Effect of Timing of Meal Intake after Squat Exercise Training on Bone Formation in the Rat Hindlimb

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Summary We hypothesized that bone acquisition was affected by the timing of meal intake after resistance exercise training. This was based on the following previous results: 1) Nutrient intake right after exercise resulted in an increase in muscle mass and a decrease in abdominal fat mass as well as muscle protein synthesis when compared to the intake of a meal later after the exercise; and 2) body composition has been proposed to be a good predictor of bone mass. To substantiate our hypothesis, 20 male rats were assigned to either a group fed a meal right after squat exercise (R) or a group fed a meal 4h after the exercise (L). The 10-wk training program consisted of approximately 70% of one repetition maximum for each animal, 15 repetitions per set, 10 sets per day, 3 d per week. As a result, hindlimb muscle mass in the R group was greater (p<0.05) than that in the L group and abdominal fat mass was less (p<0.01) in the R group as compared to the L group, regardless of there being no significant difference in body weight between the groups. Bone volume in the tibia (p<0.01) and femur (p<0.05) were both significantly greater in the R group than in the L group. Bone mineral content index (BMCI) and bone mineral density index (BMDI) in the tibia of the R group were significantly (p<0.05) greater than the corresponding values of the

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L group. The greater BMCI and BMDI in the tibia were positively and significantly ($p<0.05$) related with hindlimb muscle mass, but not with abdominal fat mass. There was no significant difference in BMCI and BMDI in the femur between the groups. These results suggest that the R regimen may contribute to increased bone acquisition in the tibia as compared to the L regimen, and this effect is partly due to the enlargement of muscle mass in the R group as compared to the L group.

**Key Words** bone mass, body composition, timing of meal intake, resistance exercise

The timing of protein intake after exercise has become an interesting issue in recent years. Biolo's study (1) demonstrated that the intravenous infusion of amino acid mixture right after exercise resulted in greater muscle protein synthesis when compared with the provision of amino acid at rest. Also, Okamura et al (2) indicated that the rate of protein synthesis in skeletal muscle was enhanced by the consumption of a mixture of amino acids and glucose right after exercise in comparison with the same supplement provided 2h after exercise. These findings imply that repeated early consumption of meals after exercise could accelerate a gain of skeletal muscle mass. Our previous study (3) using rats, in which the timing of meal intake was altered after exercise during a 10-wk period of resistance exercise training, substantiated this hypothesis. Meal intake right after squat-training increased muscle mass in the hindlimbs, accompanied by an accumulation of abdominal fat, as compared with provision of the same meal 4h after the exercise.

Bone mass has been demonstrated to be affected by body composition (4–12). Some investigators revealed body fat mass to be a good predictor of bone mass in subjects who are older, have a greater percentage of fat, and are physically inactive (5, 10, 11). Other investigations have found stronger relationships between bone mass and lean body mass (LBM) (8), and bone mass and regional muscle mass (9). Multiple regression analysis revealed LBM and regional muscle mass to be a better predictor of bone mass (8, 9). In either case, there has been a significant correlation between body composition and bone mass (4–12).

These previous studies (3, 4–12) proposed that the difference in timing of meal intake after exercise changed body composition and thereby resulted in altered bone acquisition. The purpose of the present study was to evaluate this hypothesis using the rats from a previous study (3) where the rats were fed a meal right after exercise or 4h after exercise during a 10-wk period of resistance exercise training. Thus, the data regarding bone measurement and bone mineral content and density were obtained and correlated to the difference in body composition of rats which were provided a meal at different times after exercising.
Effect of Timing of Meal Intake after Exercise on Bone Formation

MATERIALS AND METHODS

Animals. Twenty male Sprague-Dawley rats were purchased from Japan CLEA (Tokyo) at 4 wk of age. They were randomly assigned to either a group fed a meal right after weight-resistive exercise (Right eaters; R, n=10) or a group fed a meal 4 h after resistance exercise (Late eaters; L, n=10). Throughout the study, the rats were kept in a temperature (23±1°C) and humidity (60%)-controlled room with a 12 light/dark cycle (light from 07:00 to 19:00).

