Sintered dicalcium pyrophosphate increases bone mass in ovariectomized rats

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Received 7 November 2000; revised 23 April 2001; accepted 6 June 2001

Abstract: Bisphosphonates are synthetic pyrophosphate analogs that can be used for the treatment of osteoporosis. Sintered dicalcium pyrophosphate, as a pyrophosphate analog, may be useful in the clinical setting for osteoporosis. In this study, an ovariectomized rat model is used to evaluate the effects of orally administered sintered dicalcium pyrophosphate on bone mass. Thirty-six female rats were used in this study. They randomly were divided into six groups: a negative normal control group, a positive osteoporosis control group, and ovariectomized groups treated either with alendronate sodium (one group) or sintered dicalcium pyrophosphate (three groups, each at a different level). The animals were sacrificed at 4 weeks after treatment. For all the rats, whole blood samples were obtained for the biochemical study. Bone ashes of long bones were measured and studied and histologic studies of cancellous bone were carried out. The ingestion of either alendronate or sintered dicalcium pyrophosphate did not have any deleterious effect on the major visceral organs. Ingestion of alendronate or sintered pyrophosphate decreased the bony porosity and increased bone mineral contents in the long bones of ovariectomized rats. Thus sintered dicalcium pyrophosphate can increase bone mass in the ovariectomized rat. © 2001 John Wiley & Sons, Inc. J Biomed Mater Res 59: 246–253, 2002

Key words: sintered dicalcium pyrophosphate; alendronate; bone mass; ovariectomy; osteoporosis

INTRODUCTION

Postmenopausal osteoporosis is a common disorder characterized by an increase in bone resorption relative to bone formation, generally in conjunction with an increased rate of bone turnover.[1] Osteoporosis is a major health-care problem for aging communities. The progressive decrease in bone mass leads to an increased susceptibility to fractures, which, in turn, result in substantial morbidity and mortality. Of these, the most serious is hip fracture because of the high morbidity and associated mortality and their attendant high costs to health services.[2] Vertebral fractures also are important, not only because they can cause pain, kyphosis, and height loss, but also because they are predictive of subsequent, nonvertebral fractures independent of bone mineral density.[3] Fractures at a number of other sites also are more frequent in osteoporotic bones than in healthy bones, but these are less important. Although there are several risk factors for fractures, reduced bone mineral density is the strongest predictor.[4] Thus the ultimate goal of the pharmacologic treatment of women with postmenopausal osteoporosis is to reduce the risk of fractures by increasing their bone mass to a normal quality.

Bisphosphonates are synthetic pyrophosphate analogs that can inhibit osteoclastic bone resorption and, therefore, are used for the treatment of bone diseases, such as tumor-induced hypercalcemia and osteoporosis.[5,6] Recently, with advances in ceramics technology, the application of calcium phosphate materials as a bone substitute has received considerable attention. These materials are remarkably biocompatible, provoke little, if any, inflammatory response, and have a bioactive property. During the last decade, a large number of biomaterials have been proposed as artificial bone fillers for repairing bone defects. Sintered dicalcium pyrophosphate is a synthetic compound that has been proven to be quite biocompatible with bone tissue both in an *in vivo* animal model[7] and in an *in vitro* osteoblast cell culture model.[8] As a pyrophos-

