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Atorvastatin Enhances Bone Density in Ovariectomized Rats Given 17β-Estradiol or Human Parathyroid Hormone(1-34)

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We investigated the in vivo effect of atorvastatin on bone mineral density (BMD) in ovariectomized (OVX) rats. Eight-week-old female rats underwent either a sham operation or ovariectomy, and treatments with vehicle, atorvastatin, 17β-estradiol (E_2) and human parathyroid hormone(1-34) [hPTH(1-34)] were initiated 6 wk after the surgery. E_2 (10 µg/kg) treatment for 12 wk significantly increased lumbar BMD (L2–L4), whereas atorvastatin did not affect lumbar BMD. The combination of atorvastatin (2 mg/kg) and E_2 significantly enhanced the BMD of lumbar vertebrae (L2–L4) and femoral metaphyseal area (2/10–4/10 segments from the most proximal point) compared to that of either E_2 or atorvastatin alone. A low dose 1 µg/kg of hPTH (1-34) did not alter lumbar or femoral BMD, whereas a high dose 17.5 µg/kg of the peptide significantly increased BMD. Concomitant injections of atorvastatin (2 mg/kg) with hPTH(1-34) (1 µg/kg) for 8 wk significantly enhanced the BMD of lumbar vertebrae and the metaphyseal area of the femur in OVX rats. These findings demonstrate that chronic administration of atorvastatin appears to modestly enhance the BMD of the lumbar vertebrae and femoral metaphysis of OVX rats treated with submaximal doses of E_2 and hPTH(1-34).

Key Words: Statins; bone mineral density; ovariectomized rats; estrogen; parathyroid hormone.

Introduction

Postmenopausal osteoporosis, which results in pathologic bone fractures, is a major health problem in elderly women that typically is associated with low bone mass and poor trabecular architecture *(1)*. Antiresorptive agents, including estrogen and bisphosphonates, that increase bone mass and prevent further bone loss are beneficial for many such patients *(2)*. However, for patients with established osteoporosis, antiresorptives fail to fully restore bone mass *(3)*. Thus, an anabolic agent that enhances bone mass and improves trabecular architecture would be an invaluable addition to treatment of established osteoporosis *(4)*. Intermittent administration of parathyroid hormone (PTH) to osteoporotic human subjects has been shown to increase bone mass *(5)*. The ovariectomized (OVX) rat has received substantial acceptance as a model of human postmenopausal osteoporosis. Many of these animal studies have indicated that intermittent administration of PTH increases cortical and cancellous bone content and improves osseous biomechanical strength. PTH also increases bone formation activity *(6,7)*.

Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase to block conversion of HMG-CoA to mevalonate, a rate-limiting step in cholesterol synthesis *(8)*. These drugs, including atorvastatin, are potent inhibitors of cholesterol biosynthesis and are widely prescribed to lower cholesterol in hyperlipidemic patients at risk for cardiovascular disease *(9)*. Besides lowering serum lipids, statins have a wide range of other actions including inhibiting platelet aggregation and thrombus deposition; promoting angiogenesis; decreasing β-amyloid peptide (related to Alzheimer's disease); and suppressing T-lymphocyte activation *(10)*. By inhibiting the initial part of the cholesterol synthesis pathway, statins decrease availability of several important lipid intermediate compounds including isoprenoids such as geranylgeranyl pyrophosphate (GGPP). These are attached as posttranslational modifications to certain proteins, such as small G proteins including Ras and Ras-like proteins (Rho, Rap, Rab, and Ral) *(11)*.

Mundy et al. *(12)* first reported that statins stimulate in vivo bone formation in rodents and increase new bone volume in cultures from mouse calvaria. Recently, we showed that statins stimulate expression of bone anabolic factors such as vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2 (BMP-2) *(13)*, and promote osteoblast differentiation and mineralization in MC3T3-E1 cells *(14)*. Furthermore, statins have been shown to inhibit osteoclastic bone resorption by inhibiting protein prenylation, a

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Table 1
Effect of Atorvastatin on Serum Calcium, Phosphorus, and Osteocalcin in OVX Rats

Operation	Treatment (Dose)	Calcium (mg/dL)	Phosphorus (mg/dL)	Osteocalcin (ng/mL)
Experiment 1 (n = 10)				
Sham	vehicle	11.3 ± 0.3[a]	5.4 ± 0.3[a]	22.9 ± 1.2[a]
OVX	vehicle	9.8 ± 0.2	4.3 ± 0.2	30.5 ± 1.7
OVX	Ato (2 mg/kg)	9.3 ± 0.3	4.6 ± 0.4	30.2 ± 1.2
OVX	E_2 (10 μg/kg)	10.3 ± 0.2	3.9 ± 0.3	16.6 ± 1.4[a]
OVX	Ato (2 mg/kg) + E_2 (10 μg/kg)	10.6 ± 0.2[a]	5.1 ± 0.3[a]	16.8 ± 1.6[a]
Experiment 2 (n = 8)				
OVX	vehicle	10.3 ± 0.2	6.2 ± 0.3	35.1 ± 1.1
OVX	Ato (2 mg/kg)	9.6 ± 0.1[a]	5.5 ± 0.2[a]	36.2 ± 1.7
OVX	Low PTH (1 μg/kg)	9.9 ± 0.2[a]	5.5 ± 0.5	36.9 ± 1.0
OVX	Ato (2 mg/kg) + low PTH (1 μg/kg)	9.6 ± 0.2[a]	5.5 ± 0.3[a]	42.2 ± 1.1[a,b]
OVX	High PTH (17.5 μg/kg)	9.4 ± 0.1[a]	5.6 ± 0.3	42.5 ± 0.7[a,b]

OVX rats were injected subcutaneously with atorvastatin (Ato) and/or E_2 four times per week for 12 wk (Experiment 1), and with Ato and/or hPTH(1-34) (PTH), four times per week for 8 wk (Experiment 2). The data are expressed as the means ± SEM of 10 or 8 determinations.

