Effects of Voluntary Resistance Exercise and High-protein Snack on Bone Mass, Composition, and Strength in Rats Given Glucocorticoid Injections

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We examined the effects of a voluntary resistance exercise (climbing) together with high-protein snacks (60% protein) on bone mass and strength in rats given glucocorticoid-injections (2 mg/kg/day) as a model of age-related osteopenia. Fifty-two male Sprague-Dawley rats, 8 weeks age, were assigned to exercise or sedentary groups. These groups were further divided into groups that received no snack, snack during activity or a snack during rest. All groups were meal-fed 7:30–8:30 h and 19:30–20:30 h and the snack was fed 23:30–0:30 h (active) or 11:30–12:30 h (resting). Energy and protein intake were approximately equal in all groups. The exercise groups were allowed to climb a wire-mesh tower cage (Φ20 cm × 200 cm) to drink water from a bottle set at the top. Weight gain during the 8-week experimental period was inhibited by a glucocorticoid-injection. Bone mass and strength were increased by climbing exercise with a high-protein snack, while no effect of snack nor any effect of snack timing was observed. Bone weight, calcium content and protein content were positively correlated to maximum load or structural stiffness. These results suggest that resistance exercise and high-protein supplementation may be a preventive therapy for osteoporosis associated with aging.

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

Aging is a process that all living organisms experience over time, resulting in a general decline in various biological and physiological functions.1) Osteoporosis, a serious problem in elderly people, is characterized by bone loss leading to fractures and high bone turnover.2) In the elderly, 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 in elderly persons. 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. Adrenalectomy attenuates the development of age-specific effects, such as lower amino acid availability in the peripheral tissues.4,5)

Protein supplementation with a high insulinoic carbohydrate after meals should increase the 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 the diurnal amino acid concentration in glucocorticoid-injected rats.6) In addition, a high protein snack together with resistance exercise showed significant preventive effects on glucocorticoid-induced sarcopenia and osteopenia.6) However, these studies did not investigate all the effects of exercise on bone mass and strength in detail.

Many studies suggest that exercise has a beneficial effect on bone in humans7,8) and animals.9,10) Because exercise is effective in maintaining bone mineral density in early postmenopausal women, it has been proposed for a long-term prevention of osteoporosis.1) Animal exercise models 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,1) jumping,1) treadmill running,1) or voluntary climbing2,14,15) have demonstrated the beneficial effect of increased load on bone mass and mechanical properties. Voluntary tower climbing is a low resistance exercise that
creates little stress and strain and has been used in several studies.\textsuperscript{5,9,14-17} We previously demonstrated that voluntary tower climbing exercise increased bone mass and strength mainly by increasing bone formation in growing,\textsuperscript{9} orchiectomized,\textsuperscript{15} or ovariectomized osteopenic rats.\textsuperscript{16} 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.

Materials and Methods

All procedures involving animals were approved by the Experimental Animal Care Committee of Kagawa University.

Animals and experimental design. Fifty-two male Sprague-Dawley rats (3 weeks old) were purchased from Japan SLC, Inc. (Shizuoka) and were acclimatized for a week under standard laboratory conditions (22 ± 2°C, 60% humidity). The light/dark cycle was 12 h with lights on from 7:00 h to 19:00 h. Rats were housed in metal cages with a wire mesh tower (φ20 cm × 200 cm) that had two water bottles set at the top to adjust climbing exercise\textsuperscript{5,9,14-17} from 20:30 h to 7:30 h for 3 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 set drink bottles were gradually elevated to 200 cm over 1 week. At the age of 8 weeks, all rats were randomized by body weight to seven groups (range of mean initial weights: 200–207 g). One group was a saline control (C, n = 7) and the other groups were a glucocorticoid-injected sedentary group (G, n = 7), a glucocorticoid-injected climbing exercise group (GE, n = 7), a glucocorticoid-injected sedentary with snack feeding groups (GA, n = 8; GB, n = 8), and glucocorticoid-injected climbing exercise with snack feeding groups (GEA, n = 8; GEB, n = 7). 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) suspended in an isotonic vehicle (1% aqueous carboxymethylcellulose in saline)\textsuperscript{18} intraperitoneally at 8:30 h. The C, G and GE groups were fed with a mixture of 5 g of commercial rat chow (CE-2, Japan CLEA, Inc., Tokyo), 1.5 g of a high protein snack (60% casein and 40% sucrose) twice a day (7:30–8:30, 19:30–20:30 h) for 8 weeks (from 8 to 16 weeks old). CE-2 contained the following components, in grams per 100 grams: moisture, 8.8; crude protein, 24.6; crude fat, 4.7; crude fiber, 3.6; crude ash, 6.0. The GA and GEA groups were fed with 5 g of CE-2 twice a day (7:30–8:30, 19:30–20:30 h) and 3 g of a high protein snack at 22:00–0:30 h. The GB and GEB groups were fed with 5 g of CE-2 twice a day with 3 g of a high protein snack at 11:00–12:30 h. All rats were fed the experimental diets isoenergetically during the 8 weeks of the experimental period. The GE, GEA and GEB groups exercised continuously in tower-climbing cages for 8 weeks. The C, G, GA and GB groups were sedentary. At the end of the experiment (16 weeks old), the rats were killed by decapitation at 10:00 h after overnight fasting. Blood was collected to obtain serum, and bilateral femora was 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 measured by the Kjeldahl technique using an automatic nitrogen/protein measurement system (Model VS-FA-1, Mitamura Industries, Ltd., Tokyo). Bone calcium was measured by flame atomic absorption spectrophotometry (AAS Z-5000, Hitachi, Tokyo) after dry-ashing at 550°C and oxidizing at 100°C with a mixture of 4 ml of 0.5 m H\textsubscript{2}SO\textsubscript{4}, 2 ml of 0.1 m HNO\textsubscript{3}, 3 drops of concentrated HClO\textsubscript{4} (60%) and an excess (c.a. 0.3 ml) of 30 g/l KMnO\textsubscript{4}. Samples were then diluted with 0.1 m HNO\textsubscript{3} and the concentration of calcium was measured by atomic absorption spectrophotometry.

