BONE MINERAL DENSITY AND BONE STRENGTH FROM THE MANDIBLE OF CHRONICALLY PROTEIN RESTRICTED RATS

Carlos E. Bozzini, Graciela Champin, Rosa M. Alippi and Clarisa Bozzini

Department of Physiology, Faculty of Dentistry,
University of Buenos Aires Buenos Aires, Argentina

ABSTRACT

The present investigation was performed to assess the biomechanical repercussion of protein malnutrition imposed on rats between the 26th and 135th days of postnatal life on the mandible, which is not a weight-bearing bone but supports the loads related to the masticatory activity. Female Wistar rats aged 26 d (n= 14) were placed on either a 4%-protein diet (ICN 960254, P4 group) or a 20%-protein diet (ICN 960260, P20 group) and killed 111 d later. Both body weight and length were recorded regularly. The mandibles were dissected and cleaned of adhering soft tissue. Mandibular growth was estimated directly by taking measurements between anatomical points. Areal Bone Mineral Density (BMD) was estimated using a bone densitometer (LUNAR DPX-L). Mechanical properties of the right hemimandible were determined using a three-point bending mechanical test to obtain the load/deformation curve and estimate the structural properties of the bone. Results were summarized as means ± SD. Comparisons between parameters were performed by Student's t test. A 75% reduction in body weight and a 32% reduction in body length were observed in P4 rats. Like body size, mandibular weight, length, height and area (index of mandibular size) were negatively affected by P4 diet, as was the posterior part of the bone (posterior to molar III). The anterior part (alveolar and incisor alveolar process) was not affected by age or diet. The "load capacity" extrinsic properties of the mandible (load fracture, stiffness, yielding point) were between 43% and 64% of control value in protein restricted rats. BMD was similar in both groups of animals.

Conclusion: 1) Chronic protein malnutrition imposed on rats from infancy to early adulthood reduces the growth of the posterior part of the mandible without inducing changes in the anterior part, which produces some deformation of the bone in relation to age-matched rats; and 2) the significant reduction of strength and stiffness of the mandible seem to be the result of an induced loss of gain in bone structural properties as a consequence of a correlative loss of gain in both growth and mass, yet not in bone material properties.

Key words: biomechanics, mandible, protein deficiency- growth.

DENSIDAD MINERAL ÓSEA Y COMPORTAMIENTO BIOMECÁNICO DE LA MANDÍBULA DE RATA SOMETIDA A RESTRICCIÓN PROTEICA CRÓNICA

La investigación presente fue diseñada con el objeto de evaluar la repercusión biomecánica de la malnutrición proteica impuesta a ratas entre los días 26º y 135º de edad sobre la mandíbula (M), hueso que no soporta carga relacionada con el peso corporal sino con las fuerzas masticatorias. Ratas Wistar hembras de 26 d de edad (n= 14) fueron alimentadas con dietas conteniendo 4% (grupo P4) (ICN 960254) o 20% (grupo P20) (ICN 960260) de caseína y sacrificadas 111 d después. Peso y longitud corporales fueron registrados regularmente. Las mandíbulas fueron disecadas y liberadas de tejido blando. Se realizaron mediciones entre diversos puntos anatómicos para estimar la morfometría del hueso. La Densidad Mineral Osea (DMO) fue determinada en un densitómetro LUNAR DPX-L. La M derecha de cada animal fue sometida al test de flexión a 3 puntos para obtener la curva carga/deformación y estimar las propiedades estructurales del hueso mandibular. Los resultados (X±DS) fueron analizados estadísticamente mediante test t de Student. El peso y la longitud corporales fueron menores en el grupo P4 que en el P20 (-75% y -32%, respectivamente). Longitud de la base, altura y área mandibular (índice del tamaño de M) fueron afectados negativamente por la dieta P4, lo mismo que la porción posterior de M (posterior al molar III). La porción anterior (procesos alveolar e incisivo) no fueron afectadas por dieta o edad. Todas las propiedades biomecánicas de M (carga de fractura, resistencia en fase elástica, límite elástico) fueron 43-64% menores en grupo P4 que en grupo P20. El valor de DMO fue similar en ambos grupos. CONCLUSION: 1) La malnutrición proteica crónica impuesta a ratas desde la infancia hasta la adultez reduce el crecimiento de la porción posterior de la mandíbula sin inducir cambios en su porción anterior, lo que produce una cierta deformación del hueso en comparación con animales de la misma edad; y 2) la importante disminución de la resistencia a fractura y de la rigidez durante el período elástico sería el resultado de una reducción de ganancia de las propiedades estructurales óseas como consecuencia de una reducción correlativa de ganancia de masa ósea, con mantenimiento de la normalidad de las propiedades óseas intrínsecas.

