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Effect of the “protein diet” and bone tissue

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Abstract

The aim of this study is to evaluate the effect of the hyperproteic diet consumption on bone tissue.

Methods: The study was conducted during sixty days. Twenty eight *Wistar albinus* rats, adults, originated from Laboratory of Experimental Nutrition were divided in four groups: (n = 7); Control 1 (C1), Control 2 (C2), Hyperproteic 1 (HP1) e Hyperproteic 2 (HP2). The C2 and HP2 groups were submitted to 30% of food restriction. The hyperproteic diet was based on the Atkins diet and prepared to simulate the *protein diet*. At the end of the study the animals were anesthetized to perform bone densitometry analyses by DEXA and blood and tissue collection. Serum and bone minerals analyses were conducted by colorimetric methods in automated equipment.

Results: The total bone mineral density (BMD) of the pelvis and the spine of the food restriction groups (HP2 e C2) were lower (p < 0.05) than C1 e HP1 groups. While the femur BMD of the HP2 was lower (p < 0.05) related to others groups. It had been observed reduction (p < 0.05) in the medium point of the width of femur diaphysis and in bone calcium level in the hyperproteic groups (HP1 e HP2). It was observed similar effect on the osteocalcin level, that presented lower (p < 0.05) in the hyperproteic groups. The insulin level was lower only in HP2 and serum calcium of the HP1 and HP2 groups was lower than C1.

Conclusion: The *protein diet* promotes significant bone change on femur and in the hormones levels related to bone synthesis and maintenance of this tissue.

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EFEECTO DE LA “DIETA DE PROTEINA” Y EL TEJIDO ÓSEO

Resumen

El objetivo de este estudio fue evaluar el efecto de la ingesta de proteínas de la dieta sobre el hueso.

Métodos: El estudio duró 60 días. Veintiocho adultos *Wistar*, del Laboratorio de Nutrición Experimental fueron divididos en 4 grupos (n = 7), Control 1 (C1), de control 2 (C2), en la proteína 1 (HP1) y en la proteína 2 (HP2). Los grupos C2 y HP2 fueron alimentados restringido a 30%. Los niveles de proteína dietéticos fueron manipulados para simular la dieta de la proteína. Al final del experimento, los animales fueron anestesiados para los procedimientos de densitometría ósea (DEXA) y la recogida de tejidos. Análisis de mineral óseo en suero y se realizaron por el método colorimétrico utilizando un aparato automático.

Resultados: La densidad mineral ósea total (BMD), la pelvis y la columna vertebral de los grupos con la restricción dietética (HP2 y C2) fue menor (p < 0,05) que en los grupos C1 y HP1. Mientras que el grupo HP2 fémur fue menor (p < 0,05) en comparación con otros grupos. Disminución (p < 0,05) de la anchura del punto medio de la diáfisis femoral y la concentración de calcio en los grupos de hueso hiperproteicos (HP1 y HP2). Se observó un efecto similar en osteocalcina en suero, que fue menor (p < 0,05) en los grupos de hiperproteicos mientras que la concentración de insulina fue más bajo sólo en el grupo de HP2. Ya hiperproteicos de calcio en suero y los grupos C2 mostraron menor (p < 0,05) el grupo C1.

Conclusión: La dieta de la proteína promueve cambios significativos en el hueso en el fémur y la concentración de hormonas relacionadas con la formación y el mantenimiento de este tejido.

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Palabras clave: *Dieta de proteínas. Densidad mineral ósea. Remodelación ósea. Hormonas. Dieta Atkins.*

Abbreviations

C1: Control 1.
C2: Control 2.
HP1: Hyperproteic 1.
HP2: Hyperproteic 2.
DEXA: Dual Energy X-Ray Absorptiometry.
BMD: Bone Mineral Density.
AIN: American Institute of Nutrition.
UFF: Federal Fluminense University.
BMC: Bone Mineral Content.
ELISA: Enzyme-Linked Immunosorbent Assay.
COBEA: Brazilian College of Animal Experimentation.
ANOVA: Analysis of Variance.
FAPERJ: Research Foundation of the State of Rio de Janeiro.
PROPPI: Pro Rectory of Research, Graduate Studies and Innovation of the Federal Fluminense University.
CNPQ: National Research Council.

