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Effects of voluntary resistance exercise and high-protein snacks consisting of different proteins on bone mass and strength in rats given glucocorticoid-injections

Tatsuhiro MATSUO

Abstract

We examined the effects of a voluntary resistance exercise together with high-protein snacks on bone mass and strength in rats given glucocorticoid-injections as a model of age-related osteopenia. Forty-eight male Wistar rats were assigned to exercise or sedentary groups. One group was a control (C) and the other groups were glucocorticoid-injected groups. These groups were further divided into groups that received no snack (C and G), a high-protein snack containing 60% casein (GC and GEC) or containing 60% gelatin (GG and GEG). All groups were meal-fed at 8 : 30-9 : 30 am/pm. The snack was fed at 12 : 30-13 : 00 h for 9 weeks. The exercise groups (GEC and GEG) were allowed to climb. Bone mass and strength were increased by climbing with a casein snack. The gelatin snack decreased bone weight and protein content. These results suggest that resistance exercise and high-protein snacks with good amino acid-balance may protect against osteoporosis associated with aging.

Key words : resistance exercise, high-protein snack, bone mass, bone strength, glucocorticoid

Introduction

All living organisms age over time, resulting in a general decline in various biological and physiological functions. [1] Osteoporosis, characterized by bone loss leading to fractures and high bone turnover, is a serious problem for the elderly. [2] With age, more amino acids are absorbed from the digestive tracts and extracted by splanchnic tissues, which can result in a lower availability of dietary amino acids to the peripheral tissues. [3] It would be reasonable to hypothesize that in cases of low protein intake or increased protein requirement, this limited systemic availability of dietary amino acids could contribute to decreased bone protein synthesis, which could result in osteoporosis. Recently, in studies of bone protein synthesis and osteoporosis, glucocorticoid-injected rats are commonly used as a model of aging because glucocorticoid hormones are involved in the aging process. [4]

Supplementation of dietary proteins with a high insulinogenic sugar or carbohydrates after meals should increase amino acid supply to peripheral tissues. We previously reported that high protein snack feeding 3 h after regular meals increased total blood amino acid flow calculated by the area under the curve of diurnal amino acid concentration in glucocorticoid-injected rats. [5, 6] In addition, high protein snack together with resistance exercise showed significant preventive effect on glucocorticoid-induced sarcopenia and osteopenia [5, 6] However, these studies did not investigate detailed effects of exercise on bone mass and strength.

It has been suggested by many studies that exercise has a beneficial effect on bone in humans [7, 8] and animals. [9, 10] Because exercise is effective in maintaining bone mineral density in early postmenopausal women, it has been proposed for long-term prevention of osteoporosis. [7] Models of exercise for animals are used to examine the preventive or recovery effect of exercise on bone mass and strength as endpoints of an experiment. Animal studies using voluntary wheel running, [11] jumping, [12] treadmill running, [13] or voluntary climbing [9, 14, 15] have demonstrated the beneficial effect of increased load on bone mass and mechanical properties. Voluntary tower climbing is a light resistance exercise that creates little stress and strain and has been used in several studies. [5, 6, 9, 14–17] We previously demonstrated that voluntary tower climbing increased bone mass and strength mainly by increasing bone formation in growing, [14] orchidectomized, [15] ovariectomized, [16] or glucocorticoid-injected osteopenic rats. [5] The purpose of this study was to examine the preventive effects of voluntary climbing exercise together with a high-protein snack on bone mass and strength in glucocorticoid-induced aging model rats. More

over, we investigated the effect of amino acid composition of high-protein snack because bone protein consisted of many kinds of amino acids (essential and non-essential).

Materials and Methods

Animals and experimental design.