Palabras clave: biomecánica ósea, deficiencia proteica crecimiento, mandíbula.

INTRODUCTION

Several factors have been recognized to play an important physiological role in skeletal development, linear growth and maintenance of body mass. Both concentration and quality of protein in the diet are included among them. It is well known that protein malnutrition affects growth, development, and both collagen and mineral content of long bones in rats[1-9]. In 1988[10], we reported that severe protein restriction in weaning rats produced a considerable reduction in the bending strength and stiffness of femoral shafts, with severe impairment in the amount and/or architectural arrangement of bony material. These findings have been partially confirmed later by other investigators[11-13]. The above mentioned bone biomechanical properties recovered almost completely after protein refeeding[14].

Most of these studies have been conducted on bones of the axial or appendicular skeleton, which shows biomechanical properties associated with their condition of "weight bearing bones". The mandible is both morphologically and functionally different from the other bones of the axial skeleton. It also arises from a different embryonic germ layer (neuroectoderm) instead of bones of the axial and appendicular skeleton, which arise from the mesoderm. It has been shown that the mechanical loading of the mandible during mastication has an impact on the mass, density, and microarchitecture of the mandibular alveolar bone[13].

Protein is critical for mandibular growth. The effect of protein deficiency on the development of the rat mandible has been investigated by considering the bone as a whole[15] or as comprising a number of skeletal units that possess degrees of functional autonomy[16]. As mandibular units were not uniformly affected by protein deficiency, alterations in the proportions of the mandible with some deformation of bone occurred in protein-restricted rats. Bozzini et al.[17] demonstrated that a 20% dietary concentration of a protein with a high biological value (casein) is required for normal, undeformed mandibular growth.

During evolution, the skeleton of vertebrates developed an important property, *resistance to deformation*, and indirectly to fracture, which was adapted to the physiological mechanical demands of the environment. To do so, the maintenance of sufficient quantity and quality of bone is necessary throughout life to withstand ordinary stress (body weight, masticatory loading) to which skeletal components are subjected.

The mechanical properties of bones as organs (known as *structural properties*) are the *strength* (assessable as the bone's ability to support loads) and the *stiffness* (measurable as the load/deformation curve). They are determined by the so-called *material and geometric* properties. Bone material properties are unaffected by bone size or shape. They are usually evaluated by assessing two important properties, namely, the *stiffness of the mineralized tissue* (Young's modulus of elasticity), and its breaking load at failure per unit of cross-sectional area. These properties are determined by matrix mineralization as well as by other, mineralization-unrelated, microstructural factors, such as crystal size and packing and the disposition of collagen fibers[18].

We have previously shown[19] that anatomical dimensions, bone calcium, and bone strength of the female rat mandible increased linearly from day 21 to approximately day 90 of chronological age. After day 90, the rate of growth of all measurements showed a marked decline or deceleration. No statistically significant difference was found between day 90 and day 120 values. It was thus concluded that the female rat mandible attains it adult size, peak bone calcium mass, and bone structural mechanical properties at some point between 90 and 120 days of postnatal life. Bearing in mind these findings and in order to establish the effects of prolonged protein malnutrition on bone mass and bone strength in the rat mandible, the present investigation was performed on female rats that were submitted to a diet with low protein content but isocaloric to the control diet between the 26th and the 135th days of age. Thus, the bone effects of early protein undernutrition could be assessed in adulthood.

MATERIALS AND METHODS

Two groups of 7 female Wistar rats aged 26 d and weighing about 52 g at the start of the experiment were housed in stainless-steel cages under natural light-dark photoperiod in a temperature controlled (23º) room. The animals were placed on either a 4%-protein diet (ICN, cat. 960254, P4 diet) or a 20%-protein diet (ICN, cat. 960260, P20 diet). The diets were isocaloric.