Mechanical testing. A three-point bending test was done as previously described\textsuperscript{10,19} using a load tester (Rheoner, Model RE-3300S, Yamaden, Co., Ltd., Tokyo). Each specimen of a 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 midpoint 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 protein concentration, albumin concentration, albumin/globulin ratio and ALP activity were measured using kits (A/G B-test and 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 a p value of <0.05. All analyses were done 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 were significantly
Table 1. Body Weight and Structural Measurements of Femurs from Each Group of Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat number</th>
<th>Final body weight (g)</th>
<th>Femoral wet weight (mg)</th>
<th>Femoral dry weight (mg)</th>
<th>Femoral length (L) (mm)</th>
<th>Midshaft width (MW) (mm)</th>
<th>MW/L × 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>7</td>
<td>307 ± 6a</td>
<td>563 ± 11a</td>
<td>503 ± 11a</td>
<td>35.0 ± 0.3a</td>
<td>3.98 ± 0.06ab</td>
<td>11.4 ± 0.1ab</td>
</tr>
<tr>
<td>G</td>
<td>7</td>
<td>281 ± 6a</td>
<td>500 ± 17c</td>
<td>452 ± 16ab</td>
<td>33.9 ± 0.3b</td>
<td>3.84 ± 0.10bc</td>
<td>11.3 ± 0.2ab</td>
</tr>
<tr>
<td>GE</td>
<td>7</td>
<td>286 ± 5a</td>
<td>536 ± 9ab</td>
<td>483 ± 8ab</td>
<td>34.2 ± 0.2a</td>
<td>3.92 ± 0.04abc</td>
<td>11.5 ± 0.1ab</td>
</tr>
<tr>
<td>GA</td>
<td>8</td>
<td>279 ± 6a</td>
<td>487 ± 11c</td>
<td>440 ± 9b</td>
<td>33.8 ± 0.3b</td>
<td>3.76 ± 0.05c</td>
<td>11.1 ± 0.1b</td>
</tr>
<tr>
<td>GB</td>
<td>8</td>
<td>283 ± 3c</td>
<td>510 ± 16de</td>
<td>462 ± 14a</td>
<td>3.39 ± 0.3c</td>
<td>3.85 ± 0.06bc</td>
<td>11.4 ± 0.1bc</td>
</tr>
<tr>
<td>GEA</td>
<td>8</td>
<td>283 ± 3c</td>
<td>540 ± 13c</td>
<td>477 ± 15e</td>
<td>34.3 ± 0.2c</td>
<td>4.01 ± 0.05e</td>
<td>11.7 ± 0.1e</td>
</tr>
<tr>
<td>GEB</td>
<td>7</td>
<td>534 ± 6d</td>
<td>483 ± 5f</td>
<td>33.9 ± 0.2b</td>
<td>3.15 ± 0.03bc</td>
<td>11.4 ± 0.1b</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ±SE for 7-8 rats in each group.
C, control; G, glucocorticoid-injected sedentary; GE, glucocorticoid-injected climbing exercisers; GA, glucocorticoid-injected sedentary with snack feeding (23:00-03:30 h); GB, glucocorticoid-injected sedentary with snack feeding (11:00-12:30 h); GEA, glucocorticoid-injected exercise with snack feeding (23:00-03:30 h); GEB, glucocorticoid-injected climbing exercise with snack feeding (11:00-12:30 h). ns, not significant. 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. Femoral Protein and Calcium Content and Mechanical Parameters from Each Group of Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat number</th>
<th>Protein (mg)</th>
<th>Calcium (mg)</th>
<th>Calcium/Protein</th>
<th>Maximum load (N)</th>
<th>Structural stiffness (N/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>7</td>
<td>132 ± 4b</td>
<td>147 ± 7ab</td>
<td>1.11 ± 0.04</td>
<td>134 ± 5c</td>
<td>147 ± 2a</td>
</tr>
<tr>
<td>G</td>
<td>7</td>
<td>117 ± 5bc</td>
<td>137 ± 6d</td>
<td>1.18 ± 0.05</td>
<td>108 ± 10b</td>
<td>128 ± 6b</td>
</tr>
<tr>
<td>GE</td>
<td>7</td>
<td>123 ± 2ab</td>
<td>152 ± 5ab</td>
<td>1.24 ± 0.04</td>
<td>124 ± 5b</td>
<td>139 ± 6b</td>
</tr>
<tr>
<td>GA</td>
<td>8</td>
<td>114 ± 4c</td>
<td>140 ± 3f</td>
<td>1.23 ± 0.03</td>
<td>124 ± 3b</td>
<td>131 ± 5b</td>
</tr>
<tr>
<td>GB</td>
<td>8</td>
<td>118 ± 3ac</td>
<td>151 ± 4a</td>
<td>1.29 ± 0.03</td>
<td>121 ± 5b</td>
<td>129 ± 4e</td>
</tr>
<tr>
<td>GEA</td>
<td>8</td>
<td>125 ± 3bc</td>
<td>150 ± 7ab</td>
<td>1.20 ± 0.07</td>
<td>125 ± 4a</td>
<td>140 ± 4e</td>
</tr>
<tr>
<td>GEB</td>
<td>7</td>
<td>123 ± 3bc</td>
<td>159 ± 2a</td>
<td>1.30 ± 0.03</td>
<td>132 ± 4a</td>
<td>145 ± 4e</td>
</tr>
</tbody>
</table>

