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MANDIBULAR CONDYLAR PROCESS AND TIBIA BONE RESPONSES TO A PROTEIN RESTRICTION DIET. A HISTOMORPHOMETRIC STUDY IN GROWING RATS.

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Summary

This study analyze the effect of protein restriction on bone remodeling in mandibular condylar process and its impact on longitudinal bone growth, and compare this responses to that on the proximal tibia in growing rats. Wistar rats of 21 days were assigned to one of the following groups: control (fed a regular hard diet *ad libitum*) and experimental (fed a hard diet lacking protein *ad libitum*). Animals were euthanized five weeks after. Both bones were obtained and fixed in 10% formalin; the mandibles were hemisected at the symphysis. Remaining soft tissue was removed. Metallic landmarks were placed in the mandibular foramen of each hemimandible. The hemimandibles were radiographed in order to perform cephalometric studies. Tibial and condylar process length was recorded. Sections of the tibial metaphysis and mandibular condyle were obtained and stained with hematoxylin-eosin and toluidine blue. Histomorphometric determinations were performed on histologic sections of tibia and condylar process subchondral bone of both groups: protein restricted and control animals. The length of the tibia and the condylar processes was significantly lower in the experimental group. The histomorphometric analysis showed that the experimental group exhibit a significant decrease in bone formation surfaces associated with an increase in bone surfaces covered with lining cells in the tibia, and concomitant with an increase in bone resorption surfaces, in the condylar process. Tibia and condylar process bone volume was significantly lower in the experimental group. In summary, severe protein undernutrition inhibits osteogenesis in the bone remodeling process in the tibia and mandibular condyle.

Key words: protein restriction diet, bone growth, histomorphometry, endochondral ossification.

RESPUESTAS DEL PROCESO CONDYLAR MANDIBULAR Y DE LA TIBIA A LA RESTRICCIÓN PROTEICA. ESTUDIO HISTOMORFOMÉTRICO EN RATAS EN CRECIMIENTO.

Resumen

Este estudio analiza el efecto de la desnutrición proteica en la remodelación ósea y en el crecimiento longitudinal del cóndilo mandibular, y comparamos esta respuesta con la observada en la metáfisis proximal de la tibia de ratas en crecimiento. Ratas Wistar de 21 días de edad fueron distribuidas en dos grupos denominados control (alimentados con

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dieta dura regular *ad libitum*), y experimental (alimentados con dieta dura restringida en proteínas *ad libitum*). Los animales fueron sacrificados a las cinco semanas del inicio del experimento. Ambos huesos fueron disecados, fijados en formol al 10%, los tejidos blandos fueron eliminados y las mandíbulas fueron divididas en la línea media. Marcas metálicas fueron colocadas en el agujero mandibular y las hemimandibulas fueron radiografiadas para realizar estudios cefalométricos. Se registraron la longitud del proceso condilar y de las tibias. Posteriormente se realizaron cortes longitudinales del proceso condilar y de la metáfisis de la tibia y se colorearon con hematoxilina-eosina y azul de toluidina. Los cortes fueron analizados al microscopio óptico y sometidos a análisis histomorfométrico. La longitud de las tibias y del proceso condilar fueron significativamente menores en el grupo experimental. El análisis histomorfométrico mostró que el grupo experimental presenta una reducción significativa de las superficies de formación ósea asociadas a un incremento de las superficies cubiertas por osteoblastos inactivos en las tibias y de las superficies de reabsorción ósea en el proceso condilar. El volumen óseo resultó significativamente menor en ambos huesos en el grupo experimental. En síntesis, la restricción proteica inhibe la osteogénesis en la tibia y el cóndilo mandibular.