The nutritional protocol was extended until rats were 135 days old; the experimental period thus lasted 109 d. At its end, final body weight and length were established. Body length was taken as the distance between nose and rump. Rats were

euthanized by ether overdose. The mandibles were dissected, cleaned of adhering soft tissue, and split at the midline suture. Hemimandibles were then weighed in a Mettler scale and stored at -20ºC wrapped in gauze soaked with Ringer's solution in sealed plastic bags, in accordance with Turner and Burr[20].

Each bone was thawed at room temperature before analysis. Mandibular growth was estimated directly on the right hemimandible by taking measurements (to the nearest 0.05 mm) by the use of digital calipers according to Eratalay et al.[21] with some modifications[22].

Dimensions measured were as follows (Fig. 1):

a) *mandibular area* was calculated from a triangle formed between three points: the most anterior inferior bone point of the interdental spine (O), the most posterior point of the angular process (C), and the most superior point of the coronoid process (B); b) the *length of the base* of the jaw was estimated by the distance O-C; c) the *length of the mandible* was estimated by the distance A-O; and d) the *mandibular height* corresponded to the distance C-B. These specific measurements were chosen because they give information on the growth of the bone as a whole without considering its morphological units[23]. The *alveolar length* was the distance between two points on the alveolar process immediately anterior to the anterior surface of the first molar (K) and immediately posterior to the posterior surface of the third molar (L). The *interdental length* (incisor alveolar process) was the distance from the most anterior superior bone point of the interdental spine (L) to K. The mandibular length was divided into *anterior* and *posterior* parts by a vertical line drawn immediately posterior to the posterior surface of the third molar.

Areal bone mineral density (BMD) of the left hemimandible was determined using a bone densitometer (LUNAR DPX-L) and specific software for small animals designed by LUNAR General Electric Medical Systems (Madison, WI, USA). The DPX-L uses a constant potential X-ray source combined with the K-absorption edge with effective energies between 38 and 70KeV. All measurements were carried out with a fine-diameter collimator on the X-ray output. Results are expressed as g/cm^2. Precision, expresses as a CV, was 0.72 ± 0.34 (SD). It shoud be noted that DEXA-BMD is not a volumetric density. It represents the whole mass of mineral present in the bone region studied (regardless of the bone structure in that region) expressed per unit of projected bone area. The BMD can be considered as an indicator of the degree of concentration of mineral within the whole bone[24]. It does not give information concerning bone material quality or distribution.

Mechanical properties of the rat hemimandible were determined using a three-point bending mechanical test[25]. Before testing, each bone was thawed to room temperature and then placed on two lower supports (13 mm span) with the lateral aspect facing down and centered along its length. Load was applied transversally to the bone axis at a point immediately posterior to the posterior surface of the third molar. The test

