Title:

The preventive effect of calcium supplementation on weak bones caused by the interaction of exercise and food restriction in young female rats during the period from acquiring bone mass to maintaining bone mass

Names of authors:

Yuki Aikawa [1], Umon Agata [1], Yuya Kakutani [1], Shoyo Kato [1], Yuichi Noma [1],Satoshi Hattori [1], Hitomi Ogata [2], Ikuko Ezawa [3], Naomi Omi [2]

Institutions:

[1] Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

[2] Faculty of Health and Sport Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

[3] Department of food and nutrition, Japan Women's University, 2-8-1 Mejirodai, Bunkyo-ku, Tokyo 112-8681, Japan

Running head:

High Ca effect on bone in inadequate food

Address for correspondence:

Naomi Omi, PhD

Institute of Health and Sports Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8574, Japan.

(TEL) +81-29-853-6319; (FAX) +81-29-853-6507

E-mail: ominaomi@taiiku.tsukuba.ac.jp

Abstract

Increasing calcium (Ca) intake is important for female athletes with a risk of weak bone caused by inadequate food intake. The aim of the present study was to examine the preventive effect of Ca supplementation on low bone strength in young female athletes with inadequate food intake, using the rats as an experimental model. Seven- week-old female Sprague–Dawley rats were divided into four groups: the sedentary and *ad libitum* feeding group (SED), voluntary running exercise and *ad libitum* feeding group (EX), voluntary running exercise and 30% food restriction group (EX-FR), and a voluntary running exercise, 30% food-restricted and high-Ca diet group (EX-FR+Ca). To Ca supplementation, we used 1.2% Ca diet as “high-Ca diet” that contains two-fold Ca of normal Ca diet. The experiment lasted for 12 weeks. As a result, the energy availability, internal organ weight, bone strength, bone mineral density (BMD), and Ca absorption in the EX-FR group were significantly lower than those in the EX group. The bone strength and Ca absorption in the EX-FR+Ca group were significantly higher than those in the EX-FR group. However, the bone strength in the EX-FR+Ca group did not reach that in the EX group. These results suggested that Ca supplementation had a positive effect on bone strength, but the effect was not sufficient to prevent lower bone strength caused by food restriction in young female athletes.

Keywords:

Young female athlete ・ Inadequate food intake ・ High Ca diet・ Bone strength ・ Growing rat

Introduction

Adequate food intake is important to maintain health, growth, and maturation and to minimize injury and optimize sports performance [1]. However, there are athletes who restrict food intake to reduce weight for endurance, aesthetic, and weight-class sports [2]. To prevent health problems caused by inadequate food intake in athlete, nutritional supplements are taken [3]. One of such nutritional supplements is calcium (Ca), preventing bone fragility in female athletes on low amount of energy intake [4,5].

Low bone strength, including the condition caused by the combination of exercise and reduced food intake, may cause stress fractures [6], and it is an important problem to address. It is generally recognized that exercise enhances bone strength by increasing mechanical loading [7,8]. However, for exercise to exert an anabolic effect on the bones, an adequate nutritional status is essential [9]. Females who exercise with an inadequate energy intake can suffer low bone mineral density (BMD) due to a reduction in energy availability (the amount of dietary energy remaining for other body functions after exercise training) [4]. In animal study, it has been reported that energy restriction decreases Ca absorption rate in female rats [10]. Therefore, female athletes on a low amount of energy intake may be needed to intake much Ca. Moreover, food restriction in itself induces a reduction in Ca intake. Therefore, female athletes with a risk of low bone strength by inadequate food intake for weight control should augment the amount of Ca

60 intake. Ca does not increase energy intake, so Ca supplementation has an advantage that does not increase
61 body weight.

62 Ca supplementation may be particularly important in adolescent [11] because this development period
63 is critical for acquiring bone mass [1,12]. It has been reported that higher the intake of Ca is associated
64 with significant gains in hip BMD and lower stress fracture rate [13]. Stear et al. [14] have reported that
65 Ca supplementation enhances the bone mineral status in adolescent girls. Dibba et al. [15] have found that
66 increased Ca intake increases bone mineral status in children. However, the effect of Ca supplementation
67 on the bones of young female athletes with a risk of low bone strength caused by inadequate food intake
68 is still unclear.

69 Using animals for bone studies makes it possible to control the conditions and directly measure
70 the bone strength and calcium absorption in a short period of time. Some studies have reported that
71 food restriction in mature female rats under an exercise regimen lowers the bone mineral content (BMC)
72 [16,17] and BMD [18]. Moreover, we have reported that in “young” female rats, the interaction of
73 voluntary running exercise and food restriction results in lower bone strength and lower BMD than
74 exercise or food restriction alone [19].

75 The aim of the present study was to examine the preventive effect of Ca supplementation on low bone
76 strength in young female athletes with inadequate food intake, using the rats as an experimental model. In

77 the present study, to Ca supplementation, we used 1.2% Ca diet as “high-Ca diet” that contains two-fold
78 Ca of normal Ca diet. We hypothesize that the Ca supplementation would prevent low bone strength.
79
80 **Materials and Methods**
81 Experimental design
82 Female Sprague-Dawley rats (n = 29, 7 weeks old) were randomly divided into four experimental
83 groups after a one-week acclimatization period. The groups included a sedentary and *ad libitum* feeding
84 group (SED, n = 7), a voluntary running exercise and *ad libitum* feeding group (EX, n = 7), a voluntary
85 running exercise and 30% food-restricted group (EX-FR, n = 7), and a voluntary running exercise and
86 30% food-restricted and “high-Ca diet” group (EX-FR+Ca, n = 8). The experiment period was 12 weeks.
87 The rats were purchased from CLEA Japan (Tokyo, Japan) and were fed the diet described in Table 1. The
88 SED, EX and EX-FR groups were given normal diet (0.6% Ca), while the EX-FR+Ca group was given
89 “high-Ca diet” (1.2% Ca). The EX-FR and EX-FR+Ca groups were fed a 30% restricted diet that was
90 calculated to contain 70% of the mean amount consumed in the previous week by the SED group. As a
91 result, the diet in EX-FR and EX-FR+Ca groups was restricted by the mean of 35% in comparison with
92 the EX group. The present study used 1.2% Ca diet as “high-Ca diet”, because the composition is
93 over two-fold of normal diet used in our previous study [19] and the AIN-93G diet is used as a
94 standard diet of growing rat [20]. Viguet-Carrin et al. [21] also used 1.2% Ca diet as high Ca diet

