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Short Communication

Improved bone status by the β-blocker propranolol in an animal model of nutritional growth retardation

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The aim of the present research was to study if the β-blocker propranolol, which is known to increase bone mass, could reverse the adverse skeletal effects of mild chronic food restriction in weanling rats. Male Wistar rats were divided into four groups: control, control + propranolol (CP), nutritional growth retardation (NGR) and nutritional growth retardation + propranolol (NGRP). Control and CP rats were fed freely with the standard diet. NGR and NGRP rats received, for 4 weeks, 80 % of the amount of food consumed by the control and CP rats, respectively. Results were expressed as mean values and SEM. Food restriction induced detrimental effects on body and femur weight and length ($P<0{\cdot}05$) and bone structural and geometrical properties ($P<0{\cdot}001$), confirming results previously shown in our laboratory. However, the β-blocker overcame the deleterious effect of nutritional stress on load-bearing capacity, yielding load, bone stiffness, cross-sectional cortical bone area and second moment of inertia of the cross-section in relation to the horizontal axis without affecting anthropometric, histomorphometric and bone morphometric parameters. The results suggest that propranolol administration to mildly chronically undernourished rats markedly attenuates the impaired bone status in this animal model of growth retardation.

Nutritional growth retardation: Propranolol: Bone histomorphometry: Mechanical performance

Bone acquisition and maintenance are physiological processes by which bone mass is related to complex and dynamic bone modelling and remodelling mechanisms during vertebrate life, both regulated by autocrine, paracrine, endocrine and nervous mechanisms[1–5].

Bone resistance to fracture is highly correlated with both geometry and material properties[6]. Changes in the mechanical effectiveness of bone could be due to changes in the amount of mass and its spatial distribution and/or intrinsic mechanical quality of its constitutive substance[7].

Several findings have reported nervous system participation in the regulation of bone remodelling as demonstrated by the presence of adrenergic receptors in osteoblasts and osteoclasts[8], and sympathetic nerve fibres in bone tissue[9]. Furthermore, there is evidence that β-adrenergic antagonists such as propranolol increase bone formation rate, osteoblast number[10] and bone strength in rat models[11].

Many factors are determinants of bone quality, but nutritional status is one of the most important. Protein–energy malnutrition during development, mostly during critical periods of body growth, contributes to longitudinal growth failure with subsequent risk of osteoporosis and bone fragility later in life[12,13].

In our laboratory we have developed a nutritional stress model, based on clinical paediatric findings[14–16], in weanling male rats placed on a 20 % restricted balanced diet for a long time that closely resembles the suboptimal nutrition observed in children who consume inappropriate diets with insufficient total energy to sustain normal growth and weight gain[17].

Previous studies performed in our laboratory have shown impairment of bone biomechanical performance in undernourished rats (nutritional growth retardation (NGR) rats) induced by global mild chronic food restriction. The data suggested that the impaired performance of the diaphyseal shafts of NGR animals should be regarded as resulting predominantly from changes in the spatial distribution of bone material rather than its intrinsic quality[13].

Because there is a link between nutrition and bone status and it is known that bone mass regulation is related to adrenergic regulatory pathways, the aim of the present study was to

Abbreviations: BV/TV, bone volume; CP, control + propranolol; HpZTh, thickness of hypertrophic zone; NGR, nutritional growth retardation; NGRP, nutritional growth retardation + propranolol; PZTh, thickness of proliferative zone of growth plate cartilage.

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examine if the β-blocker propranolol, which is known to increase bone mass, could reverse adverse skeletal effects in the present nutritional stress model by the estimation of the biomechanical performance of the femur.

Materials and methods

Animals

Weanling male Wistar rats (mean initial body weight 46·30 ± 1·76 g) were housed and kept under 12 h light–12 h dark cycles and maintained at 21 ± 1°C with 50–60 % humidity. The experiment was conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Buenos Aires Ethics Committee.

Diet

Animals were fed with a standard diet (Purina chow) of the following composition (g/100 g): protein, 23·5; lipids, 7·09; fibre, 6·0; Ca, 1·3; P, 0·8; ash, 6·39; water, 7·96; dextrin, balance.