Palabras claves: restricción proteica, crecimiento óseo, histomorfometría, osificación endocondral

Introduction

Protein undernutrition in third world countries is associated to weaning in an extremely poor environment constituting a serious public health problem. It occurs rapidly as a result of protein deficient diets based almost solely on carbohydrates, negatively influences the ponderal curve, and therefore the growth curve of the child.[1,2]

It is well documented that bone formation during growth is related to several factors such as heredity, functional and environmental factors.[3,4] Environmental factors include nutritional deficiencies, which may appear during growth and affect bone development causing marked variations in bone shape and size.[5,6]

Dietary proteins are essential to the biosynthesis of organic matrix of bone tissue and to the different factors involved in endochondral ossification. There is evidence that longitudinal bone growth is regulated by systemic factors, such as growth (GH), thyroid, sexual, and calciotropic hormones (PTH and vitamin D),[6] and others such leptin, which is secreted by adipocytes.[7] During fetal and postnatal growth, there is a close relation between the overall nutritional status and longitudinal bone growth. Protein deprivation decreases GH production and the quantity of GH receptors in the liver, as well as liver production and plasma concentration of IGF-1 in growing rats, since IGF-1 production is associated with amino acid bioavailability,[8,9] thus resulting in impaired longitudinal bone growth. In this regard, there are a number of studies on the effects of undernourishment on development of long bones and mandibular condylar process, focused primarily on the epiphyseal growth plate.

It is well documented that protein deprivation affects the expression of the insulin like growth factor receptor, total height, as well as the number of chondrocytes per column in the proliferative and hypertrophic zones of the growth cartilage and the size of hypertrophic chondrocytes.[5,10-12]

In addition to causing morphological alterations in the epiphyseal cartilage in the rat, protein deprivation after weaning leads to low body weight,[5,10,11] lack of mandibular and craneofacial development,[13,14] and decreased long bone length.[14] This suggests that protein malnutrition causes alterations not only in the epiphyseal growth plate but also in subchondral bone remodeling.

Whether mandibular condylar process and axial or peripheral skeleton respond similarly to systemic bone loss remains a subject of controversy. There are reports in the literature of the effects of protein undernutrition on condylar fractures[15] and on the expression of insulin like growth factor receptor in the condylar cartilage.[12]

In the other hand it is well documented that there is an association between protein deprivation and osteoporosis, systemic bone mineral density (BMD) and alveolar bone BMD, this association seems to be inferior in alveolar bone, compared with that observed in other skeletal sites.[1]

In this context, it is necessary to pointed out that mandibular condylar process like alveolar bone are subjected to intermittent, abrupt and heavy forces during mastication.

However, to the best of our knowledge, there are no studies of the effect of protein undernutrition on the subchondral bone remodeling and on growth of the mandibular condylar process compared with tibia, nor about its implications in facial development.

Based on the above, the aim of the present experimental work was to assess, using radiographics and histomorphometric methods the influence of chronic protein restriction diet in longitudinal bone growth of mandibular condylar process, and bone activity in the subchondral region of the mandibular condylar process, and compare this response to that on the proximal metaphysis of tibia in growing Wistar rats. In addition, the effects of longitudinal condylar growth is analyzed in relation to facial development

Materials and methods

Twenty Wistar rats were used. The animals were weaned at the age of 21 days and assigned to one of the following groups: control group (n=10) fed ad libitum a biosynthetic regular hard diet, and protein restricted (PR) group (n=10) fed ad libitum a biosynthetic hard diet with lower protein content (7%) (Table 1).

Table 1. Centesimal chemical composition per substance expressed as a percentage (%) of chow fed to control and protein-restricted animals in the present study.

	Controls	Protein-restricted
Proteins (casein)	21	7
Carbohydrates (corn starch)	60	74
Fat (corn oil)	13	13
Vitamin mixture (ICN, Biomedicals, Inc., Ohio, USA)	2.5	2.5
Salt mixture (ICN, Biomedicals, Inc., Ohio, USA)	3.5	3.5

Body weight and food consumed by all the animals were recorded throughout the experiment.

Five weeks after (rats aged 8 weeks) the animals were euthanized, the mandibles and tibia were carefully dissected and fixed in 10% formalin, after which the soft tissue was removed.

The National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NHI publication 85-23 Rev. 1985) were followed. In order to perform the radiographic study, the mandibles were hemisected at the symphysis.

