

Differences in vertebral, tibial, and iliac cancellous bone metabolism in ovariectomized rats

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Abstract Bone histomorphometry is usually performed on the iliac bone in humans and the tibia or vertebrae in rats. Bone metabolism differences among skeletal sites may be problematic when translating experimental results from rats to humans, but data on such differences in rats are lacking. Therefore, we examined the differences in bone structure and metabolism among skeletal sites using the lumbar vertebra (LV), tibia, and iliac bone obtained from ovariectomized or sham-operated rats preoperatively and at various times from 3 days to 26 weeks postoperatively. The trabeculae were thicker in the LV, where bone metabolism was less active than at other sites, and numerous fine trabeculae were observed in the tibia, where bone metabolism was more active. The iliac bone structure and metabolism were intermediate between those of the tibia and LV. Ovariectomy induced lower bone volume and higher bone metabolism in all skeletal sites, but the changes were greatest and occurred earliest in the tibia, followed by the iliac bone and then LV. Ovariectomy caused changes in bone metabolic markers, which occurred earlier than those in bone tissue. Activation frequency (Ac.f) increased after

ovariectomy. At week 26 in ovariectomized rats, Ac.f was highest in the tibia (3.13 N/year) but similar between iliac bone (0.87 N/year) and LV (1.39 N/year). Ac.f is reportedly 0.3–0.4 N/year in the iliac bone of postmenopausal women, suggesting that bone turnover in rats is several times higher than in humans. The reference values reported here are useful for translating experimental results from rats to humans.

Keywords Ovariectomy · Rat · Histomorphometry · Bone metabolism

Introduction

Species-related differences in the metabolism of target organs and/or tissues must be considered when translating nonclinical results from the drug development stage to clinical investigations. Various countries' guidelines on the development of drugs for osteoporosis state that species-based differences in bone turnover should be considered to determine the optimal study periods and dosing protocols in animal studies and human clinical trials [1–3]. Smith et al. [4] performed a pharmacological study of ibandronate in monkeys based on this approach.

Bone histomorphometry is an excellent method for the quantification of bone formation, resorption, and calcification in bone tissue and assessment of the state of bone metabolism. Most investigators have chosen to evaluate histomorphometric changes in cancellous remodeling of the tibial metaphysis or lumbar vertebral body after ovariectomy (OVX) in rats as an animal model of postmenopausal osteoporosis [1, 2, 5]. However, iliac crest biopsies are generally used in clinical bone histomorphometric studies in humans, and differences among skeletal sites present a problem when translating nonclinical results to clinical

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investigations. Numerous studies have investigated the changes in bone metabolism at the lumbar vertebral body (LV) or tibia in rats following OVX [5, 6]. However, histomorphometric data on the iliac bone in rats are scarce [7], and the differences in bone metabolism among skeletal sites are unclear. In other words, there are insufficient reference data on the differences in bone structure and metabolism among various skeletal sites in rats, preventing studies that take species differences in bone turnover into account. Therefore, we performed a bone histomorphometric analysis of the lumbar vertebral, tibial, and iliac bone spongiosa in normal and OVX rats to obtain reference data on bone structure and metabolism and, thus, clarify the differences in bone structure and metabolism among skeletal sites and the effects of OVX over time. Changes in bone metabolic markers in the blood and urine were also investigated to study OVX-induced, and systematically observed, changes in the state of bone metabolism.

Materials and methods

Animals

Eleven-week-old female Sprague–Dawley rats (Charles River, Kanagawa, Japan) were used. The rats were maintained under a 12/12-h light/dark cycle with unrestricted access to tap water and a standard diet containing 1.2 % calcium, 0.9 % phosphorus, 22.0 % protein, and 6.2 IU vitamin D3 per gram (CRF-1; Oriental Yeast, Tokyo, Japan). The animals were allowed to acclimate to their environment for 2 weeks before the start of the experiment. The experimental protocols were approved by the experimental animal ethics committee at Asahi Kasei Pharma Corp. and conducted in accordance with guidelines concerning the management and handling of experimental animals.

Bone histomorphometric experiments

At 13 weeks of age, sexually mature female Sprague–Dawley rats underwent either OVX or sham surgery under anesthesia [8]. A double fluorochrome labeling technique was used to determine the active mineralization sites and rates of bone formation. Calcein (Dojindo Laboratories, Kumamoto, Japan) was subcutaneously injected into each rat twice at a dose of 10 mg/kg body weight before sacrifice as follows: in the groups necropsied preoperatively and 1 week postoperatively, calcein was given 2 and 6 days before necropsy (label schedule 01-03-01-01); in the group necropsied 2–13 weeks postoperatively, calcein was given 3 and 11 days before necropsy (label schedule 01-07-01-02); and in the group necropsied 26 weeks postoperatively, calcein was given 3 and 13 days before necropsy (label

schedule 01-09-01-02). This regimen was selected after considering the effects of aging and OVX, and to appropriately measure fluorochrome-based parameters.

The rats were sacrificed under anesthesia, and the fifth lumbar vertebra, right ilium, and right tibia were removed. Five rats were sacrificed on the day of surgery (day 0) to obtain baseline values. Sham and OVX rats ($n = 5$ each) were sacrificed at 3 days and 1, 2, 4, 13, and 26 weeks postoperatively. Successful OVX was confirmed by a reduction in the uterus weight at necropsy.

Bone histomorphometry

The bones were removed and dissected free of soft tissue, fixed in 70 % ethanol, stained with Villanueva bone stain, dehydrated in a graded series of ethanol, defatted in acetone, and embedded in polymethyl methacrylate (Wako Pure Chemical Industries, Osaka, Japan). Thin sections (5 μm) were prepared from a sagittal section of the central fifth LV, a coronal section of the proximal tibia (tibia), and a sagittal section of the cranial ilium (iliac bone) [7].

Bone histomorphometric parameters related to bone mass, structure, resorption, formation, and turnover were measured using an image analysis system (Histometry RT Camera; System Supply, Nagano, Japan). Histomorphometric measurements were obtained from cancellous bone tissue in the secondary spongiosa region (Fig. 1).

The following static parameters were measured: trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). The following bone resorption parameters were measured: eroded surface (ES/BS), osteoclast surface (Oc.S/BS), and osteoclast number (N.Oc/BS). The following bone formation and turnover parameters were measured: osteoid surface (OS/BS), osteoblast surface (Ob.S/BS), mineralizing surface (MS/BS, based on double plus half single label), mineral apposition rate (MAR), osteoid maturation time (Omt), bone formation rate (BFR/BV), formation period (FP), remodeling period (Rm.P), total period (Tt.P), and activation frequency (Ac.f) [9, 10].