similarly. The SED group was individually housed in normal cages (15 × 25 × 19.5cm), while the EX, EX-FR, and EX-FR+Ca groups were individually housed with free access to voluntary running exercise on a wheel in the cage (wheel circumference, 1 m; cage, 27 × 35 × 35 cm). We used the voluntary running so that the changing daily running distance could be assessed. The room was maintained at 22 ± 1ºC under a constant 12:12 h light-dark cycle (light 8:00 to 20:00). Animal care and experimental procedures were approved by the Animal Experimental Committee of the University of Tsukuba.

<The position of Table 1>

Daily data collection and specimen harvesting

The body weight and dietary intake were measured every second day, and the running distance was measured every day. On the day prior to the dissection, all rats were made to fast for 12 h. Whole blood samples were collected from the abdominal aorta using syringes under diethyl ether anesthesia. Serum samples were separated by centrifugation at 2500 rpm for 20 min at 4ºC. The serum was frozen at -80ºC for the determination of bone metabolic markers. The abdominal fat, plantaris muscle, soleus muscle, uterus, adrenal gland, thymus, femur, tibia and the lumbar spine were collected from each rat after death. Femurs were collected, the adhering connective tissues were removed, and the bone strength was immediately measured. Subsequently, the femur was dried at 100°C for 24 h in the electric furnace, and

their dry weight was measured. Next, the dried femurs were burned to ash at 600°C for 15 h, and the ash weight was measured. The tibia and lumbar spine were stored in 70% ethanol after being harvested and cleaned of soft tissue for the measurement of the BMC, bone area, and BMD. The animals were placed in individual metabolic cages (24 × 20 × 18 cm) on the 80th and 81st day, just before the end of the experimental period. Urine and fecal was collected over two 24-h periods. Urine was collected under acidic conditions using 2 mL 2N hydrochloric acid. The urine was centrifuged at 2500 rpm for 15 min to eliminate refuse.

Calculation of energy availability

The energy intake, exercise-induced energy expenditure, and energy availability was calculated as previously described [19]. Energy intake was calculated by multiplying the amount of normal diet intake (3.73 kcal/g) or "high-Ca diet" intake (3.68 kcal/g). Exercise-induced energy expenditure due to daily wheel-running was calculated as 5.0 kcal/kg body weight times the number of km run [22], as in the previous study [16] (Exercise-induced energy expenditure energy expenditure = wheel-running distance ×body weight × 5.0 kcal / kg body weight / km). Energy availability was calculated as energy intake minus exercise energy expenditure [16].

Evaluation of estrous cycle by spectral analysis of the running distance

131 The estrous cycle by spectral analysis of the running distance was evaluated as previously described
132 [19]. The analysis was performed to determine whether the running distance increased every 4 or 5 days
133 to assess the estrous cycle. To remove the slowly varying baseline from the data for the voluntary
134 wheel-running distance, we used empirical mode decomposition (EMD) [23], a new adaptive data
135 analysis method for analyzing nonlinear and non-stationary data. The signal was decomposed into several
136 basic components called intrinsic mode functions (IMFs), and the residual signal was understood as the
137 signal trend.
138 First, we used the voluntary wheel-running distance data after 30 days because, according to a
139 previous study [16], estrus dysfunction due to food restriction with running exercise appears after 30 days.
140 Second, we analyzed the data using the EMD method and re-created the data set to extract the residual
141 signal trend and the lowest-frequency IMF component from the original data. Third, we analyzed the data
142 with maximum entropy spectral analysis, using the final prediction error criterion for optimal order
143 selection. Last, to exclude the inter-individual differences in the total power affecting the local power, we
144 also computed the proportion of the power spectrum of the running distance from 0.2 to 0.3 Hz in the
145 total power spectrum, because some previous studies have shown that female rats have a 4- or 5-day
146 running cycle in association with the estrus cycle, and their running activity is high during proestrus
147 [16,22]. If the running distance increases cyclically every 4 or 5 days, the proportion of the power
148 spectrum of the running distance from 0.2 to 0.3 Hz in the total power spectrum will be high. In contrast,

149 a minimal wheel-running fluctuation has been reported in anestrous female rats [16].Therefore, if the
150 female rats are anestrous, the proportion of the power spectrum of the running distance from 0.2 to 0.3 Hz
151 in the total power spectrum will be low.
152
153 Measurement of bone strength using three-point bending test
154 The strength of the femoral mid-shaft was assessed using a three-point bending test (DYN-1255, IIO
155 DENKI, Tokyo, Japan) as previously described (distance between the fulcrums, 1 cm; plunger speed, 100
156 mm/min; full scale, 50 kg; chart speed, 120 cm/min) [24]. Breaking force refers to the loading weight
157 (gravitational acceleration) required for bone breaking. Breaking energy refers to the workload that
158 results in the breaking of the bone.
159
160 Measurement of BMC, bone area and BMD using dual-energy X-ray absorptiometry
161 The BMC, bone area, and BMD of the tibia and L3-L6 lumbar spine were measured using
162 dual-energy X-ray absorptiometry (DXA; Aloka, DCS-600R, Tokyo, Japan) as previously described [25].
163 The tibia was divided into five divisions, and the first division from the upper side was considered the
164 proximal metaphysis site. The second and third divisions from the upper side were considered the
165 diaphysis site. The tibia at the metaphysis site contains mainly cancellous bone, and the tibia at the
166 diaphysis site contains mainly cortical bone.