Introduction

The diet with high protein concentration is the most popular fad diet and has been considered to be effective in weight reduction.^{1,2,3} It is characterized by high concentration of protein, lipids and low concentration of carbohydrates.³ However, there is evidence that excessive consumption of protein may impair bone health and may contribute to the development of osteoporosis, especially among adult women.⁴

The change in bone promoted by excess of animal protein and fat consumption has been attributed to the development of metabolic acidosis, caused by excessive production of ketone bodies. This state of ketogenesis seems to stimulate bone resorption and inhibit the osteoblasts activity.^{5,6}

However, the literature is controversial concerning the effects of high protein intake and possible changes in the bone tissue. Some studies suggest that hyperproteic diets are associated with a greater propensity to bone loss.^{7,8} However, other researchers have reported beneficial effects, including increase in bone mineral density.^{9,10}

Soon, the previous studies are inconclusive and require more information about the effect of the hyperproteic diet on bone health. Its effect on the development of osteopenia and/or osteoporosis still needs investigation. The objective of this study was to evaluate the effects of the fad diet, based on higher protein and lipid concentration, on bone tissue of adult female rats.

We emphasize that the main contribution of this study is to understand the changes that occur in the body, especially in bone tissue, due to the consumption of the "protein diet". This way we can alert physicians and nutritionists regarding the negative effects of this diet on bone tissue.

Methodology

The study was conducted at the Experimental Nutrition Laboratory in the Nutrition and Dietetics Department, Nutrition College of the Federal Fluminense University, with female *Rattus, Norvegicus Wistar Albino*, with 90 days of age, weighing approximately 200 g. All animals were housed on experimentation for 60 days in individual polypropylene cages, properly identified. The environment was maintained with constant temperature ($24^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and adequate lighting with light-dark cycle of 12 to 12 hours.

The animals were divided into 4 groups ($n = 7$ per group): Control group: 1) control 1 (C1) and 2) control 2 (C2) * - fed diets consisting of 69.20% carbohydrate (59.20% of starch and 10% sugar), 11.30% protein (casein), 4.80% lipid (soybean oil), 1% vitamin mixture (Prag solutions, São Paulo, Brazil), 3.5% mineral mix (Prag Solutions, São Paulo, Brazil), 5% fiber (cellulose), 0.25% choline bitartrate and 0.18% L-cystine. The control diets were manufactured following the recommendations of the American Institute of Nutrition (AIN-93M);¹¹ Hyperproteic group: 3) hyperproteic 1 (HP1) and 4) hyperproteic 2 (HP2) * - fed diets containing 4.73% of carbohydrate (lactose), 49.77% protein (47.54% beef protein and 2.23% milk protein), 15.97% lipid (11.97% animal fat from meat and 4% soybean oil), 1% vitamin mixture (Prag Solutions, São Paulo, Brazil); 3.5% mineral mix (Prag solutions, Sao Paulo, Brazil); fiber 7% (5% cellulose and 2% agar), 0.18% L-cysteine and 0.25% choline bitartrate. Agar was used to shape ration. The groups * C2 and HP2 received 70% of the amount of feed consumed by C1 and HP1, respectively. The ingredients for the formulation of the hyperproteic diet were purchased in local market. The meat was dried, milled, sifted and mixed with the other ingredients.

The weight (g) of animals was measured using precision balance (Bioprecisa JY 50001, precision 0.1 g) and water consumption (mL) using a graduated cylinder, weekly. The water was available on free demand for all groups. The control of the supply and leftover of the diet was weekly for C1 and HP1. For the groups C2 and HP2 was done daily, due to food restriction.

