclasts (1%) [2, 3]. Osteocytes are derived from osteoblasts that have stopped the production of bone matrix and have become trapped within lacunae in the interior of the newly formed bone [2–5]. A large number of canaliculi, which are approximately 250–300 nm in length [2] and contain the cytoplasmic extensions of osteocytes, radially emerge from these lacunae. The cytoplasmic extensions come in contact with the extensions of neighboring osteocytes as well as with osteocytes of the bone surface [3, 6], and also extend up to the bone marrow and establish communications with blood vessels [2]. The osteocyte lacunae and canaliculi thus generate a functional three-dimensional interconnected network through which interstitial fluid and small molecules circulate [7, 8]. This network is known as "lacuno-canalicular network" and one of its main functions is to detect the need for remodeling of the bone tissue in response to mechanical and hormonal needs [6–17].

The strategic location of osteocytes within the mineralized bone matrix makes them excellent candidates to detect the need for remodeling during functional adaptation to mechanical loads and for repair of microcracks, and, in both cases, transmit signals to the effector cells in charge of bone formation and resorption [13–15]. Several studies have shown that osteocytes perform their function by transducing mechanical loads into biochemical signals able to influence osteoblasts and osteoclasts [6–11, 14–17].

It has been recently suggested that osteocytes are involved in calcium (Ca) and phosphorus (P) homeostasis. In this sense, the osteocyte network could also function as an endocrine regulator [9–12], since it constitutes a large surface that can be used to mobilize Ca, as for example during pregnancy, lactation, and hibernation [18, 19]. In addition, through the Dmp1, Phex and FGF23 signaling pathways, osteocytes regulate the resorption of phosphate at the renal level [9–12].

Recent studies have assessed the influence of various dietary components, especially Ca and vitamin D, on bone and mineral metabolism. However, other vitamins (such as vitamins A, B, C, and K) and trace elements (such as strontium,

✉ Alejandro A. Gorustovich
agorustovich@conicet.gov.ar

[1] Interdisciplinary Materials Group-IESIING-UCASAL, INTECIN UBA-CONICET, A4400EDD Salta, Argentina

[2] Research Nutritionist Consultant, Grand Forks, ND, USA

Published online: 04 September 2018

zinc, silicon, and boron) are also potentially important and should thus be taken into account [20–26].

Boron (B) is a bioactive trace element that satisfies several criteria to be considered essential for animals and humans, including the following: (a) it is present (at comparable concentrations) in healthy tissues of different animals, (b) it has a homeostatic mechanism of control, and (c) its deficiency results in the alteration or loss of important physiological functions [27–36]. However, it lacks a defined biochemical function. It has been suggested that the daily intake of B should be in the order of 1–13 mg/day for adults [20, 27]. The main sources of B are fruits (except citrus and berries), vegetables, legumes, nuts, and the water, cider, and beer [28, 29].

The nutritional importance of B in the remodeling and repair of cancellous bone tissue has been shown [37–40]. Regarding the effects of B on cortical bone, in previous studies, we have assessed the modeling and remodeling of the periodontal cortical of the alveolar bone of the jaw in mice [41]. However, the effects of the nutritional deficiency of B on the cortical tissue of bones of the appendicular skeleton have not yet been described.

In the last decades, there has been an increasing interest in the study of the density of osteocyte lacunae (number of lacunae/mm^2) as a histomorphometric indicator of bone formation under local and systemic variables [42–60]. Given the above background, the aims of the present study were to histomorphometrically evaluate the density of osteocyte lacunae in cortical bone of the femur of mice under conditions of nutritional deficiency of B and to analyze the effects of this deficiency on the biomechanical properties of the mouse tibia.

Materials and Methods

The study material came from 21-day-old male Swiss mice used in previous experiments [41]. All animal experiments were carried out in keeping with the guidelines of the National Institutes of Health for the care and use of laboratory animals (NIH Publication No. 85–23, Rev. 1985). The protocol was examined and approved by the Institutional Ethics Committee of the School of Dentistry, University of Buenos Aires.

