Effects of Strontium on Calcium Metabolism in Rats II. Strontium Prevents the Increased Rate of Bone Turnover in Ovariectomized Rats

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ABSTRACT—The effects of stable strontium were investigated in ovariectomized (OVX) rats by calcium balance and calcium kinetic studies, histomorphometric analysis and measurements of calcium levels in bone. After 10 days of pair-feeding with a control diet, 71-day-old female Wistar rats were either sham-operated (Sham group) or ovariectomized. The OVX rats were divided into two subgroups: those that were treated with strontium (OVX+Sr group, strontium intake; 87.5 μmol/day/rat) and those that were not (OVX group). Both groups were pair-fed their respective control or strontium diets for 2 weeks. Calcein and tetracycline were injected every 2 weeks from 1 week before ovariectomy to calculate the rate of bone formation in the diaphyseal femora cortex (% BFFC). In the OVX group, urinary calcium and % BFFC decreased, while bone resorption, bone formation and femora length increased at the end of the experiment, as compared with those in the Sham group. No such changes were observed in rats in the OVX+Sr group. The calcium balance, calcium levels in bone and trabecular bone volume in the metaphysis did not change in any of the three groups. These results suggest that strontium may be able to prevent the changes in bone turnover induced by estrogen deficiency.

Keywords: Calcium, Strontium, Bone formation, Bone resorption, Ovariectomy

Ovariectomized (OVX) animal models in a variety of species have been used to evaluate the mechanism of or to assess the effect of drugs in estrogen deficiency (1-4). Although several histomorphometric studies have been performed to investigate the mechanism of estrogen deficiency, only a few have also studied calcium metabolism. Indeed, changes in calcium metabolism are closely associated with, or induce, morphometric disorders in OVX animals. Thus, the analysis of calcium metabolism in OVX rats is necessary to evaluate the mechanism of estrogen deficiency, and calcium balance and calcium kinetic studies are useful for investigating it (5).

Our previous report showed that a large amount of strontium, more than approx. 400 mg/kg/day, disturbs calcium metabolism in intact rats and produces inhibitory effects on calcium metabolism, such as decreased intestinal calcium absorption, decreased bone formation and resorption, negative calcium balance, and decreased calcium contents in bone (6, 7). On the other hand, a strontium-containing substance (S12911) has been shown to prevent femoral osteopenia in OVX rats (8). Morphometrically, the effects of this substance can be observed within 60 days after ovariectomy, at dosages that correspond to less than approx. 100 mg/kg/day of strontium (8). It has been considered that low-dose strontium has a beneficial effect in OVX animals. However, there are few reports on calcium metabolism that investigate the effects of low-dose strontium in OVX rats.

Wronski et al. (9) demonstrated that changes in histomorphometric parameters can be detected as early as 14 days after ovariectomy by measuring the percent of cancellous bone volume in tibiae. We hypothesized that several changes in calcium metabolism might be apparent before histomorphometric changes and that a low dose of strontium might prevent changes in calcium metabolism induced by estrogen deficiency. Thus, a calcium metabolic study in OVX rats within 2 weeks after ovariectomy is necessary to investigate the beneficial effects of strontium. We examined this hypothesis and the effects of low-dose

strontium soon after ovariectomy by calcium balance and calcium kinetic studies, histomorphometric analysis and measurements of calcium levels in bone.

MATERIALS AND METHODS

Animals and diets

Eighteen female Wistar rats (Saitama Experimental Animals Supply Co., Ltd., Saitama) were given a synthetic vitamin D-deficient control diet for 10 days from when they were 61-day-old (5, 6). All of the rats were pair-fed and given 10 IU of vitamin D_3 throughout the experiment in individual metabolic cages. Vitamin D_3 dissolved in 0.1 ml of cottonseed oil was placed on each portion of the diet every day just before feeding. Six rats underwent sham operations (Sham group), and the remaining 12 rats were ovariectomized at the age of 71 days. The ovariectomized rats were separated into two subgroups: an OVX group (n=6) and an OVX+Sr group (n=6). A constant amount of calcium was fed to the rats in each group (1.75 mmol/day). The strontium-fed rats were given a diet similar to that in the control group, but to which strontium carbonate had been added (87.5 μmol/day). Body weight was noted every day throughout the experiment. The treatment of the experimental animals was approved by the Experimental Animal Committee of Showa University.

