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Basic nutritional investigation

Influence of dietary vitamin D deficiency on bone strength, body composition, and muscle in ovariectomized rats fed a high-fat diet

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ABSTRACT

Objective: Vitamin D deficiency is associated with a greater risk for osteoporosis and also influences skeletal muscle functions. The aim of this study was to investigate the influence of vitamin D restriction on ovariectomized (OVX) rats fed a high-fat diet.
Methods: Twenty-four 13-wk-old female rats were ovariectomized, and another 6 received a sham operation (Sham). The OVX rats were divided into four groups and fed experimental diets: a basic control diet (OVX-Cont), a basic control diet with vitamin D restriction (OVX-DR), a high-fat diet (OVX-F), and a high-fat diet with vitamin D restriction (OVX-FDR).
Results: At 28 d after starting the experimental diets, the fat mass was significantly increased in the OVX-F and OVX-FDR groups compared with OVX-Cont group, whereas the muscle mass was significantly decreased in the OVX-F and OVX-FDR groups compared with the OVX-Cont group. Compared with the OVX-Cont group, the bone mineral content of the femur was significantly lower in the OVX-DR and OVX-FDR groups, and the bone mineral density of the femur was significantly lower in the OVX-DR group. Myogenin is one of the muscle-specific transcription factors. The levels of mRNA expression of *myogenin* in the soleus and gastrocnemius muscles from the OVX-DR and OVX-FDR groups were reduced markedly compared with those from the OVX-Cont group.
Conclusion: We provided evidence that a high-fat diet with vitamin D restriction influences bone and muscle metabolism using OVX rats. Further studies on vitamin D deficiency in the regulation of muscle as well as bone metabolism would provide valuable data for the prevention of osteoporosis and sarcopenia.

Introduction

Osteoporosis, a disease characterized by low bone mass and the microarchitectural deterioration of bone tissue, leads to bone fragility and a consequent increase in fracture risk [1]. In women, menopause causes changes in bone remodeling, an imbalance between bone formation and bone resorption, and net bone loss [2]. To reduce the risk for osteoporosis, it may be important to maximize peak bone mass during youth and prevent the loss of bone mass in postmenopausal women and older people. Osteoporosis results from complex interactions between genetic and environmental factors. Nutrition is an environmental factor for the prevention of osteoporosis, so nutritional education targeted at promoting and maintaining bone health is indispensable for young people.

Dietary vitamin D is important for developing strong, healthy bones in children and helping to protect against osteoporosis, bone fractures, and breaks in older adults. Vitamin D deficiency causes a bone mineralization disorder and reduced bone density, and the risk for fracture is increased [3]. Vitamin D regulates bone metabolism and is known to play an essential role in enhancement of the absorption of calcium and phosphate from the intestine. In addition, vitamin D modulates muscle and bone-derived hormones, facilitating crosstalk between these tissues. Vitamin D deficiency is also associated with muscle atrophy and weakness [4], and the provision of vitamin D and an appropriate serum vitamin D concentration can help reduce fall risk and maintain physical functions [5]. Furthermore, reduced levels of vitamin D are associated with cancer, cardiovascular disease, osteoarthritis, and autoimmune disorders [6].

Metabolic syndrome is characterized by abdominal adiposity, dyslipidemia, and elevated glucose and blood pressure associated

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with a Westernized diet, surplus energy intake in the form of fat, increasing obesity, and sedentary lifestyles [7]. Obesity is associated with metabolic syndrome, and low levels of serum 25-hydroxyvitamin D_3 [25(OH)D] were frequently observed in patients with obesity [8,9]. Although the reason for the increased risk for vitamin D deficiency in obesity remains unclear, vitamin D may be less bioavailable in obese individuals because of its deposition in body fat compartments [10]. Both obesity and vitamin D deficiency have been recognized as major public health issues worldwide.

Previously, we reported the influence of dietary vitamin D restriction on bone strength, body composition, and skeletal muscle using male rats fed a high-fat diet [11]. At 28 d after starting the experimental diets, the visceral fat mass was significantly increased in the high-fat diet group compared with the control group, and the muscle mass tended to decrease in the vitamin D-restricted diet group compared with the control group [11].

In the present study, we aimed to provide further insight into the influence of a vitamin D-restricted high-fat diet on the bone strength, body composition, and skeletal muscle using ovariectomized (OVX) rats as a model for studying postmenopausal women, involving the mRNA expression of muscle-specific transcription factors.

Materials and methods

Experimental animals

The care and use of rats in the present study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals. The study protocols were approved by the Animal Care and Use Committee of Japan Women's University.