At the end of the experimental period, all animals were submitted to vaginal washing procedure to identify the estrous cycle phase. After this, those that were in the "estrus" phase were separated and fasted for six hours before sacrifice. They were anesthetized with intraperitoneal injection (with ketamine xylazine 1:1) at a dosage of 0.1 mL/200 g and bone mineral density (BMD) measured by dual energy x-ray absorptiometry (DEXA) (GE Lunar 200368 DXA instrument, Wisconsin, USA), in the Nutritional Assessment Laboratory of the Nutrition College, UFF. The analysis was performed using specific software for small animals^{12,13} (encore in 2008, 13.40 Version GE Healthcare). Bone mineral density (BMD, g/cm^3), bone mineral content (BMC, g) and bone area (cm^2) were analyzed in each

animal. After DEXA, with the animals under anesthesia, we collected blood by cardiac puncture in tubes with anticoagulant and centrifuged at 3,000 rpm for 20 minutes. Then, after the sacrifice, the right femur was removed, cleaned and weighted. Plasma samples and femur were frozen at -80°C for further analysis.

Serum concentrations of calcium, phosphorus and magnesium were determined by the colorimetric method using commercial kits (Bioclin, Belo Horizonte, Brazil) in automated equipment. Insulin, parathyroid and osteocalcin hormones were determined by enzyme linked immunosorbent assay (ELISA) using specific commercial kits (USCn, Life Science Inc) and the reading performed using Thermo Plater Reader.

The evaluation of the distance between the epiphysis and the width of the midpoint of the diaphysis was performed, in femur, with caliper and express in millimeters. Subsequent evaluation of the weight of the femur (g) were analyzed BMD (g/cm^2), BMC (g) and bone area (cm^2) using DEXA. The femur was placed (one at a time) in a container with rice to simulate soft tissue.¹⁴

The mineral composition of femur was performed from the ash production,¹⁵ which were subsequently acidified, heated and diluted in deionized water. Subsequently, calcium, phosphorus and magnesium analyzes were carried out by colorimetric method, using automated equipment.

The results are presented as mean and standard deviation. For the analysis of the comparison of means between groups were used ANOVA and Duncan as post-test. We work with a significance level of 5%. GraphPad InStat version 3.1 for NT Win/95 was used for analysis.

This project was submitted to the Ethics Committee responsible for research in animal laboratory at UFF, and has been approved under protocol number 0027/08. All animals were handled in accordance with the ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA).

Results

During the study period, it can be observed that the groups fed diets on free demand (C1 and HP1) showed increase in body weight ($P < 0.05$), while those who suffered food restriction (C2 and HP2) lost weight (fig. 1).

Regarding the daily diet intake, the control group (C1) showed higher feed intake ($P < 0.05$) compared to the other groups. While the group HP2 showed the lowest intake. The feed consumption of the group that received hyperproteic diet on free demand (HP1) was similar to the group C2 (fig. 2).

Regarding water intake, the groups that received hyperproteic diet, especially HP2, showed higher water intake ($P < 0.05$) when compared to the control group (C1). While water intake of the C2 group has

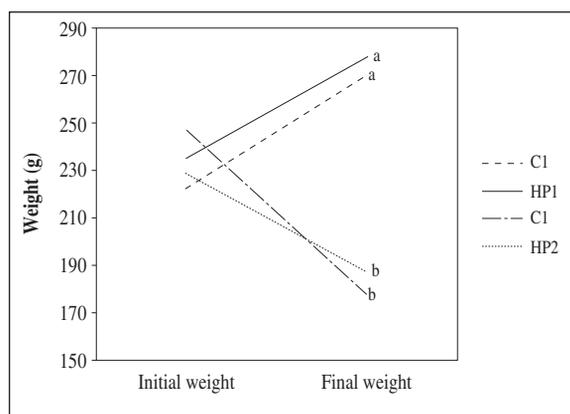


Fig. 1.—Evolution of body weight of the animals during all the experiment. Control group (C1); Hyperproteic diet (HP1). Groups C2 and HP2 submitted to 30% of food restriction. Different letters indicates statistical difference (ANOVA, $P < 0.05$). Niterói, in 2012.