Calcium or strontium metabolism

The experimental protocol is shown in Fig. 1. Four days before and 3 days after ovariectomy (1st and 2nd periods), a calcium balance study was performed over a 3-day period. To determine calcium intake (Vi), food consumption was noted. Urine and feces were harvested throughout the 3-day period. Calcium was measured in feces (VF) and urine (Vu). Net intestinal calcium absorption (Vna: Vi−VF) and calcium balance (VΔ: Vna−Vu) were calculated (5, 6). Urine and feces were harvested on a sheet of filter paper. The feces and urine-containing filter paper were ashed (640℃, 3 days). These samples were dissolved in 2.0 M HCl. The calcium in feces and the urine solution was determined by an atomic absorption spectrophotometer (Perkin Elmer Co., Ltd., Norwalk, CT, USA). Simultaneously with the calcium balance study, a strontium balance study was also performed to determine strontium intake (sVi), urinary strontium excretion (sVu) and strontium in feces (sVF), and to calculate net intestinal strontium absorption (sVna) and strontium balance (sVΔ) using the same methods described above. Ten days after ovariectomy (3rd period), a strontium balance study, a calcium balance study and a calcium kinetic study were performed over a 3-day period, using the method described by Aubert and Milhaud (10). When the rats were 81-day-old, 1.11 MBq of $^{45}CaCl_2$ solution was injected into the tail vein of each rat. Blood samples were obtained from the tail at 2, 4, 6, 25, 49 and 73 hr after the injection. The cold- and hot-calcium in feces and urine were measured. The samples of feces, urine and serum obtained from the tail vein before sacrifice were counted for ^{45}Ca by a liquid scintillation counter (Packard Instrument Company, Inc., Meriden, CT, USA). At the age of 85 days, a blood sample was obtained from each rat from the carotid artery under ether anesthesia, and the amounts of serum calcium and strontium were determined. Calcium retention in bone (Vo+), calcium release from bone (Vo−) and other calcium fluxes in the body were calculated from these data using the two-compartment model (5). In the final period, calcium or strontium balance parameters, described above, were also determined.

Bone histomorphometry

Calcein (10 mg/kg) and tetracycline (10 mg/kg) were in-

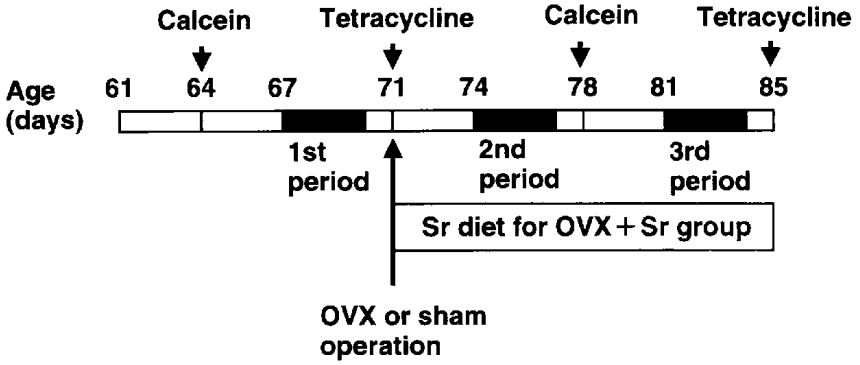

Fig. 1. Experimental protocol. Calcium balance studies were performed before (1st period) and after (2nd period) ovariectomy. The final balance study (3rd period) was combined with a calcium kinetic study. All rats were fed calcium at 1.75 mmol per day, and the rats in the OVX+Sr group were also fed strontium at 87.5 μmol per day from the day of ovariectomy to the end of the experiment. Calcein and tetracycline were injected subcutaneously every 2 weeks as time markers to calculate % BFFC.