167

168 Ca balance study

169 Ca balance study was measured as previously described [26]. All feces were burned to ash at 600°C

170 for 15 h, and the fecal matter was dissolved in 1N nitric acid. Ca content in urine and feces was measured

171 using inductively coupled plasma atomic emission spectroscopy (ICAP–AES; 575 V, Nippon Jarrell-Ash).

172 Ca absorption and Ca accumulation were calculated using Ca intake and the fecal and urinary excretion of

173 Ca. Amount of Ca absorption (mg/day) = Ca intake minus fecal Ca excretion. Rate of Ca absorption (%)

174 = amount of Ca absorption divided by Ca intake multiplied by 100. Amount of Ca accumulation (mg/day)

175 = amount of Ca absorption minus urine Ca excretion. Rate of Ca accumulation (%) = amount of Ca

176 accumulation divided by Ca intake multiplied by 100.

177

178 Statistical analysis

179 All data were expressed as mean ± standard error (SE). Statistical analysis was carried out using

180 one-way analysis of variance (ANOVA). In any analysis, if significant difference were observed, the

181 variables were analyzed using the Tukey's post-hoc comparison tests. SED group data were not included

182 in ANOVA. Unpaired t tests were used to compare results for SED group and EX group to assess the

183 effect of exercise. The significance level was set at $p<0.05$. All statistical analyses were performed using

184 SPSS Statistical Packages (Ver. 19.0; SPSS Inc., Chicago, USA).

185

186 **Results**

187

188 *<The position of Figure 1>*

189 *<The position of Table 2>*

190

191 Food intake, Ca intake, running distance, and energy availability

192 Figure 1 presents the change in food intake (Fig. 1A), running distance (Fig. 1B), and energy

193 availability (Fig. 1C) during the experimental period. The food intake, running distance, and energy

194 availability are expressed as the mean of the weekly average. Food intake in the EX-FR and EX-FR+Ca

195 groups continued was restricted throughout the experimental period. The average values of food intake,

196 Ca intake, and energy intake in the EX group were significantly higher than those in the SED group. The

197 food intake, Ca intake, energy intake, the percentage of the power spectrum of the running distance, and

198 energy availability in the EX-FR group were significantly lower than those in the EX group. The Ca

199 intake in the EX-FR+Ca group was significantly higher than those in the EX-FR group.

200

201 *<The position of Table 3>*

202

Body weight and internal organ weight

Figure 1D shows the changes in body weight during the experimental period. The body weight is expressed as the mean weight at the beginning of the week. The increase in body weight in the EX-FR and EX-FR+Ca groups were suppressed.

The body weight and internal organ weight at dissection are presented in Table 3. The body weight and abdominal fat weight in the EX group were significantly lower than those in the SED group. The soleus muscle weight in the EX group was significantly higher than those in the SED group. The body weight, abdominal fat, plantaris muscle weight, soleus muscle weight, uterus weight, and adrenal gland weigh in the EX-FR group were significantly lower than those in the EX group. There were no significant differences between these parameters in the EX-FR and EX-FR+Ca group.

<The position of Figure 2>

Bone strength, BMD, bone weight, BMC, and bone area

The breaking force and breaking energy of the femur are presented in Figure 2. The breaking energy as well as the breaking force of the femur in the EX group were significantly higher than those in the SED group. The breaking force and energy in the EX-FR group were significantly lower than those in the EX group, while the breaking energy of femur in the EX-FR+Ca group was significantly higher than that in

the EX-FR group. However, the breaking energy of femur in the EX-FR+Ca group was significantly lower than that in the EX group.

<The position of Figure 3>

The BMD of the lumbar spine, total tibia, proximal metaphysis tibia, and diaphysis tibia are presented in Figure 3. Those in the EX-FR group were significantly lower than those in the EX group. There were no significant differences between the EX-FR and EX-FR+Ca group.

<The position of Table 4>

The bone weight, BMC, and bone area are presented in Table 4. The BMC of the diaphysis tibia, bone area of the diaphysis tibia, and of total tibia in the EX group were significantly higher than those in the SED group. The dry weight of femur, ash weight of the femur, BMC of the lumbar, bone area of the lumbar, BMC of the total tibia, bone area of the total tibia, BMC of the proximal metaphysis tibia, bone area of the proximal metaphysis tibia, BMC of the diaphysis tibia, and bone area of the diaphysis tibia in the EX-FR group were significantly lower than those in the EX group. There were no significant differences between these parameters in the EX-FR and EX-FR+Ca group.

239

240 *<The position of Table 5>*

241

242 Ca balance

243 Table 5 presents the results of Ca balance analysis. The amount of Ca absorption and accumulation in

244 the EX-FR group were significantly lower than those in the EX group. The urinary Ca excretion, fecal Ca

245 excretion, amount of Ca absorption, and amount of Ca accumulation in the EX-FR+Ca group were

246 significantly higher than those in the EX-FR group.

247

248 **Discussion**

249 The aim of the present study was to examine the preventive effect of Ca supplementation on the low

250 bone strength in young female athletes with inadequate food intake, using the rats as an experimental

251 model. To Ca supplementation, we used 1.2% Ca diet as “high-Ca diet” that contains two-fold Ca of

252 normal Ca diet. Our data demonstrated that the “high-Ca diet” resulted in higher bone strength in these

253 animals in comparison with similarly exercised rats kept on a restricted diet with normal Ca levels.