Thirty Sprague-Dawley strain female rats ages 12 wk and weighing about 220 to 280 g were used. All rats were fed the control diet (AIN-93 M diet) [12] for 7 d; 24 rats were OVX, and another 6 rats received a sham operation (Sham). The OVX rats were separated into four groups after a 7-d recovery period: basic control diet (OVX-Cont), a basic control diet with vitamin D restriction (OVX-DR), high-fat diet (OVX-F), and high-fat diet with vitamin D restriction (OVX-FDR; n = 6 in each group). The control diet with vitamin D restriction (DR) had vitamin mix in the control diet replaced by vitamin mix without vitamin D_3. The high-fat diet (F) had corn starch in the control diet replaced by lard, and the lipid energy rate was adjusted to 40%. The high-fat diet with vitamin D restriction (FDR) had vitamin mix in the high-fat diet replaced by vitamin mix without vitamin D_3. The calcium and phosphorus contents were 0.5% and 0.3% (% weight/wet weight), respectively, in all five groups. All animals were housed individually in wire cages with free access to ion-exchanged distilled water, under 12-h light/dark cycles, a constant temperature (23°C ± 1°C), and constant humidity. Body weights were measured every second day.

Twenty-eight days after the beginning of the experimental diet, the animals were fasted overnight and sacrificed by bleeding from the abdominal aorta under anesthesia with phenobarbital sodium (2.6 mg/100 g body weight).

Biochemical analysis of serum

Blood was collected and centrifuged at 1,000 g for 15 min to extract the serum. Sera were collected and stored at −80°C until thawed for analysis.

Calcium was measured employing the *o*-cresol-phthalein complexion color development method [13], and inorganic phosphorus was determined using the method of *p*-methylaminophenol reduction [14]. Serum 25(OH)D was measured using a radioimmunoassay method (25-hydroxyvitamin D ^{125} I RIA Kit, DiaSorin, Stillwater, MN, USA) [15].

X-ray computed tomography

Twenty-eight days after starting the experimental diets, the body composition (fat and muscle amounts) and bone mineral density (BMD) were measured using an x-ray computed tomography (CT) system for small experimental animals with a rat mode (LaTheta LCT-100, Hitachi-Aloka Medical, Ltd., Tokyo, Japan) [16]. The visceral and subcutaneous fat volumes, computed automatically, were compared with those after the radiologist's adjustments. Figure 1A shows CT images at 1.5-mm intervals in the measurement area of the visceral and subcutaneous fat volumes, presented with two yellow lines. Bone parameters (bone volume, bone mineral content [BMC], BMD, and cortical bone thickness) were computed using the CT images at 0.5-mm intervals in the measurement area of the right femur.

Reverse transcription-polymerase chain reaction analyses of mRNA for Myf-5, MyoD, *or* myogenin *gene*

Total RNA from the right soleus and gastrocnemius muscles was extracted by the thiocyanate-phenol-chloroform extraction method (CS104 B RNA-BEE, TelTest, Inc., Friendswood, TX, USA) [17]. As a template for polymerase chain reaction (PCR), single strand cDNA was prepared from 2 μg of total RNA using the PrimeScript first strand cDNA synthesis kit (Takara, Shiga, Japan). PCR primers were used for rat *Myf-5* (Myogenic factor 5) (forward: 5′-GGAATGCAATCCGCTACATT-3′, reverse: 5′-CAGGGCAGTAGATGCTGTCA-3′) [18], rat *MyoD* (myogenic differentiation antigen1, myogenic factor 3, *Myf-3*) (forward: 5′-GACGGCTCTCTCTGCTCCTT-3′, reverse: 5′-GTCTGAGTCGCCGCTGTAGT-3′) [19], and rat *myogenin* (myogenic factor 4, *Myf-4*) (forward: 5′-TGCCACAAGCCAGACTACCCACC-3′, reverse: 5′-CGGGGCACTCACTGTCTCTCAA-3′) [20].

The PCR was carried out using SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Chelmsford, MA, USA). The amplified samples were analyzed using 5.25% polyacrylamide gel electrophoresis. The stained gels were observed with ultraviolet light. The density of the photograph was determined using Image Analysis Software (CS Analyzer 3 for Windows, ATTO, Tokyo, Japan). All values were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

Statistical analyses

Values are shown as the means ± standard error. Normal distribution and homogeneity of variance for each parameter were assessed by a Shapiro-Wilk test and a Levene's test, respectively. For comparisons between the OVX-Cont and Sham groups, the unpaired Student's *t* test was used. Comparisons between treatments (OVX-Cont versus OVX-DR, OVX-F, or OVX-FDR, and OVX-F versus OVX-FDR) were performed using Tukey's test after analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$. Analysis was conducted using SPSS version 22 (IBM Corporation, Somers, NY, USA).

Results

Animals and diets

As shown in Table 1, the final body weight (g), body weight gain (g/d), food intake (g/d), energy intake (kcal/d), and energy efficiency (body weight gain/energy intake) in the OVX-Cont group were significantly higher than in the Sham group ($P < 0.01$, $P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively).

There were no significant differences in the final body weight [ANOVA: F(3, 20) = 2.307; $P = 0.107$, not significant], body weight gain [ANOVA: F(3, 20) = 2.965; $P = 0.057$, not significant], or food intake [ANOVA: F(3, 20) = 2.870; $P = 0.062$, not significant] among the OVX groups (OVX-Cont and OVX-DR, OVX-F, or OVX-FDR, and OVX-F and OVX-FDR groups).