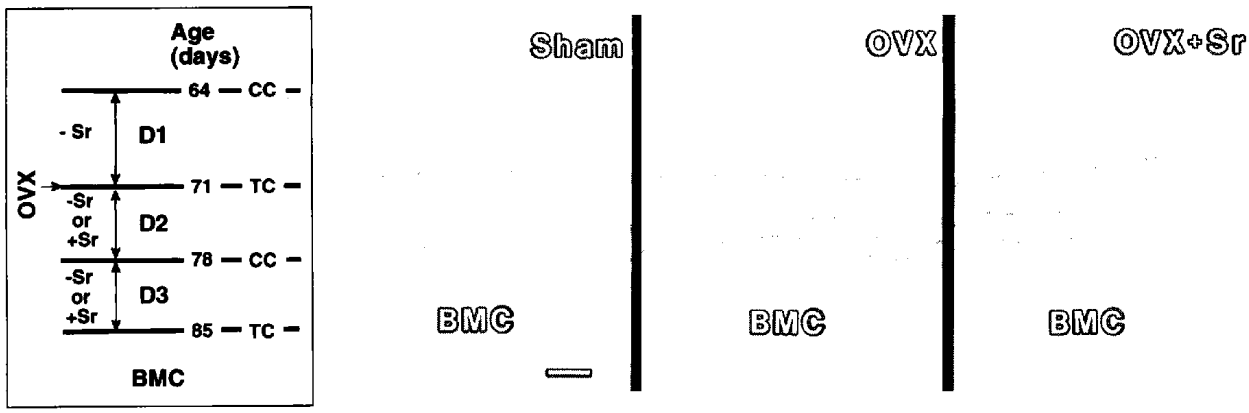

Fig. 2. The rate of bone formation in the diaphyseal femora cortex (% BFFC). Calcein (10 mg/kg) and tetracycline (10 mg/kg) were injected subcutaneously every two weeks from the age of 64 and 71 days, respectively, as time markers. The final tetracycline injection was given 6 hr before sacrifice. The distance between the first calcein and tetracycline labels (D1) reflects bone formation without ovariectomy or strontium-feeding in each rat (control period). D2 and D3 show bone formation after ovariectomy or strontium treatment. Percent BFFC was defined as D2 or D3 divided by D1 × 100. Micrograms show a part of the area in which % BFFC was evaluated in each group. Decreased bone formation was clearly observed in the OVX group. Bone marrow cavity (BMC). Horizontal bar = 100 μm.

jected subcutaneously every two weeks from the age of 64 and 71 days, respectively, for use as time markers. The final tetracycline injection was given 6 hr before sacrifice. Two weeks after ovariectomy, the right femur from each rat was removed and fixed in 70% ethanol. After measuring bone length with a slide caliper, the bones were embedded in polyester resin (Rigolac, Nissin EM Co., Ltd., Tokyo). The middle of the diaphysis was cross-sectioned (100-μm thick) and examined by fluorescence microscopy to determine the rate of bone formation in the diaphyseal femora cortex (% BFFC). Micrographs of calcein and tetracycline labels were taken inside of the endosteal front-lateral diaphysis. The distances between two adjacent lines were measured randomly at 6 points. One line was applied every week (4 lines total), and ovariectomy was performed immediately after the 2nd injection. Therefore, the bone matrix between lines 1 and 2 (D1) was formed during the week before ovariectomy (control period). Percent BFFC after ovariectomy was defined as the average percent ratio of bone formation (distance between two adjacent lines of each period, D2 or D3, to that of the control period). A schematic explanation of % BFFC is shown in Fig. 2. The distal femur metaphysis was embedded in polyester resin, divided in half longitudinally, and the section was polished with 5- and 0.3-μm alumina particles on a polishing cloth. Backscattered electron images of the section were then taken with a scanning electron microscope (S-2500CX; Hitachi, Tokyo) after coating with carbon to calculate the trabecular bone volume (TBV, percent of bone tissue). TBV was measured within a 3.7-mm-wide window on the secondary spongiosa, 1.0 and 1.9 mm from the epiphyseal growth plate, centered on the long axis of the bone, and was evaluated with image processing and analysis software (Ultimage/Pro 2; Graftek, Mirmande, France).