254 However, the body weight, estrous cycle, BMD, bone weight, BMC, and bone area were not affected by

255 “high-Ca diet”.

256 Our previous study using young female rats has determined that the voluntary running exercise resulted in
257 higher bone strength, and the interaction of voluntary running exercise and food restriction lowered
258 energy availability, internal organ weight, bone strength, and BMD in comparison with exercise or food
259 restriction alone [19]. The previous study was performed in young female rats aged 8-20 weeks. Sengupta
260 et al. suggested that female SD rats acquired peak bone mass at 3 months [27]. Therefore, it is considered
261 that the period of our previous study was from the time of acquiring bone mass to maintaining bone mass.
262 The present study newly examined the effect of a “high-Ca diet” on the bone characteristics of young
263 female rats kept under voluntary running exercise and food restriction during the period from acquiring
264 bone mass to maintaining bone mass.
265 The present study used 1.2% Ca diet as high Ca diet to Ca supplementation. It is unclear whether 30%
266 food restriction in the present study may have caused an inadequate Ca intake in young female rats under
267 exercise or sedentary. However, it was lowly probable that the food restriction caused a calcium
268 deficiency to young female rat with exercise, because they took Ca as much as the amount of 0.4% Ca
269 diet with *ad-libitum* feeding. Hunt et al. [28] have reported that 0.2% and under Ca diet impair bone
270 growth in young female rat, and this report supports our assessment. Moreover, as a result, the EX-FR+Ca
271 group fed the mean +41% Ca compared to the SED group, fed the mean +28% Ca compared to the EX
272 group, and fed the +99% Ca compared to EX-FR group. Therefore, we considered that intake of 1.2% Ca

diet in young female rats with exercise and food restriction performed not a relief of Ca deficiency but an intake of rich calcium for them.

The energy intake and energy availability were not affected by “high-Ca diet”. Energy availability is defined as energy intake minus exercise-induced energy expenditure, and low energy availability suppresses various physiological functions, including cellular maintenance and growth [29], thus decreasing the total energy expenditure [30].We concluded that the body weight and internal organ weight were not significantly different in the EX-FR and EX-FR+Ca group because of similar energy availability levels in these groups. We also considered that “high-Ca diet” did not prevent reproductive dysfunction due to similar uterus weight and the proportion of the power spectrum of the running distance from 0.2 to 0.3 Hz in the total power spectrum.

The results demonstrated that a “high-Ca diet” led to high bone strength in young female rats under voluntary running exercise and food restriction (Fig. 2). In previous studies using young female rats, Hunt et al. [26] have reported that 0.1 % Ca diet and 0.2 % Ca diet lowered the bone strength in comparison with a 0.3-0.7 % Ca diet. Viguet-Carrin et al. [21] reported that a 0.2% Ca diet has a detrimental effect on bone strength; however, 1.2 % Ca diet has a positive effect on bone strength at a constant Ca/P ratio. As previously described, we considered that food restriction didn’t cause a calcium deficiency to young female rat with exercise. Thus, it is highly probable that the positive effect of “high-Ca diet” on bone strength in exercising young female rat with food restriction was caused by the effect of high amount of

291 Ca intake similar to Hunt et al. study in sedentary *ad-libitum* feeding rats. However, the bone strength in
292 exercising young female rat with food restriction and “high-Ca diet” was lower than that in the rat with
293 ad-libitum feeding and normal Ca diet. Furthermore, the breaking energy of femur in the EX-FR+Ca
294 group was significantly higher than that in the EX-FR group; however this was not the case for the
295 breaking force of the femur. Lin et al. [31] have similarly reported that the maximal load energy of
296 the femur was significantly different between groups but the maximal load of the femur was not
297 significantly different between groups using the three-point bending test in a study to determine the
298 effects of a mechanical loading course on bone. They have also reported that the cortical area and
299 thickness of the femur were significantly difference similar to the result of the maximal load, so their
300 parameter may be factors of changing the maximal load in their study. However, the results of our
301 study on bone did not show a change with similar breaking energy; therefore, it is unclear how a
302 “high-Ca diet” caused a difference in the results of the breaking force and energy in the present study.
303 BMD is frequently used as a proxy measure of bone strength and accounts for approximately 70% of
304 bone strength [32]. BMD is the main factor that determines bone strength. In the present study, a
305 “high-Ca diet” had no effect on the BMD in young female rat with exercise and food restriction (Fig. 3).
306 This result is agreement with previous studies using young female [21] and young male rats [33].
307 Moreover, the “high-Ca diet” had no effect on the bone weight, BMC, bone area (Table 4); this result is
308 similar to those from the previous studies using young female rats [21,34]. The study of Viguet-Carrin et

309 al. has reported that a high-Ca diet has a positive effect on bone strength but does not affect the BMD or
310 bone microarchitectural parameters. The authors have concluded that a high-Ca diet has a beneficial
311 effect on the attainment of peak bone strength with no evidence of a detrimental effect on bone modeling,
312 at least in the short term. [34]. In the current study, we did not analyze the mechanisms by which the
313 “high-Ca diet” induces high bone strength. However, our results suggested that the “high-Ca diet”
314 induced high bone strength may be associated with factors other than BMD, bone weight, BMC, or bone
315 area. Bone quality also accounts for bone strength [32]; therefore, bone architecture, turnover, damage
316 accumulation, and mineralization might be altered by a “high-Ca diet”.
317 We tested the Ca balance to confirm the high Ca intake link to high Ca absorption. Food restriction
318 reduces Ca intake. In the current study, food restriction lowered amount of Ca absorption, but did not
319 lower the rate of Ca absorption in young female rat with voluntary wheel running (Table 4). Energy
320 restriction reduces fractional calcium absorption [10]. However, low Ca intake induces high Ca
321 absorption rate, and high Ca intake induce low Ca absorption rate [35,36]. We considered that food
322 restriction did not significantly lower the rate of Ca absorption in young female rat with voluntary wheel
323 running because the restriction resulted in low energy intake and low Ca intake. However, for low amount
324 of Ca intake, food restriction lowered the amount of Ca absorption. “High-Ca diet” caused high amount
325 of Ca absorption and did not cause lower the rate of Ca absorption in young female rat with voluntary
326 wheel running on food restriction. These results did not agree with the data from some of the previous