Biochemical analysis of serum parameters

The levels of serum calcium in the Sham, OVX-Cont, OVX-DR, OVX-F, and OVX-FDR groups were 11.3 ± 0.1, 10.2 ± 0.1, 10.1 ± 0.1, 9.9 ± 0.1, and 9.8 ± 0.1 mg/mL, respectively. The levels of serum phosphorus in the Sham, OVX-Cont, OVX-DR, OVX-F, and OVX-FDR groups were 7.1 ± 0.2, 7.7 ± 0.1, 8 ± 0.1, 7.5 ± 0.2, and 7.7 ± 0.3 mg/mL, respectively. There were no significant differences in the levels of serum calcium (ANOVA: F[3, 20] = 2.442; $P = 0.094$, not significant) or phosphorus (ANOVA: F[3, 20] = 1.602; $P = 0.220$, not significant) among the OVX groups.

The levels of serum 25(OH)D in the Sham, OVX-Cont, OVX-DR, OVX-F, and OVX-FDR groups were 30.3 ± 3.2, 31.3 ± 3.6, 14.3 ± 1, 29.2 ± 3.1, and 13.2 ± 1.1 ng/mL, respectively. The levels of serum 25(OH)D (ANOVA: F[3, 20] = 14.636; $P = 0.000$) in the OVX-DR and OVX-FDR groups were significantly decreased compared with OVX-Cont group ($P < 0.01$ and $P < 0.001$, respectively), and the level of serum 25(OH)D in the OVX-FDR group was significantly decreased compared with the OVX-F group ($P < 0.01$).