[a] $p < 0.01$ compared with vehicle-treated OVX rats.

[b] $p < 0.01$ compared with LowPTH- or atorvastatin-treated OVX rats.

mechanism similar to that of nitrogen-containing bisphosphonates such as alendronate *(15,16)*. One clinical study reported that statins could prevent steroid-induced osteoporosis *(17)*. Animal experiments show that statins also stimulated bone formation, which raises the possibility that these drugs may be used as anabolic agents in the management of established osteoporosis *(12)*. By contrast, Maritz et al. *(18)* reported that statins inhibit bone formation and produce a net reduction in bone mineral density (BMD) in rats. We investigated the effect of atorvastatin on lumbar and femoral BMD in OVX rats treated with an anti-resorptive agent, 17β-estradiol (E_2), and a bone-anabolic hormone, human parathyroid hormone(1-34) [hPTH(1-34)].

Results

We examined the effects of atorvastatin on regulation of bone metabolism in E_2-treated OVX rats (*Experiment 1*). OVX rats were injected subcutaneously with vehicle, E_2 and atorvastatin for 12 wk; and sham-operated rats also received vehicle for 12 wk, the serum calcium and phosphorus were measured (Table 1). Serum calcium and phosphorus concentrations of vehicle-treated OVX rats were lower than those of sham-operated rats, whereas administration of E_2 + atorvastatin in OVX rats significantly increased the calcium and phosphorus concentrations. Administration of atorvastatin or E_2 alone did not affect serum concentrations of calcium and phosphorus in OVX rats. We next tested the hypothesis that atorvastatin can enhance the bone anabolic effect induced by hPTH(1-34) in OVX rats (*Experiment 2*). After 8 wk of intermittent administration of atorvastatin and/or hPTH(1-34), the same serum markers were analyzed (Table 1). Injection of hPTH(1-34) at doses of up to 17.5 μg/kg in OVX rats decreased serum calcium concentrations, but did not affect serum phosphorus concentrations. Atorvastatin administration for 8 wk reduced serum concentrations of calcium and phosphorus. Serum osteocalcin, an osteoblast-derived marker for bone formation, was determined in these rats (Table 1). Ovarian hormone deficiency increased serum concentrations of osteocalcin. Serum osteocalcin concentrations were significantly higher in OVX rats treated with hPTH(1-34) at 17.5 μg/kg and with a combination of atorvastatin at 2 mg/kg + hPTH(1-34) at 1 μg/kg than those treated with vehicle, atorvastatin or hPTH (1-34) (1 μg/kg). Administration of atorvastatin (2 mg/kg) alone for 8–12 wk did not affect serum osteocalcin concentrations in OVX rats (Table 1).

To determine whether atorvastatin enhanced BMD, the effects of E_2 and low-dose hPTH(1-34) on lumbar and femoral BMD were tested in atorvastatin-treated OVX rats. E_2 (10 μg/kg) significantly increased lumbar BMD (L2–L4), restoring it to that of sham-operated rats, whereas atorvastatin (2 mg/kg) did not alter lumbar BMD (Fig. 1). Lumbar BMD was significantly elevated in both E_2 and atorvastatin-treated rats, and exceeded the BMD of the sham group (Fig. 1). Figure 2 shows femoral BMD of OVX rats treated with E_2 and/or atorvastatin, and sham-operated rats. E_2 administration in OVX rats increased BMD, while atorvastatin

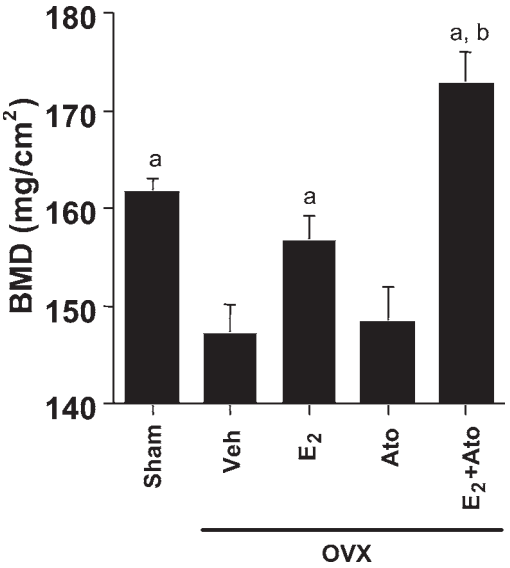

Fig.1. Effect of E_2, atorvastatin (Ato), or E_2 plus atorvastatin (E_2 + Ato) on lumbar BMD. Six weeks after OVX or sham operation (Sham), rats were injected subcutaneously with vehicle (Veh), E_2 (10 μg/kg), atorvastatin (2 mg/kg), or E_2 + atorvastatin, four times per week for 12 wk, and thereafter were killed to determine BMD of the lumbar vertebrae (L2–L4). BMD was measured using DEXA. Data are expressed as the mean ± SEM of 10 rats. [a]$p < 0.05$ compared with vehicle-treated OVX rats. [b]$p < 0.05$ compared with E_2-, or atorvastatin-treated OVX rats.