Forty-eight male Wistar rats (5 weeks old) were purchased from Japan SLC, Inc. (Shizuoka) and acclimatized for a week under standard laboratory conditions (22±2℃, 60% humidity). The light/dark cycle was 12 h with lights on from 8 : 00 h to 20 : 00 h. Rats were housed in metal cages with a wire-mesh tower (ϕ20 cm × 200 cm). Two water bottles were set at various heights in the tower to adjust the climbing exercise (5, 6, 9, 14-17). The bottles were available from 21 : 30 h to 8 : 30 h for 4 weeks. There were no bottles in the bottoms of the tower cages. At the beginning, the bottles were set at a height of 20 cm. The bottles were gradually elevated to 200 cm over 1 week. At the age of 10 weeks (body weight, 190-192 g), all rats were randomized by body weight into six groups. One group was a saline control (C, n=8). The other groups were a glucocorticoid-injected sedentary group (G, n=8); two glucocorticoid-injected sedentary groups with snacks (one with casein snacks-GC, n=8; one with gelatin snacks-GG, n=8) and two glucocorticoid-injected climbing groups with casein and gelatin snacks (GEC, n=8; GEG, n=8). Group C was given 2 ml/kg/day of saline and the other groups were given 2 mg/kg/day of prednisolone (Wako Pure Chemical Industries, Ltd., Osaka) intraperitoneally at 9 : 30 h. Groups C and G were fed a mixture of 5 g of commercial rat chow (CE-2, Japan CLEA, Inc., Tokyo), 1.5 g of a high casein protein snack (60% of casein and 40% of sucrose) twice a day (8 : 30-9 : 30, 20 : 30-21 : 30 h) for 9 weeks (from 10 to 19 weeks old). The GC and GEC groups were fed 5 g of CE-2 twice a day (8 : 30-9 : 30, 20 : 30-21 : 30 h) and 3 g of a high casein protein snack at 12 : 30-13 : 00 h. The GG and GEG groups were fed 5 g of CE-2 twice a day and 3 g of a high gelatin protein snack (60% of gelatin and 40% of sucrose) at 12 : 30-13 : 00 h. All rats received the experimental diets isoenergetically during the 9-week experimental period. The GEC and GEG groups exercised continuously in tower climbing cages for 9 weeks. The other groups were sedentary. In this study, the daily distances and time periods of climbing activity were not measured, but previous studies confirmed no significant differences among groups.[14, 15] Diurnal variations of serum amino acid levels were measured in each group between week 7 and week 8 of the dietary manipulation. Blood samples (150 ・l) were obtained from a tail artery at 04 : 00, 08 : 00, 12 : 00, 16 : 00, 20 : 00, and 24 : 00 h and put into capillary tubes for the determination of serum amino acid concentrations. At the end of the experiment (19 weeks), the rats were killed by decapitation at 10 : 00 h after overnight fasting and 24 h without exercise (GEC and GEG groups). Blood was collected to obtain serum, and bilateral femora were quickly removed and freed from connective tissues and measured for length, mid shaft width and wet weight.

Bone protein and calcium measurement.

Bone protein content was determined by Kjeldahl technique using automatic nitrogen/protein measurement system (Model VS-FA-1, Mitamura Industries, Ltd., Tokyo). Bone calcium was determined by flame atomic absorption spectrophotometry (AAS Z-5000, Hitachi, Tokyo) after dry-ashing at 550℃ and oxidizing at 100℃ with a mixture of 4 ml of 0.5 M H_2SO_4, 2 ml of 0.1 M HNO_3, 3 drops of concentrated $HClO_4$ (60%) and an excess (c. a. 0.3 ml) of 30 g/l $KMnO_4$. Samples were then diluted with 0.1 M HNO_3 and the concentration of calcium was determined by atomic absorption spectrophotometry.