Calcium and strontium in femora

At the age of 85 days, the left femur from each rat was dissected out, adherent soft tissue was carefully removed, and the bones were dried (110℃, 1 day) and ashed (640℃, 3 day). To determine the amount of calcium and strontium in bone, the dried and ashed bones were weighed by an electronic reading balance (Libror AEL160; Shimadzu Co., Kyoto) and dissolved in 2.0 M HCl. Calcium and strontium were determined as described above.

Statistical analyses

All results are expressed as the mean±S.E.M. The significance of the differences between the values in the Sham group and those in the other groups was evaluated by Student's *t*-test.

RESULTS

Calcium and strontium metabolism

No significant difference was observed in the serum calcium concentrations of the three groups. There were no significant changes in serum calcium in the control-diet groups or in the sum of serum calcium and strontium in the strontium-fed group (Table 1). Calcium intake (Vi) was well controlled, and no significant difference was observed between the groups throughout the entire experimental period (data not shown). There were no significant differences in net intestinal calcium absorption

Table 1. Serum calcium and strontium concentrations

	Calcium (Ca) (mM)	Strontium (Sr) (μM)	Ca+Sr (mM)
Sham	2.579±0.039	—	—
OVX	2.562±0.030	—	—
OVX+Sr	2.533±0.027	27.31±3.846	2.560±0.028

All values are means±S.E.M. (n=6). —, values are either not detected or not calculated.

Table 2. Net intestinal calcium absorption (Vna, mmol/day)

	Pre-OVX 1st period	Post-OVX	
		2nd period	3rd period
Sham	0.567±0.044	0.532±0.035	0.440±0.038
OVX	0.598±0.072	0.553±0.029	0.507±0.021
OVX+Sr	0.558±0.060	0.494±0.017	0.419±0.040

All values are means±S.E.M. (n=6).

Table 3. Calcium balance (VΔ, mmol/day)

	Pre-OVX 1st period	Post-OVX	
		2nd period	3rd period
Sham	0.463±0.062	0.454±0.046	0.372±0.038
OVX	0.502±0.061	0.494±0.038	0.465±0.023
OVX+Sr	0.461±0.044	0.406±0.024	0.359±0.049

All values are means±S.E.M. (n=6).

Table 4. Urinary calcium excretion (Vu, mmol/day)

	Pre-OVX 1st period	Post-OVX	
		2nd period	3rd period
Sham	0.105±0.031	0.077±0.017	0.068±0.006
OVX	0.095±0.017	0.059±0.013	0.043±0.003**
OVX+Sr	0.097±0.014	0.089±0.026	0.060±0.009

All values are means±S.E.M. (n=6). **P<0.01 vs Sham group at each period.

(Vna) or calcium balance (VΔ) between groups or between periods (Tables 2 and 3). There was no significant difference in urinary calcium (Vu) among the three groups in the 1st and 2nd periods. However, Vu in the OVX group in the 3rd period was significantly decreased, as compared with that in the Sham group (Table 4). In the calcium kinetic study, increased bone formation (Vo+) and increased resorption (Vo−) were observed in the OVX group, as compared with the Sham group (Fig. 3). In the OVX+Sr group, none of the parameters obtained from the calcium balance and calcium kinetic studies were significantly different from that in the Sham group. No significant difference was observed in net intestinal strontium absorption (sVna) between the 2nd and 3rd periods. However, strontium balance (sVΔ) significantly increased, and urinary strontium excretion (sVu) decreased in the 3rd period, as compared with those in the 2nd period in the OVX+Sr group (Table 5). Fractional stron-