Experimental Procedure

At weaning, the mice were assigned to two groups: control (B+; $n = 10$) and experimental (B−; $n = 10$). Considering that it has been established that 3 mg B/kg of diet prevents the signs associated with B deficiency in rats and mice [39, 41, 61], control animals were fed a basal diet containing 3 mg B/kg and experimental animals were fed a basal diet containing 0.07 mg B/kg (Table 1) for 9 weeks [41]. The diet was provided by the United States Department of Agriculture, Agricultural Research Service, USDA, ARS Grand Forks Human Nutrition Research Center, Grand Forks ND, USA.

Table 1 Composition of the basal diet

Ingredients	g/kg
Granulated corn prewashed with acid	713.5
Vitamin-free casein	160
Oil	75
tert-Butylhydroquinone	0.014
DL-α-Tocopherol	0.2
Choline chloride	1
L-Cystine	2
Macro minerals[a]	29.3
Vitamins[b]	4
Trace elements[c]	15
Total	1000

[a] Macro minerals (in g): $CaHPO_4$, 17; KCl, 7; and Mg $(C_2H_3O_2)_2$ $4H_2O$, 5.3

[b] Vitamins (in mg): vitamin A palmitate (500,000 IU/g), 16; thiamine HCl, 10; pyridoxine HCl, 15; nicotinic acid, 30; DL-pantholeic acid, 48; vitamin B_{12} (0.1% in mannitol), 50; folic acid, 2; biotin, 1; riboflavin, 27; vitamin K (phylloquinone), 1; inositol, 50; para-aminobenzoic acid, 5; vitamin D_3 (400,000 IU/g), 2.5; and dextrose, 3742.5

[c] Trace elements (in mg): NaCl, 2000; $Mn(C_2H_3O_2)_24H_2O$, 45; $CuSO_45H_2O$, 30; $Zn(C_2H_3O_2)_22H_2O$, 84; powdered iron (dissolved in HCl), 75; $NaHAs_47H_2O$, 5; K, 0.4; $NaSeO_35H_2O$, 1.4; $Cr(C_2H_3O_2)_32$ H_2O, 2; NH_4VO_3, 0.3; $(NH_4)_2MoO_4$, 1; NaF, 2; $NiCl6H_2O$, 3.7; $NaSiO_29$ H_2O, 50, and granulated corn (prewashed with acid), 12,700.2

During the experiment, the animals were daily fed ad libitum. Body weight was recorded every 7 days and food consumption was recorded every 24 h. The animals were sacrificed by means of an intraperitoneal injection of pentobarbital sodium (Nembutal 1%), and femora and tibiae were resected. The femora were fixed in formalin 10% and their length recorded with a digital caliper with an accuracy of 0.05 mm, as the distance between the most proximal point of the femoral head and the most distal point of the medial condyle. The tibiae were kept at − 20 °C for subsequent biomechanical studies.

Histological Procedure

The femora were stained in toto with basic fuchsin 1% according to the methodology described by Frost [62–64] and then processed for inclusion in methyl methacrylate. Histological cuts were subsequently obtained transversal to the longitudinal axis, at the level of the medial femoral diaphysis. The cross sections were ground using a grinding machine and finished manually with sandpaper to obtain sections about 50 μm thick.

Histological and Histomorphometric Evaluation

Histological and histomorphometric parameters were evaluated by means of common optical and fluorescence microscopy (Nikon Eclipse E800 fluorescence microscope equipped with a HeNe laser unit (Eclipse C1)), using an excitation of 544 nm and an emission filter of 570 nm.

The histomorphometric parameters evaluated included:

1- Outer diameter (Fig. 1a), inner diameter (Fig. 1b), and thickness of the cortical of the medial femoral diaphysis (Fig. 1c). These measurements were taken on the digital images obtained through fluorescence microscopy at × 40. The free software EZ-C1, Silver Version 3.0 (Free Viewer-Nikon) was used.

