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REPORT

Time Course of Influence by Ovariectomy and Calcium Diet on Bone Properties in Mice

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Abstract. This study was investigated the influence by ovariectomy (OVX) and calcium diet on bone properties in eighty-one female ICR strain mice with age of 5 weeks. The animals were randomly assigned to sham operation (SHAM), OVX, SHAM+low Ca intake (L.Ca) and OVX+L.Ca group. They were euthanized with lethal dose of pentobarbital sodium at day 50, 100 and 140 post-operatively. For determining the bone properties, both femur and tibial bones were excised from the hind limb, and removed off surrounding tissues. Thereafter, bone length, bone dry weight, and also mechanical strength and ash content of the bones were determined. The bone length on both femur and tibia was significantly longer in OVX group than in the other groups after 50 day of experiment, this situation was continued to the end of the experiment. Bone dry weight, mechanical strength, and ash content were significantly decreased by OVX and L.Ca over the time of the experiment, and those of OVX+L.Ca group were the lowest in all groups. OVX and L.Ca have a great potential for weakening the mechanical strength and have an additive effect when combined. OVX and L.Ca block the gain of bone mass.

Key words: low calcium diet, ovariectomy, bone properties

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Calcium (Ca) is one of the most important nutriment to gain the peak bone mass[1)2)]. Bone mass regulates the mechanical strength of bone about 80–90%, and bone loss induces the reduction of mechanical strength and increase of fracture risk. To get bone mass as much as possible is very necessary to preventing osteoporosis. Matkovic *et al.*[3)] reported that in district low Ca intake bone mineral density was reduced in all age groups of both sex, and incidence of bone fracture rate was higher in district low Ca intake than district high Ca intake. This indicates that habitual intake Ca is important to increase bone mass. Also Ca intake is the most useful in adolescence to influence bone mass in all of generations[2)4)].

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In animals, low Ca intake induces bone loss and the reduction of bone formation rate[5-7)]. The ovariectomized (OVX) animal is well known for osteoporotic model, and widely used[8)9)]. Bone loss is enhanced when OVX animals were fed low Ca diet. Though Ca intake in adolescence is important to gain bone mass, the investigation of effects of Ca intake in growth on bone mass and reports combined OVX with low Ca intake are few. Changes of bone loss and bone mass by OVX and Low Ca diet could be different between adult and young mice. OVX causes bone loss quickly in adult mice, and it is considered that bone loss is slow in young mice. Because metabolism and physiological functions in period of growth is very different from aging.

Therefore, this study investigated the time course effects and changes of OVX and Ca intake on bone in young mice.

Materials and Methods

Experimental animals care

Eighty-one female ICR mice (5 weeks old, body weight 26.7 ± 1.9 g) were used. Six mice were sacrificed as the baseline at the start of the experiment. The remaining 75 mice were either ovariectomized or sham operated under pentobarbital sodium anesthesia (50 mg/kg) and randomly divided into four groups, sham operation (SHAM), OVX, SHAM+low Ca intake (L.Ca), and OVX+L.Ca group (Table 1.). After the operation, the animals were housed in individual cages (20.5 × 31 × 13 cm) under the following conditions, temperature, 23 ± 1 °C, humidity. 50 ± 5% and 12 hour day-night cycle. Group in SHAM and OVX were fed standard laboratory food (SF : Ca-1.1g/100 g), however, group in SHAM+L.Ca and OVX+L.Ca were special low calcium food (L.Ca : Ca-6.0 mg/100 g) made by ORIENTAL YEAST CO., LTD. Food and water were available ad libitum.

This study was carried out in accordance with Guide for Animal Experimentation, Hiroshima University and Committee of Research Facilities of Laboratory Animal Science, Hiroshima University School of Medicine.

Mechanical testing

Mice were sacrificed at day 50, 100 and 140 after the operation. The femur and tibia bones excised from the hind limbs and removed off surrounding tissue.