Metallic landmarks consisting in L shaped 0.2 mm steel ligature wires were placed in

the mandibular foramens of one hemimandible of each rat (Figure 1).[16] The marked hemimandibles were positioned laterally on the film alongside a 10 mm

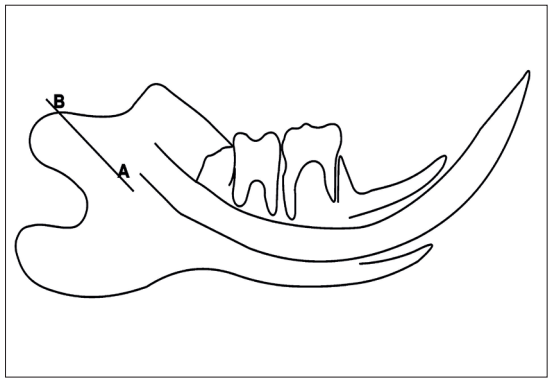

Figure 1. Reference points defined on the lateral roentgenograph of the mandible. A. Position of markers in the mandibular foramen. B. Fovea pterigoidea.

The marked hemimandibles were positioned laterally on the film alongside a 10 mm long wire and radiographed using standard X-ray equipment at 70 kV and 8 mA, 0.3 second exposure time; the focus to film distance was 40 cm. Reference points were marked on paper tracings of the projected image of the radiographs (x7 magnification) in order to determine the length of the condylar process (Figure 1). Following, the tibia and hemimandibles were decalcified in 10% EDTA, pH=7.2 and processed following routine procedures for embedding in paraffin. Longitudinal sections were obtained at the level of the mandibular condyle and proximal metaphysis of the tibia. Sections were stained with hematoxylin-eosin and toluidine blue for histomorphometric determinations (Figures 2 and 3).

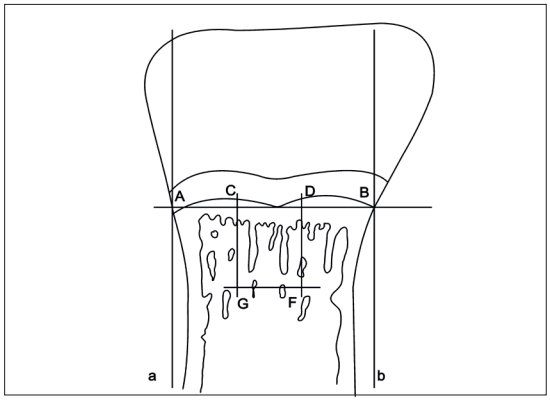

Figure 2. Diagram of a longitudinal tibia section. The studied area was determined as follows: lines **a** and **b** were drawn parallel to the longest axis of the bone, and tangential to the intersection of the growth cartilage with the cortical plates. Line **c** was drawn tangential to the lower of the growth cartilage and perpendicular to lines **a** and **b**; points **A** and **B** were thus determined at the intersection of line **c** with lines **a** and **b** respectively. Segment **AB** was divided into three equal segments: **AC**, **CD**, and **DB**. Segment **CG** was drawn parallel to lines **a** and **b**, through point **C**, and segment **DF** was drawn similarly, through point **D**; both segments were 7 cm long. Segment **GF** was drawn through point **G**, parallel to **AB**. The histomorphometric study was performed on area **CDFG.**

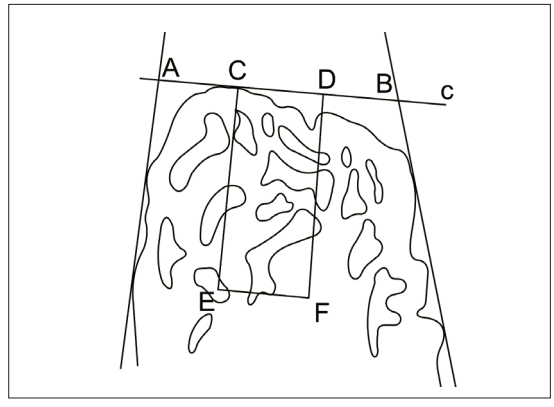

Figure 3. Diagram of a sagittal section of the mandibular condylar process. The studied area was determined as follows: lines **a** and **b** were drawn tangential to the upper anterior and lower posterior borders of the mandibular condylar process. Line **c** was drawn tangential to the upper border and perpendicular to the longitudinal axis of the mandibular condilar process, thus determining points **A** and **B** at its intersection with lines **a** and **b** respectively. Segment **AB** was divided into three equal parts: **AC**, **CD**, and **DB**. Segment **CE** was drawn through point **C**, parallel to line **a**, and segment **DF** was drawn parallel to segment **CE** through point **D**; both segments were 7 cm long. Segment **EF** was drawn through point **E**, parallel to AB. The histomorphometric study was performed on the area **CDFE**.