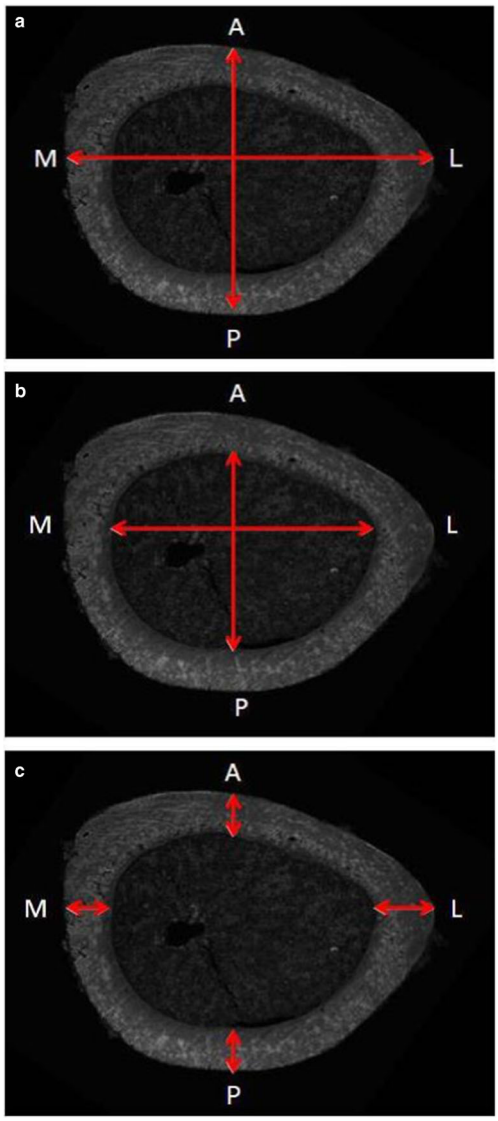

Fig. 1 **a** Outer diameter (A anterior, P posterior, M medial, L lateral). **b** Inner diameter (A anterior, P posterior, M medial, L lateral). **c** Thickness of the cortical of the medial femoral diaphysis (A anterior, P posterior, M: medial, L: lateral)

2- Greater and lesser diameters of osteocyte lacunae. These parameters were determined using a common optical microscope provided with an eyepiece micrometer and an objective of × 100.

3- Density of osteocyte lacunae. The number of osteocyte lacunae/mm^2 of cortical bone tissue was determined using images captured through fluorescence microscopy (with an objective of × 60). To this end, an area of 0.0625 mm^2 was determined in the anterior (A), posterior (P), medial (M), and lateral (L) regions of the medial femoral diaphysis. Then, by using the digital superimposition of a grid, each area was subdivided into 25 fields of 0.05 mm × 0.05 mm each (Fig. 2), and the number of osteocyte lacunae recorded in each field. The Scandium 5.0 software was used (Build 1054, Soft Imaging system GmbH).

Evaluation of Biomechanical Properties

The mechanical properties at the level of the tibial diaphysis were evaluated through a three-point flexion test. To do this, each tibia was horizontally placed on an Instron equipment model 4442, with the anterior face up, on two equally spaced supports, separated by a constant distance of 11 mm. In this position, the diaphysis was centrally loaded with a force of 50 N at a speed of 5 mm/min until its fracture. The load/deformation curves obtained allowed determining the following structural biomechanical properties associated with the resistance of the bone [65]:

1- *Bone resistance to fracture*, i.e., the effective resistance of the bone to lose its integrity as a single structure

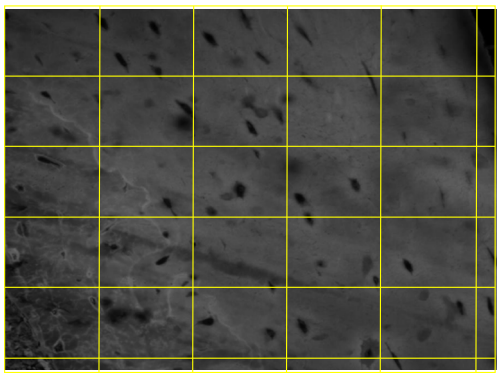

Fig. 2 Determination of the fields to count the number of osteocyte lacunae

2- *Maximum elastic resistance*, i.e., the maximum capacity of the bone in flexion to elastically resist a deforming load;
3- *Elastic energy absorption*, i.e., the capacity of the bone to absorb mechanical energy when deforming elastically
4- *Bone rigidity*, i.e., the effective resistance of the whole bone to be deformed elastically by the action of loads.

Statistical Analysis

The data are presented as mean ± standard deviation and were statistically analyzed by means of the Student's *t* test, assuming $\alpha = 0.05$ and $\beta = 0.01$.