The length of femur and tibia were measured with a micrometer. A materials testing machine (computer control system autograph AGS-1000A, SHIMADZU, Co, Japan) was used to measure and calculate the three-point bending strength of the femur and tibia. The compression head, 5 mm in diameter, loaded the center of bone at a constant vertical velocity of 0.5 mm/min. The bones were then dried, weighed and burned at 600°C for 7 hours to obtain ash content.

Statistics

Multiple analysis of variance (ANOVA) and Post hoc tests were conducted to examine the differences of groups. One-way ANOVA was used to find changes of duration of the experiment. The level of significance was established at $p<0.05$. All data were expressed as means and standard division (SD).

Results

Tables 2–1, 2–2 and 3 give the results of the experiment.

Final body weight of OVX mice was heavier than that of SHAM animals. The bone length on both femur and tibia was significantly longer in OVX group than in the other groups after 50 day of experiment, this situation was continued to the end of the experiment. Effects of Ca intake were seen during the experiment, and femur and tibia dry weight in the OVX+L.Ca and SHAM+L.Ca mice showed marked decrease compared with that of SHAM mice. In OVX+L.Ca and SHAM+L.Ca groups, decrease of bone dry weight continued from start to 50 days of the experiment and was restored after 100 days. The yield strength of femur in OVX and OVX+L.Ca was less than in baseline until 100 days of the experiment. The yield strength of femur and tibia in OVX+L.Ca showed the lowest value in all groups. Though OVX and L.Ca caused the reduction of the yield strength, the yield strength was influenced strongly by OVX rather than L.Ca. Ash content of femur and tibia in OVX+L.Ca and SHAM+L.Ca animals decreased for 50 days from start point of the experiment and was less than in baseline. L.Ca affected the ash content greatly.

Effects of OVX and Ca intake in all measured bone properties showed significant differences and interactions were not found (Table 3). The bone dry weight, mechanical strength and ash content in OVX+L.Ca group indicated the lowest values. OVX and L.Ca have a great potential for weakening the mechanical strength and have an additive effect when combined. OVX and L.Ca block the gain of bone mass.

Table 1. Group forms of experimental mice

group	duration of the experiment	N.	diet
SHAM	50 days	6	SF
	100 days	7	SF
	140 days	6	SF
OVX	50 days	6	SF
	100 days	7	SF
	140 days	7	SF
SHAM+L.Ca	50 days	6	L.Ca
	100 days	6	L.Ca
	140 days	6	L.Ca
OVX+L.Ca	50 days	6	L.Ca
	100 days	6	L.Ca
	140 days	6	L.Ca

OVX: ovariectomy+SF, SHAM: sham-operation+SF, OVX+L.Ca: ovariectomy+L.Ca, SHAM+L.Ca: sham-operation+L.Ca. SF: standard laboratory food, L.Ca: low calcium diet.

Discussion

It is very important to investigate when the effects of OVX and Ca intake appear and how long these effects continue. Because the changes of bone properties were different by given factors or duration of the experiment.