Measurement of bone metabolic markers

At 13 weeks of age, sexually mature female Sprague–Dawley rats underwent either OVX or sham surgery under anesthesia ($n = 10$ each). This experiment was performed using a group of rats independent from that used for bone histomorphometry. All rats were fasted for >6 h before blood and urine collection. Blood and urine samples were obtained preoperatively and at 3 days and 1, 2, 4, 13, and 26 weeks postoperatively. Serum samples were obtained by centrifugation of the blood samples. Serum and urine

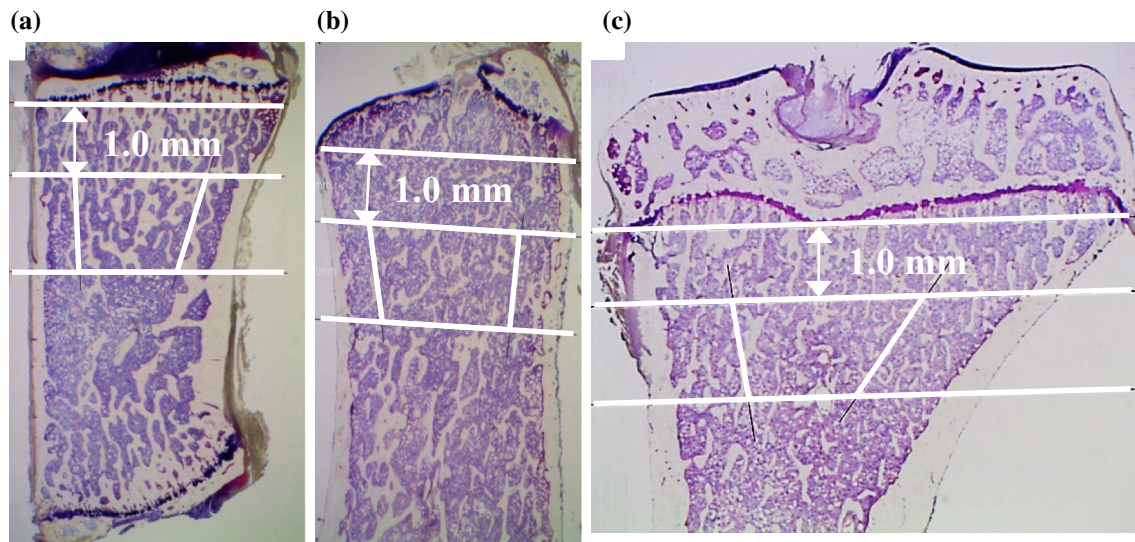


Fig. 1 Histomorphometric measurement ranges in lumbar vertebra (LV), iliac bone, and tibia. **a** LV the secondary spongiosa region of the LV was defined as the interior of the fifth lumbar vertebra 1.0 mm from the cranial and caudal growth plates and 0.5 mm from the cortical bone. The measurement range was determined by measuring the craniocaudal distance of the secondary spongiosa region of each

sample to half on the caudal side. **b** Iliac bone the secondary spongiosa region of the iliac bone was defined as the interior of the iliac bone 1.0 mm distal to the proximal growth plate and 0.5 mm from the cortical bone. **c** Tibia the secondary spongiosa region of the tibia was defined as the interior of the proximal tibial metaphysis 1.0 mm distal to the proximal growth plate and 1.0 mm from the cortical bone

samples were aliquoted and stored at -80°C until analysis. The urinary level of C-terminal telopeptide of type I collagen (CTX), a bone resorption marker, was measured using RatLaps EIA (Immunodiagnostic Systems, Boldon, UK). The urinary level of creatinine was measured using L-type Wako CRE-M (Wako Pure Chemical Industries, Osaka, Japan). The urinary level of CTX was corrected for the urinary level of creatinine. The serum level of osteocalcin (OC), a bone formation marker, was measured using an osteocalcin rat ELISA system (GE Healthcare Bioscience, Tokyo, Japan). All assays were performed according to the manufacturers' instructions.

Statistical analysis

All data are presented as the mean \pm SD. The effects of OVX and time were investigated using two-way ANOVA. Variables showing a difference between the sham and OVX conditions and between the preoperative and postoperative states were then analyzed with Student's *t* test at each time point (post hoc). Statistical significance was defined as $p < 0.05$.

Results

There were no deaths during surgery. The rats awoke from anesthesia ~3 h after surgery and began feeding. There were no clear behavioral differences after surgery between

the OVX and sham groups. Uterine weight at necropsy revealed uterine atrophy in all rats in the OVX group, confirming that the procedure had been performed correctly.

Bone histomorphometry

The sham group exhibited no changes in either BV/TV or Tb.Th in the LV, iliac bone, or tibia from day 0 to week 26 postoperatively (Table 1; Supplementary Fig. S1a–f). Although neither Tb.N nor Tb.Sp changed in the LV or tibia, both parameters were lower from day 3 onward than at day 0 in the iliac bone (Table 1; Supplementary Fig. S1g–l). In the OVX group, BV/TV decreased in all skeletal sites and was lower than that in the sham group at week 26 postoperatively in the LV and from week 4 onward and week 2 onward in the iliac bone and tibia, respectively. Tb.Th showed no substantial changes in any skeletal site in the OVX group; however, Tb.N in the iliac bone and tibia decreased markedly in the OVX group, and Tb.Sp was higher in the OVX group than in the sham group from week 13 onward in the LV and from week 4 onward in the iliac bone and tibia (Table 1; Supplementary Fig. S1).

The bone resorption parameters ES/BS, Oc.S/BS, and N.Oc/BS in the LV and tibia of rats in the sham group were temporarily lower soon after surgery than at day 0, whereas no clear change was observed in the iliac bone (Table 2; Supplementary Fig. S2). ES/BS was higher in the OVX group than in the sham group at week 4 in each skeletal site and at week 26 in the iliac bone and tibia. Oc.S/BS and

