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mRNA of cytokines in bone marrow and bone biomarkers in response to propranolol in a nutritional growth retardation model

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ABSTRACT

Background: The aim of this study was to assess mRNA of IL-6, TNFα and IL-10 cytokines in bone marrow, possible mediators involved in altered bone remodeling with detrimental consequences on bone quality in NGR (Nutritional growth retardation) rats.
Methods: Weanling male Wistar rats were assigned either to control (C) or experimental group (NGR) (n = 20 each). C and NGR groups were assigned to 2 groups according to receiving saline solution (SS) or propranolol hydrochloride (P): C, C + P (CP), NGR or NGR + P (NGRP). For 4 weeks, NGR and NGRP rats received 80% of the amount of food consumed by C and CP, respectively, the previous day, corrected by body weight. P (7 mg/kg/day) was injected *ip* 5 days/week, for 4 weeks in CP and NGRP rats. Body weight and length were recorded. After 4 weeks, blood was drawn. Femurs were dissected for RNA isolation from bone marrow and mRNA of cytokines assays.
Results: Food restriction induced a significant negative effect on body growth in NGR and NGRP rats ($p < 0.001$). P had no effects on zoometric parameters ($p > 0.05$). CTX-I increased in NGR rats *vs.* C ($p < 0.001$), but diminished in NGRP ($p < 0.01$). Serum osteocalcin, PTH, calcium and phosphate levels remained unchanged between groups ($p > 0.05$). In NGR, bone marrow IL-6 mRNA and IL-10 mRNA levels were low as compared to other groups ($p < 0.05$). In contrast, bone marrow TNF-α mRNA levels were significantly high ($p < 0.05$).
Conclusions: This study provides evidences that NGR outcomes in a bone marrow proinflammatory microenvironment leading to unbalanced bone remodeling by enhancement of bone resorption reverted by propranolol.

Introduction

An optimal bone status in adulthood is a consequence of multiple factors that regulate bone quality and quantity mainly acquired during childhood and adolescence [1]. Bone mass and shape are determined by continuous remodeling performed by a coordinated activity between osteoclasts and osteoblasts [2]. During childhood, *modeling* allows individual bones to grow in size and to shift in space for the formation of new bone. Bone's geometry mainly depends on bone modeling which is determined by the uncoupled periostial and peritrabecular apposition and trabecular or endostial resorption [2]; however, much of the cellular activity in a bone occurs by remodeling throughout life.

Although there is evidence of compensatory mechanisms in energy metabolism and body composition in response to mild chronic food restriction [3], both animal and human studies indicate that protein–energy malnutrition can be detrimental for both acquisition of bone mass during growth and its conservation during adulthood. Therefore, malnutrition during development can lead to impairment of biomechanical competence [4,5].

In our laboratory, we have developed a nutritional stress model in weanling male rats placed on a 20% restricted balanced diet for a

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long time (nutritional growth retardation rats, NGR rats) that closely resembles the suboptimal nutrition observed in children who consume inappropriate diets with insufficient total energy to sustain normal growth and weight gain [3]. The nutritional status model is based on clinical pediatric findings related to infant feeding practices, such as hypocaloric foods that are given to infants postlactation [6] due to parental misconceptions, health practices and beliefs [7,8].

In previous studies, we have observed a physiological decrease in energy expenditures in NGR rats [9,10] as an adaptive mechanism to suboptimal nutrition leading to nutritional growth retardation, as well as to inhibition of somatotrophic and reproductive axes in postweaning male rats [9,11]. However, these adaptive responses to caloric restriction also showed impaired mechanical femoral competence in NGR rats that may result from altered bone mass and architectural distribution, rather than from its intrinsic quality [4,5].

In congruence with the osteoporosis concept defined by the World Health Organization (WHO) [12], our previous reports suggested that bone health in NGR rats is compromised and, then, increased risk of fractures.

Several findings evidenced nervous system bone metabolism control as the sympathetic nervous system has been implicated in the regulation of bone formation and bone mass through β2-adrenergic receptors expressed in osteoblasts and the presence of sympathetic nerve fibers in bone tissue [13,14]. Furthermore, there is evidence that β-adrenergic antagonists as propranolol increase bone formation rate, osteoblast number [13] and bone strength in rat models [15].