Experimental design

Rats were randomly assigned to four groups: control, control + propranolol (CP), NGR and NGR + propranolol (NGRP). Control and CP rats were fed freely with the standard diet. NGR and NGRP rats received, for 4 weeks, 80 % of the amount of food consumed by the control and CP rats, respectively, the previous day, corrected for body weight. All rats had free access to water. Propranolol (7 mg/kg per d; Richmond Laboratory, Buenos Aires, Argentina) was injected intraperitoneally 5 d per week, for 4 weeks, in the CP and NGRP rats. Propranolol regimen administration and dose concentration were chosen according to previous studies[10]. The control and NGR rats received saline injections in an identical dosage regimen. Body weight and dietary intake were recorded daily; body length was recorded every 4 d. A Mettler PC 4000 scale (Mettler-Toledo, Zurich, Switzerland) was used to measure body weight with an accuracy of ± 1 mg. For length measurements, animals were anaesthetised slightly with diethyl ether in an anaesthetic induction chamber. Body length was determined with a scaled ruler in mm from the nose tip to the last hairs of the tail base. Food consumption was measured by using special feeders, which allowed the recovery of spilled food. Food intake was weighed daily with a Mettler scale (accuracy ± 1 mg). Animals were killed under anaesthesia after 4 weeks of food restriction. Femurs and tibiae from each animal were dissected avoiding periostial lesion and weighed, and their length was determined with a digital calliper from the tip of the greater trochanter to the distal surface of the lateral-medial condyle. A Mettler PE 600 scale (Mettler-Toledo) was used to measure femur weight expressed in g. Femurs were used for mechanical studies, whereas tibiae were used for histomorphometric studies. Additionally, ten rats were killed for initial measurements on the day the experiment began (day 0).

Histomorphometry

Tibiae were resected, fixed in buffered formalin, decalcified in 10 % EDTA (pH 7) for 25 d, and embedded in paraffin. Longitudinally oriented sections of the tibiae were obtained and stained with haematoxylin–eosin. Histomorphometric evaluation of the decalcified bone section was performed on digitalised microphotographs by employing Image-Tool software (University of Texas Health Science Center at San Antonio; UTHSCA). The following static parameters were evaluated in the middle area of the subchondral bone[18]: thickness of the proliferative zone of the growth plate cartilage (PZTh) (μm); thickness of the hypertrophic zone (HpZTh) (μm), both calculated as the means of thirteen different measurements performed at thirteen locations randomly chosen on each section; bone volume as a fraction of bone tissue in total volume (BV/TV) (%). Total volume was taken as bone tissue plus bone marrow.

Biomechanical tests on femurs

The whole bones were submitted to a three-point bending test in a computerised Instron Universal Testing Machine (model 4442; Instron, Canton, MA, USA). The breaking force was applied perpendicularly to the long axis of the bone at midshaft. Bones were placed lying horizontally with the anterior aspect facing down on two supports equidistant from their ends, separated by a constant distance, and loaded (50 N) centrally at a speed of 5 mm/min. The plots of load *v.* deformation obtained were analysed to determine the following structural mechanical properties: load-bearing capacity (N), yielding load (N), yielding deformation (mm) and stiffness in elastic conditions (N/mm). Because bone segments between the supports were closely comparable with elliptical cylinders, the micromorphometry of the horizontal and vertical external (H and V) and internal (h and v) diameters of the fracture sections enabled us to calculate the following geometric properties: cross-sectional cortical bone area (mm^2) and moment of inertia of the cross-sectional area (mm^4). A stress–strain curve was determined from the previous force–deformation curve using engineering formulae. Material properties calculated were: yield stress (N/mm^2) and Young's modulus of elasticity (N/mm^2)[6].

Statistical analysis

Results are expressed as mean values with their standard errors. Data were analysed by one-way ANOVA followed by the Student–Neuman–Keuls test. Differences were considered significant if $P<0·05$[19]. Statistical analysis was performed with the Graphpad Prism statistical package (version 3; Graphpad Software, San Diego, CA, USA).

Results

As shown in Table 1, the body weight and length of the NGR and NGRP rats were 39·7 and 79·8 %, respectively, as compared with the control and CP rats after 4 weeks of food restriction ($P<0·001$). Femoral growth and tibia growth were also negatively affected in undernourished rats at the end of the restrictive period ($P<0·01$). Nevertheless, propranolol had no effects on the anthropometric and bone morphometric parameters measured.