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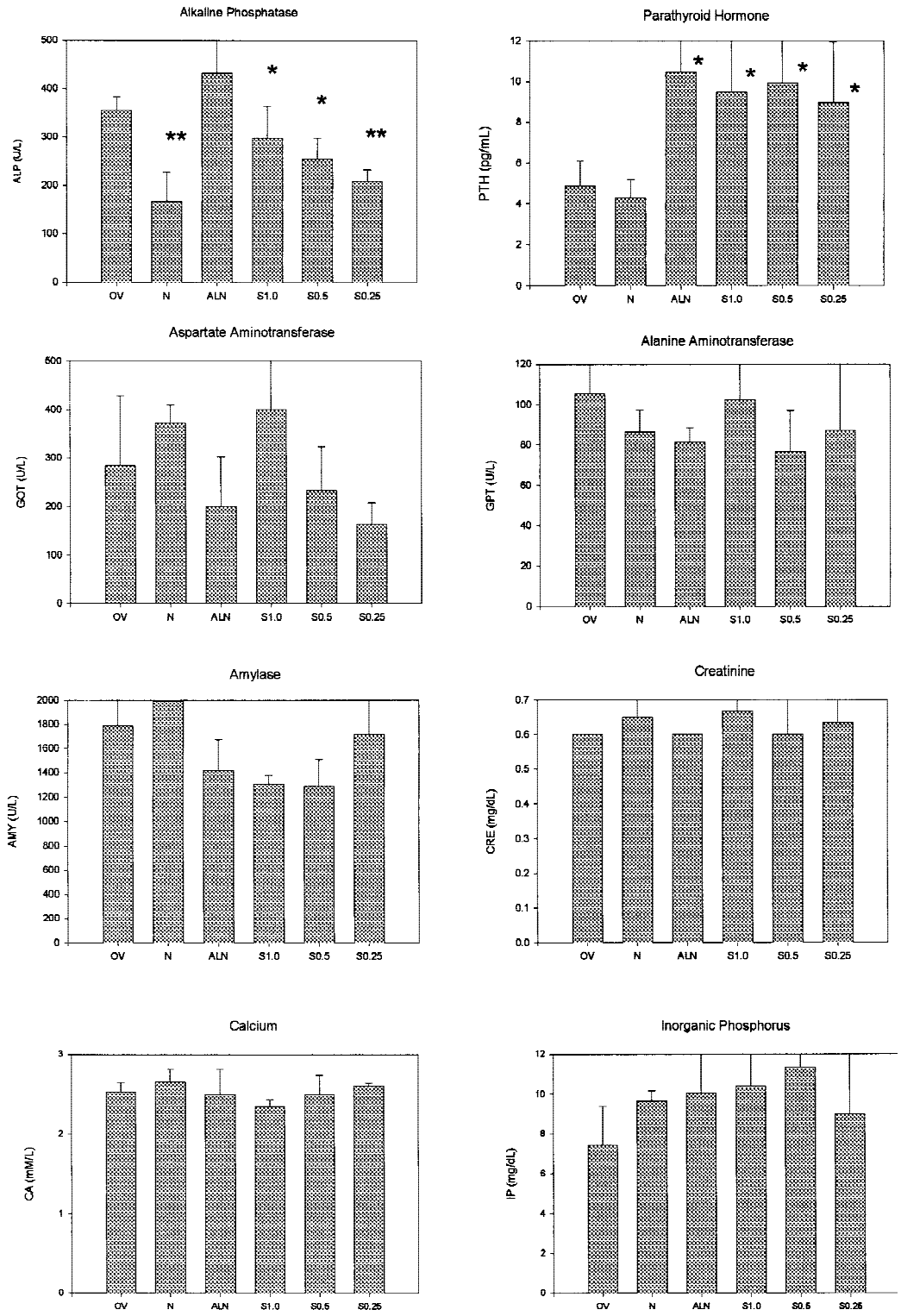

Figure 1. Biochemical parameters after ovariectomy with and without alendronate or sintered pyrophosphate treatment. After ingestion of either alendronate or sintered dicalcium pyrophosphate for 1 month, most of the biochemical parameters in serum did not show any significant change while the markers for bone metabolism, including alkaline phosphatase and parathyroid hormone, did change. After ovariectomy, the alkaline phosphatase titer in the serum of the ovariectomized rats was higher than that of the normal rats. Ingestion of alendronate did not affect the increase in alkaline phosphatase titer following ovariectomy, but the ingestion of sintered pyrophosphate did decrease the alkaline phosphatase titer in the ovariectomized rats. After ovariectomy, the parathyroid hormone in the serum did not show any statistical change compared to that of the normal rats. Ingestion of alendronate or sintered pyrophosphate increased the parathyroid hormone titer in the ovariectomized rats. OV: ovariectomy; N: sham-operated normal control; ALN: alendronate, 1.0 mg/kg/day; S 1.0: sintered dicalcium pyrophosphate, 1.0 mg/kg/day; S 0.5: sintered dicalcium pyrophosphate, 0.5 mg/kg/day; S 0.25: sintered dicalcium pyrophosphate, 0.25 mg/kg/day. *: $P < 0.05$; **: $P < 0.005$ compared with ovariectomy group.