Table 1 Structural parameters of lumbar vertebra, iliac bone, and tibia

Variable (units)	Operation	Day 0	Day 3	Week 1	Week 2	Week 4	Week 13	Week 26	p value (OVX) ^a	p value (time) ^b
BV/TV (%)										
LV	Sham	37.4 ± 6.4	36.4 ± 5.4	34.5 ± 3.7	35.7 ± 4.0	35.1 ± 4.7	37.0 ± 4.9	41.8 ± 6.2		
	OVX	–	33.8 ± 1.3	36.6 ± 3.4	34.8 ± 5.7	37.5 ± 11.5	30.1 ± 6.3	25.9 ± 5.0*	0.015	0.899
Iliac bone	Sham	36.7 ± 4.8	35.0 ± 5.6	38.2 ± 2.7	32.1 ± 5.5	33.3 ± 6.6	32.2 ± 5.1	29.2 ± 5.6		
	OVX	–	34.9 ± 5.4	29.7 ± 8.2*	33.5 ± 5.3	24.0 ± 5.5*†	22.5 ± 4.8*†	21.0 ± 6.5†	<0.001	0.001
Tibia	Sham	32.2 ± 5.6	30.7 ± 3.7	33.0 ± 1.6	32.4 ± 1.4	32.4 ± 8.2	27.1 ± 5.2	26.9 ± 6.1		
	OVX	–	27.1 ± 4.5	26.5 ± 6.5	25.4 ± 2.6*†	21.1 ± 4.4*†	12.2 ± 2.9*†	12.1 ± 8.1*†	<0.001	<0.001
Tb.Th (µm)										
LV	Sham	76.8 ± 10.4	80.7 ± 11.7	77.8 ± 12.3	75.3 ± 4.1	79.0 ± 14.0	79.7 ± 5.8	86.7 ± 8.4		
	OVX	–	69.8 ± 6.7	76.1 ± 6.1	75.6 ± 10.2	83.4 ± 13.4	78.4 ± 12.8	72.7 ± 16.2	0.139	0.7353
Iliac bone	Sham	66.7 ± 13.5	74.6 ± 16.0	78.1 ± 7.6	69.6 ± 9.1	73.3 ± 4.6	77.5 ± 11.7	65.9 ± 12.1		
	OVX	–	69.1 ± 9.3	68.6 ± 18.7	73.5 ± 5.0	74.6 ± 18.3	76.5 ± 9.0	76.5 ± 9.3	0.940	0.885
Tibia	Sham	53.7 ± 2.7	56.1 ± 3.5	56.7 ± 4.3	58.1 ± 5.2	54.8 ± 3.7	50.7 ± 3.8	53.2 ± 5.3		
	OVX	–	54.7 ± 2.6	51.0 ± 3.3	49.1 ± 4.4	55.2 ± 3.2	59.6 ± 4.8	60.3 ± 12.6	0.973	0.810
Tb.N (N/mm)										
LV	Sham	4.87 ± 0.43	4.53 ± 0.45	4.47 ± 0.42	4.75 ± 0.58	4.47 ± 0.28	4.63 ± 0.41	4.81 ± 0.37		
	OVX	–	4.87 ± 0.50	4.85 ± 0.67	4.61 ± 0.48	4.40 ± 0.84	3.83 ± 0.49	3.59 ± 0.29	0.066	0.069
Iliac bone	Sham	5.56 ± 0.42	4.74 ± 0.46†	4.90 ± 0.26†	4.60 ± 0.32†	4.52 ± 0.65†	4.16 ± 0.40†	4.43 ± 0.31†		
	OVX	–	5.04 ± 0.30	4.35 ± 0.29*†	4.55 ± 0.46†	3.23 ± 0.32*†	2.92 ± 0.31*†	2.71 ± 0.58*†	<0.001	<0.001
Tibia	Sham	5.98 ± 0.88	5.46 ± 0.45	5.85 ± 0.62	5.61 ± 0.51	5.86 ± 1.11	5.32 ± 0.76	5.02 ± 0.84		
	OVX	–	4.95 ± 0.77	5.16 ± 0.93	5.20 ± 0.66	3.81 ± 0.68*†	2.03 ± 0.34*†	1.87 ± 0.93*†	<0.001	<0.001
Tb.Sp (µm)										
LV	Sham	130.0 ± 21.9	141.8 ± 21.0	147.7 ± 15.0	137.6 ± 24.9	145.3 ± 8.6	137.8 ± 24.0	122.3 ± 21.7		
	OVX	–	137.0 ± 13.4	133.4 ± 24.0	143.5 ± 26.0	152.6 ± 70.0	186.0 ± 36.8*	207.2 ± 21.2*	0.008	0.214
Iliac bone	Sham	114.0 ± 6.4	137.7 ± 13.3†	126.3 ± 7.8†	148.7 ± 20.8†	151.7 ± 35.4†	164.5 ± 23.9†	160.5 ± 19.7†		
	OVX	–	129.7 ± 15.8	162.1 ± 22.4*†	148.1 ± 24.9†	237.6 ± 33.8*†	268.4 ± 39.9*†	305.4 ± 82.3*†	<0.001	<0.001
Tibia	Sham	117.1 ± 31.6	127.8 ± 16.5	115.6 ± 13.4	121.4 ± 11.5	120.6 ± 35.1	140.4 ± 28.2	150.8 ± 40.4		
	OVX	–	150.8 ± 30.5	147.0 ± 31.5	145.7 ± 21.4	216.2 ± 64.0*†	443.2 ± 83.6*†	599.1 ± 337.7*†	<0.001	<0.001

Data are expressed as the mean ± SD, n = 5

The time-courses of changes in these parameters are presented in Supplementary Fig. S1

BV/TV trabecular bone volume, Tb.Th trabecular thickness, Tb.N trabecular number, Tb.Sp trabecular separation, LV lumbar vertebra, OVX ovariectomy

* p < 0.05 vs. Sham group (unpaired Student's t test)

† p < 0.05 vs. week 0 (unpaired Student's t test)

^a p value between Sham and OVX groups (two-way ANOVA)

^b p value among time points (two-way ANOVA)