In agreement with bone remodeling control and its association with β-adrenergic control *via* sympathetic nervous system, previous studies performed in our laboratory showed overall increase of hypothalamic noradrenergic activity in NGR rats [16]. Moreover, we have demonstrated that propranolol treatment exerts a preventive effect on detrimental consequences on bone status in NGR rats by an increase in cortical bone mass with an improvement in its spatial distribution [17].

Since bone continuously undergoes significant turnover by modeling/remodeling process [18], imbalance in bone turnover due to suppression of osteoblast recruitment and stimulation of osteoclastogenesis can result from a proinflammatory microenvironment in bone marrow [19,20].

Previous studies evidenced that upregulated proinflammatory cytokines including tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are recognized as important factors in the pathogenesis of metabolic disorders as osteoporosis [21,22]. They may promote osteoclast differentiation and bone resorption through modifying the receptor activator of NF-κB (RANK)/RANK ligand/osteoprotegerin pathway [23].

Besides, other reports supported that male sex steroids, acting through the androgen-specific receptor, inhibit the expression of the IL-6 gene; and this cytokine mediates the upregulation of osteoclastogenesis and, therefore, the bone loss caused by androgen deficiency [24]. The suppressive effects of sex hormones on osteoclastogenesis have been extended to include TNFα [25]. Both, IL-6 and TNFα, among other cytokines, seem to exert their effects by stimulating the expression of RANKL.

Due to disturbance in gonadotropic axe with delayed puberty in NGR rats previously observed by us [11], we have hypothesized that the detrimental bone status could be linked, at least in part, to a proinflammatory microenvironment in bone marrow in this hypogonadotropic hypogonadism model.

The aim of this study was to assess the expression of IL-6, TNFα and IL-10 cytokines in bone marrow as possible mediators involved in altered bone remodeling with detrimental consequences on bone quality in NGR rats. Otherwise, serum bone turnover biomarkers, C-terminal crosslinking telopeptid of type I collagen (CTX-I) and osteocalcin were assessed. Additionally, since chronic elevation of parathyroid hormone (PTH) exerts catabolic effect on the skeleton [26] and noradrenergic hyperactivity increase PTH release [27], serum PTH, phosphate and calcium levels were determined. Likewise, due to the knowledge that propranolol increases biomechanical competence and reduces the risk of bone fractures in NGR rats [17], the expression of bone marrow cytokines and biomarkers of bone status were studied under β-blocker administration.

Experimental methods

Animals and diet

Forty weanling male Wistar rats (21–23 days old; mean initial body weight 44.40 ± 0.14 g) were provided by the Animal Resources of the Department of Biochemistry, School of Dentistry, University of Buenos Aires, Argentina. Animals were fed a standard diet (Purina chow) of the following composition (g/100 g): protein, 22.7; lipids, 7.09; fiber, 6.0; Ca, 1.3; P, 0.8; ashes, 6.50; water, 7.96; dextrin, balance. The rats were housed and kept under 12 h light–12 h dark cycles. Room temperature was maintained at 21 ± 1 °C with 50–60% humidity. The experiment was conducted in accordance with the principles and procedures outlined in the USA National Institutes of Health Guide for the Care and Use of Laboratory Animals [28], and approved by the University of Buenos Aires Ethic Committee.

Experimental design

Animals were randomly assigned either to control (C) or experimental group (NGR) of 20 animals each. C and NGR groups were subdivided in 2 groups according to receiving saline solution (SS) or propranolol hydrochloride (P). The four groups were: C, C + P (CP), NGR and NGR + P (NGRP). C and CP rats were fed freely with the standard diet. For 4 weeks, NGR and NGRP rats received 80% of the amount of food consumed by C and CP, respectively, the previous day, corrected by body weight (food intake in g/100 g body weight/day). All rats had free access to water. Propranolol hydrochloride (7 mg/kg/day; Richmond Laboratory, Argentina) was injected *ip* 5 days/week, for 4 weeks in CP and NGRP rats, C and NGR received saline injections at an identical dosage regimen. The choice of propranolol hydrochloride dose (7 mg/kg/day) was based on our previous studies and was coincident with Takeda et al. [13]. No effects on biochemical competence were observed under 3.5 mg/kg/day or 14.0 mg/kg/day of propranolol hydrochloride. Seven and 10.5 mg/kg/day of propranolol hydrochloride markedly attenuated the impaired bone status improving bone cortical mass and architecture design in NGR rats, with a maximum response at 7 mg/kg day [29]. The present study was conducted in growing rats, whereas others have used lower doses, but on adult animal models and with other skeletal disorders [15,30–32].