Tibia length was measured with a precision caliper, using the intercondyloid eminence and the medial malleolus as a reference points.

The length of the condylar process was determined on paper tracings of the radiographs of the hemimandibles (Figure 1), according to a method described elsewhere.[16]

Histomorphometric determinations

The following parameters were measured in mandibular condyle subchondral bone and tibia of control and protein restricted animals following standard stereological methods.[17]

1. Trabecular surface covered by osteoblasts (Obl S/BS%): indicating active bone formation.
2. Trabecular surfaces with resorption areas (with or without osteoclasts) (RS/BS%): indicating bone resorption.
3. Trabecular surface covered by lining cells (Flat Obl S/BS%): indicating bone at rest.
4. Trabecular bone volume to total volume of the measured area (BV/TV%).

Experimental data were expressed as mean±SD.

Single comparisons between groups were assessed by Student's *t* test.

Differences were considered significant if $p<0.05$.

Results

Body Weight

A slight increase in body weight was observed in both groups throughout the first ten days of the study, after which no further increase was detected in the PR group.

Body weight was significantly lower in PR rats as compared to controls at the end of the experimental period (Figure 4).

There was no significant difference in the amount of food consumed between the control and PR groups (Figure 5).

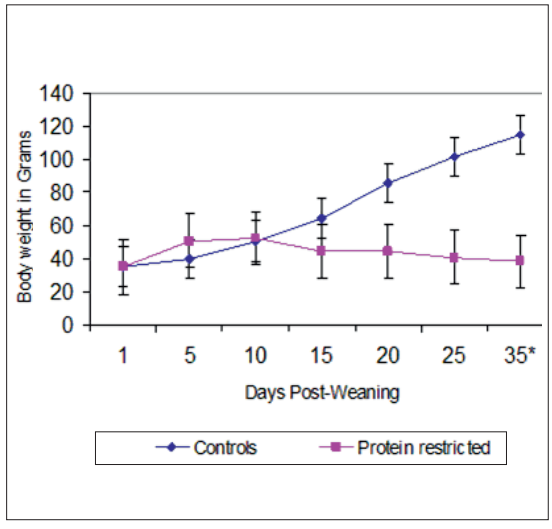

Figure 4. Body weight of control and protein-restricted groups. *p<0.05. Students t test.

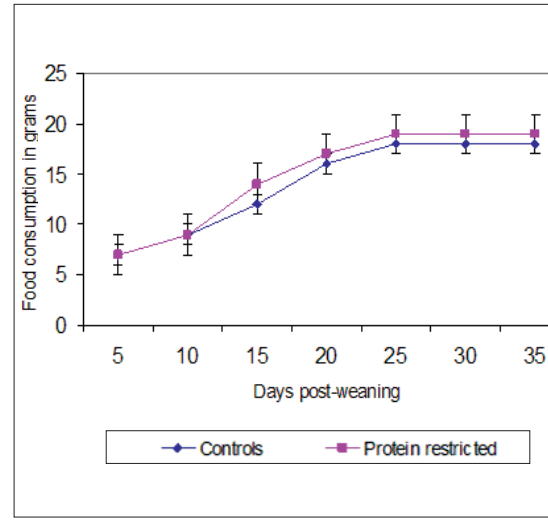

Figure 5. Food consumption in controls and protein restricted groups.

Histomorphologic analysis

The tibia and condylar process histologic sections of control animals exhibited normal bone trabeculae as regards number and thickness. The primary trabeculae had a cartilage core and mostly cuboidal osteoblasts on their surface (Figures 6 and 7).

Sections corresponding to protein restricted group showed alterations in the subchondral bone of the proximal metaphysis of the tibia and of the condylar process, exhibiting fewer, shorter, and thinner bone trabeculae compared to those observed in control sections. In addition, none of the sections of the protein restricted group presented bone trabeculae with a stained cartilage core, nor osteoid on their surface. The few osteoblasts that were encountered failed to exhibit the typical cuboidal shape, and were irregular.