did not influence femoral BMD (Fig. 2A). The combination of E_2 and atorvastatin did not enhance BMD of the total area of the femur compared with E_2-treated group (Fig. 2A). To examine whether the effects of E_2 and atorvastatin differ between trabecular and cortical bone BMD at the metaphyseal area, which is abundant in trabecular bone and the diaphyseal area, consisting of mostly cortical bone, was compared in these rats. The E_2 + atorvastatin group had a significantly increased metaphyseal BMD compared with the atorvastatin groups (Fig. 2B), whereas the diaphyseal BMD was not significantly different in the E_2, atorvastatin, or E_2 + atorvastatin groups (Fig. 2C). Concomitant injections of atorvastatin with a low dose of hPTH(1-34) significantly increased BMD in the lumbar vertebrae (L2–L4) (Fig. 3). The high PTH group showed increased BMD of the total, metaphyseal, and diaphyseal areas in OVX rats (Fig. 4). Femoral BMD in the low PTH and atorvastatin groups was similar to that of the vehicle-treated group, while the low PTH + atorvastatin group revealed significantly higher total or metaphyseal BMD compared with the vehicle, or low PTH groups (Fig. 4A,B). Atorvastatin administration did not significantly enhance diaphyseal BMD in OVX rats treated with low PTH (Fig. 4C).

Figure 5 shows representative histologic sections of epiphyseal and metaphyseal areas of tibiae from OVX rats. Administration of either E_2 or atorvastatin to OVX rats did not significantly affect trabecular bone volume, whereas

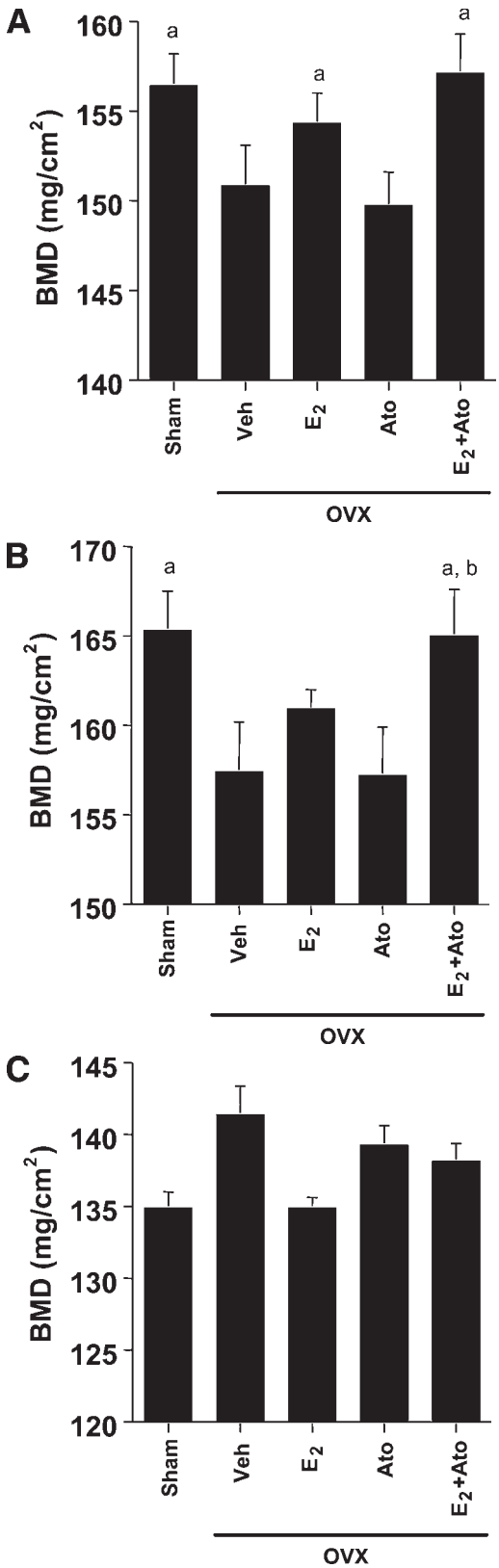

Fig. 2. Effect of E_2, atorvastatin (Ato), or E_2 plus atorvastatin (E_2 + Ato) on femoral BMD. The animal protocol was the same as for Fig. 1. The BMD of femurs was measured in three ways (A, total area ; B, metaphyseal area ; C, diaphyseal area). Data are expressed as the mean ± SEM of 10 rats. [a]$p < 0.05$ compared with vehicle (Veh)-treated OVX rats. [b]$p < 0.05$ compared with atorvastatin-treated OVX rats.

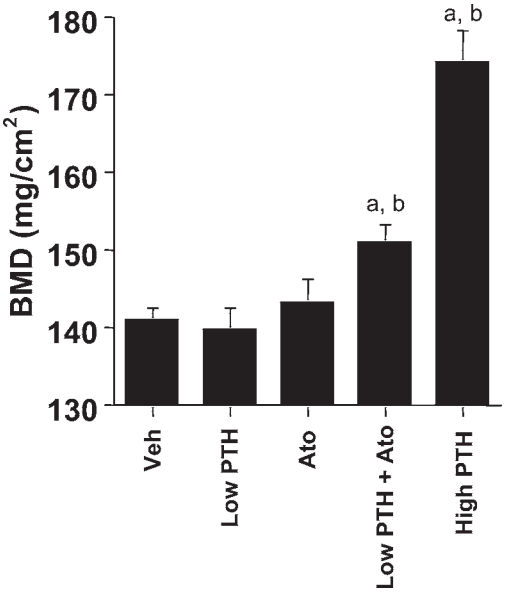

Fig. 3. Effect of hPTH(1-34), and/or atorvastatin on lumbar BMD of OVX rats. Six weeks after OVX, rats were injected subcutaneously with vehicle (Veh), low dose (1 μg/kg) of hPTH(1-34) (LowPTH), atorvastatin (2 mg/kg) (Ato), LowPTH + Ato, or high dose (17.5 μg/kg) of hPTH(1-34) (HighPTH), four times per week for 8 wk, and thereafter were killed to determine lumbar BMD (L2–L4). BMD was measured using DEXA. Data are expressed as the mean ± SEM of 8 rats. [a]$p < 0.05$ compared with vehicle-treated OVX rats. [b]$p < 0.05$ compared with LowPTH- or atorvastatin-treated OVX rats.