Body weight was recorded every day in the morning before food distribution, and body length, every 4 days. Growth data were compared over time. Dietary intake was registered daily. Animals were euthanized under anesthesia 0.1 ml of ketamin hydrochloride (100 mg/ml, Holliday Lab.)/100 g body weight was mixed with 0.02 ml of xylazine (100 mg/ml, Konig Lab.)/100 g body weight by intramuscular injection [33], after 4 weeks of food restriction (w4). Immediately, blood was drawn by cardiac puncture, allowed to clot and the serum was stored at −20 °C until biochemical assays were performed. Femurs from each animal were dissected, weighed and bone marrow was extracted for RNA isolation and RT Real Time

PCR and stored at −80 °C until mRNA of cytokines assays were performed.

Nutritional status

Zoometry

Body weight and length were recorded serially during the experimental period (daily and 4 days, respectively), after a 2–4 h fasting period. A Mettler PC 4000 scale (Zurich, Switzerland) was used to measure body weight with an accuracy of ±1 mg. For length measurements, animals were immobilized by isoflurane anesthesia gas. Body length was determined with a scaled ruler in mm from the nose tip to the last hairs of the tail base.

Dietary intake

Throughout the experimental period, food intake was calculated daily as the difference of two consecutive days' consumption using a Mettler scale (accuracy ±1 mg).

Biochemical determinations

Serum C-telopeptide fragments of collagen type I (CTX-I; ng/ml) and osteocalcin (ng/ml) were determined by RatLaps™ EIA and Rat-MID™ Osteocalcin EIA, respectively, Immunodiagnostic Systems Limited (IDS Ltd.), UK.

Intact parathyroid hormone (PTHi; pg/ml) was determined by electrochemoluminiscence (ECLIA), Roche, SA. Calcemia (mg/dl) was assessed by spectrophotometer of atomic absorption. Serum phosphorus (mg/dl) was determined by spectrophotometer UV (Wiener, SA).

RNA isolation and RT real time PCR

Total RNA was extracted from bone marrow with the TRIzol® reagent (Invitrogen). RNA (2 μg) was pre-treated with RNase-free DNAse I, heated at 70 °C for 10 min, placed on ice for 1 min, and then incubated with a mixture containing 0.5 mmol/l dNTPs mix, 25 ng/μl random primers, 1X first-strand buffer, 25 units of RNAse inhibitor, 200 units of MMLV reverse transcriptase and water in a final volume of 25 μl, for 1 h at 42 °C. The reaction was stopped by heating at 90 °C for 5 min and the mixture was brought to 100 μl with diethylpyrocarbonate-treated water and stored at −70 °C. In selected tubes, reverse transcriptase was omitted as a genomic contamination control. Amplifications by real-time PCR were carried out in a StepOnePlus RealTime PCR Systems (AB Applied Biosystems) and performed using 0.5 μl cDNA in a final volume of 25 μl in the following reaction mixture: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 500 nM of each specific oligonucleotide primer, 0.625 U GoTaq® polymerase and 0.96 × EvaGreen. The sequence for the oligonucleotide primers for IL-6 (NM_012589), IL-10 (NM_012854) and TNF-α (NM_012675) mRNAs were generated using the NBCI primer design tool as follows: IL-6: forward 5′-TCCTACCCCAACTTCCAATGCTC-3′ reverse 5′-TTGGATGGTCTTGGTCCTTAGCC-3′, IL-10: forward 5′-AAAGCAAGGCAGTGGAGCAG-3′ reverse 5′-TCAAACTCATTCATGGCCTTGT-3′, TNF-α: forward 5′-CACTTCACAAGTCGGAGGCT-3′ reverse 5′-TCTGACAGTGCATCATCGCT-3′. IL-6, TNF-α, IL-10 mRNA amounts were standardized against the amount of GAPDH mRNA and expressed according to the $2^{\Delta\Delta Ct}$ method [34].