studies in sedentary rats [35,36]. Our data suggest that the effect of “high-Ca diet” on Ca absorption is different in sedentary rats with ad-libitum feeding compared to exercising rats on food restriction. We also found that “high-Ca diet” prevented the lower amount of Ca accumulation in young female rat with voluntary wheel running and food restriction. Nevertheless, the bone weight and BMC did not significantly differ between the normal Ca diet and “high-Ca diet” in young female rat with voluntary wheel running and food restriction. These results might suggest that absorbed Ca is accumulated to organs excepting bone in young female rats eating a “high-Ca diet” with voluntary wheel running and food restriction.

In conclusion, this is first study to examine the preventive effect of Ca supplementation on low bone strength in young females actively exercising on a restricted diet, using the rat as an experimental model and high-Ca diet. Food restriction caused lower energy availability, internal organs weight, bone strength, BMD, bone weight, BMC, bone area, Ca absorption, and Ca accumulation in these rats. “High-Ca diet” induced higher bone strength, Ca absorption, and Ca accumulation in comparison with the normal Ca diet in the rats. However, this bone strength did not reach the bone strength in the rat with *ad-libitum* feeding and normal diet. These results suggest that Ca supplementation had a positive effect on bone strength, but the effect was not sufficient to prevent lower bone strength caused by inadequate food intake in young female athletes during the period from acquiring bone mass to maintaining bone mass.

345 **Conflict of interest**

346 The authors declare that they have no conflict of interest.

347

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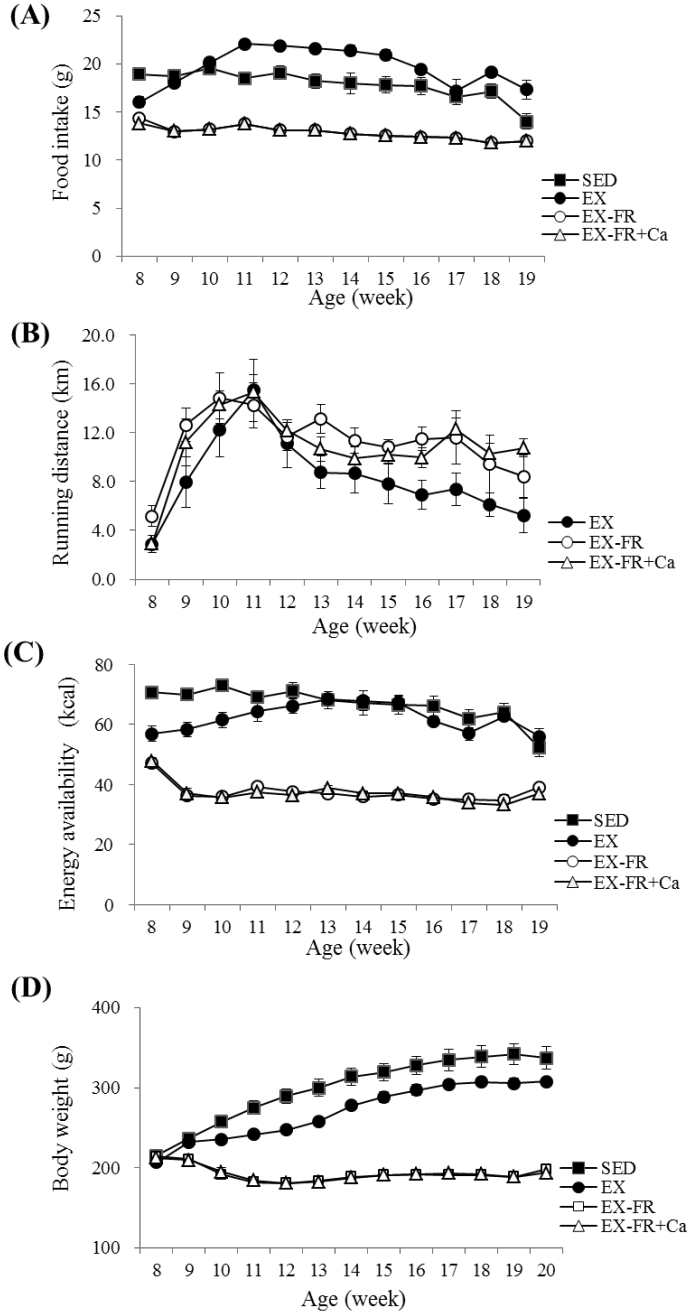
449 **Figure legends**

Figure. 1



450

451 **Figure 1. Changes in food intake (A), running distance (B), energy availability (C), and body weight**

452 **(D).**

SED: sedentary group. EX: exercise group. EX-FR: exercise + food restriction group. EX-FR+Ca: exercise + food restriction + high-Ca diet group. Values are means ± SE. The small SEs may not be visible. Body weight, food intake, and energy availability were measured every other day, and running distance was measured every day. The values of food intake, running distance and energy availability are expressed as the means of the weekly averages for each parameter. The body weight is expressed as the mean weight at the beginning of every week.