Table 2 Bone resorption parameters of lumbar vertebra, iliac bone, and tibia

Variable (units)	Operation	Day 0	Day 3	Week 1	Week 2	Week 4	Week 13	Week 26	<i>p</i> value (OVX) ^a	<i>p</i> value (time) ^b
ES/BS (%)										
LV	Sham	3.62 ± 1.49	3.03 ± 1.11	3.52 ± 1.06	6.28 ± 1.38 [†]	3.74 ± 0.78	3.38 ± 0.74	2.15 ± 1.07		
	OVX	–	2.33 ± 0.76	3.29 ± 0.96	8.06 ± 3.91 [†]	5.39 ± 1.29*	5.76 ± 1.86*	3.25 ± 1.01	0.026	0.899
Iliac bone	Sham	4.52 ± 2.71	3.73 ± 1.27	3.03 ± 0.90	5.57 ± 1.14	4.76 ± 1.53	4.01 ± 1.08	2.96 ± 0.96		
	OVX	–	5.50 ± 2.41	3.89 ± 1.12	7.57 ± 2.16	9.34 ± 2.23** [†]	4.86 ± 2.13	6.56 ± 2.23*	<0.001	0.001
Tibia	Sham	6.78 ± 2.71	3.39 ± 1.04 [†]	3.77 ± 1.20	9.31 ± 1.43	6.82 ± 1.55	6.13 ± 1.59	2.51 ± 1.14 [†]		
	OVX	–	3.65 ± 1.45	4.30 ± 1.05	9.25 ± 1.60	9.03 ± 0.74*	6.90 ± 2.98	10.84 ± 4.32*	<0.001	<0.001
Oc.S/BS (%)										
LV	Sham	1.86 ± 0.80	0.81 ± 0.53 [†]	0.62 ± 0.62 [†]	2.81 ± 0.87	1.14 ± 0.40	0.79 ± 0.26 [†]	0.58 ± 0.31 [†]		
	OVX	–	0.91 ± 0.45 [†]	0.66 ± 0.39 [†]	3.69 ± 2.40	1.90 ± 0.52*	2.02 ± 0.79*	0.63 ± 0.31 [†]	0.034	<0.001
Iliac bone	Sham	2.16 ± 1.51	1.59 ± 0.69	1.45 ± 0.60	1.87 ± 0.75	1.68 ± 0.81	1.51 ± 0.38	0.72 ± 0.36		
	OVX	–	2.17 ± 1.34	1.50 ± 0.53	3.27 ± 0.81*	3.09 ± 0.84*	2.03 ± 1.15	1.45 ± 0.79	<0.001	0.001
Tibia	Sham	3.71 ± 1.83	1.50 ± 0.50 [†]	1.22 ± 0.36 [†]	4.54 ± 0.37	2.93 ± 0.98	2.67 ± 0.93	0.77 ± 0.70 [†]		
	OVX	–	1.42 ± 0.50 [†]	1.99 ± 0.66	3.94 ± 0.88	3.29 ± 0.74	3.76 ± 2.34	2.00 ± 0.86	0.070	<0.001
N.Oc/BS (N/mm)										
LV	Sham	0.767 ± 0.329	0.423 ± 0.197	0.282 ± 0.245 [†]	1.029 ± 0.346	0.419 ± 0.166	0.321 ± 0.104 [†]	0.240 ± 0.109 [†]		
	OVX	–	0.678 ± 0.489	0.271 ± 0.154 [†]	1.343 ± 0.809	0.592 ± 0.143	0.702 ± 0.239*	0.254 ± 0.077 [†]	0.030	<0.001
Iliac bone	Sham	0.784 ± 0.468	0.611 ± 0.208	0.560 ± 0.252	0.626 ± 0.206	0.502 ± 0.169	0.583 ± 0.167	0.280 ± 0.164		
	OVX	–	0.928 ± 0.581	0.552 ± 0.165	0.997 ± 0.183*	0.915 ± 0.165*	0.641 ± 0.286	0.548 ± 0.311	0.001	0.016
Tibia	Sham	1.374 ± 0.642	0.649 ± 0.177 [†]	0.482 ± 0.160 [†]	1.674 ± 0.278	0.884 ± 0.316	0.951 ± 0.238	0.313 ± 0.252 [†]		
	OVX	–	0.683 ± 0.207	0.730 ± 0.296	1.397 ± 0.453	1.002 ± 0.197	1.258 ± 0.740	0.854 ± 0.483	0.083	<0.001

Data are expressed as mean ± SD, *n* = 5

The time-courses of changes in these parameters are presented in Supplementary Fig. S2

ES/BS eroded surface, Oc.S/BS osteoclast surface, N.Oc/BS osteoclast number, LV lumbar vertebra, OVX ovariectomy

* *p* < 0.05 vs. Sham group (unpaired Student's *t* test)

[†] *p* < 0.05 vs. week 0 (unpaired Student's *t* test)

^a *p* value between Sham and OVX groups (two-way ANOVA)

^b *p* value among time points (two-way ANOVA)

Table 3 Bone formation parameters of lumbar vertebra, iliac bone, and tibia

Variable (units)	Operation	Day 0	Day 3	Week 1	Week 2	Week 4	Week 13	Week 26	<i>p</i> value (OVX) ^a	<i>p</i> value (time) ^b
OS/BS (%)										
LV	Sham	2.86 ± 2.92	3.43 ± 1.79	5.55 ± 2.86	3.44 ± 1.17	5.06 ± 2.19	3.79 ± 2.01	2.78 ± 1.65		
	OVX	–	1.82 ± 1.69	2.34 ± 1.45*	7.60 ± 4.03	14.72 ± 4.23**†	13.23 ± 2.21**†	16.09 ± 4.35**†	<0.001	<0.001
Iliac bone	Sham	7.04 ± 3.57	6.27 ± 2.84	8.31 ± 4.98	8.41 ± 4.67	8.13 ± 4.73	2.33 ± 2.08†	3.97 ± 1.43		
	OVX	–	6.56 ± 4.04	4.82 ± 2.31	8.50 ± 7.55	31.33 ± 3.61**†	24.22 ± 4.80**†	22.46 ± 6.33**†	<0.001	<0.001
Tibia	Sham	10.22 ± 7.46	14.23 ± 5.26	15.13 ± 7.94	14.92 ± 9.74	12.86 ± 9.68	4.92 ± 2.25	3.13 ± 3.29		
	OVX	–	12.63 ± 3.36	14.24 ± 6.08	17.54 ± 9.84	27.08 ± 1.41*	26.54 ± 13.88*	27.96 ± 5.08*	<0.001	0.497
Ob.S/BS (%)										
LV	Sham	0.43 ± 0.23	1.65 ± 0.98†	1.83 ± 0.95†	0.43 ± 0.35	1.28 ± 1.20	0.83 ± 0.22†	0.62 ± 0.32		
	OVX	–	0.85 ± 0.39	0.46 ± 0.27*	1.24 ± 0.70**†	2.83 ± 1.61†	2.82 ± 1.03**†	3.76 ± 2.60**†	0.003	0.044
Iliac bone	Sham	3.12 ± 1.93	3.17 ± 2.17	2.25 ± 2.10	1.55 ± 1.87	2.19 ± 1.62	0.79 ± 0.57†	0.92 ± 0.64†		
	OVX	–	2.78 ± 1.99	1.12 ± 0.79	2.13 ± 2.54	10.46 ± 2.20**†	6.14 ± 1.46**†	4.04 ± 1.51*	<0.001	<0.001
Tibia	Sham	5.39 ± 4.38	4.91 ± 1.46	4.43 ± 4.54	1.76 ± 2.09	1.88 ± 1.51	0.86 ± 0.45	0.65 ± 0.77		
	OVX	–	4.33 ± 1.38	3.62 ± 3.00	4.17 ± 3.97	6.91 ± 1.88*	7.28 ± 6.75	7.12 ± 2.41*	<0.001	0.880

Data are expressed as mean ± SD, *n* = 5

The time-course of changes in these parameters is presented in Supplementary Fig. S3

OS/BS osteoid surface, Ob.S/BS osteoblast surface, LV lumbar vertebra, OVX ovariectomy