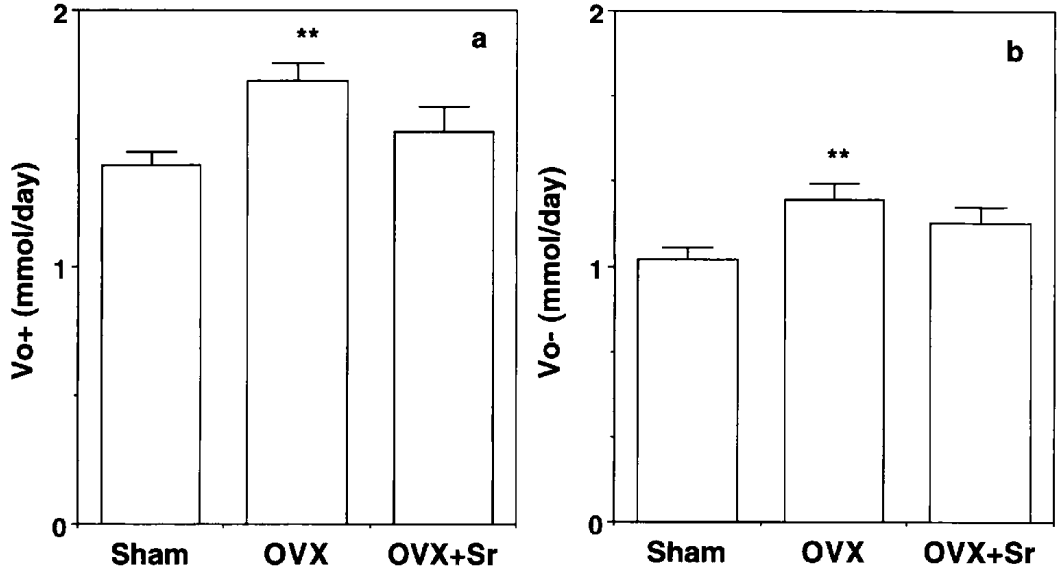

Fig. 3. Effects of strontium and/or ovariectomy on Vo+ (a) and Vo− (b). Vo+ and Vo− indicate calcium retention in bone and calcium release from bone, respectively. Each reported value is a mean±S.E.M. (n=6). **P<0.01 vs the Sham group.

Table 5. Strontium intake, net intestinal strontium absorption, urinary strontium excretion and strontium balance (sVi, sVna, sVu and sVΔ, respectively, μmol/day)

	Post-OVX	
	2nd period	3rd period
sVi	84.43±1.69	87.13±0.04
sVna	19.23±1.58	21.78±3.29
sVu	7.31±0.55	3.48±0.35**
sVΔ	11.91±2.10	18.30±3.40*

All values are means±S.E.M. (n=6). *P<0.05, **P<0.01 vs the 2nd period.

tium retention in bone (sVΔ/sVna×100) increased from approximately 60% in the 2nd period to 82% in the 3rd period (P<0.01).

Histomorphometric study

As shown in Fig. 4, a time-dependent decrease in the rate of bone formation in the diaphyseal femora cortex (% BFFC) was observed in the OVX group, as compared with that in the Sham group. In the OVX+Sr group, % BFFC decreased 1 week after ovariectomy, but no further decrease was observed at the end of the experiment. In the area in which % BFFC was evaluated, calcein and tetracycline labels were observed without breaks, i.e.,