Figure. 2

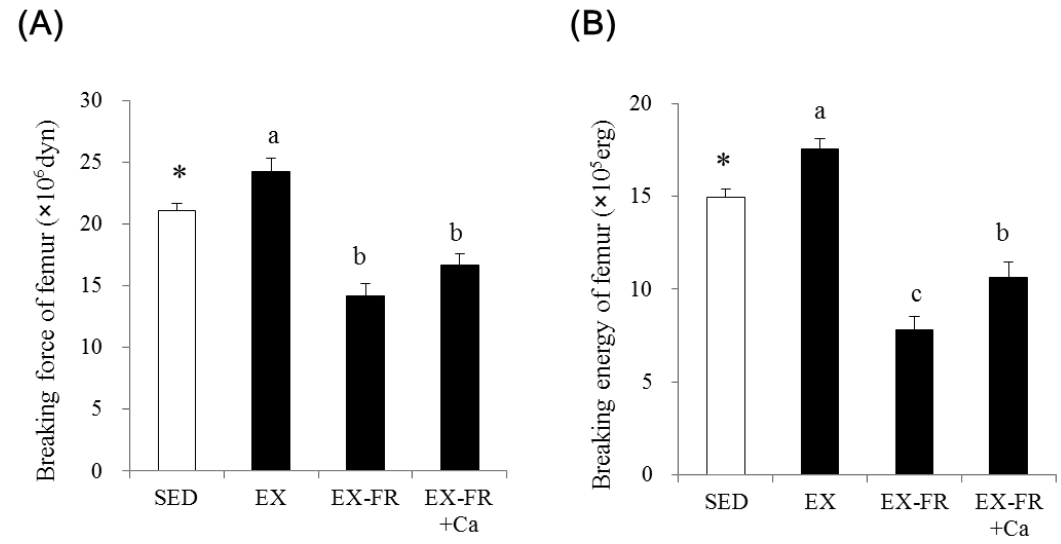


Figure 2. Breaking force and breaking energy of femur.

(A): Breaking force of femur. (B): Breaking energy of femur.

SED: sedentary group. EX: exercise group. EX-FR: exercise + food restriction group. EX-FR+Ca: exercise + food restriction + high-Ca diet group. Values are expressed as means ± SE.

Unpaired t tests were used to compare results for SED group and EX group to assess the effect of exercise.

*$p<0.05$ vs. EX group.

Data in EX, EX-FR, and EX-FR+Ca groups were analyzed using the Tukey's post-hoc comparison test.

Means with unlike alphabet are significantly different.

Figure. 3

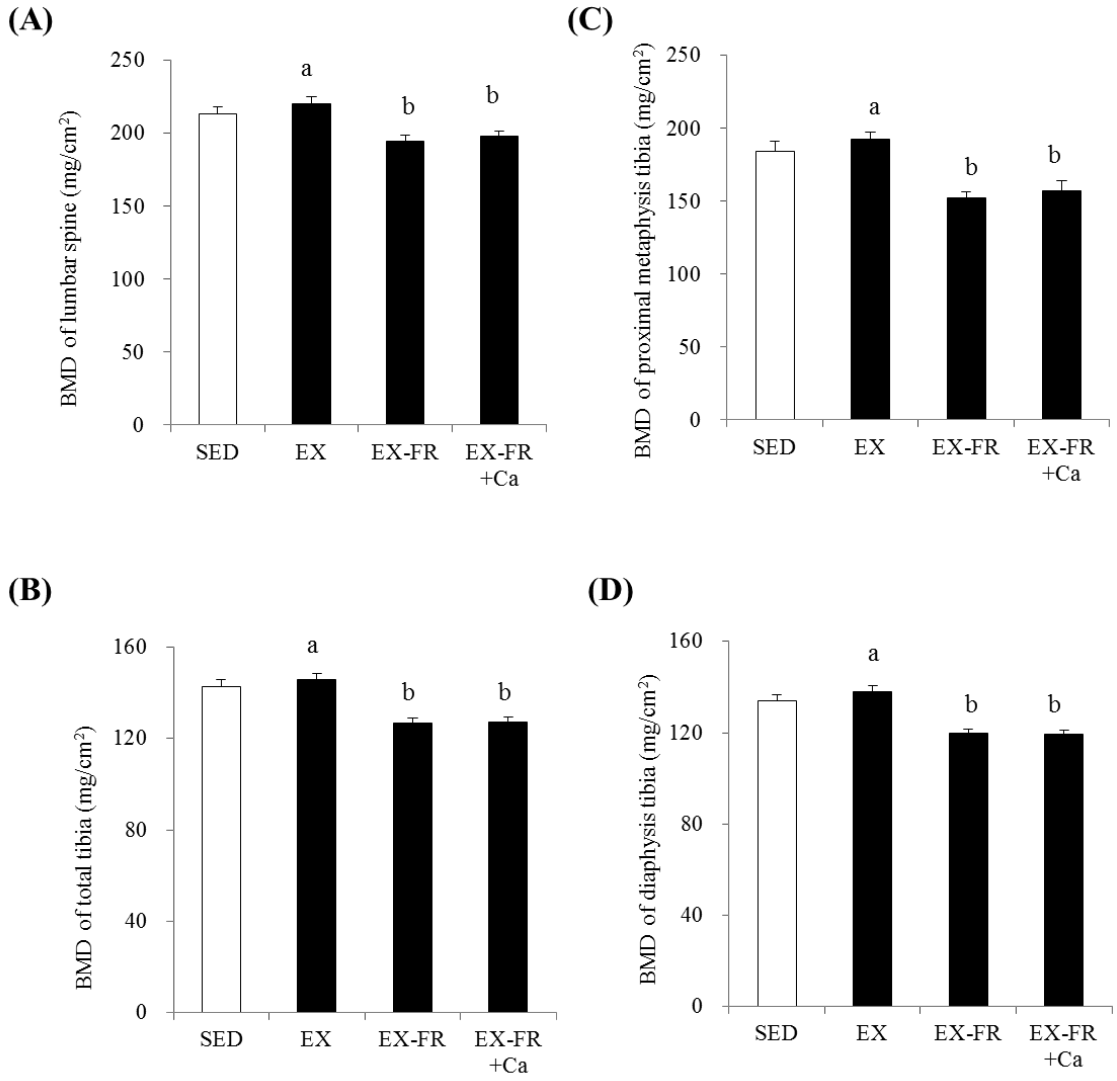


Figure 3. Bone mineral density (BMD) of lumbar spine and tibia.