combined treatment of E_2 and atorvastatin showed a tendency to increase the trabecular bone area (Fig. 5A–D). Administration of low PTH and atorvastatin to OVX rats augmented the trabecular bone volume compared to that of low PTH (Fig. 5E–G), although changes in the tibia may not apply to other skeletal sites. We next performed a μCT analysis of femurs (Fig. 6). Images from sham-operated rats showed a greater trabecular bone area than those with ovariectomy. In OVX rats, combined treatment of E_2 and atorvastatin revealed a clear increase of trabecular bone area compared to that of E_2 or atorvastatin alone. By contrast, the diaphyseal bone area did not differ in animals of experiment 1 (Fig. 6). Images of μCT from experiment 2 showed that the trabecular bone area of OVX rat femurs was elevated by combined treatment of hPTH(1-34) (1 μg/kg) with atorvastatin, compared to single treatment. The trabecular

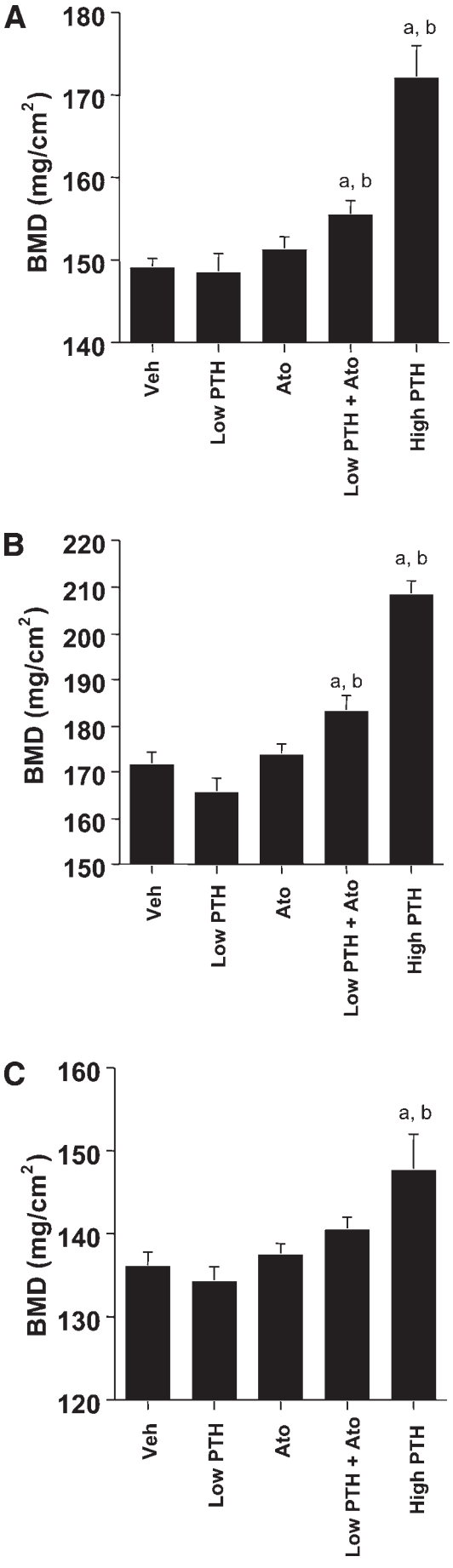

Fig. 4. Effect of hPTH(1-34), and/or atorvastatin on femoral BMD of OVX rats. Six weeks after OVX, rats were injected subcutaneously with vehicle (Veh), low dose (1 μg/kg) of hPTH(1-34) (LowPTH), atorvastatin (2 mg/kg) (Ato), LowPTH+Ato, or high dose (17.5 μg/kg) of hPTH(1-34) (HighPTH), four times per week for 8 wk. The BMD of femurs was measured in three ways (A, total area ; B, metaphyseal area ; C, diaphyseal area). Data are expressed as the mean ± SEM of 8 rats. [a]$p < 0.05$ compared with vehicle-treated OVX rats. [b]$p < 0.05$ compared with LowPTH-treated OVX rats.