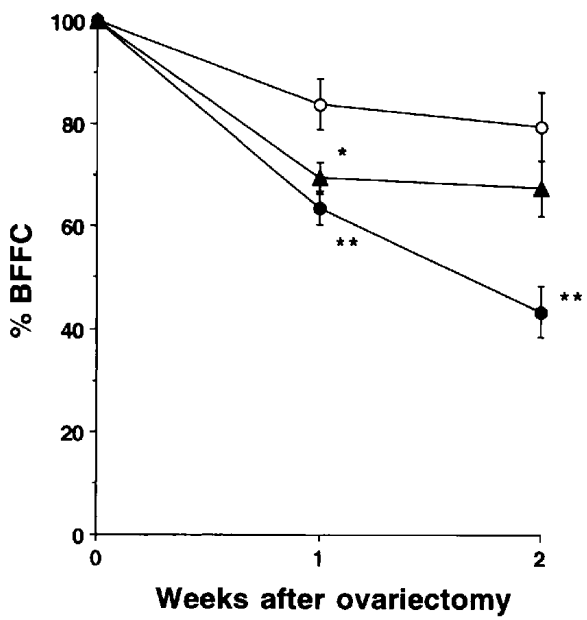

Fig. 4. Effects of strontium and/or ovariectomy on the rate of bone formation in the diaphyseal femora cortex (% BFFC). % BFFC is described in Fig. 2. Open circles, closed circles and closed triangles show the results in the Sham, OVX and OVX+Sr groups, respectively. The values represent the mean±S.E.M. (n=6). *P<0.05, **P<0.01 vs the Sham group.

Table 6. Body weight, calcium and strontium in femora, femur length and trabecular bone volume (TBV)

	Sham	OVX	OVX+Sr
Initial body weight (g)	165.3 ±2.3	170.5 ±3.0	167.8 ±1.5
Final body weight (g)	196.5 ±2.4	205.5 ±3.4*	203.2 ±2.8
Dried bone weight (mg)	377.97±6.70	389.55±5.80	380.28±7.18
Ashed bone weight (mg)	237.20±3.36	244.22±4.73	235.70±5.00
% Ca/dried bone	25.27±0.24	24.20±0.76	24.71±0.42
% Ca/ashed bone	40.25±0.27	38.61±1.17	39.86±0.48
Femur length (mm)	30.6 ±0.2	32.0 ±0.4*	30.8 ±0.5
% TBV	24.19±3.15	23.91±2.44	23.66±3.13
% Sr/Ca (molar ratio)	—	—	0.51±0.01

All values are means±S.E.M. (n=6). *P<0.05 vs Sham group. —, values are not calculated.

ossification was observed in the endosteum without bone resorption in each group. Trabecular bone volume (TBV) in the femur did not change in any of the groups (Table 6).

Body weight and calcium or strontium contents in bone

Body weight in the OVX group was higher than that in the Sham group at the end of the experiment. Femur length in the OVX group increased significantly (P<0.05), as compared with that in the Sham group. However, the femur length in the OVX+Sr group did not increase. The calcium contents in the femur did not change in any of the groups. The percent molar ratio of strontium to calcium in the OVX+Sr group was approximately 0.5% (Table 6).

DISCUSSION

The administration of a large amount of strontium has been reported to inhibit 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) production (11, 12), and to subsequently decrease intestinal calcium absorption (6, 12) and induce rachitic bone lesions (13, 14). One possible mechanism of these phenomena is that they result from an increase in the sum of serum calcium and strontium levels in strontium-fed animals (15). Unfortunately, we did not measure $1,25(OH)_2D_3$ levels in this study. However, it has been reported that a low dose of strontium, given in a strontium chloride solution of less than 0.4%, does not inhibit $1,25(OH)_2D_3$ production (16). Furthermore, in the present study, strontium did not suppress net intestinal calcium absorption, and there were no significant differences between the serum calcium concentrations in the Sham and OVX groups and the sum of the calcium and strontium concentrations in the OVX+Sr group. Even when rats are fed large doses of strontium (1.75

mmol/day/rat), there is no change in serum parathyroid hormone (PTH) or calcitonin (CT). On the other hand, ovariectomy does not alter serum $1,25(OH)_2D_3$ (17), CT (18) or PTH (17) levels in rats. Therefore, the effects of strontium on calcium metabolism in the OVX groups, at least within 2 weeks, might not be associated with the levels of calcium-regulating hormones.

In the present study, we found that both Vo+ and Vo− simultaneously increased within 2 weeks in the OVX group, while there was no difference in calcium balance (VΔ), trabecular bone volume (TBV) or calcium contents between the groups. These findings support the notion that an increased rate of bone turnover occurs before trabecular bone loss in OVX animals (2, 19). On the other hand, neither Vo+ nor Vo− in the OVX+Sr group were increased. These findings may indicate that strontium prevents the increase in bone turnover induced by estrogen deficiency.