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Table 1. Body, femur and tibia weight and length, thickness of proliferative zone of growth plate cartilage, thickness of hypertrophic zone and tibiae volume in control, nutritional growth retardation (NGR), control + propranolol (CP) and NGR + propranolol (NGRP) groups

(Mean values with their standard errors for ten animals per group)

Group...	Control		NGR		CP		NGRP	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Body weight (g)	243·20	13·20	96·60*†	5·80	239·10	8·90	95·50*†	10·60
Body length (cm)	21·04	0·30	16·80*†	0·37	20·60	0·25	16·70*†	0·59
Femur weight (g)	0·72	0·06	0·46*†	0·02	0·64	0·02	0·48*†	0·02
Femur length (mm)	27·90	0·25	23·90*†	0·30	27·43	0·38	25·10*†	0·20
Tibia weight (g)	0·58	0·01	0·38*†	0·02	0·55	0·02	0·40*†	0·01
Tibia length (mm)	32·90	0·28	28·80*†	0·34	32·73	0·28	30·82*†	0·44
Thickness of proliferative zone of growth plate cartilage (tibia) (μm)	253·14	6·51	163·79*†	3·72	255·46	4·52	196·14*†	3·24
Thickness of hypertrophic zone (tibia) (μm)	163·79	8·28	112·61*†	3·32	156·64	4·21	101·89*†	2·43
Bone volume (tibia) (%)	64·00	3·28	27·30*†	3·68	67·70	8·80	36·10*†	4·10

* Mean value was significantly different from that of the control group ($P<0·05$).
† Mean value was significantly different from that of the CP group ($P<0·05$).

Histomorphometric evaluation of longitudinally oriented sections of the tibiae exhibited a thinning of growth plate cartilage in NGR and NGRP rats as compared with the control and CP groups, respectively. After 4 weeks of food restriction, the PZTh and HpZTh of the NGR group were 35·30 and 31·20 % less ($P<0·001$), respectively, as compared with the control group, and the PZTh and HpZTh of the NGRP group were 23·20 and 35·00 % less ($P<0·001$), respectively, as compared with the CP group. When bone volume was calculated, the BV/TV of the NGR and NGRP groups were 57·35 and 47·00 % less than those of the control and CP groups, respectively ($P<0·001$). Nevertheless, NGRP animals showed no significant difference as compared with the NGR group (Table 1).

As previously reported[13], structural properties, i.e. load-bearing capacity, yielding load and diaphyseal stiffness were negatively affected in the NGR rats after food restriction ($P<0·05$). Cross-sectional cortical area and moment of inertia of the fracture section were significantly reduced ($P<0·05$) after 4 weeks of food restriction in the post-weaning period as compared with the control rats. Strikingly, food-restricted propranolol-treated rats revealed changes in both structural and geometrical properties whose values were significantly higher ($P<0·05$) than their respective controls. When outside (H, horizontal; V, vertical) and inside (h, horizontal; v, vertical) diameters were analysed, horizontal cortical wall thickness (ΔH − h) was about 44 % higher in the NGRP animals as compared with the NGR animals ($P<0·05$). Nevertheless, significant differences in material bone quality, assessed by yield stress and modulus of elasticity, were not evident between groups. However, this β-blocker did not modify biomechanical competence parameters when the CP group is compared with the control group (Table 2).

Discussion

Although poverty and food shortage in developing countries remain the most common cause of NGR, inappropriate eating habits can also lead to children's failure to thrive among the middle-to-upper socio-economic class[20,21]. NGR refers to a pattern of growth characterised by subnormal body and length growth where weight-for-height deficit and alterations in the biochemical markers of malnutrition are not evident[22].

An optimal bone status in adulthood is a consequence of multiple factors that regulate bone quality and quantity mainly acquired during childhood and adolescence by continuous coordinated activity between osteoclasts and osteoblasts in the modelling and remodelling processes[23].

In the past few years, the possibility that the nervous system may control bone metabolism has been raised, as the sympathetic nervous system has been implicated in the regulation of bone formation and bone mass through β_2-adrenergic receptors expressed in osteoblasts[5,24–26].

In order to provide a better understanding of the sympathetic nervous system as a negative regulator of bone status, we have evaluated the effects of propranolol on the biomechanical competence of a long weight-bearing bone in a stress-induced food-restriction model of growth retardation. In the present study, the food restriction imposed was severe enough to decrease the normal growth rate in the NGR and NGRP animals. In the femur and tibia, a similar result was observed in the undernourished groups. However, when propranolol was administered to the treated groups, no significant differences were observed on anthropomorphometric parameters between the CP *v.* control and NGRP *v.* NGR groups, respectively. These results are in agreement with other authors that reported changes in bone mass induced by the β-blocker while body weight remained unchanged[10].