All rats were fed on an AIN-93G diet (Japan CLEA, Tokyo) twice a day. The animals in the R group were fed from 07:00 to 08:00 and 19:00 to 20:00, while the animals in the L group were fed from 11:00 to 12:00 and 19:00 to 20:00. The resistance exercise was performed from 06:00 to 07:00. A pair feeding system was employed to equalize food intake. Food intake and body weight were measured daily.

Exercise training. The resistance exercise (squatting) model developed for rats was employed. The exercise apparatus was basically prepared as previously reported (13). The apparatus allowed 10 rats to do exercise at the same time. All rats were gradually acclimated to the apparatus during the 3 wk before onset of the exercise training. One repetition maximum (1 RM) was measured for each rat. The initial load was 0.7 kg, which was about 70% 1 RM in the weakest rat. The squat exercise was conducted by giving electrical stimulation (10 V, 100 Hz for 0.3 s with 2-s intervals) to the tails of rats. The training consisted of 10 sets of 15 repetitions a day. Each set was separated by a 2-min resting period. The training was performed 3 times a week every other day for 10 wk. The 1 RM was measured every 2 wk to determine the optimal load. All procedures were conducted in conformity with the “Guide for the Care and Use of Laboratory Animals (1996).”

Muscle and bone preparation. Tissue collection was conducted immediately following euthanasia by decapitation 24 h after the last training session. Hindlimbs were removed at the hip joint trunk and skinned. Muscle samples such as soleus, gastrocnemius, plantaris, tibialis anterior, extensor digitorum longus and quadriceps were removed and trimmed of any visible connective tissue and fat. Each muscle tissue was quickly weighed using an electronic balance (HF-200, A & D, Tokyo, Japan). The right hindlimb was immersed in a 4% formaldehyde solution.

The right femur and tibia were isolated by removing the remaining soft tissues. These bones were dehydrated by immersion in 70, 90, 95 and 99% ethanol solution every 24 h, and thereafter dried at 45°C in an incubator for 5 d.

Gross bone measurements. Bone dry weight was measured on an electronic balance. Bone volume was determined using the water replacement method. Femoral and tibial length were measured with a caliper (Digimatic Solar, Mitsutoyo, Tokyo, Japan) from the greater trochanter to the distal medial condyle and the proximal intercondylar area to the medial aspect of the distal epiphysis, respectively. Maximal bone widths of the femur and tibia were measured at the midshaft point with the caliper.

Measurement of bone mineral content and density index. Bone mineral content
index (BMCI) and density index (BMDI) of the femur and tibia were measured by the analysis of digital images obtained with soft X-rays. This method, developed for rodent bone (14), was basically the same as the Microdensitometry method (15, 16). The bones were placed directly on printing paper (FUJIBRO WP FM3) and X-ray photographs of the bones were taken using a soft X-ray apparatus (SRO-405C, SOFRON, Tokyo, Japan). With respect to the conditions for taking photographs, the voltage and electric current of the beryllium radiation source, distance from radiation source to printing paper and exposure time for radiation were 40 kV, 3 mA, 450 mm and 180 s, respectively. Two aluminum ten-step wedges (0.25 mm thickness each step and 0.5 mm thickness each step) were also placed on printing paper to calibrate the density among X-ray photographs and thereby estimate BMCI. Images of soft X-ray photographs obtained were introduced into a personal computer (PC-9801 RA, NEC, Tokyo, Japan) via an image scanner (GT-6000, EPSON, Tokyo). Digital image processing and the border following method were used in this analysis. This method provides the bone length, bone area, BMCI and BMDI of each bone (14). BMCI was computed by summing up the degrees of light and shade from a total of 256 degrees in each pixel distributed in the bone area. BMDI was calculated by dividing BMCI by the number of pixels in the bone area. The BMCI and BMDI were measured for the whole bone and proximal 1/5, midshaft 3/5 and distal 1/5 areas of the bone. The coefficient of variation with this method in our laboratory was 2% for BMCI and 3% for BMDI.