phate analog, sintered dicalcium pyrophosphate should be useful in clinical setting characterized by abnormal bone resorption, such as osteoporosis.[9]

In this study, we used an ovariectomized rat model to evaluate orally administered sintered dicalcium pyrophosphate relative to its ability to reverse the effects of osteoporosis. Our hypothesis is that sintered dicalcium pyrophosphate can increase bone mass in the ovariectomized rat.

MATERIALS AND METHODS

Thirty-six female Wistar rats (Animal Center of National Taiwan University Hospital) weighing 250–350 g were used in this study. They were randomly divided into six groups. The members of group A, the negative normal control group, were subjected to a sham operation without ovariectomy. The rats in groups B to F received bilateral ovariectomies via a bilateral retroperitoneal approach. In group B, the positive osteoporosis control group, no further treatment was given. In groups A and B, the rats were given starch. In groups C to F, the experimental groups, the rats were treated with experimental medications. Group C received Fosamax [alendronate sodium (ALN), 4-amino- 1- hydroxybutylidene-1, 1-bisphosonic acid, trihydrate sodium salt; Merck & Co. Inc., Whitehouse, NJ]; 1.0 mg/kg/day, as described by Guy et al.[10] Groups D to F received sintered dicalcium pyrophosphate[11] (sintered β-Ca2P207 with 5% Na4P207·10H20) at levels of 0.25 (group D), 0.5 (group E), and 1.0 (group F) mg/kg/day.

In a pilot study, we demonstrated that osteoporosis can be established within 3 weeks after ovariectomy. In this study, all rats were fed without medication for 1 month to establish the osteoporosis changes in the ovariectomized rats. Then the medications were given via a feeding tube through the mouth once a day in the early morning after 12 h of starvation. During the daytime, the animals were fed Purina Laboratory Chow *ad libitum* and housed in a temperature-, humidity-, and light-controlled environment. Surgical procedures and experimental protocols were approved and supervised by the Medical College's Animal Research Committee of the National Taiwan University. The animals were sacrificed at 4 weeks after treatment (i.e., 8 weeks after surgery).

For all rats, whole blood samples were obtained with plastic syringes via intracardiac puncture immediately following sacrifice. The blood samples were stored on ice, and then the serum was separated by centrifugation, divided into 500-μL aliquots in Eppendorfs, and deep-frozen at −80°C until further analysis.