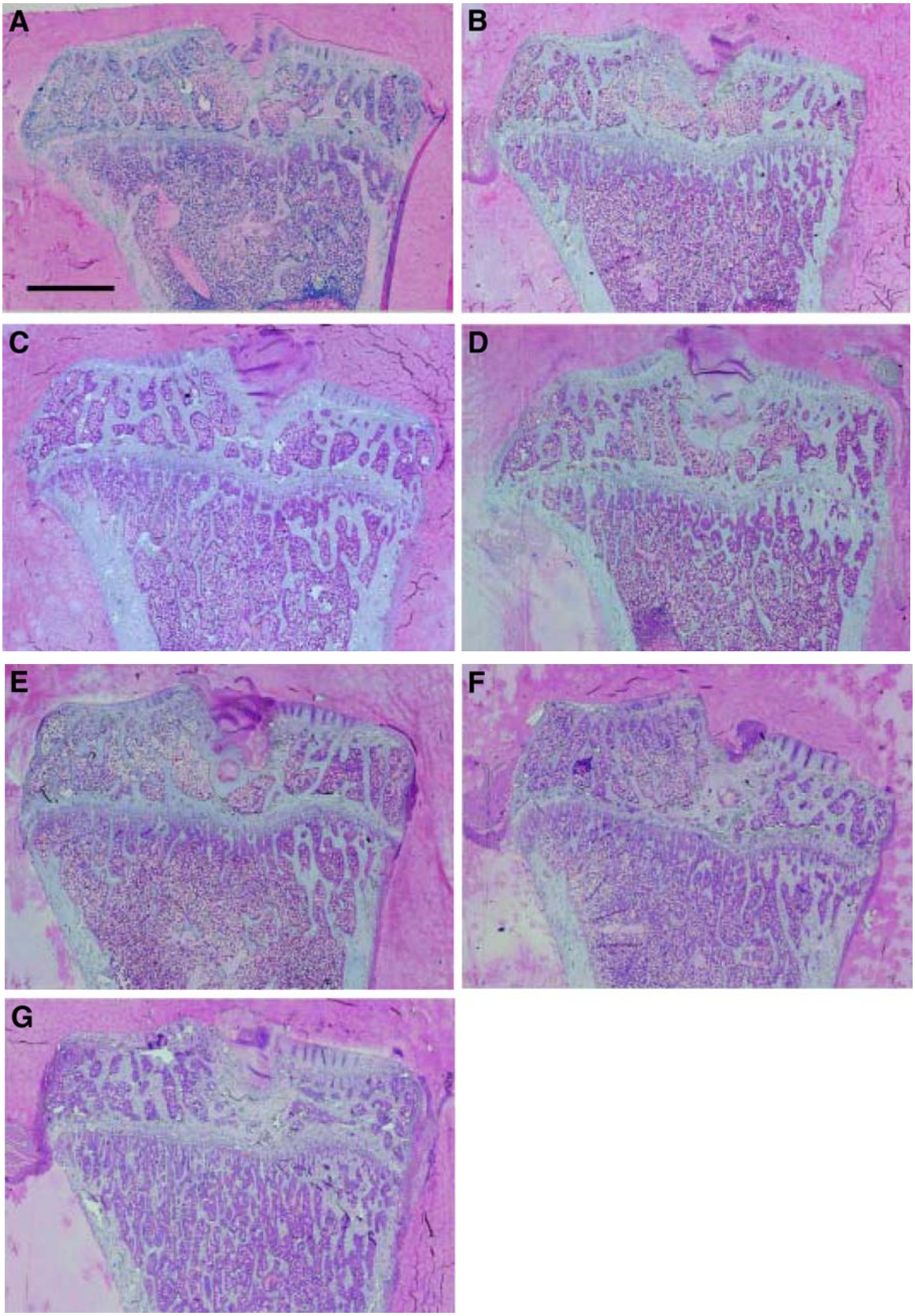

Fig. 5. Histologic analysis of proximal tibiae of OVX rats. Undecalcified sections were stained with hematoxylin and eosin. (**A**) vehicle-treated rat. (**B**) E_2 (10 μg/kg)-treated rat. (**C**) atorvastatin (2 mg/kg)-treated rat. (**D**) E_2 (10 μg/kg) + atorvastatin-treated rat. (**E**) LowPTH [1μg/kg of hPTH(1-34)]-treated rat. (**F**) atorvastatin (2 mg/kg)-treated rat. (**G**) LowPTH + atorvastatin (2 mg/kg)-treated rat. The animal protocols of panels A–D and E–G were the same as for Fig. 1 (Experiment 1) and Fig. 3 (Experiment 2), respectively. Three sections per group were made, and a representative sample at each group was photographed. Bar in (**A**) = 1 mm.