Statistical analysis

The results were expressed as mean values with their standard deviations (SEM). One-way analysis of variance (ANOVA) was used to compare data among groups. When a statistically significant difference was encountered, a Student–Newman–Keul's test was performed. In all analyses, Bartlett's test for homogeneus variances was done. Significance was set at the $p < 0.05$ level. The Statistical Product and Service Solutions for Windows 9.0 (SPSS Inc., Chicago, IL) and the Graphpad Prism (version 5.0) (Graphpad Software, San Diego, CA, USA) were used for statistical analyses.

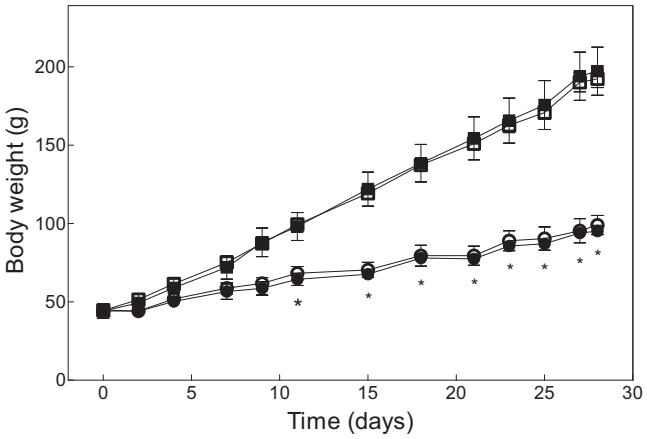

Fig. 1. Body weight over time. Control (■), control + propranolol (□), NGR (●), NGR + propranolol (○) groups. Mean value ± SEM; 10 animals/group. *: indicates significant differences between NGR *vs.* control and NGRP *vs.* CP groups ($p < 0.05$).

Results

As shown in Figs. 1 and 2, food restriction induced a highly significant negative effect in body growth in NGR and NGRP rats, as compared to C and CP rats, respectively, after 4 weeks ($p < 0.001$). However, no differences on zoometric parameters were found between C *vs.* CP and NGR *vs.* NGRP groups ($p > 0.05$).

Biochemical bone turnover markers of the four studied groups are shown in Fig. 3a and b. CTX-I concentrations showed a significant increase in the NGR rats as compared to C group ($p = 0.001$) (Fig. 3a). Nevertheless, bone resorption biomarker decreased significantly in NGRP animals as compared to NGR group ($p < 0.01$). Not significant differences were observed in CTX-I levels among NGRP, CP and C rats ($p > 0.05$). Also, osteocalcin serum levels remained unchanged between rats under conditions of suboptimal nutrition and rats fed *ad libitum* with or without Propranolol treatment (Fig. 3b) ($p = 0.3569$).

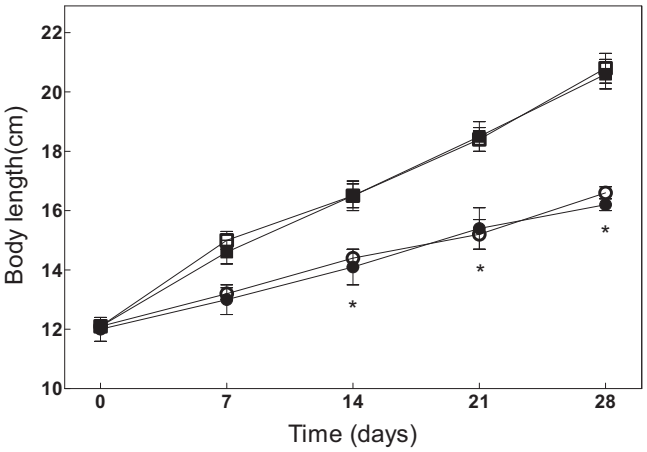

Fig. 2. Body length over time. Control (■), control + propranolol (□), NGR (●), NGR + propranolol (○) groups. Mean value ± SEM; 10 animals/group. *: indicates significant differences between NGR *vs.* control and NGRP *vs.* CP groups ($p < 0.05$).