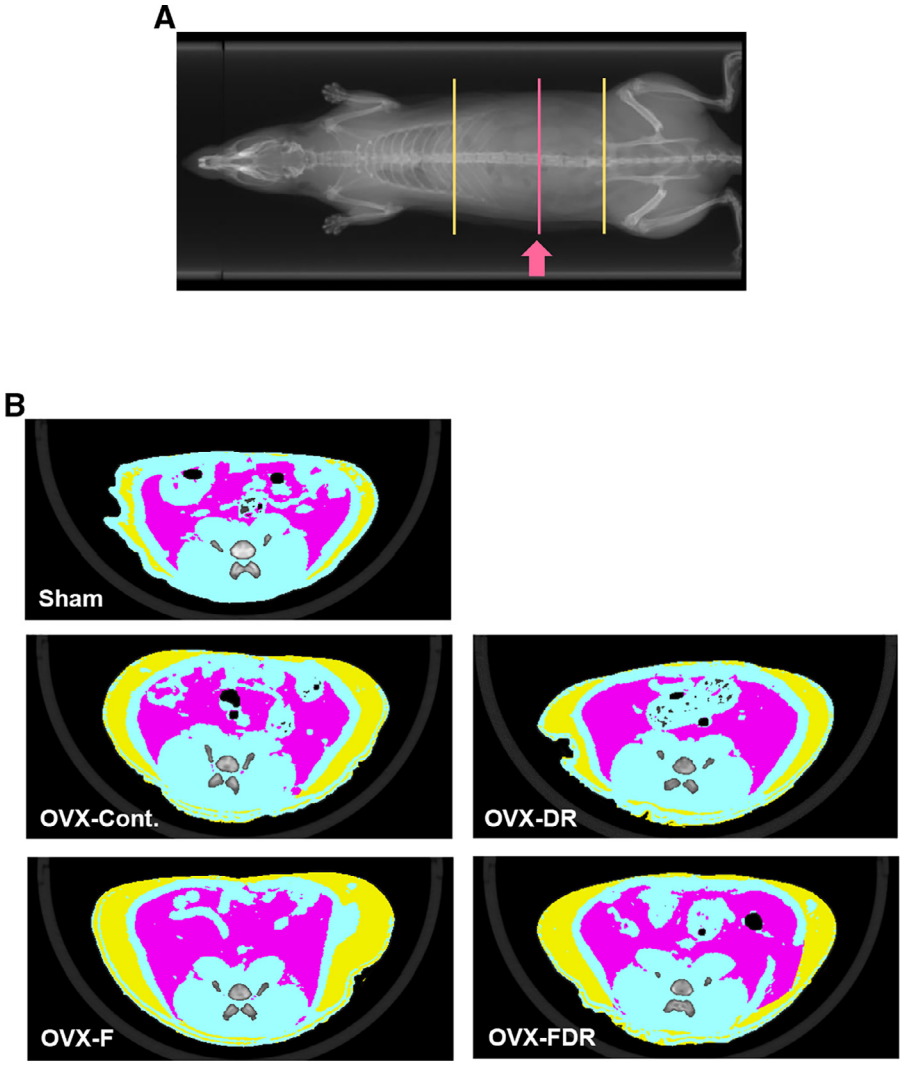

Fig. 1. X-ray CT 28 d after starting the experimental diets in the Sham, OVX-Cont, OVX-DR, OVX-F, and OVX-FDR groups. **(A)** Representative x-ray CT images of the whole bodies of rats. For body composition measurements, images were acquired at 1.5-mm intervals in the measurement area, presented as 2 yellow lines. **(B)** Cross-sectional appearance of rats in the Sham, OVX-Cont, OVX-DR, OVX-F, and OVX-FDR groups. Tomographic x-ray CT images of the same quartered lumbar vertebral regions shown with a pink line in panel A. The areas indicated in pink, yellow, and light blue are visceral fat, subcutaneous fat, and muscle, respectively. Cont, basic control diet group; CT, computed tomography; DR, vitamin D-restricted basic diet group; F, high-fat diet group; FDR, vitamin D-restricted high-fat diet group; OVX, ovariectomized; Sham, sham-operated group.

X-ray CT of the fat area and the weight of fat and muscles

Scout and tomographic x-ray CT images are shown in Figure 1. Figure 1B shows tomographic images of the same quartered lumbar vertebral regions indicated with the pink line in Figure 1A. The areas indicated with pink, yellow, and light blue are visceral fat, subcutaneous fat, and muscle, respectively. Compared with the OVX-Cont group, the visceral fat areas

Table 1
Final body weight, body weight gain, food intake, energy intake, and energy efficiency

	Sham	OVX				*P*-values (ANOVA)
		Cont	DR	F	FDR	
Final body weight (g)	326.7 ± 9.4**	369.3 ± 5.6	368.0 ± 4.2	381.0 ± 2.2	378.3 ± 4.3	0.107
Body weight gain (g/d)	1.2 ± 0.2***	3.1 ± 0.1	2.9 ± 0.2	3.4 ± 0.1	3.4 ± 0.2	0.057
Food intake (g/d)	16.1 ± 0.4***	20.7 ± 0.3	20.4 ± 0.1	20.0 ± 0.3	19.7 ± 0.3	0.062
Energy intake (kcal/d)	60.1 ± 1.4***	77.1 ± 1.1	76.1 ± 0.5	90.6 ± 1.1***	89.2 ± 1.3***	0.000
Energy efficiency (Body weight gain/energy intake)	0.020 ± 0.003***	0.041 ± 0.001	0.038 ± 0.003	0.038 ± 0.001	0.038 ± 0.002	0.655

ANOVA, analysis of variance; Cont, basic control diet group; DR, vitamin D-restricted basic diet group; F, high-fat diet group; FDR, vitamin D-restricted high-fat diet group; OVX, ovariectomized; Sham, sham-operated group.
Results are the means ± standard error of 6 animals. One-way ANOVA test followed by Tukey's post hoc test.
Final body weights were measured on the 28th day after starting the experimental diets.
**Significant difference relative to OVX-Cont group $P < 0.01$.
***Significant difference relative to OVX-Cont group $P < 0.001$.

Table 2
Weights of total fat pad, visceral fat, subcutaneous fat, muscles, body fat rate, and the right soleus and gastrocnemius muscles

Weight (g/100 g body weight)	Sham	OVX				*P*-values (ANOVA)
		Cont.	DR	F	FDR	
Total fat pad	5.8 ± 0.3**	7.7 ± 0.4	7.7 ± 0.5	11.2 ± 0.5***	10.1 ± 0.2**	0.000
Visceral fat	5.0 ± 0.3	5.0 ± 0.2	5.8 ± 0.3	7.8 ± 0.4***	7.3 ± 0.3***	0.000
Subcutaneous fat	0.8 ± 0.1**	1.9 ± 0.2	1.9 ± 0.3	3.3 ± 0.3**	2.7 ± 0.2	0.003
Muscles	21.0 ± 0.2	20.7 ± 0.4	18.9 ± 0.4	18.6 ± 0.7*	16.8 ± 0.4***	0.000
Body fat rate (%)	21.6 ± 1.1	24.3 ± 2.0	27.6 ± 1.4	39.4 ± 1.7***	36.2 ± 0.8***	0.000
Soleus muscle	0.048 ± 0.001	0.045 ± 0.001	0.040 ± 0.001*	0.044 ± 0.002	0.039 ± 0.001*	0.012
Gastrocnemius muscle	0.674 ± 0.015	0.663 ± 0.006	0.612 ± 0.015*	0.620 ± 0.010	0.592 ± 0.013**	0.002

ANOVA, analysis of variance; Cont, basic control diet group; DR, vitamin D-restricted basic diet group; F, high-fat diet group; FDR, vitamin D-restricted high-fat diet group; OVX, ovariectomized; Sham, sham-operated group.
Results are the means ± standard error of six animals. One-way ANOVA test followed by Tukey's post hoc test.
**Significant difference relative to OVX-Cont group $P < 0.01$.
***Significant difference relative to OVX-Cont group $P < 0.001$.
*Significant difference relative to OVX-Cont group $P < 0.05$.

increased in the OVX-F and OVX-FDR groups, shown in the lower area of Figure 1B.