Results

Body Weight and Food Consumption

No significant differences were observed in body weight or food consumption between groups (Table 2).

Morphometric Evaluation

No statistically significant differences were observed in the length of the femur ($p > 0.05$) between both groups (Table 2).

Histological and Histomorphometric Parameters

The histomorphometric analysis of the outer and inner diameter and thickness of the cortical of the medial femoral diaphysis showed no statistically significant differences between both groups ($p > 0.05$) (Tables 3, 4, and 5).

The histological evaluation showed no differences in the size of the osteocyte lacunae between groups (Table 6), whereas fluorescence microscopy showed absence of microcracks in the corticals of both groups.

The histomorphometric evaluation showed that the density of osteocyte lacunae was lower in the experimental mice (B−) than in control mice (B+) (Fig. 3) (25% lower in the medial surface and 18% lower in the posterior surface) (Table 7). Although the density of osteocyte lacunae was also lower in the anterior and lateral surfaces, the difference between the two groups was not statistically significant ($p > 0.05$).

Table 2 Study parameters at 9 weeks after treatment

Groups	B (+)	B (−)
Body weight (g)	30 ± 2	31 ± 1*
Food consumption (g)	15 ± 2	15 ± 1*
Femoral length (mm)	15.42 ± 0.43	15.68 ± 0.44*

(mean ± SD, *$p > 0.05$)

Table 3 Outer diameter (mm)

		Anterior-posterior	Medio-lateral
Outer diameter (mm)	B (+)	1.23 ± 0.04 *	1.72 ± 0.13 *
	B (−)	1.27 ± 0.02	1.75 ± 0.12

(mean ± SD, *$p > 0.05$)

Biomechanical Properties

The structural biomechanical properties obtained at the level of the medial tibial diaphysis are shown in Fig. 4a–d. The maximum force required for the fracture (Fig. 4a), the load at the point of fracture or elastic limit (Fig. 4b), and the energy accumulated during the elastic deformation (Fig. 4c) did not differ between the groups. However, the diaphyseal rigidity (Fig. 4d) was significantly lower (19%) in the experimental group (B−) than in the control group (B+) ($p < 0.05$).

Discussion

It has been established that the density of osteocyte lacunae represents the number of osteoblasts which have become incorporated during the formation and mineralization of the bone matrix [66–68]. In this sense, the lower density of osteocyte lacunae observed in the present study suggests that a nutritional deficiency of B would determine a decrease in the number of osteoblasts that will finally be differentiated to osteocytes. In general, around 29% of osteoblasts develop into osteocytes, 6% develop into bone lining cells, and the remaining 65% die by apoptosis [3, 4].

Previous studies by our research group have shown that the nutritional deficiency of B affects both the osteogenesis of the alveolar bone in rats [39] and the modeling and remodeling of the periodontal cortical of the alveolar bone of the jaw of mice due to a marked decrease in bone formation [41]. In addition, both experimental models showed a statistically significant decrease in the percentage of osteoblastic bone surfaces and an increase in the quiescent surfaces associated with bone

Table 4 Inner diameter (mm)

		Anterior-posterior	Medio-lateral
Inner diameter (mm)	B (+)	0.85 ± 0.07 *	1.14 ± 0.01 *
	B (−)	0.90 ± 0.02	1.16 ± 0.06

(mean ± SD, *$p > 0.05$)

Table 5 Cortical thickness (mm)

		Anterior	Posterior	Medial	Lateral
Cortical thickness (mm)	B (+)	0.19 ± 0.01 *	0.20 ± 0.02 *	0.19 ± 0.01 *	0.41 ± 0.11 *
	B (−)	0.20 ± 0.01	0.20 ± 0.02	0.20 ± 0.02	0.39 ± 0.12