Angiogenesis was clearly evident in the condylar process; a large number of trabeculae had no lining cells on the surface. The number of osteoclasts was markedly higher, but no differences in osteoclast morphology were observed between the control and protein restricted groups (Figures 8 and 9).

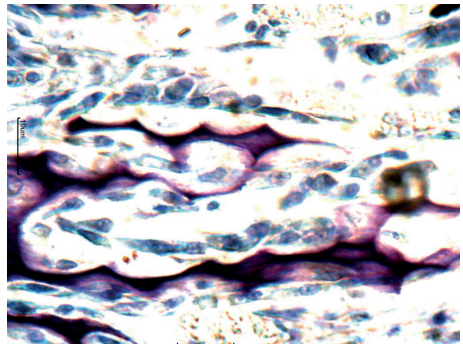

Figure 6. Light micrographs of trabecular tibial bone in control animals, showing cuboidal osteoblasts with rich cytoplasm on the osteoid layer along the bone surface . The trabeculae shows a methachromatic cartilague core. Toluidine blue 40x.

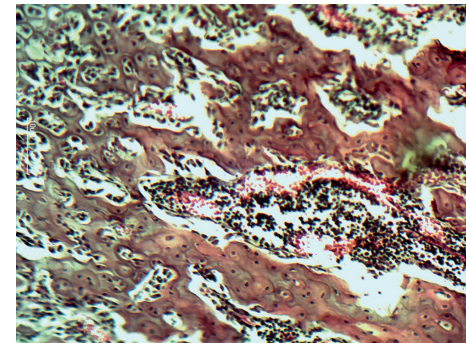

Figure 7. Light micrographs of the subchondral bone in condylar process in control group showing normal bone trabeculae in terms of number and thickness. Cuboidal osteoblasts can be observed along the trabecular bone surface. Hematoxylin and eosin 10x.

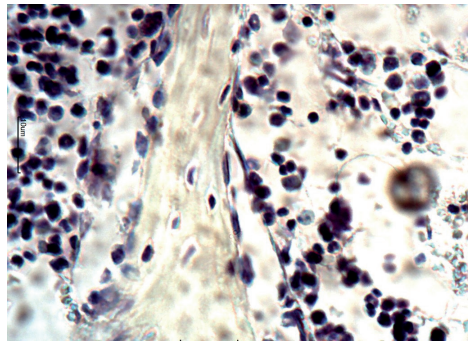

Figure 8. Light micrographs of trabecular tibial bone in protein-restricted animals showing thin trabeculae without osteoid and osteoblastic cells. Note the absence metachromasia in the center of the bone trabeculae. Toluidine blue 40x.

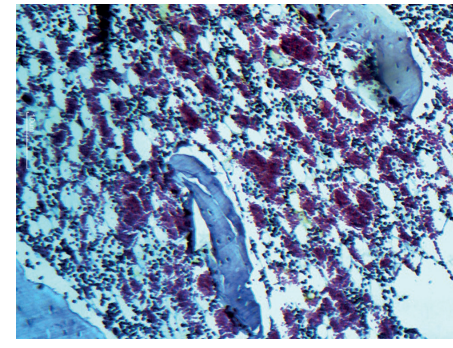

Figure 9. Condylar process in the protein-restricted group showing thin and disorganized trabeculae. Osteoid and typical osteoblastics cells cannot be observed. Angiogenesis was clearly evident. Hematoxylin and eosin 10x.

Tibia and mandibular dimensions

The length of the tibia was significantly lower in PR group (Control: 28±1 mm vs. PR: 25±0.7 mm; $p<0.05$).

The condylar process length in tracings was significantly shorter in PR animals (Control: 21±2 mm vs. PR: 17±3 mm; $p<0.05$).

Histomorphometric analysis

The histomorphometric analysis of bone activity in the proximal metaphysis of the tibia showed that the protein restricted group exhibited a statistically significant decrease in bone formation surfaces, associated with an increase in bone areas covered with lining cells. Bone volume was significantly lower in the protein restricted group.