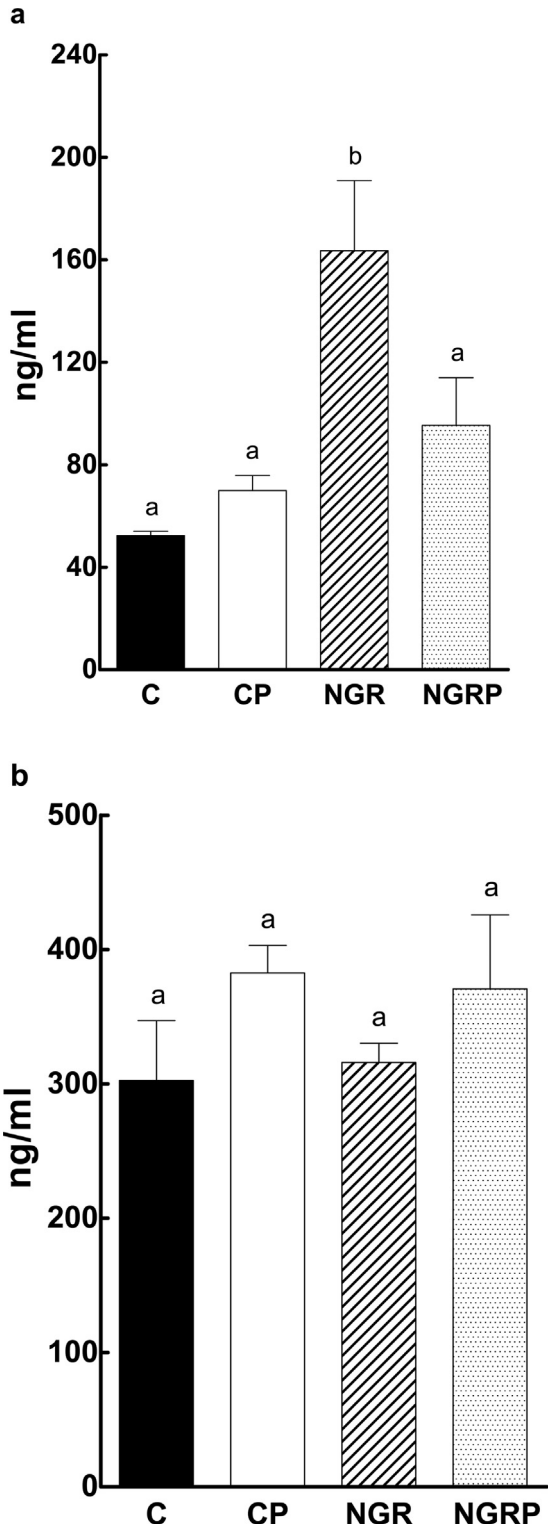

Fig. 3. Bone modeling/remodeling biomarkers. Serum CTX-I (a) and osteocalcin (b) in control (C), control + propranolol (CP), experimental NGR (NGR) and experimental NGR + propranolol (NGRP) groups at w4. Mean value ± SEM; 10 animals/group. Different letters means significant differences between groups ($p < 0.05$).

Fig. 4a–c shows serum PTH, calcium and phosphate levels, respectively, for experimental and control groups. Not significant differences between groups were observed ($p = 0.979$, $p = 0.071$ and $p = 0.903$, respectively).