* *p* < 0.05 vs. Sham group (unpaired Student's *t* test)

† *p* < 0.05 vs. week 0 (unpaired Student's *t* test)

^a *p* value between Sham and OVX groups (two-way ANOVA)

^b *p* value among time points (two-way ANOVA)

Table 4 Kinetic parameters of lumbar vertebra, iliac bone, and tibia

Variable (units)	Operation	Day 0	Day 3	Week 1	Week 2	Week 4	Week 13	Week 26	p value (OVX) ^a	p value (time) ^b
MS/BS (%)										
LV	Sham	4.60 ± 3.13	4.71 ± 1.62	4.29 ± 3.24	3.68 ± 2.57	3.96 ± 2.02	4.04 ± 1.59	1.91 ± 1.19		
	OVX	–	2.69 ± 2.11	0.94 ± 0.51 [†]	6.15 ± 2.59	12.09 ± 3.96** [†]	9.15 ± 2.22** [†]	7.36 ± 2.29*	<0.001	<0.001
Iliac bone	Sham	10.31 ± 6.10	6.31 ± 3.71	4.41 ± 3.28	8.93 ± 4.68	7.43 ± 4.78	1.43 ± 1.36 [†]	1.28 ± 0.40 [†]		
	OVX	–	6.99 ± 4.67	1.72 ± 1.18 [†]	5.31 ± 3.91	24.19 ± 3.86** [†]	9.82 ± 1.76** [†]	6.17 ± 3.93*	<0.001	<0.001
Tibia	Sham	16.57 ± 12.21	17.68 ± 9.16	13.39 ± 6.37	14.51 ± 5.16	11.79 ± 7.23	4.04 ± 1.78	2.05 ± 1.96 [†]		
	OVX	–	16.41 ± 5.38	10.68 ± 3.58	11.81 ± 7.19	29.72 ± 3.71*	13.38 ± 7.02*	14.71 ± 2.20*	<0.001	<0.001
MAR (µm/day)										
LV	Sham	1.26 ± 0.14	1.12 ± 0.34	1.15 ± 0.37	1.13 ± 0.41	1.19 ± 0.28	1.02 ± 0.40	0.89 ± 0.35		
	OVX	–	1.31 ± 0.32	0.90 ± 0.43	0.72 ± 0.12	1.04 ± 0.24	1.09 ± 0.31	0.88 ± 0.11	0.283	0.250
Iliac bone	Sham	1.74 ± 0.26	1.64 ± 0.33	1.57 ± 0.42	1.13 ± 0.30 [†]	1.41 ± 0.34	0.85 ± 0.19 [†]	0.78 ± 0.15 [†]		
	OVX	–	1.87 ± 0.52	1.44 ± 0.41	0.91 ± 0.20 [†]	1.75 ± 0.33	1.00 ± 0.20 [†]	0.81 ± 0.12 [†]	0.415	<0.001
Tibia	Sham	1.61 ± 0.33	1.62 ± 0.27	1.56 ± 0.27	1.16 ± 0.26 [†]	1.18 ± 0.22 [†]	1.08 ± 0.20 [†]	1.06 ± 0.43		
	OVX	–	1.54 ± 0.15	1.43 ± 0.15	1.18 ± 0.35	1.76 ± 0.15	1.12 ± 0.21 [†]	0.96 ± 0.44 [†]	0.459	<0.001
Omt (days)										
LV	Sham	2.96 ± 0.43	3.31 ± 1.01	3.70 ± 1.64	3.27 ± 1.31	3.15 ± 0.41	3.82 ± 1.20	6.58 ± 3.80		
	OVX	–	2.98 ± 0.61	4.93 ± 2.75	4.61 ± 0.66 [†]	3.64 ± 0.47 [†]	3.51 ± 0.70	5.02 ± 0.75	0.738	0.009
Iliac bone	Sham	2.90 ± 0.59	2.51 ± 0.47	2.73 ± 0.88	3.48 ± 0.79	3.00 ± 0.45	5.80 ± 1.82 [†]	6.03 ± 1.09 [†]		
	OVX	–	2.38 ± 0.73	3.19 ± 0.82	3.94 ± 0.77 [†]	2.71 ± 0.42	4.80 ± 0.68 [†]	5.77 ± 0.91 [†]	0.586	<0.001
Tibia	Sham	3.00 ± 0.49	2.82 ± 0.49	2.69 ± 0.53	3.31 ± 1.30	3.66 ± 0.79	3.33 ± 0.54	4.01 ± 1.54		
	OVX	–	2.87 ± 0.30	2.77 ± 0.21	3.34 ± 1.02	2.56 ± 0.27	3.69 ± 0.58	5.66 ± 3.45	0.585	0.007
BFR/BV (%/year)										
LV	Sham	58.5 ± 46.9	55.1 ± 36.5	51.7 ± 42.4	36.3 ± 20.6	41.8 ± 16.8	38.6 ± 23.2	14.6 ± 10.4		
	OVX	–	35.9 ± 25.9	8.9 ± 8.7 [†]	42.8 ± 18.1	110.7 ± 42.0*	119.7 ± 86.0	98.5 ± 51.7*	67.9 ± 27.1*	0.009
Iliac bone	Sham	208.5 ± 155.8	103.2 ± 67.4	71.2 ± 55.0	111.6 ± 70.0	119.7 ± 86.0	12.4 ± 12.8 [†]	11.2 ± 3.7 [†]		
	OVX	–	146.1 ± 126.3	26.7 ± 18.8 [†]	55.6 ± 49.1	444.2 ± 195.3*	444.2 ± 195.3*	96.2 ± 31.5*	52.5 ± 40.7	0.003
Tibia	Sham	405.9 ± 368.1	364.4 ± 165.2	276.6 ± 162.4	222.1 ± 120.8	193.0 ± 133.6	66.1 ± 32.7	27.5 ± 27.7		
	OVX	–	342.7 ± 130.0	228.7 ± 106.7	216.8 ± 140.1	697.8 ± 126.6*	190.9 ± 129.6	171.6 ± 76.2*	<0.001	<0.001
FP (days)										
LV	Sham	10.36 ± 1.69	14.64 ± 5.28	48.49 ± 63.27	23.47 ± 13.04	22.77 ± 4.90 [†]	19.33 ± 11.16	29.16 ± 6.05 [†]		
	OVX	–	11.83 ± 7.32	54.17 ± 25.86 [†]	37.90 ± 17.45 [†]	27.94 ± 11.33 [†]	25.54 ± 8.24 [†]	45.16 ± 14.25 [†]	0.196	0.008
Iliac bone	Sham	8.81 ± 4.40	11.53 ± 4.12	25.85 ± 16.94	13.59 ± 4.56	20.70 ± 13.43	47.80 ± 16.83 [†]	72.83 ± 36.15 [†]		
	OVX	–	9.93 ± 4.13	41.00 ± 24.12 [†]	26.51 ± 6.69** [†]	19.22 ± 8.49 [†]	59.94 ± 20.39 [†]	118.08 ± 56.87 [†]	0.026	<0.001
Tibia	Sham	8.01 ± 2.55	8.49 ± 3.54	12.52 ± 3.70	16.68 ± 1.90 [†]	18.41 ± 4.27 [†]	22.27 ± 6.47 [†]	23.69 ± 9.43 [†]		
	OVX	–	9.24 ± 3.64	18.68 ± 5.31 [†]	26.31 ± 8.04** [†]	10.92 ± 2.50*	42.27 ± 8.94** [†]	40.70 ± 21.16 [†]	<0.001	<0.001