A statistically significant decrease in bone formation surfaces covered with active osteoblasts associated with an increase in bone resorption surfaces was observed in the condylar process of protein restricted animals.

Bone volume was significantly lower in this group.

The results of the morphometric study are summarized in Tables 2 and 3.

Table 2. Histomorphometric determinations in the tibia accodring differents diets (Mean ± SD).

	Controls	Protein restricted	Student's *t* test
Obl S/BS%	68±6	18±14	p<0.05
Flat Obl S/BS%	20±7	59±25	p<0.05
RS/BS%	12±6	24±15	ns
BV/TV%	60±10	30±10	p<0.05

Obl S/BS%: Trabecular surface covered by osteoblasts, indicating active bone formation. Flat Obl S/BS%: Trabecular surface covered by lining cells, indicating bone at rest. RS/BS%: Trabecular surfaces with resorption areas (with or without osteoclasts) Indicating bone resorption. BV/TV% : Trabecular bone volume to total bone volume of the area measured ratio.

Table 3. Histomorphometric determinations of the condylar process accodring differents diets (Mean ± SD).

	Controls	Protein restricted	Student's *t* test
Obl S/BS%	55±19	18±9	p<0.05
Flat Obl S/BS %.	15±7	12±5	NS
RS/BS %.	24±7	70±9	p<0.05
BV/TV%	70±6	20±3	p<0.05

Obl S/BS%: Trabecular surface covered by osteoblasts, indicating active bone formation. Flat Obl S/BS%: Trabecular surface covered by lining cells, indicating bone at rest. RS/BS%: Trabecular surfaces with resorption areas (with or without osteoclasts) Indicating bone resorption. BV/TV%: Trabecular bone volume to total bone volume of the area measured ratio.

Discussion

The results obtained in the present work show that dietary protein deficiency following weaning causes alterations in subchondral bone remodeling in the tibia and mandibular condylar process; these alterations are characterized by a decrease in bone surfaces covered by active osteoblasts, indicating a severe decrease in bone formation.

Protein undernutrition induced by feeding weanling rats a diet lacking in proteins results in bone growth arrest and alterations in the size and biomechanical properties of the mandible and long bones.[13,14,18] A number of histomorphometric studies of the epiphyseal cartilage have demonstrated height and cell density of the proliferative and hypertrophic zones to be decreased.[6,10,11] Nevertheless, how and to what extent protein malnutrition affects subchondral bone remodeling in long bones and the mandibular condyle remains to be clarified. In the present study the percentage of protein in the diet of the experimental group, as well as weight loss achieved in PR group are consistent with those reported by other authors.[1,12,13,15,18]

Our results showed that dietary protein deficiency clearly alters the bone remodeling process. According to the histomorphometric study of subchondral bone activity in the tibia, these alterations feature a statistically significant decrease in trabecular surfaces covered with active osteoblasts, associated with an increase in bone surfaces covered with lining cells. However, no significant differences in trabecular surfaces with resorption areas were observed between groups. These results may indicate that dietary protein deficiency would cause that the osteoblast precursor cells failure to differentiate to active osteoblasts. This alteration in cell differentiation would have a negative impact on osteogenetic activity, and its most immediate, obvious consequence is the significant decrease in BV/TV. However, it also has a direct effect on bone growth, as evidenced by the significant difference in tibia length of control and experimental animals.

Longitudinal growth of long bones is mainly due to endochondral ossification of the epiphyseal growth plate. Firstly, it is essential that chondrocytes proliferate in this cartilage as a result of mitotic activity in the proliferative zone. Secondly, they must differentiate by changing in shape and volume in the hypertrophic zone, and finally terminal chondrocyte maturation and vascular invasion must take place.[11] Hypertrophic chondrocytes synthetize vascular-endothelial growth factor (VEGF), which promotes vascular invasion prior to ossification,[19] and core binding factor a 1 (Cbfa 1), a transcription factor that is essential for cells differentiation from osteoprogenitor cells to osteoblasts.[20] It is well documented that protein undernutrition severely affects gene expression, protein synthesis, and cell differentiation.[21] It is therefore likely that in the present study, protein restriction affected chondrocyte proliferation and differentiation at the end of the cell cycle, and during the transition to hypertrophic chondrocytes.[6] Given that hypertrophic chondrocytes express high levels of RNAm for VEGF[19], alterations in their differentiation would imply alterations in vascular invasion in the active ossification zone. Undoubtedly, this situation would alter the microenvironment that osteoblast precursor cells require to differentiate and begin to synthetize organic matrix. Hypertrophic chondrocytes also synthetize Cbfa1.[19,20] It has been demonstrated that a lack of this factor leads to a total lack of bone formation in mice, since osteoblast maturation is blocked.[22] It is highly probable that synthesis and secretion of these factors was inhibited or altered in the present study, contributing to failure of osteoblast precursor cells to complete differentiation to active osteoblasts.