Table 2 shows the results of body composition measurements using an x-ray CT system for laboratory animals. After 28 d, the total and subcutaneous fat weights (g/100 g body weight) were significantly higher in the OVX-Cont group compared with Sham group ($P < 0.01$, respectively). The fat weights (g/100 g body weight) of total (ANOVA: F[3, 20] = 17.368; $P = 0.000$) and visceral (ANOVA: F[3, 20] = 20.697; $P = 0.000$) were significantly higher in the OVX-F and OVX-FDR groups compared with the OVX-Cont group (OVX-F: $P < 0.001$ and $P < 0.001$ and OVX-FDR: $P < 0.01$ and $P < 0.001$, respectively), whereas the muscle weights (g/100 g body weight) (ANOVA: F[3, 20] = 10.941; $P = 0.000$) were significantly lower in the OVX-F and OVX-FDR groups compared with the OVX-Cont group ($P < 0.05$ and $P < 0.001$, respectively; Table 2). The right soleus muscle weight (ANOVA: F[3, 20] = 4.703; $P = 0.012$) was significantly lower in the OVX-DR and OVX-FDR groups than in the OVX-Cont group ($P < 0.05$, respectively), and the right gastrocnemius muscle weight (ANOVA: F[3, 20] = 6.821; $P = 0.002$]) was also significantly lower in the OVX-DR and OVX-FDR groups than in the OVX-Cont group ($P < 0.05$ and $P < 0.01$, respectively; Table 2).

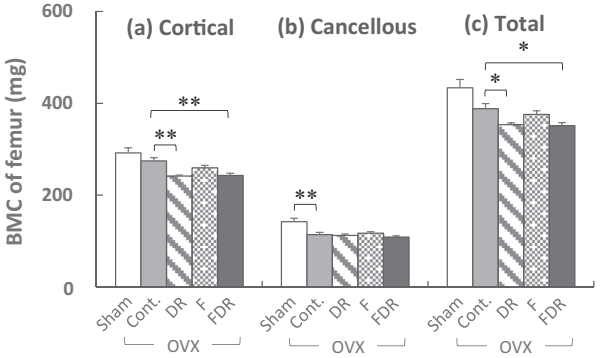

Fig. 2. BMC of the femur measured by x-ray CT 28 d after starting the experimental diets. Results are the means ± standard error of 6 animals. One-way ANOVA test followed by Tukey's post hoc test. Significant difference relative to OVX-Cont group (**$P < 0.01$; *$P < 0.05$). (**A**) Cortical BMC (ANOVA: F[3, 20] = 8.684; $P = 0.001$). (**B**) Cancellous BMC (ANOVA: F[3, 20] = 1.041, $P = 0.396$). (**C**) Total BMC (ANOVA: F[3, 20] = 4.968; $P = 0.010$). ANOVA, analysis of variance; BMC, bone mineral content; Cont, basic control diet group; CT, computed tomography; DR, vitamin D-restricted basic diet group; F, high-fat diet group; FDR, vitamin D-restricted high-fat diet group; OVX, ovariectomized; Sham, sham-operated group.

Bone parameters of the femur

As shown in Table 3, the cortical bone volumes of the femur (ANOVA: F[3, 20] = 5.754; $P = 0.005$) in the OVX-DR and OVX-FDR groups were significantly decreased compared with OVX-Cont group (OVX-DR [$P < 0.05$] and OVX-FDR [$P < 0.01$], respectively). The cortical bone thickness of the femur (ANOVA: F[3, 20] = 8.021; $P = 0.001$) in the OVX-DR and OVX-FDR groups was significantly decreased compared with OVX-Cont group ($P < 0.01$ and $P < 0.05$, respectively; Table 3).

As shown in Figure 2, the cancellous BMC of the femur was significantly lower in the OVX-Cont group compared with Sham group ($P < 0.01$). The cortical (ANOVA: F[3, 20] = 8.684; $P = 0.001$) and total BMC (ANOVA: F[3, 20] = 4.968, $P = 0.010$) of the femur in the OVX-DR group were significantly decreased compared with the

Table 3
Volume and cortical bone thickness of the femur

	Sham	OVX				*P*-values (ANOVA)
		Cont.	DR	F	FDR	
Volume (cm^3)						
Cortical	0.25 ± 0.01	0.24 ± 0.01	0.21 ± 0.00*	0.23 ± 0.00	0.21 ± 0.00**	0.005
Cancellous	0.28 ± 0.01	0.26 ± 0.02	0.29 ± 0.01	0.28 ± 0.01	0.27 ± 0.01	0.539
Total	0.53 ± 0.02	0.50 ± 0.03	0.50 ± 0.01	0.51 ± 0.01	0.48 ± 0.01	0.740
Cortical bone thickness (mm)						
	0.500 ± 0.000	0.500 ± 0.010	0.450 ± 0.000**	0.480 ± 0.010	0.470 ± 0.010*	0.001

ANOVA, analysis of variance; Cont, basic control diet group; DR, vitamin D-restricted basic diet group; F, high-fat diet group; FDR, vitamin D-restricted high-fat diet group; OVX, ovariectomized; Sham, sham-operated group.
Results are the means ± standard error of six animals. One-way ANOVA test followed by Tukey's post hoc test.
*Significant difference relative to OVX-Cont group $P < 0.05$.
**Significant difference relative to OVX-Cont group $P < 0.01$.