Mechanical testing

A three-point bending test was performed as previously described[5, 18, 19] using a load tester (Rheoner, Model RE-33005, Yamaden, Co. Ltd., Tokyo). Each specimen of left femur was placed on a holding device with supports located at a distance of 12 mm, with the lesser trochanter proximal to, and in contact with, the proximal transverse bar. The mid point served as the anterior (upper) loading point. A bending force was applied by the crosshead at a speed of 0.1 mm/sec until fracture occurred. The breaking load (N) and structural stiffness (N/mm) were obtained directly from the load-deformation curves that were recorded continually in a computerized monitor linked to the load tester.

Serum analysis.

Serum amino acid concentrations were requested from Otsuka Pharmaceutical Co. (Saga, Japan). Serum cortisol

concentrations were determined using kits (Enzyme immunoassay for cortisol, Oxford Biomedical Research, Oxford, MI, USA). Serum alkaline phosphatase (ALP) activity was measured using kits (K-test, Wako Pure Chemical Industries, Ltd., Osaka).

Statistical analysis.

All values are expressed as mean±SE. Data were assessed by one-way ANOVA and Fisher's PLSD test. Statistical significance was set at p value of <0.05. All analyses were performed with a commercially available statistical package (StatView J-5.0, SAS Institute Inc., Cary, NC).

Results

Body weight, muscle weight and bone measurements

Final body weight in group C was significantly greater than those in the other groups (C, 282±7 ; G, 252±11 ; GC, 244±12 ; GG, 234±7 ; GEC, 258±16 ; GEG, 244 ±12 g). Gastrocnemius muscle weight in group C was also significantly greater than those in the other groups (C, 2.55±0.08 ; G, 2.32±0.09 ; GC, 2.24±0.15 ; GG, 2.17±0.07 ; GEC, 2.39±0.16 ; GEG, 2.23±0.10 g). Bone weight and structural measurements are shown in Table 1. Femoral dry weight was significantly heavier in group C, GEC and GEG than in the G, GC and GG groups (Table 1). Femoral midshaft width was significantly decreased but length was not influenced by glucocorticoid injections (Table 1). Chronic climbing significantly enhanced bone weight and midshaft width but did not alter bone length in the glucocorticoid injected groups (Table 1). High protein snack, whether containing casein or gelatin, influenced no structural parameters (Table 1).

Maximum load and structural stiffness

Femoral maximum load and structural stiffness were higher in group C than in group G (Table 1). Chronic climbing significantly enhanced bone maximum load and structural stiffness in the glucocorticoid injected groups (Table 1). In the sedentary groups, high-protein snacks, whether conteining casein or gelatin, had no influence on any mechanical parameters (Table 1). Femoral structural stiffness was highest in the GEC group (Table 1). Gelatin snack decreased the effects of climbing on bone strength (Table 1).

Bone protein and calcium content

Femoral protein and calcium content were higher in group C than in group G (Table 1). Climbing prevented the loss

Table 1 Femoral structual measurements, mechanical parameters and composition from each group of rats

Group	Rat number	Dry weight (mg)	Length (mg)	Midshaft width (mm)	Maximum load (N)	Structural stiffness (N/mm)	Protein (mg)	Calcium (mg)
C	8	423 ± 22^{a}	34.0 ± 0.4^{a}	$3.29\pm0.12^{a\,b}$	107 ± 8^{a}	$134\pm8^{a\,b}$	102 ± 6^{a}	105 ± 5^{a}
G	8	$398\pm24^{b\,c}$	$33.5\pm0.5^{a\,b}$	3.17 ± 0.10^{c}	$100\pm4^{b\,c}$	$129\pm11^{b\,c}$	$92\pm7^{b\,c}$	$99\pm6^{b\,c}$
GC	8	$393\pm26^{b\,c}$	$33.1\pm0.7^{b\,c}$	$3.19\pm0.11^{b\,c}$	97 ± 5^{c}	$128\pm8^{b\,c}$	$91\pm7^{c\,d}$	107 ± 7^{a}
GG	8	380 ± 21^{c}	32.8 ± 0.5^{c}	$3.19\pm0.09^{b\,c}$	$99\pm5^{b\,c}$	126 ± 9^{c}	88 ± 7^{d}	98 ± 7^{c}
GEC	8	420 ± 21^{a}	$33.6\pm0.5^{a\,b}$	3.35 ± 0.10^{a}	106 ± 6^{a}	141 ± 10^{a}	99 ± 5^{a}	106 ± 6^{a}
GEG	8	$413\pm15^{a\,b}$	$33.2\pm0.3^{b\,c}$	$3.28\pm0.08^{a\,b}$	$103\pm5^{a\,b}$	$131\pm11^{b\,c}$	$96\pm4^{a\,b}$	$104\pm5^{a\,b}$