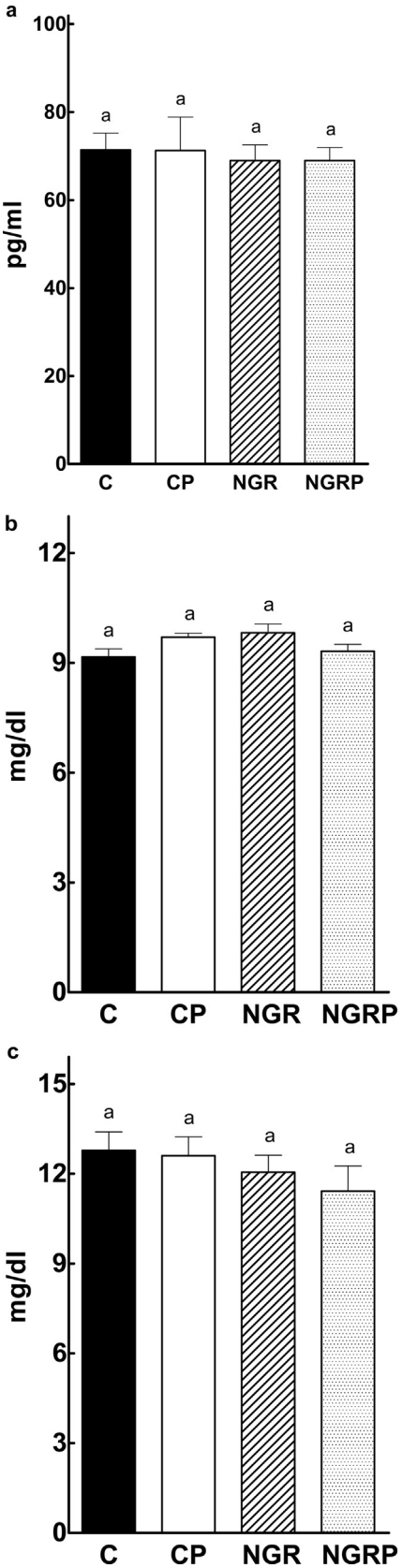

Fig. 4. Metabolic/endocrine environment. Serum PTHi (a), calcium (b) and phosphorus (c) in control (C), control + propranolol (CP), experimental NGR (NGR) and experimental NGR + propranolol (NGRP) groups at w4. Mean value ± SEM; 10 animals/group. Different letters means significant differences between groups ($p < 0.05$).

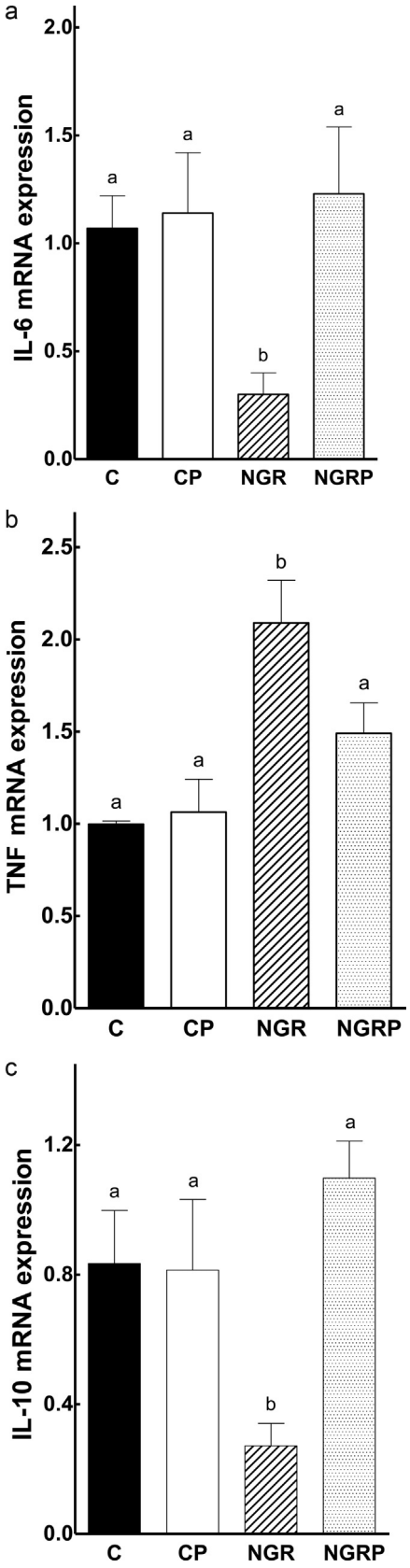

Fig. 5. Bone marrow pro-anti-inflammatory environment. Bone marrow IL-6 (a), TNFα (b) and IL-10 (c) mRNA expressions in control (C), control + propranolol (CP), experimental NGR (NGR) and experimental NGR + propranolol (NGRP) groups at w4. Mean value ± SEM; 10 animals/group. Different letters means significant differences between groups ($p < 0.05$).

IL-6 mRNA, TNF-α mRNA and IL-10 mRNA expressions are shown in Fig. 5a–c, respectively. Bone marrow IL-6 mRNA expression was significantly different among groups ($p = 0.033$), being NGR significantly low as compared to the NGRP ($p < 0.01$), C ($p < 0.05$) and CP ($p < 0.05$) (Fig. 5a).