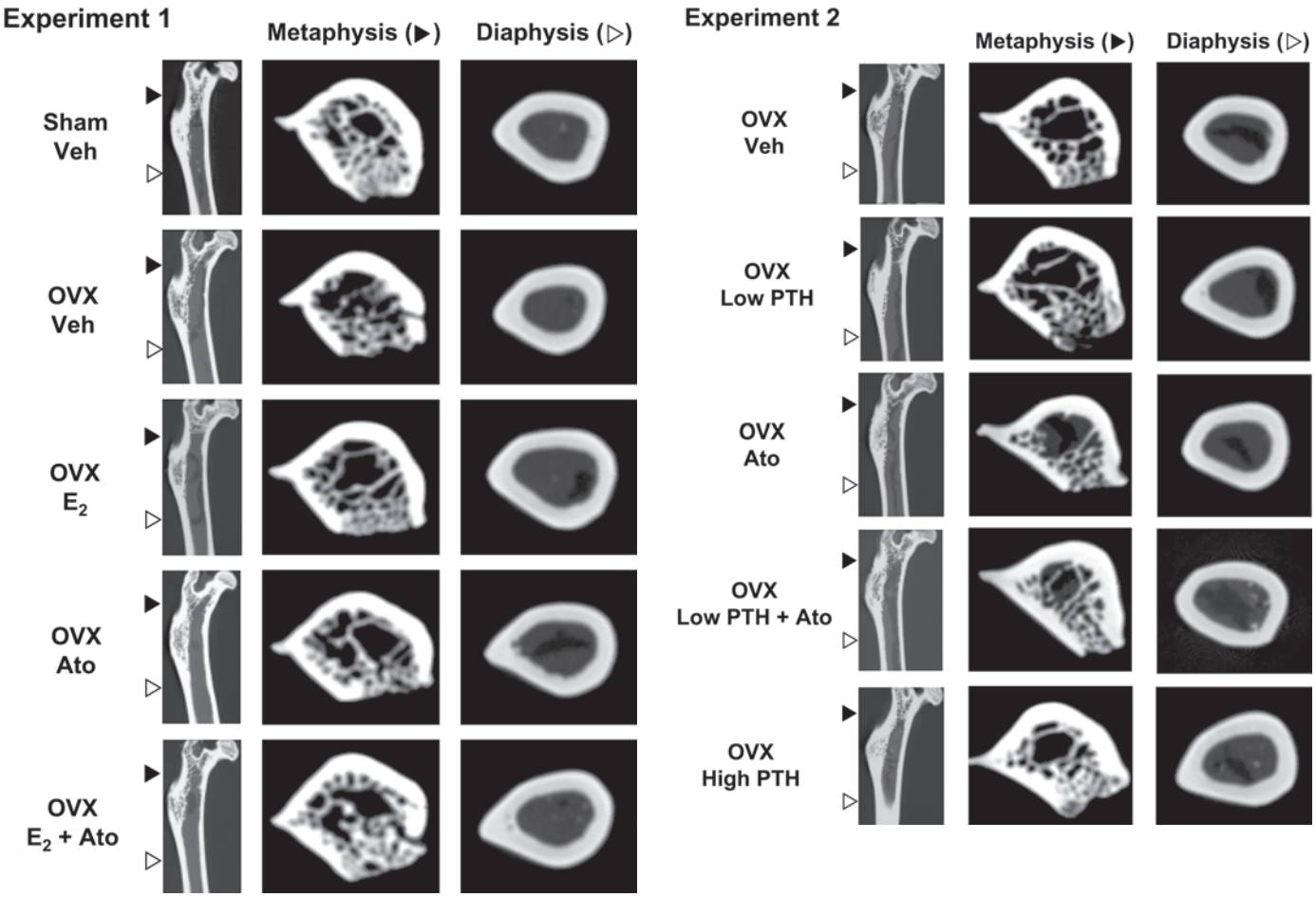

Fig. 6. μCT analysis of rat femurs. Representative vertical images of proximal femurs, and representative transverse images of metaphysis and diaphysis are shown. Animal protocols of Experiments 1 and 2 are described in Figs. 1 and 3, respectively. Veh, vehicle; Ato, atorvastatin (2mg/kg); E_2, 17β-estradiol (10 μg/kg); LowPTH, hPTH(1-34) (1μg/kg); HighPTH, hPTH(1-34) (17.5 μg/kg).

bone area was clearly increased in rats treated with a high dose (17.5 μg/kg) of hPTH(1-34) compared to vehicle treatment. The diaphyseal bone area was elevated by the treatment of a high dose of hPTH(1-34), but did not alter in other rats (Fig. 6).

Discussion

We demonstrated that the chronic administration of atorvastatin appears to modestly enhance the effects of E_2 (10 μg/kg) and hPTH(1-34) (1 μg/kg) on BMD of the lumbar vertebrae and femoral metaphysis in OVX rats. These data suggest that combination therapies using statins with appropriate antiresorptives or with anabolic agents may stop or reverse established osteoporosis in patients with hypercholesterolemia.

Since trabecular bone is more sensitive to hormones and drugs than compact bone *(19–21)*, it is likely that any positive effect of atorvastatin on BMD would be observed in bone abundant with trabecular bone (such as lumbar vertebrae and the metaphyseal area of the femur). It has been shown that statins promote bone formation in rat calvariae maintained in organ culture *(12)*, suggesting that these drugs have a beneficial effect on bone health. We demonstrated that statins potently induce osteoblast differentiation markers such as BMP-2, alkaline phosphatase, and collagenases in MC3T3-E1 nontransformed cells *(14,22)*. Furthermore, simvastatin has been shown to augment VEGF expression in MC3T3-E1 cells, suggesting that statins stimulate bone growth and repair by increasing angiogenesis via locally acting angiogenic factors such as VEGF *(13,22)*.

One in vivo study showed inhibiting effects of statins on bone resorption *(23)*. In mice, simvastatin promoted healing of bone fractures *(24)*. Clinical studies based on retrospective and observational analysis reported that elderly patients treated with statins had a markedly reduced incidence of fracture *(25,26)*. Postmenopausal osteoporotic and diabetic patients receiving statin therapy have higher BMD than untreated patients *(27,28)*. In contrast, Maritz et al. *(18)* reported that orally administered statins inhibited

bone formation and produced a net reduction in BMD in rats. Rejnmark et al. *(29)* did not demonstrate a positive effect of long-term statin treatment on BMD in postmenopausal women. Moreover, a study by Sirola et al. *(30)* showed no protection against early postmenopausal bone loss. Thus, we investigated whether atorvastatin with parenteral dosing has a bone anabolic effect in vivo in an animal model of osteopenia, OVX rats.