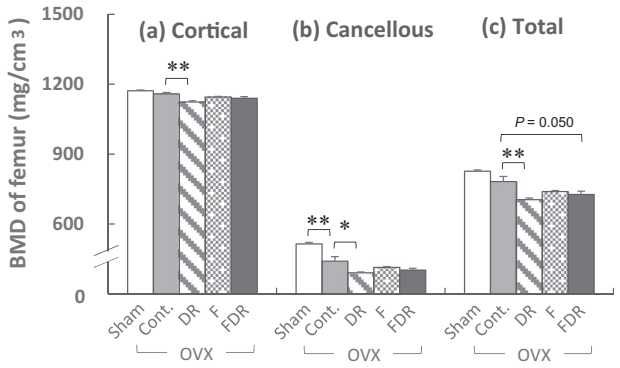

Fig. 3. Bone mineral density (BMD) of the femur measured by x-ray CT 28 d after starting the experimental diets. Results are the means ± SE of 6 animals. One-way ANOVA test followed by Tukey's post hoc test. Significant difference relative to OVX-Cont group ($^{**}P < 0.01$; $^{*}P < 0.05$). (**A**) Cortical BMD (ANOVA: F[3, 20] = 6.004; $P = 0.004$). (**B**) Cancellous BMD (ANOVA: F[3, 20] = 3.156; $P = 0.047$). (**C**) Total BMD (ANOVA: F[3, 20] = 5.540; $P = 0.006$). ANOVA, analysis of variance; BMD, bone mineral density; Cont, basic control diet group; CT, computed tomography; DR, vitamin D-restricted basic diet group; F, high-fat diet group; FDR, vitamin D-restricted high-fat diet group; OVX, ovariectomized; SE, standard error; Sham, sham-operated group.

OVX-Cont group ($P < 0.01$ and $P < 0.05$, respectively), and the cortical and total BMC of the femur in the OVX-FDR group were significantly decreased compared with the OVX-Cont group ($P < 0.01$ and $P < 0.05$, respectively; Fig. 2).

As shown in Figure 3, the cancellous BMD of the femur was significantly lower in the OVX-Cont group compared with the Sham group ($P < 0.01$). The BMD of cortical (ANOVA: F[3, 20] = 6.004, $P = 0.004$), cancellous (ANOVA: F[3, 20] = 3.156, $P = 0.047$), and total (ANOVA: F[3, 20] = 5.540, $P = 0.006$) of the femur were significantly lower in the OVX-DR group compared with OVX-Cont group ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively).

RT-PCR analyses

As we obtained the result that the soleus and gastrocnemius muscle weights in the OVX-DR and OVX-FDR groups decreased significantly compared with those of the OVX-Cont group 28 d after the beginning of the experimental diets (Table 2), we compared the mRNA expression of muscle-specific transcription factors such as *Myf-5, MyoD*, and *myogenin* in the soleus and gastrocnemius muscles.

As shown in Figure 4, the relative density of the PCR products for *myogenin* from the soleus muscle (ANOVA: F[3, 20] = 8.898; $P = 0.001$) and gastrocnemius muscle (ANOVA: F[3, 20] = 7.593; $P = 0.001$) in the OVX-DR and OVX-FDR groups were significantly reduced compared with the OVX-Cont group day 28 after starting the experimental diets (OVX-DR: $P < 0.01$ and $P < 0.05$ and OVX-FDR: $P < 0.01$ and $P < 0.01$, respectively). The relative density of the PCR products for *myogenin* from the soleus and gastrocnemius muscles in the OVX-FDR group were significantly reduced compared with the OVX-F group ($P < 0.05$, respectively).

As shown in Table 4, we also revealed that the relative density of the PCR products for *Myf-5* from the gastrocnemius muscle (ANOVA: F[3, 20] = 7.067; $P = 0.002$) in the OVX-DR and OVX-FDR groups were significantly reduced compared with the OVX-Cont group ($P < 0.05$ and $P < 0.01$, respectively), and the relative density of the PCR products for *Myf-5* from the gastrocnemius muscle in the OVX-FDR group were significantly reduced compared with the OVX-F group ($P < 0.05$).