Values are means±SD for 8 rats in each group. C, control ; G, glucocorticoid-injected sedentary; GC, glucocorticoid-injected sedentary with casein snack feeding ; GG, glucocorticoid-injected sedentary with gelatin snack feeding ; GEC, glucocorticoid-injected climbing exercise with casein snack feeding ; GEG, glucocorticoid-injected climbing exercise with gelatin snack feeding. Means with different superscripts within a column are significantly different at $p<0.05$ calculated by one-way ANOVA and Fisher's PLSD tests.

Table 2 Diurnal variation in serum essential amino acid concentrations for each group of rats

Group	Essential amino acid concentration (nmol/ml)						IAUC
	8 : 00 h	12 : 00 h	16 : 00 h	20 : 00 h	0 : 00 h	4 : 00 h	(nmol·h/ml)
C	1955 ± 120^{a}	2158 ± 151^{a}	2040 ± 259^{b}	1688 ± 155^{b}	2025 ± 148^{a}	1888 ± 252^{a}	94 ± 2097^{b}
G	1858 ± 236^{a}	2026 ± 195^{a}	1829 ± 186^{c}	$1539\pm204^{b\,c\,d}$	$1827\pm207^{b\,c}$	$1730\pm248^{a\,b}$	-1349 ± 2391^{b}
GC	1699 ± 145^{b}	1565 ± 154^{c}	2381 ± 279^{a}	2299 ± 361^{a}	$1717\pm176^{c\,d}$	1603 ± 171^{c}	4883 ± 3722^{a}
GG	1598 ± 215^{b}	$1630\pm65^{b\,c}$	1566 ± 189^{d}	1372 ± 152^{d}	1548 ± 125^{d}	1494 ± 166^{c}	-1523 ± 5856^{b}
GEC	1591 ± 163^{b}	1569 ± 73^{c}	2314 ± 87^{a}	2340 ± 237^{a}	$1757\pm151^{b\,c}$	$1622\pm158^{b\,c}$	6588 ± 3038^{a}
GEG	1649 ± 186^{b}	1758 ± 96^{b}	1614 ± 117^{d}	$1464\pm130^{c\,d}$	$1707\pm156^{c\,d}$	1551 ± 173^{c}	-609 ± 2943^{b}

Values are means±SD for 8 rats in each group. IAUC, Increment of Area under the curve based at 8 : 00 h. Means with different superscripts within a column are significantly different at $p<0.05$ calculated by one-way ANOVA and Fisher's PLSD tests.

of bone protein and calcium due to glucocorticoid injections (Table 1). Calcium content was higher in the GC group than in GG group (Table 1).

Serum cortisol and ALP concentration

Serum cortisol concentration obtained after sacrifice was not different among the groups (C, 103±34 ; G, 108±20 ; GC, 97±33 ; GG, 90±29 ; GEC, 100±19 ; GEG, 100±23 ng/ml). Serum ALP activity did not differ among the groups (C, 40.5±3.4 ; G, 38.1±2.0 ; GC, 42.2±3.3 ; GG, 39.5±2.9 ; GEC, 41.1±1.9 ; GEG, 40.6±2.3 K-A units).