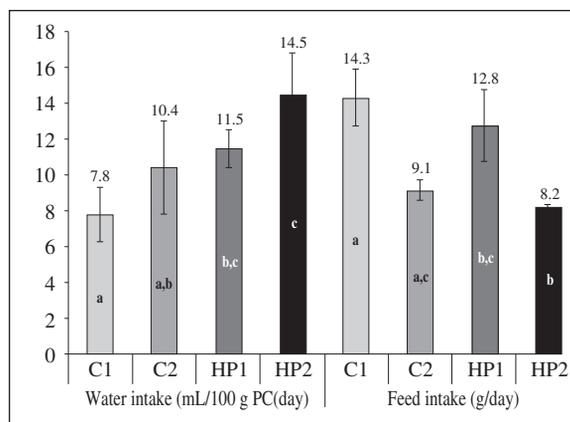


Fig. 2.—Daily feed intake and water intake of the animals. Control group (C1); Hyperproteic group 1 (HP1). Groups submitted to food restriction (30%), Control 2 (C2) and Hyperproteic 2 (HP2). Different letters denote statistical difference (ANOVA, $P < 0.05$). Niterói, in 2012

been similar to the groups that received diet on free demand (C1 and HP1) (fig. 2). It was observed also that the groups that received the “protein diet” showed diuresis higher than the other groups (observational data, not presented).

Serum level of the minerals and hormones are presented in table I. It can be observed that the groups that received hyperproteic diet (HP1 and HP2) showed lower serum calcium ($P < 0.05$) than C1 group. Regarding insulin, it was observed that the HP2 group showed lower ($P < 0.05$) serum level compared to the other groups and the osteocalcin was lower ($P < 0.05$) in the groups receiving hyperproteic diet (HP1 and HP2). The magnesium, phosphorus and parathyroid hormone level were similar between groups.

The densitometry of the total body shows that C2 and HP2 groups presented lower ($P < 0.05$) BMD, BMC and bone area compared to C1 and HP1 groups. When analyzing specific regions such as the pelvis, it

Table I
Minerals and hormones concentration at the end of the study

	C1	HP1	C2	HP2
Calcium (mg/dL)	8.22 ± 0.20 ^a	7.43 ± 0.17 ^b	7.76 ± 0.14 ^{ab}	7.15 ± 0.11 ^b
Magnesium (mg/dL)	1.84 ± 0.15	2.13 ± 0.25	2.27 ± 0.43	1.98 ± 0.10
Phosphorus (mg/dL)	5.08 ± 0.31	4.21 ± 0.20	4.12 ± 0.25	4.47 ± 0.31
Insulin (pg/mL)	1.45 ± 0.33 ^a	0.91 ± 0.39 ^a	0.64 ± 0.14 ^a	0.35 ± 0.13 ^b
Osteocalcin (ng/mL)	1.21 ± 0.47 ^a	0.38 ± 0.12 ^c	0.73 ± 0.13 ^b	0.33 ± 0.15 ^c
Parathormone (pg/mL)	8.48 ± 1.16	8.70 ± 1.43	9.04 ± 1.54	7.35 ± 1.59

Control group 1 (C1) and Hyperproteic group 1 (HP1), received diet on free demand. Control group 2 (C2) and Hyperproteic group 2 (HP2), restriction of food consumption (30%). Different superscript letters denote statistical difference (ANOVA, P < 0.05). Niterói, 2012.

Table II
Bone mineral density, bone mineral content and total area of the pelvis, spine and femur of the animals studied

	C1	HP1	C2	HP2
<i>Total body</i>				
BMD (g/cm ²)	0.16 ± 0.01 ^a	0.15 ± 0.01 ^a	0.14 ± 0.01 ^b	0.14 ± 0.01 ^b
BMC (g)	8.80 ± 0.36 ^a	8.28 ± 0.14 ^a	6.40 ± 0.15 ^b	6.82 ± 0.03 ^b
AREA (cm ²)	52.40 ± 1.80 ^a	52.60 ± 0.40 ^a	45.80 ± 0.91 ^b	46.60 ± 0.87 ^b
<i>Pelvis</i>				
BMD (g/cm ²)	0.16 ± 0.01 ^a	0.16 ± 0.01 ^a	0.13 ± 0.01 ^b	0.14 ± 0.01 ^b
BMC (g)	2.34 ± 0.16 ^a	1.98 ± 0.11 ^a	1.70 ± 0.11 ^b	1.84 ± 0.11 ^a
AREA (cm ²)	14.80 ± 0.58	12.20 ± 0.37	13.20 ± 1.07	13.0 ± 0.83
<i>Vertebral column</i>				
BMD (g/cm ²)	0.15 ± 0.01 ^a	0.15 ± 0.01 ^a	0.12 ± 0.01 ^b	0.12 ± 0.01 ^b
BMC (g)	1.78 ± 0.20 ^a	1.74 ± 0.05 ^a	1.12 ± 0.13 ^b	1.12 ± 0.12 ^b
AREA (cm ²)	12.00 ± 1.0	11.40 ± 0.50	9.40 ± 0.87	9.00 ± 0.83
<i>Femur</i>				
BMD (g/cm ²)	0.156 ± 0.01 ^a	0.146 ± 0.01 ^a	0.145 ± 0.01 ^a	0.132 ± 0.01 ^b
BMC (g)	0.400 ± 0.01 ^a	0.380 ± 0.02 ^a	0.370 ± 0.01 ^a	0.360 ± 0.03 ^b
AREA (cm ²)	2.80 ± 0.1	2.80 ± 0.1	2.71 ± 0.1	2.66 ± 0.2