Table 4 continued

Variable (units)	Operation	Day 0	Day 3	Week 1	Week 2	Week 4	Week 13	Week 26	p value (OVX) ^a	p value (time) ^b
Rm.P (days)										
LV	Sham	35.8 ± 22.1	31.5 ± 18.4	89.2 ± 106.1	70.2 ± 44.1	41.2 ± 11.3	40.9 ± 17.8	103.2 ± 140.5		
	OVX	–	41.9 ± 27.6	152.2 ± 93.7	78.6 ± 34.9	38.3 ± 14.4	36.9 ± 12.2	54.3 ± 16.5	0.785	0.022
Iliac bone	Sham	14.9 ± 8.9	18.8 ± 5.1	38.2 ± 26.0	25.3 ± 10.5	45.1 ± 45.8	165.6 ± 82.9 [†]	127.5 ± 62.9 [†]		
	OVX	–	19.0 ± 7.7	78.2 ± 40.4 [†]	87.4 ± 67.6 [†]	24.7 ± 10.2	72.4 ± 25.3 [†]	152.9 ± 65.5 [†]	0.842	<0.001
Tibia	Sham	15.3 ± 7.1	10.7 ± 4.3	16.3 ± 6.1	31.7 ± 8.2 [†]	33.5 ± 10.7 [†]	55.0 ± 24.7 [†]	52.6 ± 22.8 [†]		
	OVX	–	12.1 ± 5.4	24.6 ± 6.1	47.6 ± 25.2 [†]	14.6 ± 3.6	57.6 ± 18.9 [†]	58.1 ± 34.3 [†]	0.582	<0.001
Tt.P (days)										
LV	Sham	608.3 ± 384.9	606.3 ± 567.1	1305.8 ± 1767.5	745.3 ± 494.7	501.3 ± 177.7	631.8 ± 420.5	2518.8 ± 3691.7		
	OVX	–	1391.6 ± 1368.2	2990.1 ± 1689.5 [†]	542.9 ± 145.7	205.8 ± 101.7	206.1 ± 102.1	297.8 ± 107.4	1894.2 ± 814.4 [†]	0.751
Iliac bone	Sham	186.6 ± 196.9	206.7 ± 91.7	516.8 ± 616.8	210.1 ± 136.4	531.1 ± 751.6	3255.7 ± 2139.7 [†]	1894.2 ± 814.4 [†]		
	OVX	–	253.3 ± 257.6	1029.6 ± 730.7 [†]	917.6 ± 1149.3	61.5 ± 27.0	257.0 ± 104.3 [*]	580.5 ± 337.6 [*]	0.009	<0.001
Tibia	Sham	149.1 ± 170.7	64.4 ± 23.6	101.2 ± 52.9	159.3 ± 102.4	219.3 ± 139.7	622.2 ± 547.4	1272.3 ± 640.2		
	OVX	–	80.9 ± 53.1	149.3 ± 78.5	236.5 ± 225.0	40.6 ± 10.7	215.8 ± 144.7	148.1 ± 75.2 [*]	<0.001	<0.001
Ac.f (N/year)										
LV	Sham	1.01 ± 1.00	1.01 ± 0.67	0.98 ± 0.86	0.69 ± 0.42	0.82 ± 0.35	0.80 ± 0.51	0.38 ± 0.25		
	OVX	–	0.61 ± 0.48	0.20 ± 0.20	0.73 ± 0.26	2.28 ± 1.40	2.17 ± 1.12 [*]	1.39 ± 0.58 [*]	0.015	0.008
Iliac bone	Sham	3.70 ± 2.60	2.10 ± 1.02	1.63 ± 1.21	2.47 ± 1.47	2.09 ± 1.53	0.21 ± 0.19 [†]	0.22 ± 0.07 [†]		
	OVX	–	3.40 ± 3.55	0.62 ± 0.59 [†]	1.22 ± 1.06	6.85 ± 2.61 [*]	1.62 ± 0.62 [*]	0.87 ± 0.55 [*]	0.018	<0.001
Tibia	Sham	5.65 ± 5.22	7.05 ± 4.78	4.66 ± 2.72	3.15 ± 1.83	2.40 ± 1.52	0.89 ± 0.48	0.49 ± 0.54		
	OVX	–	5.67 ± 2.38	2.86 ± 1.04	2.80 ± 2.10	9.54 ± 2.74 [*]	2.46 ± 1.66	3.13 ± 1.88 [*]	0.030	<0.001

Data are expressed as mean ± SD, n = 5

The time-course of changes in these parameters is presented in Supplementary Figs. S4-1 and S4-2

MS/BS mineralizing surface, MAR mineral apposition rate, Omt osteoid maturation time, BFR/BV bone formation rate, FP formation period, Rm.P remodeling period, Tt.P total period, Ac.f activation frequency, LV lumbar vertebra, OVX ovariectomy

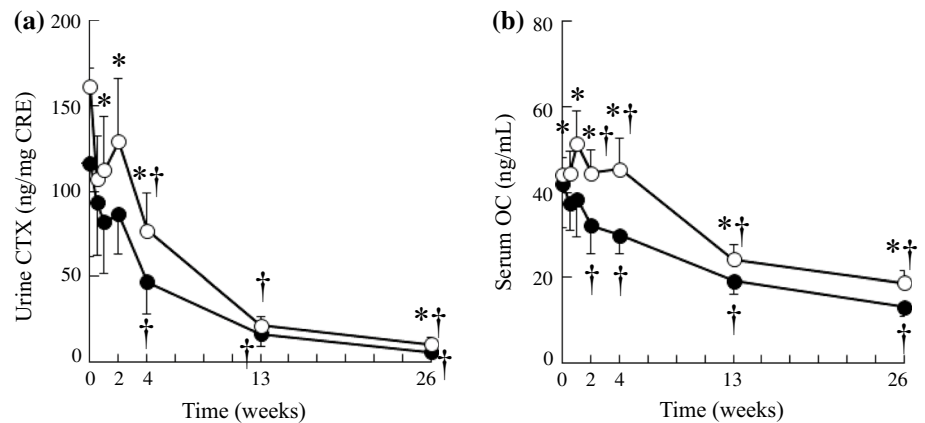
* p < 0.05 vs. Sham group (unpaired Student's t test)