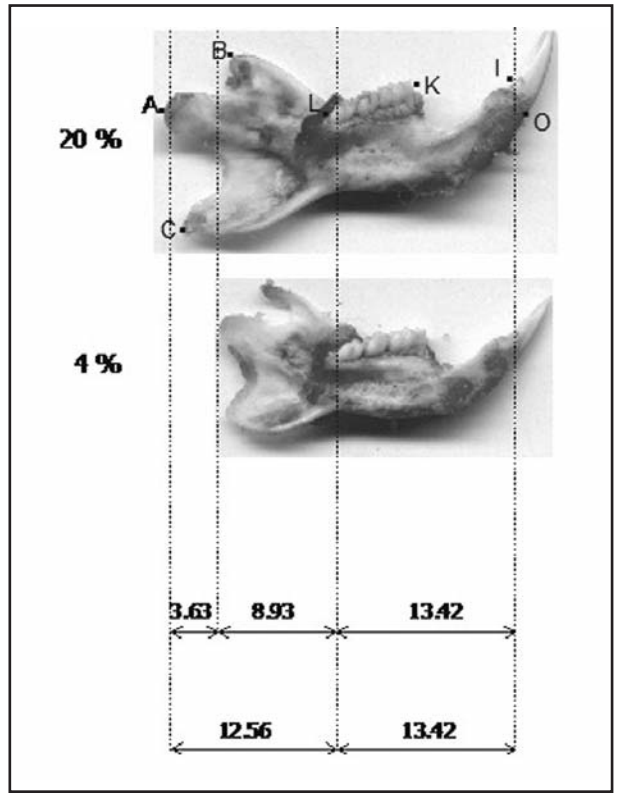

Fig. 1: Medial aspect of the left hemimandible obtained from rats fed diets containing either 20% or 4% casein. Bones were divided into anterior and posterior parts by a vertical line drawn immediately posterior to the posterior surface of the third molar. Dimensions are expressed in mm. Letters indicate the bony points between which measurements were taken. A: the most posterior point of the condyloid process; B: the most superior point of the coronoid process; C: the most posterior point of the angular process; I: the most anterior superior bone point of the interdental spine; K: bone point on the alveolar process immediately anterior to the anterior surface of the first molar; L: bone point immediately posterior to the posterior surface of the third molar; O: the most anterior inferior bone point of the interdental spine.

machine (Instron model 4442, Instron Corp., Canton, MA, USA) was operated in stroke control at a rate of 5.00 mm/min. The following structural mechanical properties that refer to the whole bone and thus reflect the combined effects of bone size and shape in addition to tissue material properties (20) were determined from the test:

a) Stiffness (Wy/dy, N/mm) – The slope of the force-displacement curve in the linear region was calculated from the best fit linear regression.

b) Maximal elastic strength (load at the yielding point) (Wy, N) – This was the value of the force at the upper extent of the linear region.

c) Ultimate strength (load at fracture) (Wf, N) – This was the maximum value of the force recorded during the test and represent the load at which the bone actually breaks.

d) Elastic energy absorption (EEA, N/mm) – The total energy absorbed by the specimen up to the yielding point was calculated as the area under the force-displacement curve.

Results were summarized as means ± SD and are considered statistically significant at the level of $P < 0.05$. Comparisons between parameters were performed by Student's *t* test. It was performed by using GraphPad Prism Software (GraphPad Software Inc., San Diego, CA, USA).

The experiment was conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Management of Laboratory Animals, and approved by the University of Buenos Aires Ethic Committee.

RESULTS

As expected, P4 rats failed to obtain normal weight gain compared with P20, age matched rats (Table 1). Body weight increased 1.49 times and 7.72 times during the entire experimental period in P4 and P20 rats, respectively ($P < 0.001$). The 75% reduction in final body weight found in P4 animals was accompanied by a significant, 32% reduction in body length. Like body size, mandibular length, height and area (an index of mandibular size) were significantly lower in P4 than in P20 rats at the end of the experimental period (Table 1). Both alveolar and incisor alveolar process lengths were unaffected by

Table 1: General and mandibular anthropometry and mandible structural mechanical properties in rats fed diets containing either 20% or 4% casein

	C-20	C-4	P
General Anthropometry			
Body weight (g)	350.50 ± 31.02	87.49 ± 19.36	< 0.001
Body length (cm)	24.40 ± 0.88	16.60 ± 0.75	< 0.001
Mandible Anthropometry			
Mandible weight (mg)	512.4 ± 80.2	268.1 ± 20.3	<0.001
Mandible length (mm)	26.74 ± 1.01	22.77 ± 0.63	< 0.001
Mandible height (mm)	12.48 ± 0.56	10.41 ± 0.36	< 0.001
Mandible area (mm^2)	137.58 ± 8.61	94.34 ± 10.25	< 0.001
Posterior part (mm)	12.81 ± 0.96	9.13 ± 0.70	< 0.001
Anterior part (mm)	13.93 ± 0.39	13.64 ± 0.21	> 0.05
Bone Structural Properties			
Elastic limit (N)	52.26 ± 12.7	25.65 ± 2.70	< 0.001
Ultimate load (Wf) (N)	62.50 ± 14.6	32.01 ± 3.30	< 0.001
Stiffness (N/mm)	188.31 ± 41.69	106.95 ± 46.36	< 0.005
EAC (N/mm)	9.50 ± 1.90	3.46 ± 0.68	< 0.05
Standard densitometry			
BMD (mg/cm^2)	156.16 ± 33.65	135.77 ± 28.43	> 0.05

All data are expressed as means ± SD.

treatment. When the length of the bone was divided into an anterior and posterior part by a vertical line drawn immediately posterior to the posterior surface of the third molar, it was remarkable that the growth of the posterior part, but not of the anterior part, was reduced by treatment (Fig. 1).

The "load capacity" extrinsic properties of the mandible of control and protein restricted rats are also shown in Table 1. All of them were reduced by between 43 and 64 %. Areal bone mineral density was similar in both groups of rats.