Control group 1 (C1) and Hyperproteic group 1 (HP1), received diet on free demand. Control group 2 (C2) and Hyperproteic group 2 (HP2), restriction of food consumption (30%). Different superscript letters denote statistical difference (ANOVA, P < 0.05). Niterói, 2012.

was observed that the C2 and HP2 groups also had lower ($P < 0.05$) BMD when compared to C1 and HP1, whereas the pelvis BMC was lower ($P < 0.05$), only in the C2 group. For the region of the spine, the groups who suffered food restriction (C2 and HP2) showed lower ($P < 0.05$) BMD and BMC than the groups with free demand diet (C1 and HP1). However, when analyzing the femoral BMD and BMC of the HP2 group it can be observed that this was lower ($P < 0.05$) than the other groups (table II).

When analyzing the biometrical parameters of the femur it can be observed that there was no significant statistical difference in the weight and distance between the epiphysis. However, there was a reduction ($P < 0.05$) in the width of the midpoint of the femoral diaphysis in HP1 and HP2 groups, when compared with C1 and C2 groups. Similar results were found

when evaluating the mineral composition of the femur, HP1 and HP2 groups presented lower ($P < 0.05$) concentration of calcium in femur. Higher concentrations of ($P < 0.05$) magnesium was found in the groups receiving hyperproteic diet in relation to groups C1e C2 (table III).

Discussion

Professionals of the health area who recommend the “protein diet” claim that persons who use this type of diet may ingest more energy, and still lose weight. However, there is no scientific evidence to confirm that this diet has metabolic advantages over conventional diets for weight reduction and maintenance of bone health.¹⁶

Table III
Biometry measurements and mineral concentration of the femur

	<i>C1</i>	<i>HP1</i>	<i>C2</i>	<i>HP2</i>
Weight (g)	0.92 ± 0.01	0.93 ± 0.05	0.92 ± 0.01	0.84 ± 0.02
Distance between the epiphysis (mm)	3.15 ± 0.05	3.20 ± 0.05	3.20 ± 0.05	3.08 ± 0.04
Midpoint of the diaphysis (mm)	0.51 ± 0.02 ^a	0.45 ± 0.05 ^b	0.51 ± 0.02 ^a	0.46 ± 0.06 ^b
<i>Femur composition:</i>				
Calcium (mg/dL)	17.11 ± 2.63 ^a	12.38 ± 0.24 ^b	16.89 ± 3.42 ^a	11.59 ± 0.3 ^b
Magnesium (mg/dL)	4.00 ± 0.33 ^a	8.2 ± 1.09 ^b	3.88 ± 0.37 ^a	8.19 ± 1.42 ^b
Phosphorus (mg/dL)	7.37 ± 1.87	6.10 ± 0.97	5.08 ± 1.77	5.76 ± 1.03

Control group 1 (C1) and Hyperproteic group 1 (HP1), received diet on free demand. Control group 2 (C2) and Hyperproteic group 2 (HP2), restriction of food consumption (30%). Different superscript letters denote statistical difference (ANOVA, $P < 0.05$). Niterói, 2012.