† p < 0.05 vs. week 0 (unpaired Student's t test)

^a p value between Sham and OVX groups (two-way ANOVA)

^b p value among time points (two-way ANOVA)

Fig. 2 Changes in urine type I collagen (CTX) (a) and serum osteocalcin (OC) (b). Closed circles Sham group, open circles ovariectomy (OVX) group. * $p < 0.05$, OVX group vs. Sham group (Student's *t* test). † $p < 0.05$, for each time point vs. 0 weeks (Student's *t* test)



N.Oc/BS were higher in the OVX group than in the sham group at week 13 in the LV and from weeks 2 to 4 in the iliac bone. In the tibia, however, Oc.S/BS was lower in the OVX group than in the sham group at day 3 postoperatively. No clear differences were seen between the two groups thereafter (Table 2; Supplementary Fig. S2).

The sham group exhibited no clear changes in the bone formation parameter OS/BS from day 0 to week 26 postoperatively in the LV, iliac bone, or tibia (Table 3; Supplementary Fig. S3a–c). Ob.S/BS was temporarily higher in the LV after surgery than on day 0 and lower in the iliac bone from weeks 13 to 26, but no clear changes in the tibia were apparent from day 0 to week 26 (Table 3; Supplementary Fig. S3d–f). OS/BS was higher in the OVX group than in the sham group in the LV, iliac bone, and tibia from weeks 4 to 26. Additionally, Ob.S/BS was higher in the OVX group than in the sham group in the LV from week 2 onward and in the iliac bone and tibia from week 4 onward (Table 3; Supplementary Fig. S3).

MS/BS tended to decrease over time in all skeletal sites in the sham group and was lower from week 13 onward in the iliac bone and at week 26 in the tibia than at day 0 (Table 4; Supplementary Fig. S4-1a–c). MAR was lower in the iliac bone and tibia from week 2 onward than at day 0. Omt was higher in the iliac bone from weeks 13 to 26 than at day 0, but no clear changes were observed in the LV or tibia. BFR/BV tended to decrease over time in all skeletal sites and was lower from week 13 onward in the iliac bone than at day 0 (Table 4; Supplementary Fig. S4-1d–l). FP, Rm.P, and Tt.P tended to be higher in each skeletal site from weeks 13 to 26 than at day 0 (Table 4; Supplementary Fig. S4-2a–i). Ac.f did not change significantly in the LV or tibia from day 0 to week 26, but was lower from week 13 onward in the iliac bone (Table 4; Supplementary Fig. S4-2j–l). MS/BS and BFR/BV were higher in the OVX group than in the sham group from week 4 onward in each skeletal site. No clear differences in MAR or Omt in any skeletal site were observed between the OVX and sham groups (Table 4; Supplementary Fig. S4-1). FP was higher

in the OVX group than in the sham group from weeks 2 to 13 in the tibia and at week 2 in the iliac bone, but showed no clear differences between the OVX and sham groups in the LV (Table 4; Supplementary Fig. S4-2a–c). No differences were observed in Rm.P between the OVX and sham groups in any of the skeletal sites. Tt.P was lower in the OVX group than in the sham group from week 13 onward in the iliac bone and at week 26 in the tibia. Ac.f tended to be higher in the OVX group than in the sham group in each skeletal site from week 4 or 13 onward (Table 4; Supplementary Fig. S4-2j–l). At week 26 in OVX rats, Ac.f was highest in the tibia (3.13 N/year) and similar between the iliac bone (0.87 N/year) and LV (1.39 N/year).

Biomarkers

In the sham group, urine CTX decreased with age and reached 6 % of the preoperative level at week 26 postoperatively (Fig. 2a). In the OVX group, however, urine CTX transiently decreased at day 3 postoperatively, then increased at week 2 and was higher from weeks 1 to 4 than in the sham group. Urine CTX then decreased over time to 7 % of the preoperative level at week 26 postoperatively.

In the sham group, serum OC decreased with age and reached 33 % of the preoperative level at week 26 postoperatively (Fig. 2b). In the OVX group, however, the serum OC increased to 117 % of the preoperative level at week 1 postoperatively and was higher from day 3 to week 4 than in the sham group. Serum OC then decreased over time to 43 % of the preoperative level at week 26 postoperatively, but remained higher in the OVX group than in the sham group throughout the study.

Discussion

In this study, we examined the bone structure and metabolism of the LV, tibia, and iliac bone in sham and OVX rats using bone histomorphometry. This report describes these

bone-related parameters in three skeletal sites in the same rat, and also provides important reference values that could be useful for future research in this setting.

Three-month-old rats (at day 0) were determined to have the largest Tb.Th in the LV, followed by the iliac bone, and then the tibia. The reverse was true for Tb.N, which was highest in the tibia, followed by the iliac bone, and then the LV. The changes in ES/BS, OS/BS, and Ac.f, which are parameters of bone resorption, formation, and turnover, respectively, indicate that bone metabolism was the most active in the tibia, followed by the iliac bone, and then the LV. Li et al. [11] encountered a similar phenomenon in the proximal tibial metaphysis and fourth lumbar vertebral body in intact aged rats. Additionally, our study revealed that the bone structure and metabolism in the iliac bone were intermediate between those in the tibia and LV.

OVX induced a decrease in BV/TV and Tb.N and enhanced bone metabolism in the LV, iliac bone, and tibia, but the degree and timing of these changes differed depending on the skeletal site. The OVX-induced decreases in BV/TV and Tb.N occurred earlier in the tibia and the iliac bone than in the LV. The OVX-induced changes in bone volume and structure were greatest in the tibia and smallest in the LV, and were intermediate in the iliac bone. These results suggest that OVX had the greatest effects on bone volume and structure in the tibia, followed by the iliac bone, and the LV. In the tibia, BV/TV dropped to about 10 % at 6 months after OVX. When the bone volume is extremely small, the state of the bone tissue may not be properly reflected in the data because of an insufficiency of tissue volume and cell population available for analysis. Therefore, long-term studies using OVX rats should focus on assessing the LV or iliac bone, where the bone volume is better retained. Wronski et al. [5, 6] reported a significantly lower cancellous bone volume in OVX rats than in control rats 14 days post-OVX in the tibia and 52 days post-OVX in the LV, and our study showed similar results. Hodsmann et al. [7] reported that the BV/TV was 35 % lower in an OVX rat pelvis than in a sham control pelvis, but was 65 % lower in an OVX rat tibia than in a sham control tibia. Similarly, in our study, OVX resulted in a larger decrease in bone volume in the tibia than in the iliac bone. Like the findings reported by Tanizawa et al. [8], our results suggest that the OVX-induced decrease in Tb.N causes an increase in Tb.Sp and a decrease in BV/TV in all skeletal sites. Tb.N and Tb.Th decrease in patients with osteoporosis, but Tb.N reportedly plays a greater role in the loss of bone volume [12], and a similar phenomenon might occur in rats. The weight-bearing sites differ between humans and rats because of their different postures. It is well known that a difference in mechanical stress affects the changes in bone structure and metabolism after OVX in rats [13]. However, there are similarities between the lumbar vertebrae of OVX