Blood biochemical parameters. Blood samples were centrifuged and the serums obtained were frozen at −40°C until analysis. All blood parameters were determined as serum levels. Alkaline phosphatase (ALP) activity was measured using phenylphosphate as the substrate. 1,25-Dihydroxycholecalciferol (1,25-(OH)2D3) levels were determined by a radioreceptor assay (Nichols Institute Diagnostics, San Juan Capistrano). Osteocalcin levels were measured by the RIA method with a goat antibody specific to rat osteocalcin and highly purified rat osteocalcin as the standard and 125I-labeled tracer (Biomechanical Technologies, Stoughton, MA, USA). Calcitonin was assayed by an RIA kit (Calcitonin RIA “Mitsubishi,” Mitsubishi Chemical, Tokyo, Japan) with highly purified rat calcitonin as the standard. Intact parathyroid hormone (PTH) was determined by an immunoradiometric assay (Rat PTH Kit, IMMUTOPIC, San Clemente, CA, USA).

Statistical analyses. The values are represented by the means ± SD. Student’s t-test was used to test for significant differences between the two groups. The relationships between bone mass and body constituents were analyzed using simple correlations. All statistical analyses were computed using Stat Flex software (SFP version 2.0, JIP, Tokyo, Japan). Statistical significance was set at the 0.05 level.

RESULTS

Table 1 indicates gross measurements of the tibia and femur in the R and L groups. There was a tendency of greater dry bone weight for both tibia (p = 0.06)
Table 1. Gross measurements of tibia and femur of rats fed a meal right (R) or later (L) after exercise.

|                | R            | L            | p value |
|----------------|--------------|--------------|---------|
| Tibia          |              |              |         |
| Dry weight (mg)| 822 ± 43     | 784 ± 43     | 0.064   |
| Volume (μL)    | 600 ± 28     | 551 ± 43     | 0.008   |
| Length (mm)    | 44.4 ± 0.8   | 43.8 ± 0.9   | 0.148   |
| Midshaft width (MW)(mm) | 2.90 ± 0.10 | 2.79 ± 0.14 | 0.056   |
| MW/length × 100| 6.54 ± 0.23  | 6.37 ± 0.29  | 0.189   |
| Femur          |              |              |         |
| Dry weight (mg)| 992 ± 48     | 955 ± 43     | 0.090   |
| Volume (μL)    | 798 ± 48     | 741 ± 52     | 0.020   |
| Length (mm)    | 40.1 ± 0.6   | 39.6 ± 0.7   | 0.101   |
| Midshaft width (MW)(mm) | 5.03 ± 0.16 | 4.88 ± 0.23 | 0.119   |
| MW/length × 100| 12.6 ± 0.4   | 12.4 ± 0.6   | 0.407   |

Values are means ± SD for 10 rats in each group.

and femur (p = 0.09) in the R group in comparison with the L group. Bone volumes in both the tibia (p < 0.01) and femur (p < 0.05) were significantly greater in the R group than in the L group. Only midshaft width in the tibia tended to be greater (p = 0.06) in the R group than the L group. Except for this value, no tendency of difference or significant difference was observed in the bone length, midshaft width or midshaft width/length ratio of the tibia and femur between the two groups.

The whole and different areas of BMCI and BMDI in the tibia and femur are demonstrated in Table 2. The significantly (p < 0.05) greater whole BMCI in tibia of the R group as compared to the L group was accompanied by a significantly greater whole BMDI in the tibia of the R group (p < 0.05). Analyses of BMCI and BMDI at different areas of the tibia indicated that there was a significantly greater BMCI (p < 0.05), a tendency of greater BMDI (p = 0.06) in the mid area and a tendency of greater BMCI (p = 0.06) in the proximal area for the R group as compared with the corresponding data for the L group. With respect to femur BMCI and BMDI, only whole BMCI tended to be greater (p = 0.09) in the R group. Except for this value, there was no tendency nor significant difference between the groups for BMCI and BMDI in the whole femur as well as proximal, mid and distal areas.

Table 3 indicates several serum biochemical parameters relating to bone metabolism. There was no significant difference in the activity of ALP or levels of calcitonin, 1,25-(OH)₂D₃, PTH or osteocalcin between the groups.