In tibiae, the histomorphometric studies showed that the thickness of the growth plate cartilage was negatively affected in the NGR rats after food restriction as compared with the control rats. The lack of effect of propranolol on growth plate thickness is consistent with the unaltered linear bone growth rates noted in the propranolol-treated rats.

Whole-bone biomechanical changes are determined by both mass and its spatial distribution and/or intrinsic quality of its constitutive substance[7]. An important negative effect on femoral stiffness and strength was evident in response to external loading after the food-restriction period in the NGR and NGRP rats. In congruence with mechanical properties, geometrical parameters were significantly reduced in the NGR and NGRP animals when compared with the control and CP rats, respectively. When outside and inside diameters were analysed, cortical wall thickness was about 44 % higher

Table 2. Load-bearing capacity, yielding load, stiffness in elastic conditions, cross-sectional area, moment of inertia, horizontal and vertical cortical thickness, yield stress and elastic modulus in control, nutritional growth retardation (NGR), control + propranolol (CP) and NGR + propranolol (NGRP) groups

(Mean values with their standard errors for ten animals per group)

Group...	Control		NGR		CP		NGRP	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Load-bearing capacity (N)	53·36	2·58	24·16*†	2·56	45·47	3·29	43·80*‡	1·00
Yielding load (N)	41·56	3·90	19·58*†	2·54	32·76	1·52	35·74*‡	1·20
Stiffness in elastic conditions (N/mm)	93·92	5·62	41·20*†	6·38	98·82	7·14	77·47*‡	16·52
Cross-sectional area (mm^2)	9·39	0·56	5·74*†	0·39	8·00	0·60	7·60*‡	0·52
Moment of inertia (mm^4)	10·42	0·67	5·19*†	0·48	9·78	0·83	8·31*‡	0·66
Horizontal cortical thickness (mm)	1·98	0·17	1·27*†	0·12	1·81	0·14	1·81‡	0·19
Vertical cortical thickness (mm)	1·51	0·16	1·18	0·12	1·24	0·10	1·22	0·15
Yield stress (N/mm^2)	18·63	0·98	18·16	1·84	17·40	0·81	21·14	1·22
Elastic modulus (N/mm^2)	241·83	28·79	231·00	54·50	285·00	19·10	259·00	56·40

* Mean value was significantly different from that of the control group ($P<0·05$).
† Mean value was significantly different from that of the CP group ($P<0·05$).
‡ Mean value was significantly different from that of the NGR group ($P<0·05$).

in the NGRP animals as compared with the NGR rats ($P<0·05$), making evident an increment in bone mass. These results are in agreement with other studies about bone remodelling and its association with β-adrenergic control via the sympathetic nervous system[27].

Since there is evidence about different responses of intramembranous or endochondral ossification to central control of bone mass[28] and propranolol could not prevent the negative effect of food restriction on growth plate cartilage and BV/TV, but horizontal cortical wall thickness was higher in the NGRP compared with the NGR group, it is possible to hypothesise that this β-blocker exerts effects mainly on intramembranous ossification by bone apposition under the condition of nutritional stress. This could be the result of a differential sensitivity of osteoblast and/or osteoclast to different signalling mechanisms relative to nutritional status.

On the other hand, the material quality of bone was not affected either by food restriction as seen in previous studies performed in our laboratory[13] or by β-blocker administration. It is remarkable that propranolol administration to the mildly chronically undernourished rats strongly attenuated the impaired biomechanical competence in this animal model of growth retardation, exerting its effects in the spatial distribution of bone material rather than its intrinsic quality. In fact, load-bearing capacity, yielding load and stiffness were 80·0, 82·5 and 88·0 % higher, respectively, when NGRP animals were compared with NGR animals.

These later results suggest that propranolol treatment exerts a preventive effect against the detrimental consequences to bone status in mildly chronically food-restricted growing rats by an increment in cortical bone and by improving its spatial distribution. The results also emphasise that the β-blocker propranolol potentially benefits bone structure under conditions of nutritional stress.

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C. E. L. was responsible for anthropometric data registration, collection of food intake data, drug administration and histomorphometric evaluation and data interpretation. M. I. O. performed biomechanical studies, interpreted the data and wrote the paper. C. B. performed biomechanical studies and statistical analysis. P. M. was responsible for histomorphometric evaluation and data analysis. R. M. A. cooperated with biomechanical assay interpretation. P. M. B. designed and coordinated this specific study, performed the statistical analysis, interpreted the data and wrote the paper. The authors thank Graciela Champin for her technical assistance. The present study was supported by research grants from the University of Buenos Aires (UBACyT O010).

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