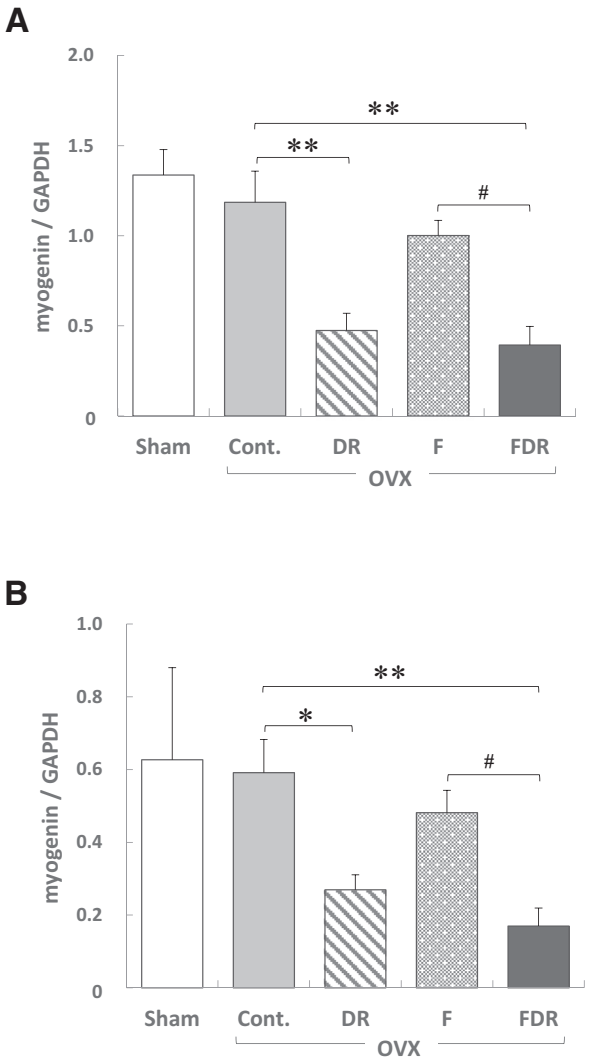

Fig. 4. Relative expression level of mRNA for rat *myogenin* from the (**A**) soleus (ANOVA: F[3, 20] = 8.898; $P = 0.001$) and (**B**) gastrocnemius (ANOVA: F[3, 20] = 7.593; $P = 0.001$) muscles. All values were normalized to the housekeeping gene *GAPDH*. Results are the means ± standard error of six animals. One-way ANOVA test followed by Tukey's post hoc test. Significant difference relative to OVX-Cont group ($^{*}P < 0.05$; $^{**}P < 0.01$). Significant difference between OVX-FDR and OVX-F groups ($^{\#}P < 0.05$). ANOVA, analysis of variance; Cont, basic control diet group; DR, vitamin D-restricted basic diet group; F, high-fat diet group; FDR, vitamin D-restricted high-fat diet group; OVX, ovariectomized; Sham, sham-operated group.

Discussion

In the present study, we demonstrated the influence of dietary vitamin D deficiency and a high-fat diet on the bone strength, body composition, and muscle in OVX rats fed a high-fat diet.

Estrogen deficiency induces body weight gain and bone loss, and OVX rats are the most commonly used animal model of postmenopausal osteoporosis. The final body weight, body weight gain, weight of the total fat pad, and subcutaneous fat mass were significantly higher in the OVX-Cont group than in the Sham group at 28 d after starting the experimental diets. Also, the cancellous BMC and BMD of the femur were significantly lower in the OVX-Cont group than in the Sham group. These findings are consistent with previous studies demonstrating that menopause is associated with body weight gain and changes in the body composition, such as a

Table 4
Relative expression level of mRNAs for rat *Myf-5* and *MyoD* of the soleus and gastrocnemius muscles

	Sham	OVX				*P*-values (ANOVA)
		Cont.	DR	F	FDR	
Soleus muscle						
Myf-5	1.94 ± 0.36	1.93 ± 0.38	1.31 ± 0.43	1.83 ± 0.16	0.65 ± 0.05	0.043
MyoD	0.17 ± 0.04	0.46 ± 0.21	0.30 ± 0.10	0.27 ± 0.07	0.13 ± 0.03	0.402
Gastrocnemius muscle						
Myf-5	0.71 ± 0.10	0.54 ± 0.08	0.26 ± 0.03*	0.48 ± 0.06	0.22 ± 0.04$^{**,\#}$	0.002
MyoD	0.22 ± 0.06	0.21 ± 0.05	0.12 ± 0.03	0.31 ± 0.07	0.20 ± 0.07	0.211

ANOVA, analysis of variance; Cont, basic control diet group; DR, vitamin D-restricted basic diet group; F, high-fat diet group; FDR, vitamin D-restricted high-fat diet group; OVX, ovariectomized; Sham, sham-operated group.
Results are the means ± standard error of six animals. One-way ANOVA test followed by Tukey's post hoc test.
All values were normalized to the housekeeping gene *GAPDH*.
*Significant difference relative to OVX-Cont group $P < 0.05$.
**Significant difference relative to OVX-Cont group $P < 0.01$.
$^{\#}$Significant difference between the OVX-F and OVX-FDR groups $P < 0.05$.

decline in the BMC, a loss of lean body mass, and an increase in the fat mass [21–23].