Values are means ±SE for 7-8 rats in each group. ns, not significant. Means with different superscripts within a column are significantly different at p < 0.05 calculated by one-way ANOVA and Fisher’s PLSD tests.

greater than those in the other groups (Table 1). Gastrocnemius muscle in group C was also significantly greater than those in the other groups (C, 3.19 ± 0.12; G, 2.87 ± 0.07; GE, 2.91 ± 0.05; GA, 2.85 ± 0.06; GB, 2.88 ± 0.07; GEA, 2.80 ± 0.06; GEB, 2.88 ± 0.07 g). Femur wet and dry weights were significantly heavier in group C than in the G, GA and GB groups (Table 1). Femoral lengths were decreased but midshaft width and MW/L were not influenced by glucocorticoid injections (Table 1). Chronic climbing exercise significantly increased bone weight and midshaft width but did not alter femoral length in the glucocorticoid injected groups (Tables 1). High protein snack feeding, whether fed in active or resting phases, influenced no structural parameters for the femur (Table 1).

Bone protein and calcium content
Bone protein content was significantly higher in group C than in group G, whereas bone calcium did not differ between groups C and G (Table 2). Femoral calcium/protein ratio did not differ among all groups (Table 2). Climbing prevented the loss of bone protein due to glucocorticoid injections (Table 2). Femoral calcium content was significantly increased by both climbing and high protein snacks (Table 2). No differences in bone calcium content were found between groups GA and GB, or groups GEA and GEB (Table 2).

Maximum load and structural stiffness
The results of mechanical tests are shown in Table 2. Femoral maximum load and structural stiffness were significantly higher in group C than in group G. Chronic climbing significantly enhanced bone maximum load and structural stiffness in the glucocorticoid injected groups. High-protein snacks, whether in active phase or resting phase, had no influence on any mechanical parameters.