rats and women with postmenopausal osteoporosis, including in the remaining trabecular bone, which is oriented in the craniocaudal direction. This suggests that the changes in bone structure and bone metabolism due to estrogen deficiency are similar in rats and humans.

Analysis of bone resorption and formation parameters confirmed an increase in ES/BS and OS/BS from week 4 after OVX onward in each skeletal site, particularly in the tibia at week 26. The OVX-induced changes in OS/BS were also greatest in the tibia, followed by the iliac bone, and then the LV. These findings suggest that OVX-induced activation of bone resorption and formation was most potent in the tibia, followed by the iliac bone, and then the LV. A previous study reported an increase in the osteoclast and osteoblast surface from day 14 after OVX onward in the tibia [5] and from day 35 after OVX onward in the LV [6]. Overall, these findings suggest that OVX-induced bone resorption and formation occur earlier in the tibia than in the LV or iliac bone.

Ac.f is considered to be a comprehensive indicator of bone turnover based on remodeling, because it is the inverse of Tt.P and indicates the frequency of the remodeling cycle from the quiescent phase to the resorption phase, formation phase, and then back to the quiescent phase. Ac.f was increased by OVX in each skeletal site, with the largest increase in the tibia, followed by the iliac bone, and then the LV. Ac.f in the iliac bone (0.87 N/year) was similar to that in the LV (1.39 N/year) at week 26 in OVX rats. In contrast, the Ac.f in human iliac bone was reported to range from 0.3 to 0.4 N/year [14–16]. Because Ac.f is an index of the frequency of bone turnover, bone turnover in rats occurs several times faster than it does in humans.

There are several possible explanations for the differences in the effects of OVX on bone resorption, bone formation, and bone turnover among the skeletal sites. The first explanation relates to the mechanical loading on each site. The tibia receives a greater mechanical load than does the LV or iliac bone because the rat is a tetra-pedal animal. It is conceivable that sites with greater mechanical loading, such as the tibia, are characterized by more active bone turnover. Indeed, there is a strong relationship between OVX-induced bone loss and the bone turnover rate in rats [5, 11]. Therefore, the changes in bone structure after OVX may be greater in the tibia than in the LV and iliac bone. The second explanation relates to the differences in the populations of osteoclasts and osteoblasts among the skeletal sites. In particular, according to N.Oc/BS, Oc.S/BS, and Ob.S/BS at baseline, there were more osteoclasts and osteoblasts located on the surface of cancellous bone in the tibia than in the LV or iliac bone. These findings suggest that skeletal sites, such as the tibia, that contain numerous bone-related cells owing to their fine cancellous bone with a large trabecular bone surface could be more sensitive to

estrogen deficiency than other sites with fewer bone-related cells.

Bone formation, resorption, calcification, and turnover tended to be temporarily suppressed in the early stage after OVX (weeks 1–2 postoperatively). Tanizawa et al. [8] also reported that a transient reduction in bone formation in the tibia might have been related to inflammatory changes evoked by OVX. We observed a phenomenon in the tibia that could also occur in the iliac bone and the LV.

Bone metabolic markers increased similarly to the parameters of bone resorption and formation in bone tissue after OVX, suggesting that these markers reflect bone resorption and formation in bone tissue, and that the OVX-induced decreases in bone mineral density are caused by accelerated bone metabolism. Although urine CTX and serum OC decreased at the same rate in the OVX and sham groups from weeks 4 to 26 postoperatively, they remained higher in the OVX group. These results suggest that the rate of bone metabolism remained higher in the OVX group than in the sham group from postoperative week 4 onward. Rapid changes in post-OVX bone metabolism thus occurred up to postoperative week 4 and then stabilized from week 13 onward. Therefore, a period of 3–6 months after OVX in rats (6–9 months old) could be the suitable period for evaluating bone metabolism or drugs for osteoporosis. Furthermore, the bone structure and bone metabolism of a specific skeletal site, e.g., iliac bone, LV, or tibia, can be directly compared between rats and humans. Additionally, the structure and metabolism of the iliac bone in rats stabilized about 3–6 months after OVX and were similar to those of the LV. Therefore, the LV could be used instead of the iliac bone in rodent studies.

Although the 13-week-old rats that were used in the present study are sexually mature, they are still growing. Studies have shown that the speed and magnitude of the skeletal response to OVX can vary between skeletal sites and with the age of the rat at the time of OVX [17, 18]. However, because the rate of the decrease in bone volume and the magnitude of changes in bone resorption and formation parameters in the present study did not differ greatly from those in rats that underwent OVX at a later age, the changes may be considered a result of OVX [17–19].

Our study revealed that the OVX-induced changes in bone metabolic markers occurred earlier than the tissue changes. One reason for this phenomenon may be that bone metabolic markers reflect the systemic and real-time activity of osteoclasts and osteoblasts, which are responsible for bone resorption and osteoid formation, respectively; thus, more time is required to detect tissue changes by bone histomorphometry.

In conclusion, the novel findings of this study are that aging or OVX in rats affects bone structure and metabolic parameters regardless of the skeletal site, but the degree and

timing of these changes differ between skeletal sites. Additionally, the present results have clarified the rate of bone turnover in the iliac cancellous bone in OVX rats (Ac.f: 0.87 N/year). In comparison, Ac.f in human iliac bone is reportedly 0.3–0.4 N/year, suggesting that bone turnover in rats is several times higher than that in humans. The data on bone structure and metabolism in rats obtained in this study are expected to be useful as reference values for the translation of experimental results in rats to humans.

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Conflict of interest Aya Takakura, Ryoko Takao-Kawabata, Yukihiro Isogai, and Toshinori Ishizuya are employees of Asahi Kasei Pharma Corporation. Makoto Kajiwara and Hisashi Murayama are employees of Kureha Special Laboratory. Sadakazu Ejiri has no conflicts of interest to report.

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