Bone marrow TNF-α mRNA expressions were significantly different among groups ($p = 0.001$), being NGR significantly high as compared to NGPR ($p < 0.05$), C ($p < 0.001$) and CP ($p < 0.001$) and (Fig. 5b).

Bone marrow IL-10 mRNA expressions were significantly different among groups ($p = 0.001$), being NGR significantly low as compared to NGRP ($p < 0.001$), C ($p < 0.05$) and CP ($p < 0.05$) (Fig. 5c).

Discussion

Bone acquisition and maintenance are physiological processes by which bone mass is related to complex and dynamic bone modeling and remodeling mechanisms during vertebrate life [18,35].

Bone status depends on peak bone mass achieved, rate of bone loss and maintenance of their micro-architecture, all related to genetics, body weight and composition, neuroendocrine activity, and quality and quantity of food consumption throughout life [36,37].

Different animal models have been used to study the effect of altered nutrition on postnatal skeletal growth, bone density and biomechanical parameters. In general, the authors assessed the effect of either a protein-deficient diet fed *ad libitum* [38] or severe or chronic limitation of energy requirements for variable periods of time in infantile rats [39].

The nutritional stress model used in the present study resembles the suboptimal nutrition of children who consume inappropriate diets to sustain normal growth [7,8]. NGR refers to a pattern of growth defined by the anthropometric indices of the Wellcome classification system [40], characterized by subnormal body and length growth where weight-for-height deficit and alterations in the biochemical markers of malnutrition are not evident [7].

In the present study, food restriction imposed was severe enough to decrease normal growth rate in NGR and NGRP animals. However, when propranolol was administered, no significant differences were observed on zoometric parameters between CP *vs.* C and NGRP *vs.* 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 [13].

Since there is evidence about different responses of intramembranous or endochondral ossification to central control of bone mass [41] and previous studies performed in our laboratory showed that propranolol did not prevent the negative effect of food restriction on growth plate cartilage and bone volume but cortical wall thickness was wider in NGRP *vs.* NGR group [17], it is possible to hypothesize that this β-blocker exerts effects mainly on intramembranous ossification by bone apposition under nutritional stress condition. This could be the result of a differential sensitivity of osteoblast and/or osteoclast to different signaling mechanisms relative to nutritional status.

Bone remodeling is maintained by two key cell populations, osteoblasts and osteoclasts, which are regulated by cytokines, hormones, and growth factors [42]. Under physiological conditions, a balance between bone formation and resorption is maintained for skeletal homeostasis. In the present nutritional stress model, this balance is disrupted in favor of osteoclast-mediated bone resorption. Activation of osteoclasts requires complex interactions between cells of the osteoclast lineage and

mesenchymal cells and lymphocytes [43] regulated by a large number of cytokines. Among these, IL-6, TNF-α and IL-10 are known to modulate both osteoblast and osteoclast function [19,44].

There are certain evidences that cytokines enhance osteoclastogenesis indirectly by upregulating RANKL expression [45]. IL-6 can upregulate RANKL and thus indirectly support osteoclast formation *via* the interaction with mesenchymal cells [46]. In fact, IL-6 is believed to play a positive regulatory role in osteoclast differentiation by inducing the expression of receptor activator of NF-κB ligand (RANKL) on the surface of osteoblasts, which interacts with RANK expressed on osteoclast progenitors, inducing osteoclast differentiation. Also, some authors have demonstrated that IL-6 and TNF-α induced osteoclast differentiation directly, in a RANKL-independent manner [47,48]. However, there is evidence that the direct effects of IL-6 on osteoclasts are inhibitory rather than stimulatory [49]. Moreover, it was demonstrated that IL-6 can also directly act on osteoclast progenitors to suppress their differentiation *via* an inhibition of RANK signaling pathways [49]. Likewise, *in vitro* results showed that IL-6 decreases osteoclastogenesis in mouse bone marrow-derived macrophages in a dose-dependent manner, by disturbing osteoclast formation [50].