Serum protein and enzyme activity
Serum albumin concentration was lower in groups C than in the other groups (C, 3.80 ± 0.04; G, 3.88 ± 0.05; GE, 3.86 ± 0.07; GA, 3.88 ± 0.07; GB, 3.81 ± 0.03; GEA, 4.03 ± 0.07; GEB, 4.06 ± 0.09 mg/100 ml). Serum total protein concentration and albumin/globulin ratio did not differ for any groups (serum protein concentration: C, 6.46 ± 0.11; G, 6.45 ± 0.14; GE, 6.38 ± 0.14; GA, 6.33 ± 0.21; GB, 6.12 ± 0.08; GEA, 6.54 ± 0.23; GEB, 6.43 ± 0.21 mg/100 ml, albumin/globulin ratio: C, 1.45 ± 0.06; G, 1.52 ± 0.05; GE, 1.54 ± 0.04; GA, 1.54 ± 0.08; GB, 1.54 ± 0.08; GEA, 1.66 ± 0.11; GEB, 1.73 ± 0.06). Serum ALP activity did not differ among any groups (C, 41.5 ± 4.3; G, 34.5 ± 4.3; GE, 35.1 ± 2.8; GA, 40.4 ± 3.6; GB, 46.7 ± 5.0; GEA, 39.5 ± 2.5; GEB, 40.5 ± 6.4 K-A units).

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**Relationship between structural parameters and mechanical test results**

Dry weight, midshaft width, calcium content, and protein content of femur were positively correlated to maximum load or structural stiffness (Fig. 1). Femoral length was not correlated to any mechanical test results (data not shown).

**Discussion**

This study demonstrated that climbing with a high-protein snack prevented femoral bone loss and loss of mechanical strength in glucocorticoid-injected rats. However, only the high-protein snack alone 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. These results support, at least in part, our previous findings, though the differences of effects of climbing and high-protein snacks on glucocorticoid-induced osteopenia remain between two studies. In this study,
the high-protein snack contained just 60% casein and 40% sucrose. These were no trace elements such as vitamins and minerals. The high-protein snack we used previously contained the following ingredients, in grams per kilogram: casein, 591.8; L-methionine, 9.0; mineral mixture, 70.0; choline bitartrate, 3.0; vitamin mixture 7.0; fiber, 50.0; corn starch 201.5; soybean oil, 67.7.6 This snack provided 60, 20, and 20% of energy as protein, fat, and carbohydrate, respectively. The discrepancy of snack composition between our studies might be due to the differences between the high-protein snacks. We offered the high-protein snack 3 h after regular meals everyday because trace nutrients could be present in blood or stages of metabolism in the rat body. Some studies22,23 report the importance of the time element for nutrients, with delayed supplementation of deficient nutrients failing to improve suppression of animal growth. A detailed study will be required to clarify this discrepancy.

We have demonstrated that glucocorticoid in rats decreases body weight gain, which is caused by skeletal muscle atrophy. On the other hand, bone weights, protein, and calcium contents in the GE, GEA and GEB groups did not differ from those in group C. These results suggest that 8 weeks of climbing prevented glucocorticoid-induced osteopenia but did not prevent glucocorticoid-induced muscle atrophy. Some researchers indicated that resistance exercise initiated with or before glucocorticoid administration attenuates the subsequent muscle atrophy but does not prevent it.22-24 To stimulate resistance exercise in animals, skeletal muscles were surgically removed and the effects of overload on the synergistic muscles were examined.22-24 Using this ablation model of functional overload, Goldberg and Goodman23 and Kurowski et al.26 demonstrated significantly less atrophy in the rat skeletal muscle with simultaneous exercise and glucocorticoid. In addition, weight-lifting in rats induced by electric stimulation reduces glucocorticoid-induced muscle atrophy in the gastrocneumius muscle.25 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 done on the role of glucocorticoid in osteopenia and osteoporosis. Glucocorticoid induced osteoporosis is the results of a number of factors that adversely affect calcium homeostasis.26-29 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.30,31 In this study, bone calcium content and serum ALP activity were not reduced by glucocorticoid injections, although bone weight and protein contents were markedly reduced. These results may be due to amount of glucocorticoid injected. Because the effect of glucocorticoid hormone increases dose-dependently,32 we previously examined rats given 1-10 mg/kg/day prednisolone (data not shown), leading to a dose of 2 mg/kg being selected. However, the amount of glucocorticoid injected should be reconsidered.

In conclusion, we show in this study that voluntary resistance exercise together with high-protein snacks increases bone mass and strength in rats given glucocorticoid-injection, while no effect of the snack only nor any effect of snack timing was observed. These results suggest that resistance exercise and high protein supplementation may be an effective preventive therapy for osteoporosis associated with aging. However, further studies will be required to address several unsolved problems.

References

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