DISCUSSION

Infant and young animals can be seen as evolving metabolic systems as they go though a series of critical periods during the process of growth and maturation[26]. This process, which is governed by major determinants, can be influenced by several factors. Among them, the effect of dietary protein restriction on both the dimensions and structural biomechanical properties of the rat mandible is relevant to the present discussion.

Protein restriction may be imposed at any phase of the growth of the organism, i.e. suckling, weaning or later growth periods. Specific effects in each period may or may not be similar and/or reversible. The results of this study provide details of how protein undernutrition affects the mechanical properties of the mandible in young rats, as derived from determinations performed in early adulthood. Healthy bones at this stage of life are dependent on the development during the younger years of a healthy structure and an adequate bone mass.

The present study began with very young animals and the effects of treatment on mandible morphometrics and bone biomechanics were assessed in adulthood. We have previously shown[19] that the rat mandible attains its adult size, bone calcium mass and bone biomechanical competence at some point between 90 and 120 d of postnatal life. The observation period in the present study was extended to day 135 of life.

As expected, the condition caused marked growth retardation in treated rats, as derived by changes in body weight and body length. Growth retardation associated with protein undernutrition has been previously reported[3-9]. Both the final mandibular weight and the mandible general anthropometry were undoubtedly affected by growth retardation. The rat mandible can be arbitrarily partitioned into an *anterior* and *posterior* part by a vertical line drawn immediately posterior to the posterior surface of the third molar[19]. The former comprises the alveolar and the symphyseal regions, while the condyloid, the coronoid and the angular process compose the latter. In the weaning rat, the length of the *posterior* part of the mandible is about one half that of the *anterior* part. From this time on, the relative increase of the *posterior* part of the bone is more than two times higher than that of the *anterior* part, because the condyle, the growth cartilage of the mandible, is situated posteriorly. The difference in the rates of growth between the *anterior* and *posterior* parts of the bone is responsible for the observation that both portions show almost equal lengths at adulthood[19]. In the present study, rats started their protein restricted regimen when the growth of the *anterior* part of the mandible was almost finished. Therefore, no significant difference was encountered between P4 and P20 rats in relation to the length of the *anterior* part of the bone at the end of the studied period. In relation to the *posterior* part, protein undernutrition produced a depression of growth, as evidenced by the lower value found in P4 than in P20 rats. Therefore, the "*anterior part/posterior part ratio"* in P4 animals (1.49) was different from that found in P20 rats (1.09), which indicates that protein restriction induced a deformation of the mandible relative to age. This concept is illustrated in Fig. 1, in which one representative mandible of P20 (upper) and P4 (lower) rats at the end of the studied period are shown. It is evident that the posterior part of the bone was clearly negatively influenced by the low protein diet, an effect that was not seen in the anterior part.

The alterations in mandibular morphometrics induced by protein restriction were paralleled by a weakening of the bone, shown by the impairment of ultimate strength (Wf) and stiffness (Wy/dy ratio). The other extrinsic mechanical properties were also adversely affected in P4 rats.

Densitometric analysis allowed the determination of the areal BMD of the mandibular bone, a variable that is usually regarded as an indicator of the mechanical quality of bone. In fact, it represents at least one (the mineral amount expressed per unit of projected bone area) of the most relevant determinants of that property[24]. In support of that assumption, a close correlation between the chemically assessed volumetric bone mineral density (ash content per unit of bone volume) and the mechanically determined elastic modulus of bone tissue has been verified repeatedly[27, 28]. BMD was not affected by protein restriction. Thus, it could

be assumed that the moderate dietary protein restriction imposed on rats in the present study did not alter the normal rigidity of mandibular bone material.

In conclusion, we have described a number of alterations in both morphological and biomechanical variables in the rat mandible resulting from protein restriction from weaning to early adulthood. The clear differences in strength and stiffness of the bone seemed to be the result of an induced loss of gain in bone structural properties as a consequence of a correlative loss of gain in both growth and mass, yet not in bone material properties.

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CORRESPONDENCE

Carlos E. Bozzini
Cátedra de Fisiología, Facultad de Odontología, UBA
M.T. de Alvear 2142 - (1122) Buenos Aires, Argentina
cebozi@fisio.odon.uba.ar

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