We previously demonstrated using an analysis similar to that in the present study that strontium accumulates in bone in a dose-dependent manner in intact rats after 4 weeks of strontium-feeding (7). Such feeding had no effect on Vo− when the strontium content in bone was less than 1% (% Sr/Ca in terms of molarity). The serum strontium concentrations in the rats in the previous study were twofold higher than those in the present experiment. Nevertheless, in this experiment, % Sr/Ca in the femur of the rats in the OVX+Sr group was approximately 0.5%, and this strontium accumulation in bone had an inhibitory effect on the enhanced Vo− induced by ovariectomy. We previously found that strontium inhibits ^{45}Ca release from calvaria in which 1% strontium had accumulated when incubated with PTH (12). Thus, a small amount of strontium in bone might act locally to inhibit the enhanced bone resorption induced by estrogen deficiency. Although the mechanism by which accumulated strontium in bone inhibits enhanced bone resorption at the cellular level is still unclear, optimal levels of strontium in bone or bone fluid restrict the osteoclasts and osteoblasts.

Ovariectomy induces an increase in longitudinal bone growth at 14 days after surgery (9). In the present study, body weight, Vo+ and femur length in the OVX group increased compared to those in the Sham group, while those in the OVX+Sr group did not. These increases may have been due to enhanced growth hormone (GH) secretion. In fact, it has been demonstrated that GH secretion in OVX rats is greater than that in intact female rats during the night (20). Furthermore, GH also regulates the production of insulin-like growth factor-1 (IGF-1) in vitro (21). Recently, Kalu et al. (22) demonstrated that the biological activity of IGF-1 is increased in OVX rats. Furthermore, they suggested that the IGF-1 may play a role in the pathogenesis of the increased bone turnover that occurs early in ovarian hormone deficiency (22). Therefore, the effects of strontium on increased Vo+ and bone length might be associated with IGF-1 and/or GH.

It is well known that growth of the diaphysis results in an increase in the diameter of the shaft due to periosteal bone formation and enlargement of the marrow cavity mainly due to cortical endosteal bone resorption (23). However, in the area in which % BFFC was evaluated, ossification occurred without bone resorption. If estrogen deficiency causes hyper-growth of long bone, one would expect that ossification of the endosteum in the front-lateral diaphysis might be suppressed, so as to enlarge the bone marrow cavity. In fact, we found a decreased % BFFC in the OVX group. Strontium had no effect on the decrease in % BFFC at 1-week post-surgery (D2), but it did prevent a further decrease in this parameter during the 3rd histomorphometric period (D3). These findings suggest that strontium contents in the endosteal surface of the mid-shaft of the femur might be increased to an effective amount during this period. This hypothesis may explain the results of the strontium balance study. There were no significant differences in strontium intake (sVi) or net intestinal strontium absorption (sVna) between the 2nd and 3rd periods. However, strontium balance (sVΔ) may have increased in the 3rd period due to a decrease in urinary strontium excretion (sVu). Fractional strontium retention in bone (sVΔ/sVna × 100) significantly increased from 60% in the 2nd period to 82% in the 3rd period. Strontium which is deposited on newly formed bone is supplied either from food or previously formed bone that contains strontium. In the early period of strontium feeding (2nd period), resorbed bone contains little strontium, while bone that is resorbed in the 3rd period should contain more strontium. Thus, an increased supply of strontium from resorbed bone should accelerate the increase in strontium contents in newly formed bone. Increased bone resorption and formation induced by estrogen deficiency may further accelerate this phenomenon.

In conclusion, a low dose of strontium prevented changes induced by estrogen deficiency in growing rats, such as increased femur length, bone formation, bone resorption and decreased urinary calcium excretion, within 2 weeks post-surgery. However, all of these changes do not necessarily indicate that strontium reduces bone loss as the result of estrogen deficiency. Our results suggest that strontium may be able to prevent the changes in bone turnover induced by estrogen deficiency.

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