Our study did not compare the effect of different statins on BMD of OVX rats. We chose atorvastatin for these experiments, because atorvastatin has a long half-life compared with other statins. The dose of atorvastatin used (2 mg/kg) was based on clinically accepted biologically equivalent doses in humans *(31)*. Atorvastatin alone did not improve BMD or trabecular bone volume in OVX rats. Administration of simvastatin and atorvastatin at doses similar to our study in OVX rats did not influence lumbar and femoral BMD *(18)*; similarly no effect was observed in our rats injected with high-dose (10 mg/kg) atorvastatin for 10 wk (data not shown). We found that atorvastatin clearly enhanced the BMD of lumbar vertebrae and femoral metaphyseal trabecular area in OVX rats treated with E_2 or a low dose of hPTH(1-34). Analysis of μCT also demonstrated that atorvastatin treatment in combination with E_2 or hPTH(1-34) at a subeffective dose increased the bone area of metaphysis but not of diaphysis of OVX rat femurs. Our results suggest that atorvastatin can significantly enhance trabecular bone formation induced by antiresorptive agents, such as E_2, and bone anabolic drugs, such as hPTH(1-34), at submaximal doses. DEXA and μCT analyses showed that long-term treatment of effective doses of hPTH(1-34) for bone formation markedly increased not only trabecular bone but also compact bone such as femoral diaphysis, consistent with previously reported results *(32)*.

In the present experiments on estrogen replacement treatment (Experiment 1), E_2 at a dose of 10 μg/kg did not fully restore the lumbar and femoral BMD of OVX rats to that of sham-operated rats. Marked decreases in volume and mineral density of trabecular bone occurred within 2–4 weeks after ovariectomy in young adult rats *(33,34)*. Since we ovariectomized female rats at the age of 8 weeks, these young rats underwent substantial declines in trabecular BMD after the surgery. Thus, we could not detect weak drug effects to reduce bone resorption, and E_2 treatment might not fully prevent the bone loss induced by ovariectomy in the present study.

Although previous in vitro studies demonstrated that statins stimulate BMP-2 expression and promote the differentiation and mineralization in cultured osteoblasts *(13,14, 22)* and increase bone formation in calvarial organ culture *(12)*, the effect in vivo is not as significant in other's *(18)* or our studies. The reason for the difference between experimental results in vivo and in vitro is unclear. A possible explanation for the weak effect on bone metabolism in the present study is a low in vivo drug concentration in bone tissue, while the in vitro findings were obtained from a direct effect of statins on bone cells such as osteoblasts. Orally administered statins are recycled in the enterohepatic circulation, and are mostly metabolized in liver by the first circulation. A study of the deposition and metabolism of atorvastatin in rats showed that 73% of the oral dose of the statin was excreted in bile and multiple dose administration did not alter bilary metabolic profiles *(35)*. Therefore, their effective concentration in extrahepatic tissues such as bone is low, although the lipid-lowering effect of statins is high. Since we injected atorvastatin subcutaneously into OVX rats, the bioactivity of atorvastatin was higher in the present study than that at the other study with oral administration into animals *(18)*. Furthermore, atovastatin at high enough doses (2 and 10 mg/kg) with parenteral injections failed to reduce concentrations of serum cholesterol. These findings may suggest that there are unknown factors in the extracellular fluid of rats that prevent statins from reaching target tissues such as bone. This notion is, however, to be investigated.

We showed that ovariectomy significantly decreased serum concentrations of calcium and phosphorus, although this finding is consistent with previously reported results *(36)*. Ovarian hormone deficiency after surgical ovariectomy in rats, is characterized by increased bone turnover, resulting in a net loss of bone *(33)*. Furthermore, estrogen deficiency associated with ovariectomy resulted in the decreased intestinal absorption of calcium *(37)*. The OVX-induced decrease in intestinal calcium absorption was restored by supplementation with E_2 *(38,39)*. Kidneys also play an important role in the systemic homeostasis of mineral ions. In fact, glucocorticoids, steroid hormones, decrease tubular calcium reabsorption, making it likely that estrogen affects renal handling and urinary excretion of calcium and inorganic phosphate in OVX rats. Intermittent treatment of hPTH (1-34) at 1 and 17.5 μg/kg did not affect the serum concentrations of calcium and phosphate in OVX rats. The last injection was carried out 3 d before killing. It is likely that the serum concentrations of calcium and phosphate returned to those of OVX rats at the time of exsanguination.

OVX rats were found to have a significant increase in serum osteocalcin, an osteoblast-derived marker for bone formation, relative to sham-operated rats, and estrogen treatment of OVX rats restored the osteocalcin concentrations in agreement with previous observations by Shiraishi et al. *(36)*, indicating that estrogen reduces bone formation. Concurrent treatment of hPTH(1-34) at a subeffective dose and atorvastatin significantly increased serum osteocalcin concentrations in OVX rats, whereas activity of serum tartrate-resistant acid phosphatase, a kind of bone resorption marker, did not change in these treatments (data not shown). Long-term treatment of atorvastatin alone did not alter the serum concentrations. Taken together, atorvastatin acts on bone to augment bone formation modestly rather than to suppress bone resorption.

A cross-sectional study showed that long-term treatment with statins including atorvastatin did not affect body composition and BMD at the lumbar spine, hip, forearm, and whole body of postmenopausal women *(29)*. However, randomized, controlled studies are mandatory to establish statins as pharmaceutical agents for improving bone mass. In a recent study using a 1-yr randomized, controlled trial in postmenopausal women, simvastatin treatment at a dose of 40 mg/d did not affect BMD at the lumbar spine, total hip, femoral neck, or whole body *(40)*. Furthermore, in an analysis from the Women's Health Initiative (WHI) observational study, BMD at the lumbar spine, total hip, or whole body did not differ between 422 statin users and 6020 nonusers after adjustment for multiple confounders *(41)*. In contrast, the trial with a double-blinded design demonstrated a significant increase in BMD in response to simvastatin at the forearm *(40)*. Montagnani et al. *(42)* found that simvastatin treatment at a dose of 40 mg/d for 1 yr significantly increased BMD at the lumbar spine and femoral neck in 30 postmenopausal hypercholesterolemic women compared to 30 non-hypercholesterolemic, statin-untreated postmenopausal women. In other clinical studies, treatment with statins was associated with an increased BMD *(27,28)*. The reasons for these conflicting results regarding the effects of statins on BMD must be elucidated.