(mean ± SD, *$p > 0.05$)

lining cells [39, 41]. So far, there is very limited information regarding the effects of B at the cellular and molecular level [30, 69]. Fu et al. [69] showed that a concentration of B ≤ 0.65 mM has mitogenic effects on bone marrow mesenchymal cells in vitro, as well as on MLO-A5 cells, a cell line of the osteoblast/osteocyte lineage. In addition, B supplementation at 1 or 10 ng/mL increased mineralized nodule formation and bone tissue-associated mRNA expression of type I collagen, bone sialoprotein, osteopontin, osteocalcin, run-related transcription factor 2, and bone morphogenetic protein-4, 6, and 7 levels by cultured MC3T3-E1 osteoblasts [70]. Boron also was found to increase calcium deposition, alkaline phosphatase activity, and enhance the expression of osteogenic markers in cultured human bone marrow stromal cells [71]. Recently, Capati et al. [72] demonstrated the acceleration of the Ca^{2+} influx and efflux by 0.1 mM B supplementation in cultured NOS-1 osteoblastic cells. Results indicated that cell membrane stability may be related to the mechanism by which a very low concentration of B promotes the proliferation and differentiation in mammalian osteoblastic cells in vitro [72].

Several authors have evaluated the normal density of osteocyte lacunae [46, 48–53, 66, 73–79] but their results are very variable, probably associated with different factors such as the species studied, the age of the animal, and the anatomical location and/or type of bone (cortical or spongy) analyzed. In the present study, the density of osteocyte lacunae determined in the cortex of the femur of mice fed a diet sufficient in boron (3 mg B/kg) was in the order of ~ $13 \times 10^2/mm^2$, similar to that observed in the ulna and femur of other species of laboratory mice [74, 79]. In contrast, mice fed a diet deficient in B for 9 weeks showed a decrease in the density of osteocyte lacunae, but no microcracks in the cortex of the femur. These results are different from those reported by Vashishth et al. [46] and Qiu et al. [52], who studied samples of human cortical bone tissue and found microcracks associated with regions with lower density of osteocyte lacunae. Considering the important roles of osteocytes and their lacuno-canalicular network, the analysis of the functional relevance of the decrease in the density of osteocyte lacunae in different regions of the cortical bone tissue is a topic of interest for future studies.

It has been widely documented that the longitudinal growth of long bones in the postnatal stage can be affected by multiple factors, including nutritional, pharmacological, and mechanical stimuli [25, 80, 81].

Studies using rats fed diets deficient in protein or rats subjected to a restriction of 20% in the intake of food immediately after the lactation period have shown a marked decrease in the bone mass and alterations in morphometric (e.g., length) and biomechanical variables of the femoral diaphysis [82–84]. In this study, we found no significant differences in the length of the femur between the two groups. These observations are consistent with other studies showing a nutritional deficiency having not effect on femur length. Fong et al. [85], who found that mice fed a diet poor in zinc (2.5 mg/kg) for 9 weeks after weaning did not show alterations in the postnatal longitudinal growth of the tibia and femur.

Previous studies have shown that a nutritional deficiency of B negatively affects the biomechanical properties of bones of the axial and appendicular skeleton [38, 61, 86–88, 92]. In the present study, among the structural biomechanical properties evaluated, only the bone rigidity was affected, since the tibia of the mice fed a B-deficient diet showed lower resistance to the deformation during the period of elastic bone behavior.

Table 6 Size of osteocyte lacunae (μm)

		Larger diameter	Smaller diameter
Size of osteocyte lacunae (μm)	B (+)	9.42 ± 1.70 *	3.94 ± 0.73 *
	B (−)	8.91 ± 1.70	4.08 ± 0.79

(mean ± SD, *$p > 0.05$)

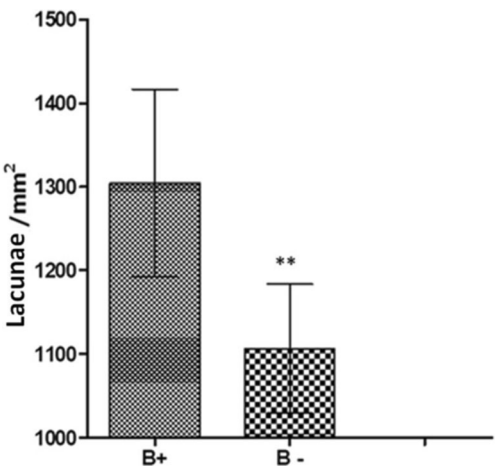

Fig. 3 Total density of osteocyte lacunae (mean of 4 regions of the medial femoral diaphysis) (mean ± SD **$p < 0.05$)

Table 7 Density of osteocyte lacunae (no. of lacunae/mm^2)