At the end of the present study, as shown in the previous study (3), body weight didn’t differ between the R (484 ± 15 g) and L (477 ± 25 g) groups. However, a significant difference was observed for hindlimb muscle weight (p < 0.05) as well as abdominal fat tissue weight (p < 0.05) between the R and L groups. The abdominal
Table 2. Bone mineral content index (BMCI) and bone mineral density index (BMDI) of the tibia and femur of rats fed a meal right (R) or later (L) after exercise.

|                  | R                | L                | p value |
|------------------|------------------|------------------|---------|
| **Tibia**        |                  |                  |         |
| Whole BMCI (×10³) | 177.0±8.5        | 164.8±10.6       | 0.011   |
| Whole BMDI       | 62.1±2.7         | 59.4±2.4         | 0.027   |
| Proximal BMCI (×10³) | 52.2±4.2       | 48.8±3.4         | 0.059   |
| Proximal BMDI    | 65.6±4.7         | 63.2±2.8         | 0.181   |
| Midshaft BMCI (×10³) | 31.3±1.8        | 29.3±2.1         | 0.029   |
| Midshaft BMDI    | 62.8±4.3         | 59.6±2.8         | 0.062   |
| Distal BMCI (×10³) | 32.6±1.8        | 31.4±2.1         | 0.183   |
| Distal BMDI      | 66.1±3.4         | 65.5±2.2         | 0.657   |
| **Femur**        |                  |                  |         |
| Whole BMCI (×10³) | 223.5±15.6       | 212.9±10.2       | 0.087   |
| Whole BMDI       | 70.8±5.2         | 70.5±2.4         | 0.853   |
| Proximal BMCI (×10³) | 48.8±4.5        | 46.1±2.5         | 0.114   |
| Proximal BMDI    | 71.4±5.6         | 70.1±3.3         | 0.539   |
| Midshaft BMCI (×10³) | 39.0±1.9        | 37.9±3.2         | 0.359   |
| Midshaft BMDI    | 71.3±4.8         | 72.3±3.6         | 0.600   |
| Distal BMCI (×10³) | 57.7±5.2        | 55.5±2.8         | 0.255   |
| Distal BMDI      | 80.9±4.9         | 78.8±2.2         | 0.216   |

Values are means±SD for 10 rats in each group. BMCI and BMDI were measured by analysis of digital images obtained by soft X-rays. BMCI was computed by summing up the degrees of light and shade from a total of 256 degrees in each pixel distributed in the bone area. BMDI was calculated by dividing BMCI by the number of pixels in the bone area.

Table 3. Blood biochemical parameters in rats fed a meal right (R) or later (L) after exercise.

|                  | R                | L                | p value |
|------------------|------------------|------------------|---------|
| Alkaline phosphatase (IU/L) | 242.2±46.8  | 251.9±54.1       | 0.673   |
| 1,25(OH)₃ cholecalciferol (pg/mL) | 77.7±14.9    | 79.7±22.0        | 0.812   |
| Parathyroid hormone (pg/mL)    | 26.2±8.7      | 20.6±7.0         | 0.130   |
| Calcitonin (pg/mL)             | 101.0±24.9    | 96.9±34.1        | 0.759   |
| Osteocalcin (ng/mL)            | 61.8±9.8      | 62.7±9.4         | 0.845   |

Values are means±SD for 10 rats in each group.

Fat tissue weight, in terms of sum of epididymal, perirenal and mesenteric fat tissue weight in the L group, was significantly greater (p<0.05) than that in the R group (27.7±7.1 vs. 21.1±4.1 g). In contrast, hindlimb muscle weight in terms of sum of soleus, gastrocnemius, plantaris, tibialis anterior, extensor digitorum longus and quadriceps was significantly greater in the R group than in the L group (14.9±0.8 vs. 14.0±1.0 g, p<0.05).
DISCUSSION

The major finding in the present study was that the greater tibia BMCI and BMDI in the R group, as compared to the L group, was accompanied by increased hindlimb muscle mass and decreased abdominal fat mass. This result is consistent with those from investigations which demonstrated that body composition influences bone mass (4–12).

So far, it has been demonstrated that body composition, including fat mass and muscle mass, is an important contributor to bone mass (4–12). Some investigators (5, 10, 11) have shown a strong relationship between fat mass and bone mineral density (BMD) in subjects who are older, have a greater percentage of fat, and are physically inactive. Reid and associates (10, 11) have found total fat mass to be the best predictor of total bodily BMD and lumbar and femoral neck BMD. However, these studies also demonstrated a relationship between body weight and BMD. Increased body fat is often associated with increased body weight. The greater body weight has also been thought to result in greater bone mass by increasing the mechanical stress placed on the skeleton (11, 17, 18). Madsen’s study (8) also indicated that a significant positive correlation was found between fat mass and BMD as well as body weight and BMD in sedentary subjects. However, with the inclusion of trained athletes who often had greater muscle mass and less fat mass, none of the correlations between fat mass and BMD were significant. In accordance with this finding, tibia BMDI in our trained rats was not greater in the L group than in the R group, although the L group possessed more fat mass than its counterpart. This suggests that fat mass was not a determinant of tibia BMDI in the trained rats of the present study.