To our knowledge, this is the first report showing that long-term treatment with a statin enhances bone mass in the presence of E_2 and hPTH(1-34) in an osteopenic animal model. As our studies *(13,14,22)* and those of others *(12, 23,24)* have shown, statins have a profound effect on bone metabolism. The statins are potent drugs that lower serum cholesterol and have other pleiotropic effects affecting the arterial wall *(43)* and angiogenesis *(44)*. These drugs are widely and increasingly used in a population in whom osteoporosis is of concern. However, because the effects of the currently available statins on bone formation in vivo is not marked, development of new statins more specific for bone will greatly improve the usefulness of these drugs for the treatment of osteoporosis.

Materials and Methods

Animals and Experimental Design

Experimental protocols were approved by the Institutional Animal Care and Use Committee of Ohu University and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals. Six-week-old virgin Sprague–Dawley female rats (Clea Japan, Tokyo) were maintained with *ad libitum* access to water and to rodent chow (containing 1.42% calcium, 1.16% phosphorus, and 1.6 IU/g vitamin D_3). A constant temperature of 24°C was maintained, as well as a 14 h light:10 h darkness cycle. At the age of 8 wk, rats were anesthetized with sodium pentobarbital (50 mg/kg) and underwent either ovariectomy by a dorsal approach or sham operation. Sham-operated and OVX rats were maintained with rodent chow for 6 wk until experiments began.

Experiment 1. Ten sham-operated rats and 40 OVX rats were used. OVX rats were divided into four groups (10 rats per group), such as vehicle-treated control, E_2 (Sigma, St. Louis, MO): atorvastatin (Yamanouchi Pharmaceutical Co., Tokyo, Japan), and E_2 + atorvastatin. In the sham and OVX control groups, rats were received the vehicle four times a week for 12 wk. E_2 dissolved in corn oil at a dose of 10 μg/kg, and/or atorvastatin dissolved in 20% DMSO at a dose of 2 mg/kg were injected subcutaneously four times a week for 12 wk in OVX rats.

Experiment 2. Forty OVX rats were divided into five groups (8 rats per group): such as vehicle-treated control, hPTH(1-34) at a dose of 1 μg/kg (LowPTH), atorvastatin at a dose of 2 mg/kg, atorvastatin + LowPTH, and hPTH (1-34) at a dose of 17.5 μg/kg (HighPTH). Atorvastatin dissolved in vehicle and hPTH(1-34) dissolved in saline containing 0.1 *M* acetic acid, were injected subcutaneously four times a week for 8 wk in OVX rats. The last injection of hormone, statin, or vehicle was carried out 3 d before killing. After the completion of the treatment, rats were killed by exsanguination from the aorta under ether anesthesia. Serum concentrations of total calcium and inorganic phosphate were measured colorimetrically using commercial kits (total calcium; Wako Calcium C, OCPC Method, and inorganic phosphate; Wako Phosphor C, *p*-methylaminophenol reduction method) manufactured by Wako Chemical (Osaka, Japan). Femurs and vertebrae were excised. Serum concentrations of rat osteocalcin were measured using a commercial kit (Rat Osteocalcin IRMA Kit, Immutopics, Inc., San Clemente, CA).

Bone Mineral Density Measurements and Histology

BMD (mg/cm^2) of the second through fourth lumbar vertebrae (L2–L4) was measured by dual energy X-ray absorptiometry (DEXA) with a DCS-600 densitometer (Aloka, Tokyo, Japan), using software designed for small animal measurements. BMD of the left femur was measured with the DEXA, starting scans in the most proximal area and ending in the most distal area. During data analysis, the femur was divided into 10 equal segments along its major axis. BMD of the total femur, metaphyseal area (the second through fourth segments from the most proximal segment), and a diaphyseal area (the sixth and seventh segments from the most proximal segment) were measured *(6)*. Left tibiae were fixed with 70% ethanol and embedded in glycol methacrylate without decalcification. Undecalcified sections were stained with hematoxylin and eosin. Three sections per group were made and representative samples were shown.

Microcomputed Tomography (μCT) Analysis of Femurs

The left femurs were collected at the end of experiments and kept in 70% ethanol at 4°C. Areas of femurs with 70%

from the most proximal point were scanned using a μCT scanner (Shimadzu, Kyoto, Japan). The raw data were reconstructed, and the resulting two-dimensional (2D) images were displayed using image analysis software (CT-Solver, Shimadzu). Four hundred (42-μm-thick) transverse images were made. The sections of interest were 90th and 300th transverse images from the most proximal point, which were covered with the proximal metaphysis and diaphysis, respectively. Three samples per group were analyzed and representative images were shown.

Statistical Analysis

The data are represented as means ± SEM. Multiple comparisons of BMD (Figs. 1–4) were evaluated by Dunnett's procedure. Statistical analysis was performed using SAS statistical software (SAS Institute, Inc., Cary, NC). Statistical analysis of serum data (Table 1) was performed using analysis of variance (ANOVA) followed by Fisher's protected least significant difference test (Statview 4.02, Abacus Concepts Inc., Berkeley, CA). A p value less than 0.05 was considered to indicate statistical significance.

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