The inhibitory effects of IL-6 can be explained by the fact that IL-6 *per se* is inhibitory to osteoclasts; however, the complex of IL-6 and its soluble receptor IL-6R is stimulatory to osteoclastogenesis by activating trans-signaling through the IL-6R-associated adaptor protein gp130 [51]. IL-6 together with soluble IL-6 receptor act to induce the expression of receptor activator of RANKL on the surface of osteoblasts. RANKL interacts with RANK, which is expressed on the surface of osteoclast progenitors. This interaction is indispensable for the differentiation of osteoclast progenitors into mature osteoclasts [46]. *In vitro* studies suggest that IL-6-gp130-STAT3 signaling stimulates osteoblasts, induces RANKL expression on the surface of an osteoblastic cell line and supports osteoclastogenesis [52].

It was proposed that the effects of IL-6 on bone formation and resorption *in vivo* are determined by the concentrations of IL-6 and soluble IL-6 receptor that are present in the areas around the sites of interaction between osteoblasts and osteoclast progenitors [53]. It is believed that IL-6 usually suppresses bone resorption by inhibiting the differentiation of osteoclast progenitors without affecting osteoblasts, whereas under inflammatory conditions, when soluble IL-6 receptor is available, IL-6 acts on osteoblasts to induce their differentiation and to facilitate the osteoclast-inducing activity [46,53].

Considering the complexity of the effects of this multifunctional cytokine in bone remodeling process, the endogenous IL-6 expression in NGR rats could promote an environment predominantly inflammatory-resorptive. Even though the mechanism of action still needs to be defined, the impaired bone status found in NGR rats could be attributed to the low IL-6 mRNA in bone marrow associated to IL6R.

It is recognized that IL-10 is a cytokine with potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1 by activated macrophages [54].

Previous studies by others demonstrated that recombinant mouse IL-10 has the ability to suppress release of TNF-α by macrophages [55,56].

In the present study, the low levels of bone marrow IL-10 mRNA in NGR rats could induce a poor anti-inflammatory microenvironment that supports the TNFα deleterious effects on bone. The significant increase of TNFα mRNA levels in bone marrow in NGR rats suggests that this cytokine could mediate, at least in part, bone loss that follows the hypogonadism status previously reported by us [11,57]. TNFα could exert its effects by stimulating osteoclast activity in the NGR group as established by the highly increase of CTX-I biomarker.

Our findings are in agreement with the enhanced osteoclastogenesis in cultures of bone marrow macrophages deficient in IL-10 production [58] followed by the development of osteoporosis and increase mechanical fragility [59] reported by others.

However, NGR rats modified the cytokines expression by enhancing IL-6 mRNA and IL-10 mRNA and suppressing TNFα production in response to propranolol administration. Bone resorption biomarker levels suggest an osteoclastic activity inhibition in NGRP. The β-blocker administration to NGR rats markedly attenuate the impaired bone status observed in our previous studies by the inhibition of bone resorption process [17].

The unchanged osteocalcin serum levels, an osteoblast-specific product whose synthesis closely correlates with bone formation observed in all groups, evidences that the dysregulation of bone remodeling process is due to the enhancement of bone resorption in NGR rats.

It is well known that parathyroid hormone is a major regulator involved in the control of calcium homoeostasis in mammals, whose synthesis and secretion are regulated by the extracellular calcium concentration, monitored by the calcium-sensing receptor in the parathyroid gland [60]. In addition to calcium homoeostasis, PTH also regulates blood phosphate concentration by the inhibition of the tubular phosphate reabsorption in the kidneys [60,61]. In summary, PTH is secreted in response to systemic demands for calcium and phosphorus [61].

In the present study, although differences in serum PTHi, calcium and phosphorus are not evident between groups with/without propranolol treatment at the time of autopsy, it is known that endocrine-metabolic environment disturbances subrogate bone status to mineral balance [62].

Our previous studies demonstrated hypothalamic LHRH pulsatile release profile with low FSH, LH and testosterone in accordance with immature rats, and low STH and IGF-I serum levels, in congruence with delayed growth [4,11,57].

These results suggest that NGR outcomes in a bone marrow proinflammatory microenvironment leading to unbalanced bone remodeling by enhancement of bone resorption. Propranolol under the dosage regimen administrated could provide the appropriated bone marrow milieu necessary for an adequate bone status in the present nutritional stress model.

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Conflict of interest

None of the authors had any financial or personal conflict of interest.

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