(A): BMD of lumbar spine. (B): BMD of total tibia. (C): BMD of proximal metaphysis tibia. (D): BMD of diaphysis tibia.

SED: sedentary group. EX: exercise group. EX-FR: exercise + food restriction group. EX-FR+Ca: exercise + food restriction + high-Ca diet group. Values are expressed as means ± SE.

485 Unpaired t tests were used to compare results for SED group and EX group to assess the effect of

486 exercise. *p<0.05 vs. EX group.

487 Data in EX, EX-FR, and EX-FR+Ca groups were analyzed using the Tukey's post-hoc comparison

488 test. Means with unlike alphabet are significantly different.

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503 **Table 1. Compositions of the experimental diets.**

Constituents	normal (g)	high-Ca (g)
Glucose monohydrate	62.37	60.87
Casein[1]	18.0	18.0
Cystine	0.2	0.2
Cottonseed oil	10.0	10.0
$CaCO_3$	1.490	2.988
KH_2PO_4	1.158	1.158
K_2HPO_4	1.482	1.482
Roughage	3.0	3.0
Choline chloride	0.2	0.2
Water-soluble vitamin mixture[2]	0.1	0.1
Oil-soluble vitamin mixture	()[3]	()[3]
Ca- and P-free salt mixture[4]	2.0	2.0
Energy (kcal/100 g)	373	368

504

505 Crude protein, 18.0 %; Ca, 0.6 %; P, 0.6 %

506 [1]Casein contained 0.22 mg calcium/g and 4 mg phosphorus/ g.

507 [2]The water-soluble vitamin mixture(in%): thiamin, 0.5; riboflavin, 0.5; pyridoxine, 0.5; calcium
508 pantothenate, 2.8; nicotinamide, 2.0; inositol, 20.0: folic acid, 0.02; vitamin B_{12}, 0.002; biotin, 0.01; and
509 glucose monohydrate, 73.7.

510 [3]The rats received a supplement of the following oil –soluble vitamins in cottonseed oil three times a
511 week: β-carotene, 70 μg; 2-methyl-1.4-naphthoquinone, 105 μg; α-tocopherol, 875 μg; and vitamin D_3,
512 525 IU.

513 [4]Ca- and P-free salt mixture(in%): KCl, 57.7; NaCl, 20.9; $MgSO_4$, 17.9; $FeSO_4 \cdot 7H_2O$, 3.22; $CuSO_4 \cdot$
514 $5H_2O$, 0.078; NaF, 0.133; $CoCl_2 \cdot 6H_2O$, 0.004; KI, 0.01; $MnSO_4 \cdot 5H_2O$, 0.06; $ZnSO_4 \cdot 7H_2O$, 0.44; and
515 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.005.

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Table 2. Food intake, Ca intake, running distance, and energy availability.

	SED	EX	EX-FR	EX-FR+Ca
Food intake (g/day)	17.8 ± 0.6*	19.6 ± 0.3[a]	12.6 ± 0.1[b]	12.6 ± 0.1[b]
Ca intake (mg/day)	107 ± 3*	118 ± 2[b]	76 ± 0[c]	151 ± 0[a]
Energy intake[1] (kcal/day)	66.4 ± 2.1*	73.3 ± 1.2[a]	47.2 ± 0.1[b]	46.4 ± 0.1[b]
Wheel running distance (km/day)	-	8.7 ± 1.3	11.2 ± 0.6	10.9 ± 0.7
Percentage of the power spectrum of the running distance[2] (Rate)	-	0.44 ± 0.04[a]	0.17 ± 0.04[b]	0.18 ± 0.03[b]
Exercise-induced Energy expenditure[3] (kcal/day)	-	11.4 ± 1.6	10.7 ± 0.5	10.3 ± 0.5
Energy availability[4] (kcal/day)	66.4 ± 2.1	61.9 ± 1.4[a]	36.5 ± 0.5[b]	36.1 ± 0.5[b]

SED: sedentary group. EX: exercise group. EX-FR: exercise + food restriction group. EX-FR+Ca: exercise + food restriction + high-Ca diet group. Values are expressed as means ± SE. The values are expressed as the means of average of entire experimental period.

Unpaired t tests were used to compare results for SED group and EX group to assess the effect of exercise. *$p<0.05$ for vs. EX group.

Data in EX, EX-FR, and EX-FR+Ca groups were analyzed by the Tukey's post-hoc comparison test. Means with unlike alphabet are significantly different.

[1] Energy intake was calculated by multiplying the amount of normal diet intake (normal diet, 3.73 kcal/g; high-Ca diet, 3.68 kcal/g).

[2] To quantify the periodic component at about 4- or 5- day observed in the temporal profile of the running distance, we estimated the power in the frequency band from 0.2 to 0.3 Hz using spectral analysis of the detrended time series. Moreover, to exclude the interindividual difference in the total power affecting the local power, we also computed the proportion of the power spectrum of the running distance from 0.2 to 0.3 Hz in the total power spectrum.