In the present study, it can be noted that the hyperproteic diet seems to promote satiety when consumed on free demand. However, as this diet has higher energy content per gram, the lower feed intake by the HP1 group was not sufficient to promote weight loss; and when this diet is associated with dietary restriction, the result is similar to the conventional hypocaloric diet. It shows that it is not the “protein diet” that leads to weight loss, but the energy restriction imposed on the animals. Corroborating our results, studies show that in humans the consumption of “protein diet” on free demand, appears to increase satiety. However, in according to these authors this type of the diet can lead to weight loss, only, after prolonged periods.^{17,18} Some researches try explain the changes in metabolism that lead to the higher satiety. Studies suggest that lower carbohydrate intake may be related to excessive formation of ketone bodies, due to the change of the substrate used in the metabolism. Other factors suggested are that high protein intake leads to increase in serum concentration of amino acids that stimulate the release of anorexigenic hormones that act on satiety, and by the stimulus given by the amino acids to the cholecystokinin (CCK) secretion that could favor the reduction of food intake.^{1,19,20}

Metabolically, the largest precursor of ketone bodies is the Acetyl-Coenzyme A produced by the oxidation of fatty acids and by the ketogenic amino acids. Ketosis produced by this kind of diet can increase the osmolality and thus trigger the sensation of thirst and increase the water consumption.²¹ This fact was observed in the present study, where the animals receiving the “protein diet” had higher water intake than the control group, indicating that consumption of hyperproteic diet associated with energy restriction may promote increased production of ketone bodies, triggering higher water consumption and increasing the diuresis.

The protein and calcium are the major components of the bone tissue, so to maintain the process of bone remodeling and the synthesis of this tissue is necessary an adequate supplies of mineral and protein. Observa-

tional study found associations between protein intake and bone mass and show both, positive and negative effect.²² The bone densitometry realized in the present study show that the groups who were submitted to food restriction had a lower density and bone mineral content in the pelvis and spine, probably due to the high formation of ketone bodies and to the lower calcium intake, fact that is in accordance to the literature data. However, unlike what was observed in the pelvis and spine, the femur, of the hyperproteic group with dietary restriction was the more affected bone with a lower density and total bone mineral content and with reduction in the width of the midpoint of the femoral diaphysis; besides of the lower calcium content. This suggests that the high protein intake associated with dietary restriction increases the negative effects of diet on the bone tissue, even in adult animals due to the stimulation of bone calcium removal in order to minimize the effects of acidosis.

Some researchers suggest that the excessive dietary protein appears to have an adverse effect on the bone, only under conditions of low calcium intake,²³ what is in accordance with the negative effects observed in the hyperproteic group with dietary restriction. The physiological adaptations that may be associated to it is the hypercalciuria, due to decreased in tubular calcium resorption promoted by acidosis, increase in bone resorption and, consequently, the bone demineralization.^{22,24} However, contrary to our results, a study observed significant reduction in BMD and BMC of the femur in mice receiving hypocaloric diet.²⁵ This can also points to possible negative effects of hypocaloric diets with low calcium concentration.

Recent studies show that osteoblast have receptors for insulin that increase glucose uptake and the production of anabolic bone markers. However, it is known that low carbohydrate diets lead to lower insulin release, which may reflect in bone metabolism.²⁶ It may explain the negative results observed in the group receiving hyperproteic diet associated with dietary restriction (HP2), where the low concentration of carbohydrate in the diet decreased the stimulus to

insulin production and hence of the osteocalcin, due to the smaller stimulus for osteoblast activity, reflecting in the decrease in the width of the femur.

A study in rats shows that energy intake is a key factor for the maintenance of adequate concentrations of osteocalcin and bone formation.²⁷ Another study which emphasizes high fat diets showed that this type of diet is also able to inhibit bone formation and osteoblast activity.²⁸ Therefore, the “protein diet” has two important characteristics that can negatively influence the metabolism and bone formation: high concentration of proteins and lipids. However, the exact mechanisms by which these characteristics promote such changes still need to be better studied.

Conclusion

The results suggested that the “protein diet” promotes significant changes in femur and in the concentration of hormones related to the formation and maintenance of this tissue. These results may suggest that adult women, especially those in the premenopause and menopause, can expect to develop osteopenia and osteoporosis increased, due to the consumption of this type of diet.

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