Diurnal rhythm of serum amino acid levels

Diurnal variations in serum essential amino acid concentrations appear in Table 2. Casein snack increased serum essential amino acids dramatically, whereas serum essential amino acids were decreased by gelatin snack (Table 2). Increments of area under the curve at 8 : 00 am for essential amino acids were significantly higher in casein snack groups (GC and GEC) (Table 2).

Discussion

This study demonstrated that climbing with a high-protein snack containing casein prevented femoral bone loss and loss of mechanical strength in glucocorticoid-injected rats. However, the high-protein snack alone, even when well balanced, did not increase any preventive effect. These results suggest that chronic voluntary climbing is more effective than dietary protein supplementation in rats given glucocorticoid-injection. We previously demonstrated that climbing or high-protein snacks alone could not suppress glucocorticoid effects, but climbing together with snacks showed significant preventive effects on glucocorticoid-induced osteopenia.[5] These results support our previous findings.

We have demonstrated that glucocorticoid decreases rat body weight gain, which is caused by skeletal muscle atrophy. On the other hand, bone weights, protein and calcium contents in the GEC and GEG groups did not differ from those in group C. These results suggest that 9 weeks of climbing prevented glucocorticoid-induced osteopenia but did not avert glucocorticoid-induced muscle atrophy. Some researchers have indicated that resistance exercise initiated with or before glucocorticoid administration attenuates the subsequent muscle atrophy but does not prevent it.[20-22] To stimulate resistance exercise in animals, skeletal muscles were surgically removed and the effects of overload on the synergistic muscles were examined.[20-22] Using this ablation model of functional overload, Goldberg and Goodman[21] and Kurowski *et al.*[22] demonstrated significantly less atrophy in the rat skeletal muscle with simultaneous exercise and glucocorticoide. In addition, weight-lifting in rats induced by electric stimulation reduces glucocorticoid-induced muscle atrophy in the gastrocunemius muscle.[23] The discrepancies between our results and others could be due to the magnitude of the load on skeletal muscles or the length of the experimental period. The maximal load in our climbing exercise was rat body weight. This level of exercise may be too light to prevent glucocorticoid-induced muscle atrophy.

Many studies have been performed on the role of glucocorticoid in osteopenia and osteoporosis. Glucocorticoid induced osteoporosis is the result of a number of factors that adversely affect calcium homeostasis.[24-27] Systemic effects resulting in abnormalities in gonadal hormone secretion, calcium absorption, and renal handling of calcium and specific effects of glucocorticoids on bone all contribute to bone loss.[28, 29] In this study, serum ALP activity and cortisol concentration were not reduced by glucocorticoid injections, although bone weight and protein content were markedly reduced. These results may be due related to the amount of glucocorticoid injected. Because the effect of glucocorticoid hormone increases dose-dependently,[30] we previously examined rats given 1-10 mg/kg/day prednisolon (data not shown), leading to a dose of 2 mg/kg being selected. However, the amount of glucocorticoid injected should be reconsidered.

Gelatin is a mixture of small and large peptides with a typical amino acid composition of 30% glycine, 30% proline and hydroxyproline and absence of tryptophan.[31] There are few studies of the effects of dietary gelatin on lipid metabolism.[32-35] Aust *et al.*[32] described serum triacylglycerols lowering the effect of a mixture of casein and gelatin in rats. Gibney[33] found a hypocholesterolemic effect in rabbits. Oliveira *et al.*[34] reported that a diet containing 10% casein plus 10% gelatin accelerates atherogenesis in rats. Popescu *et al.*[35] reported a decrease of hepatic triacylglycerols, total- and free-cholesterol in rats fed a diet with gelatin (12%) and casein (8%) as the protein source. Notwithstanding the consumption of gelatin