[3] Exercise induced energy expenditure from daily wheel running was calculated as 5.0 kcal/kg body weight times kilometers run [22].

[4] Energy availability was calculated as energy intake minus exercise energy expenditure.

538 **Table 3. Body weight and internal organ weight**

	SED	EX	EX-FR	EX-FR+Ca
Body weight (g)	340 ± 15*	295 ± 5[a]	194 ± 4[b]	187 ± 4[b]
Abdominal fat weight (g)	26.0 ± 5.3*	12.4 ± 1.7[a]	1.6 ± 0.2[b]	1.0 ± 0.1[b]
Plantaris muscle weight (g)	0.34 ± 0.02	0.34 ± 0.01[a]	0.25 ± 0.13[b]	0.23 ± 0.08[b]
Soleus muscle weight (g)	0.111 ± 0.005*	0.138 ± 0.005[a]	0.095 ± 0.006[b]	0.086 ± 0.005[b]
Uterus weight (g)	0.53 ± 0.03	0.62 ± 0.04[a]	0.32 ± 0.07[b]	0.27 ± 0.06[b]
Adrenal gland weight (g)	0.037 ± 0.001	0.043 ± 0.003[a]	0.033 ± 0.002[b]	0.034 ± 0.002[b]
Thymus weight (g)	0.29 ± 0.03	0.23 ± 0.08[a]	0.18 ± 0.05[ab]	0.15 ± 0.13[b]

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541 exercise + food restriction + high-Ca diet group. Values are expressed as means ± SE. The values are
542 expressed as the means of average of entire experimental period.
543 Unpaired t tests were used to compare results for SED group and EX group to assess the effect of exercise.
544 *$p<0.05$ for vs. EX group.
545 Data in EX, EX-FR, and EX-FR+Ca groups were analyzed by the Tukey's post-hoc comparison test.
546 Means with unlike alphabet are significantly different.
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553 **Table 4. Bone weight, BMC, and bone area.**

	SED	EX	EX-FR	EX-FR+Ca
Dry weight of femur (g)	0.61 ± 0.01	0.60 ± 0.01[a]	0.46 ± 0.01[b]	0.49 ± 0.01[b]
Ash weight of femur (g)	0.43 ± 0.01	0.42 ± 0.01[a]	0.30 ± 0.01[b]	0.31 ± 0.01[b]
BMC of lumbar (mg)	558 ± 26	545 ± 8.9[a]	378 ± 13[b]	406 ± 17[b]
Bone area of lumbar (cm^2)	2.61 ± 0.07	2.48 ± 0.03[a]	1.94 ± 0.04[b]	2.05 ± 0.06[b]
BMC of total tibia (mg)	284 ± 11	309 ± 7[a]	225 ± 6[b]	235 ± 9[b]
Bone area of total tibia (cm^2)	1.97 ± 0.05*	2.15 ± 0.02[a]	1.79 ± 0.02[b]	1.85 ± 0.05[b]
BMC of proximal metaphysis tibia (mg)	92 ± 4	101 ± 3[a]	65 ± 3[b]	71 ± 5[b]
Bone area of proximal metaphysis tibia (cm^2)	0.50 ± 0.02	0.53 ± 0.01[a]	0.43 ± 0.02[b]	0.45 ± 0.02[b]
BMC of diaphysis tibia (mg)	108 ± 4*	121 ± 2[a]	91 ± 2[b]	92 ± 2[b]
Bone area of diaphysis tibia (cm^2)	0.80 ± 0.01*	0.90 ± 0.02[a]	0.77 ± 0.01[b]	0.78 ± 0.05[b]

554

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556 exercise + food restriction + high-Ca diet group. Values are expressed as means ± SE. The values are
557 expressed as the means of average of entire experimental period.
558 Unpaired t tests were used to compare results for SED group and EX group to assess the effect of exercise.
559 *$p<0.05$ for vs. EX group.
560 Data in EX, EX-FR, and EX-FR+Ca groups were analyzed by the Tukey's post-hoc comparison test.
561 Means with unlike alphabet are significantly different.

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Table 5. Ca balance study.

	SED	EX	EX-FR	EX-FR+Ca
Urine Ca excretion (mg/day)	0.89 ± 0.11	1.17 ± 0.25^{b}	1.61 ± 0.29^{b}	3.99 ± 0.97^{a}
Fecal Ca excretion (mg/day)	32.5 ± 4.0	32.6 ± 7.2^{b}	36.3 ± 5.2^{b}	73.7 ± 3.6^{a}
Amount of Ca absorption (mg/day)	59.4 ± 2.9	79.4 ± 14.3^{a}	35.1 ± 5.1^{b}	69.1 ± 4.8^{a}
Rate of Ca absorption (%)	65.1 ± 3.4	66.5 ± 10.9	49.1 ± 7.3	48.3 ± 2.6
Amount of Ca accumulation (mg/day)	58.5 ± 2.9	78.3 ± 14.2^{a}	33.5 ± 5.3^{b}	66.4 ± 4.6^{ab}
Rate of Ca accumulation (%)	64.2 ± 3.5	65.5 ± 10.8	46.9 ± 7.4	46.6 ± 3.2

SED: sedentary group. EX: exercise group. EX-FR: exercise + food restriction group. EX-FR+Ca: exercise + food restriction + high-Ca diet group. Values are expressed as means ± SE. The values are expressed as the means of average of entire experimental period.

Unpaired t tests were used to compare results for SED group and EX group to assess the effect of exercise. *$p<0.05$ for vs. EX group.

Data in EX, EX-FR, and EX-FR+Ca groups were analyzed by the Tukey's post-hoc comparison test. Means with unlike alphabet are significantly different.