Most recently, we examined the influence of dietary vitamin D restriction on bone strength, body composition, and skeletal muscle using male rats fed a high-fat diet [11]. Male rats (11 wk old) were divided into four groups and fed experimental diets: a basic control diet (Cont), a basic control diet with vitamin D restriction (DR), a high-fat diet (F), and a high-fat diet with vitamin D restriction (FDR), of which the compositions were similar to those of the OVX-Cont, OVX-DR, OVX-F, and OVX-FDR group diets, respectively, in the present study. At 28 d after starting the experimental diets, no significant differences in cortical, cancellous, or total BMC of the femur in the DR and FDR groups were observed compared with the Cont group in male rats [11]. In the present study, ovariectomy reduced the cancellous BMC of the femur, and the cortical and total BMC of the femur were significantly lower in the OVX-DR and OVX-FDR groups than in the OVX-Cont group. These results suggest that the vitamin D-restricted diet and high-fat vitamin D-restricted diet markedly impaired bone metabolism in OVX rats.

Mammalian skeletal muscles are heterogeneous, and there are two main types of muscle fiber: type I and type II. Type I muscle fibers are also known as slow-twitch or red muscle fibers, and they are responsible for long-duration, low-intensity activity such as walking or other aerobic activities. On the other hand, type II muscle fibers are known as fast-twitch or white muscle fibers and they are responsible for short-duration, high-intensity activity. The soleus muscle contains predominantly type I muscle fibers, whereas the gastrocnemius muscle contains predominantly type II muscle fibers. Several studies suggested that vitamin D deficiency induced muscle wasting and decreased the type II fiber area as a marker of muscle atrophy [4,24]. Emerging evidence has shown that vitamin D administration improves muscle performance and reduces falls in vitamin D-deficient older adults.

Because the soleus and gastrocnemius muscles weights were significantly lower in the OVX-DR and OVX-FDR groups than in the OVX-Cont group, we examined the levels of mRNA expression of *Myf-5, MyoD*, and *myogenin* of skeletal muscles (soleus and gastrocnemius muscles) between the OVX-Cont and the Sham, OVX-DR, OVX-F, or OVX-FDR groups. On day 28 after starting the experimental diets, the relative density of the PCR products for *myogenin* from the soleus or gastrocnemius muscles in the OVX-DR and OVX-FDR groups was significantly decreased compared with that from the OVX-Cont group. Interestingly, the relative density of the PCR products for *myogenin* from the gastrocnemius muscle in the OVX-FDR group reduced by 71% compared with the OVX-Cont group, and there was also a significant difference in the levels of mRNA expression of *Myf-5* between the OVX-Cont and OVX-DR or OVX-FDR groups.

Myf-5, MyoD, and *myogenin* are members of muscle-specific transcription factors and they convert multipotential mesodermal cells to myoblasts. *Myf-5* is transiently expressed at an early developmental stage of quiescent satellite cell myogenesis. The satellite cell activation is marked by the rapid onset of *MyoD* expression, whereas *myogenin* later marks the commitment to differentiation [24]. It was reported that *myogenin*-mutant mice died immediately after birth and showed a marked reduction of all skeletal muscles [25,26]. However, knockout mice carrying null mutations in either *Myf-5* or *MyoD* genes exhibited normal skeletal muscle [27]. Based on these experiments using knockout mice, *myogenin* is essential for the development of functional skeletal muscle [24]. Although we were not able to clarify whether satellite cells play any role in the maintenance of skeletal muscles in the present study, we considered that the significant differences in the levels of mRNA expression of *myogenin* between the OVX-Cont and OVX-DR or OVX-FDR groups might be attributable to the altered expression in satellite cells. The vitamin D receptor (VDR) is an intracellular hormone receptor that specifically binds to the biologically active form of vitamin D, 1α, 25-dihydroxyvitamin D_3, and interacts with specific nucleotide sequences (response elements) of target genes to produce a variety of biological effects. It was reported that *VDR* gene-deleted mice exhibited abnormal skeletal muscle development and the abnormalities were accompanied by the deregulated expression of myogenic transcription factors such as *Myf-5, MyoD*, and *myogenin*. These findings suggested that there are direct vitamin D actions on muscle through VDR because a similar influence was demonstrated by the treatment of a VDR-positive myoblast cell line, C2C12, with 1α, 25-dihydroxyvitamin D_3 *in vitro* [28].

Conclusion

We provided evidence that a high-fat diet with vitamin D deficiency influences bone and muscle metabolism, using OVX rats. Further studies are necessary to confirm the results, including histologic analysis; bone morphometric analysis, such as bone formation rates; and analyses of lipids and vitamin D metabolism pathways for the prevention and treatment of osteoporosis and sarcopenia.

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