Table 2-1. The results of measured properties in the experiment

group		body weight (g)	bone length (mm)		bone dry weight (mg)	
			femur	tibia	femur	tibia
baseline		26.7 ± 1.9	15.4 ± 0.4	17.8 ± 0.7	49.6 ± 6.4	38.4 ± 4.4
sham	50	34.7 ± 2.9	16.4 ± 0.5	19.1 ± 0.5	65.0 ± 4.9	47.4 ± 2.3
	100	40.0 ± 5.1	16.9 ± 0.5	19.9 ± 0.3	74.3 ± 8.2	54.0 ± 2.0
	140	42.3 ± 2.9	17.0 ± 0.4	20.2 ± 0.3	75.2 ± 7.8	58.5 ± 6.3
ovx	50	42.8 ± 2.7*	16.9 ± 0.5*	19.5 ± 0.4*	60.9 ± 5.8*	46.1 ± 2.9
	100	50.3 ± 4.6*	17.5 ± 0.6*	20.6 ± 0.3*	71.0 ± 6.1*	49.0 ± 4.8*
	140	52.9 ± 4.9*	17.5 ± 0.4*	20.7 ± 0.6*	73.0 ± 4.8	53.7 ± 3.3*
sham+L.Ca	50	32.0 ± 2.4	16.2 ± 0.5	19.0 ± 0.3	48.0 ± 1.9#	39.6 ± 0.7#
	100	35.2 ± 2.3	16.7 ± 0.5	19.7 ± 0.3	55.6 ± 3.0#	45.0 ± 4.7#
	140	41.1 ± 5.0	16.9 ± 0.4	20.0 ± 0.4	60.3 ± 7.0#	47.0 ± 2.1#
ovx+L.Ca	50	38.3 ± 2.3*#	16.6 ± 0.4*	19.4 ± 0.4*	45.9 ± 4.0*#	37.2 ± 2.0*#
	100	43.0 ± 4.0*#	17.0 ± 0.4*	20.5 ± 0.6*	51.8 ± 5.1*#	43.6 ± 2.2*#
	140	47.0 ± 2.1*#	17.1 ± 0.6	20.6 ± 0.4*	59.1 ± 4.7#	46.2 ± 2.1#

*Significantly different from sham-operated group ($p<0.05$).

#Significantly different from standard laboratory food group ($p<0.05$).

Table 2-2. The results of measured properties in the experiment

group		maximum load (kgf)		ash content (mg)	
		femur	tibia	femur	tibia
baseline		1.70 ± 0.10	1.27 ± 0.05	28.8 ± 2.9	20.4 ± 1.6
sham	50	2.21 ± 0.51	1.62 ± 0.36	36.7 ± 2.9	25.5 ± 2.1
	100	2.34 ± 0.25	1.67 ± 0.20	42.0 ± 5.3	30.0 ± 2.9
	140	2.49 ± 0.24	1.71 ± 0.24	44.8 ± 3.7	31.0 ± 1.9
ovx	50	1.58 ± 0.17*	1.43 ± 0.20*	35.0 ± 2.9*	25.0 ± 1.0*
	100	1.60 ± 0.19*	1.45 ± 0.18*	40.1 ± 3.1*	25.4 ± 2.2*
	140	2.10 ± 0.35*	1.53 ± 0.21*	40.8 ± 3.0*	26.8 ± 2.0*
sham+L.Ca	50	1.85 ± 0.28#	1.52 ± 0.21#	26.0 ± 1.4#	20.9 ± 0.8#
	100	2.02 ± 0.26#	1.53 ± 0.17#	29.0 ± 1.5#	23.8 ± 1.2#
	140	2.16 ± 0.52#	1.55 ± 0.27#	33.0 ± 3.3#	25.7 ± 0.5#
ovx+L.Ca	50	1.49 ± 0.20*	1.35 ± 0.17*#	24.0 ± 1.8*#	19.1 ± 1.5*#
	100	1.50 ± 0.47*#	1.41 ± 0.11*	26.9 ± 3.2*#	22.1 ± 3.2#
	140	1.80 ± 0.18*#	1.50 ± 0.09*	30.3 ± 1.4*#	24.9 ± 1.3*#

*Significantly different from sham-operated group ($p<0.05$).

#Significantly different from standard laboratory food group ($p<0.05$).

OVX rats and mice are widely used as bone loss models and OVX induces deficiency of estrogen. Osteoblasts and osteoclasts have estrogen receptors and estrogen regulates bone formation and resorption by various actions[9-11]. Estrogen deficiency causes high turn over in early stages[12)13)]. Bone reduction of proximal tibia lasts from 14 days to 100 days after OVX and then stabilizes[12)]. Osteoclasts significantly increase at 3 days after OVX[13)14)]. Thus bone loss by OVX appears soon. The yield strength was reduced by OVX rapidly. This indicated that mechanical strength was regulated by bone mass. Bone mass decreased rapidly because of high bone resorption. Longitudinal growth of bone is endochondral ossification at growth plate. As there are many osteoblasts in calcified cartilage under growth plate, bone formation is very active in this area. In addition, bone turn over is high after OVX and bone length of OVX mouse is longer than that of SHAM mouse. This phenomenon revealed the acceleration