		Anterior	Posterior	Medial	Lateral
Density of osteocyte lacunae (no. of lacunae/mm^2)	B (+)	1230 ± 278 *	1285 ± 148 **	1302 ± 130 **	1397 ± 100 *
	B (−)	1097 ± 157	1056 ± 83	978 ± 107	1294 ± 223

(mean ± SD, *$p > 0.05$;**$p < 0.05$)

During evolution, vertebrate skeletons have developed resistance to deformation, and thus indirectly to fracture, as an adaptation to the requirements of their environment and within the physiological limits of the mechanical demands [18, 89]. This development would have been achieved by optimizing the two properties that determine the resistance of any solid structure: (1) the mechanical quality of the material of which it is composed and (2) the spatial distribution of that material [90]. In the case of bones, the material properties correspond to the quantity and quality of collagen and the degree of bone mineralization, while the spatial distribution of this material determines the architectural properties of each bone [89].

The mechanism of action by which the nutritional deficiency of B negatively affects the biomechanical properties of the bone has not yet been elucidated. However, given that it has been demonstrated that this deficiency does not determine alterations in the mineralization of rat bone tissue [61], the deleterious effects observed are likely to be related to changes in the bone collagen matrix associated with an increase in the plasma levels of homocysteine [91], as previously demonstrated by Nielsen [92] in rats fed a B-deficient diet. Therefore, it would be interesting in future studies to characterize the collagen of cortical bone tissue at the molecular level by means of special analytical techniques such as infrared spectroscopy (FTIR) [93].

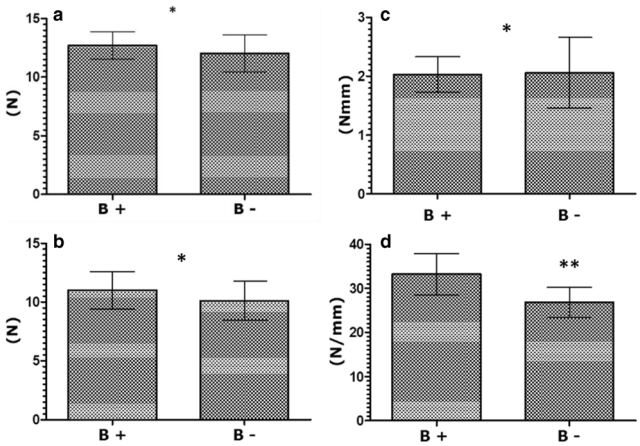

Fig. 4 **a** Bone resistance to fracture; N = force expressed in Newtons; (mean ± SD, *$p > 0.05$). **b** Maximum elastic resistance; N = force expressed in Newtons; (mean ± SD, *$p > 0.05$). **c** Elastic energy absorption; N = force expressed in Newtons; (mean ± SD, *$p > 0.05$). **d** Bone rigidity; N = force expressed in Newtons; (mean ± SD, **$p < 0.05$)

Adequate bone quality in the adult is the consequence of multiple factors that regulate the acquisition of bone mass, both qualitatively and quantitatively, during childhood and adolescence [94, 95]. In this study, B nutritional deficiency was imposed on prepubertal mice, immediately after the lactation period, and maintained during the critical periods of bone mass acquisition during growth. In this sense, our findings would be relevant to clinical medicine, because they highlight the importance of B in infant and adolescent nutrition. In future studies, it will be of interest to evaluate whether the deleterious effects of the nutritional deficiency of B on the cortical bone tissue of long bones of the skeleton of adult mice can be reversed by returning mice to a diet containing a suitable level of B in its composition.

Conclusion

The results of this study show that a nutritional deficiency of B adversely affects the bones of the appendicular skeleton, determining lower bone rigidity and a decrease in the density of osteocyte lacunae in cortical bone tissue.

Acknowledgements The authors wish to acknowledge the technical assistance of Jim Lindlauf (USDA ARS, Grand Forks Human Nutrition Research Center) for the preparation of the animal diets and Lic. Pablo Do Campo (IByME-CONICET) for the confocal laser scanning microscopy.

Funding Information This study was supported by the United States Department of Agriculture, Agriculture Research Service USDA, ARS Extramural Agreement 58-5450-4N-F038.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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