the world over, very few studies deal with the effect of its ingestion on protein and amino acid metabolism. Thus, there is no consensus about its role in preventing osteopenia. Pitkanen *et al*.[36] demonstrated that the decrease in serum essential amino acid concentration is associated with decreased energy and protein intake with aging. Volpi *et al*.[37] showed that the phyenylalanine net balance increased from the basal state, with no differences after ingestion of 18 g essential amino acids or 40 g balanced amino acids (18 g essential amino acids plus 22 g nonessential amino acids). They concluded that essential amino acids are primarily responsible for the amino acid-induced stimulation of body protein anabolism in the elderly. These findings suggest that dietary essential amino acids are necessary for stimulating bone protein formation. We found in this study that gelatin snacks did not increase serum essential amino acids. Moreover, it was shown that gelatin snacks decreased femoral protein and calcium content. Gelatin is not an appropriate source of snack protein because it lacks essential amino acids.

High-protein (casein) snacks increased serum essential amino acid concentrations in groups GC and GEC. Increments of the area under the curve of these amino acids are higher in groups GC and CEC than in other groups. However, total area under the curve was not different among all groups (data not shown). These results suggest that an abrupt increase in serum amino acid might stimulate bone protein synthesis. However, few studies have examined the relationship between serum amino acid and bone formation. Bohe *et al*.[38] reported that 162 mg of mixed amino acid (kg body weight)$^{-1}$h^{-1} infused intravenuously for 6 h in healthy human caused muscle protein synthesis to respond rapidly to the increased availability of amino acids but was then (after 2 hours) inhibited, despite continued amino acid availability. Arnal *et al*.[39−42] demonstrated that pulse protein feeding (protein consumed mainly (80%) in one meal) restored stimulation of muscle protein synthesis during the feeding period in old rats and elderly women better than a spread protein feeding pattern (spreading daily protein intake over four meals). Our present findings do not contradict these previous results, although the mechanisms of bone protein synthesis may not be the same as those of muscle protein synthesis.

In conclusion, we show in this study that voluntary resistance exercise together with high-casein snacks increases bone mass and strength in rats given glucocorticoid injections, while high-gelatin snacks had little effect. These results suggest that resistance exercise and well-balanced high-protein supplementation may be an effective preventive therapy for osteoporosis associated with aging. Further studies will be required to address other unsolved problems.

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自発的レジスタンス運動と異なるタンパク質からなる高タンパク質間食がグルココルチコイド投与ラットの骨重量と骨強度に及ぼす影響

松尾達博

要　　旨

自発的レジスタンス運動（クライミング）と異なるタンパク質からなる高タンパク質間食が，グルココルチコイドを投与した老化モデルラットの骨重量と骨強度に及ぼす影響について検討した．５週齢Wistar系雄ラット48匹を６群に分け，その内の２群を運動群，残りを非運動群とした．非運動群の一群を対照群（C）として生理食塩水投与を投与し，残りの群にグルココルチコイド（プレドニソロン２mg/kg/day）を投与した．これらの群を間食なし群（C，G），60％カゼイン間食群（GC，GEC），60％ゼラチン間食群（GG，GEG）に分類した．全群のラットに８：30－９：30時，20：30－21：30時に食餌を摂取させ，間食摂取群には12：30－13：00時にそれぞれ２種類の高タンパク質間食を摂取させた．飼育室を8：00－20：00時に明期として，９週間飼育した．摂取エネルギー量と摂取タンパク質量が各群で等しくなるように摂食量を調節した．運動群（GEC，GEG）のラットについては，上部に飲水瓶を設置したタワーケージ

（φ20cm×200cm）に入れ，毎日９：30－20：30時にクライミング運動を実施させた．体重増加量はグルココルチコイド投与によって抑制された．骨量と骨強度はクライミング運動とカゼイン間食で増加した．ゼラチン間食は骨重量，骨長および骨タンパク質含量を低下させた．これらの結果から，クライミング運動とアミノ酸組成の良い高タンパク質間食は老化に伴なう骨減弱化を防止する可能性があることが示された．