The condyle is the main growth center of the lower jaw. The present study showed that the decrease in bone formation surfaces in subchondral bone of the condyle in the

protein restricted group was associated with an increase in erosive surfaces, whereas acquiescent surfaces were not significantly different compared to those of the control group. Our results indicate that protein undernutrition affects bone remodeling in the condyle and in the tibia through different mechanisms. However, its impact on bone growth is similar, as shown by the finding that the condylar process was significantly smaller in the protein-restricted group. The increase in erosive areas in bone tissue under conditions of protein undernutrition may be explained by the fact that osteoblasts play a mayor role in their onset.[23] Thus, the increase observed in bone resorption surfaces may be due to damage to the osteoblasts, which in turn may release soluble mediators of bone resorption,[24] or lose contact with the bone surface as was observed in the present study, resulting in the presence of erosive surfaces.[25] Although a quantitative analysis of the osteoclast population was not performed, the presence of functional, well-differentiated osteoclasts in the mandibular condyle under conditions of severe protein undernutrition was evident. It could therefore be inferred that osteoclastogenesis regulation in protein-deprived animals may be affected by other factors, besides osteoblast-osteoclast interaction mediated by RANK-RANKL (receptor activator of nuclear factor-Kappa B-RANK ligand) [26] and TGF□$_1$ (transforming growth factor β1) associated to an increase in RANK RNAm and protein.[27] In addition, hypocalcemia, which is typically associated with protein undernutrition and is caused by a decrease in calcium-transporting blood proteins, may induce an increase in osteoclast number and bone resorption surfaces, probably via parathormone[28]. However, further studies are needed to elucidate the underlying mechanisms of the effects of protein undernutrition on physiological bone remodeling. According to the results obtained in this study, isocaloric protein restriction not only negatively affects the growth curve of the child, but also it could be stated that a lack of development of the condylar process in malnourished children may be found. In this context these changes could have an immediate impact on facial development. During normal facial growth the mandibular symphysis moves down and forward with respect to the other facial structures along the Rickett's facial axis.[29,30] This movement is the result of vertical and sagittal growth vectors. However, the vertical growth vector has a crucial effect on the sagittal (anteroposterior) growth direction of the mandibular symphysis. This growth pattern results from the descent of the glenoid fossae and, essentially, from the increase in condyle length[30]. The development of both components compensates simultaneously the vertical growth of the upper jaw and the upper alveolar process as well as of the lower alveolar process.[30,31] In this context the lack of development of the condylar process in undernourished subjects, may alter the normal pattern of facial growth. This alteration could be characterized by the loss of vertical control and the loss of the spatial position of the mandible with respect to the base of the skull, a growth pattern characterized by a downward and backward displacement of the mandibular symphysis and a posterior rotation of the mandible with an increase in the angle to the base of the skull and a downward inclination of the occlusal plane, resulting in a dolichofacial growth pattern. Under these conditions in which there is a marked occlusal curve it would be plausible to posit that the lower incisors would drift upward and forward to compensate the posterior rotation of the mandible. In addition, it is likely that the lack of condylar growth would result in premature occlusal contact, thus an anterior open bite could be a frequent finding in this type of nutrition deficiency.[18] Severe protein undernutrition inhibits osteogenesis in the bone remodeling process in the tibia and mandibular condyle having a negative impact on stature, as well as on complex processes involved in facial development.

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Conflicts of interest: The authors report no conflict of interest.

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