Several investigators (4, 6, 8, 12) have found that LBM is a better predictor of BMD than fat mass. Bevier et al (6) observed that only LBM was a significant predictor of lumbar spine BMD in regression analysis. Sowers and associates (12) also found that the greater the amount of LBM in the subjects, the greater the BMD, regardless of their fat mass. Large amounts of LBM resulted in greater femoral neck BMD in the subjects with both low and high amounts of fat mass. In the present study, the amount of LBM might have been greater in the R group than in the L group; abdominal fat mass was significantly lighter and hindlimb muscle was significantly heavier in the R group as compared to the L group despite no significant difference in body weight between the groups. The greater LBM in the R group might have contributed to the greater tibia BMCI and BMDI in this group.

Regional lean tissue mass (LTM) may be a more important contributor to regional BMD (7, 9). In cadavers, a significant positive correlation between psoas muscle weight and the ash weight of lumbar vertebrae has been found (7). Additionally, Nichols’s study (9) observed that LTM, as opposed to regional fat mass, was a better predictor of BMD. Regional LTM in both arm and leg was found to be significantly and positively correlated to the corresponding regional
BMD. Furthermore, multiple regression analysis resulted in only regional LTM being a significant predictor of corresponding BMD. In the present study, hindlimb muscle mass was significantly greater in the R group than in the L group. Also, a positive and significant correlation was found between hindlimb muscle mass and tibia BMCI ($r = 0.48; p < 0.05$) and hindlimb muscle mass and tibia BMDI ($r = 0.45; p < 0.05$), when the simple correlation coefficient was calculated. These results suggest that the significantly greater tibia BMDI in the R group may be, in part, explained by the significantly greater muscle mass in the R group. However, this relationship was not observed between hindlimb muscle mass and femur bone mass. Factors such as magnitude of the mechanical forces placed on the skeleton (17, 18) and muscle strength (19, 20) have also been shown to influence osteogenesis. When rats were trained using our apparatus, the mechanical forces might have been placed more on the tibia vs. femur, and/or muscular power might have been produced more in the crural muscle than the femoral muscle. This should be clarified by further investigation.

Bone strength has been indicated to depend not only upon bone mass but also on its architecture (21). If two bone cylinders have equal bone mass, the bone whose mass is distributed further away from the bending axis causes increased resistance to bending along the axis. The present study indicated that the tibia in the R group had not only a significantly greater bone volume ($p < 0.01$) but a tendency of greater midshaft width ($p = 0.06$) as compared with the corresponding data of the L group. This may imply that resistance to bending along the tibial axis is greater in the R group than in the L group.

Blood biochemical data in the present study could not explain why bone mass in the hindlimb was significantly greater in the R group than in the L group because there was no significant difference in these data between the groups. The biochemical data were obtained from the blood of the animals sacrificed 24 h after the last training session. Analyses of the blood sampled during a few hours after the resistance exercise would probably have been more important to investigate the mechanisms regarding the difference in bone acquisition between the R and L groups. Additionally, the measurement of testosterone, growth hormone and insulin-like growth factor I (IGF-I) in the blood is needed in further study because these biochemicals, which stimulate bone formation (22, 23), have been indicated to be elevated right after resistance exercise (24, 25).

With respect to muscle protein synthesis (1–3), nutrient intake right after exercise has been demonstrated to be more anabolic than when ingested at some later time. The present study suggests that this finding may be applicable to bone acquisition too. However, further investigation is needed to obtain detailed information regarding the mechanism.

In summary, the consumption of a meal right after resistance exercise training resulted in a greater BMCI and BMDI in the tibia as compared with the ingestion of a meal 4 h after the exercise training. Greater BMCI and BMDI in the tibia of the R group was associated with greater muscle mass in the hindlimb. The
exact mechanisms for these effects remain to be elucidated, and thus further studies are warranted.

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