Table 3. ANOVA among operation, food and duration

	body weight	bone length	bone width	dry bone weight	maximum load	ash content
op	<0.0001	<0.0001/<0.0001	<0.0001/<0.0001	0.1102/ 0.0045	<0.0001/ 0.0007	0.0141/<0.0001
food	<0.0001	<0.0001/ 0.0296	0.0063/ 0.0002	<0.0001/<0.0001	<0.0001/ 0.0478	<0.0001/<0.0001
duration	<0.0001	<0.0001/<0.0001	<0.0001/<0.0001	<0.0001/<0.0001	<0.0001/ 0.2206	<0.0001/<0.0001
op * food	0.1690	0.0624/ 0.5315	0.4753/ 0.9177	0.5914/ 0.1810	0.1402/ 0.2820	0.8392/ 0.0595
op * duration	0.7817	0.8822/ 0.4352	0.5152/ 0.4711	0.8296/ 0.8042	0.1902/ 0.7016	0.5819/ 0.2369
food * duration	0.5355	0.6531/ 0.9199	0.8175/ 0.1884	0.4325/ 0.4915	0.6906/ 0.9969	0.2490/ 0.1522
op * food * duration	0.8048	0.7597/ 0.7799	0.6278/ 0.6264	0.9433/ 0.4223	0.6569/ 0.8428	0.8352/ 0.0506

op: operation (OVX), food: Ca intake (L.Ca), duration: 50, 100, and 140 days. p-values are shown by femur/tibia.

of bone resorption and formation, and was the evidences that decrease of bone mass and increase of osteoblasts caused in early stages after OVX.

Ca intake is important to gain peak bone mass and bone mass of humans whose Ca sufficiently is high[4]. Ca intake is an environmental factor and depends on eating habits and region. This is due to getting little bone mass rather than decrease it. Breaking load is concerned with Ca content and 25% Ca content decrease causes 50% breaking load reduction and maximum compression load of vertebral body is proportional to the square of Ca content[15)16)]. Thus the relationship of mechanical strength and Ca has been studied in vertebra. In the case of the femur, the relationship of mechanical strength to bone mineral density or structure has been reported.

There are many factors of bone loss related to each other and are physiological or environmental condition. Ca intake, exercise, loading and life style affect bone mass. Osteoporosis advances with OVX combining immobilization compared with independent factor model. Bone mass declines by OVX and L.Ca in adult animals. However, bone properties such as mechanical strength, dry bone weight and ash content in young mice increased slowly and reach certain level. These properties did not showed only the reduction. This could be due to growth. Bone grows with aging but bone properties was blocked by OVX and L.Ca. This suggested that growth of bone properties need estrogen and calcium.

In the present study, effects of OVX and Ca intake differed in properties and the degree, appearance time and continuous period of these effects were various in properties. Changes of bone properties by OVX and L.Ca appeared rapidly at from 50 to 100 days after the operation. Not only bone loss but decrease in $1,25(OH)_2D_3$ receptor in intestine by OVX[17)18)] and differences of Ca supplement effect in Ca intake condition were noted. These factors thus affect mechanical strength. Both factors influenced bone properties, and some properties were effected by OVX mainly and others were effected by L.Ca.

Mechanical strength, dry bone weight and ash content of OVX+L.Ca group showed the lowest values. These factors may act additively as interactions were not found statistically. OVX and L.Ca have a great potential for weakening the mechanical strength and have an additive effect when combined.

This study demonstrated that OVX and L.Ca caused the reduction of mechanical strength, and effected additively when combined with these factors. Ca intake may thus help to prevent bone loss by OVX. The effects of OVX appeared early and low Ca had influence for a long term. These factors affected bone properties rapidly or slowly from the start and differed in according to the duration.

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