After killing the rats, the long bones of the four limbs

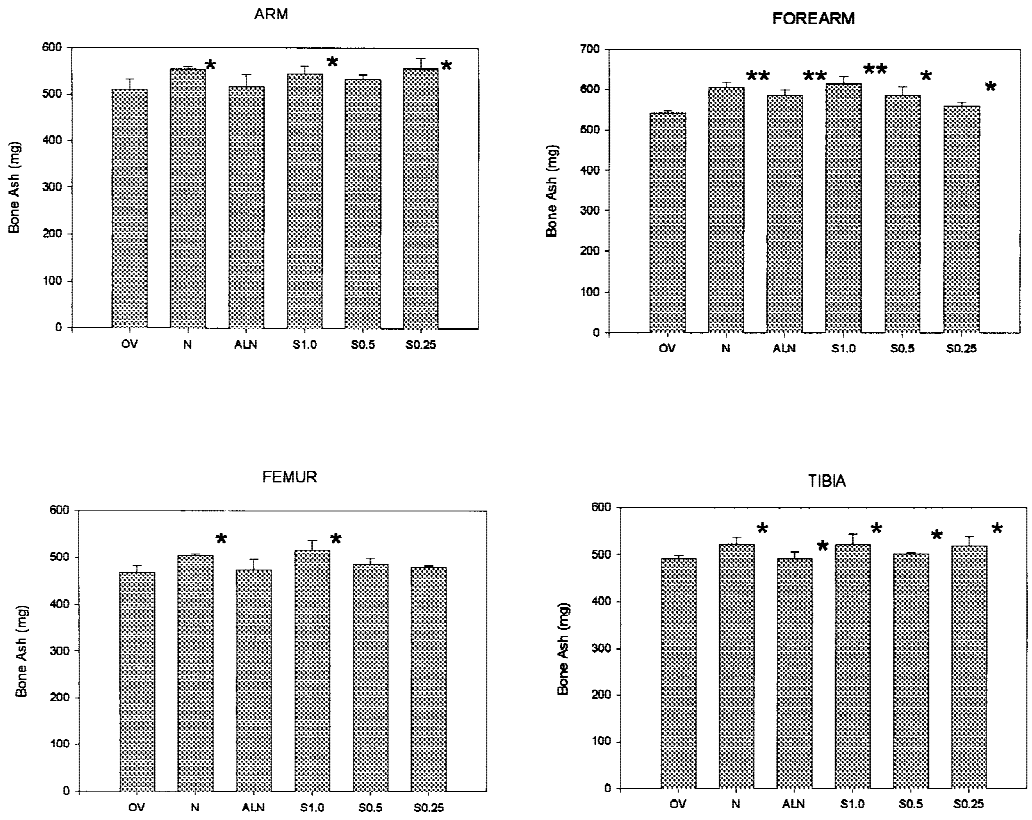

Figure 2. Weight of long bone ashes after ovariectomy with and without alendronate or sintered pyrophosphate treatment. After ovariectomy, the bone ashes of the long bones in the limbs decreased significantly. OV: ovariectomy; N: sham-operated normal control; ALN: alendronate, 1.0 mg/kg/day; S 1.0: sintered dicalcium pyrophosphate, 1.0 mg/kg/day; S 0.5: sintered dicalcium pyrophosphate, 0.5 mg/kg/day; S 0.25: sintered dicalcium pyrophosphate, 0.25 mg/kg/day. *: $P < 0.05$; * *: $P < 0.005$ compared with ovariectomy group.

(including femur, tibia, humerus, and forearm) and the thoracolumbar spine were harvested rapidly. The long bones of the four limbs were trimmed of soft tissue and then burned. The weight of the bone ashes of each long bone was measured for further analysis. A histologic study of cancellous bone was made on the lumbar vertebrae. After necropsy, the lumbar vertebrae were dissected out and fixed with 4% formaldehyde in a phosphate-buffered solution for 18 h, followed by decalcification, dehydration in alcohol, clearing in xylene, and, finally, embedding in paraffin. Sections (5 to 7 μm in thickness) were cut and stained with hematoxylin and eosin. Representative sections were photographed using light microscopy. The thickness and interconnections between the trabeculation were evaluated.

To evaluate the bony trabeculae area precisely, the image was observed by light microscopy at 40× magnification. The outline of each bony trabecula was traced and then these traces were measured by a MICD image analyzing system (MICD software series, Image Research Inc., Catharine, Ontario, Canada). The total area of bony trabeculae was evaluated by adding together each area of bony trabeculae. The percentage of bone porosity for each treatment was calculated as 100% minus the area of total bony trabeculae divided by the total area measured. The mean ± SD (standard deviation) was calculated from six replicate measurements.

Biochemical indices of skeletal metabolism from the serum, such as alkaline phosphatase (ALP; procedure no. ALP-10, Sigma Co.), aspartate aminotransferase, and alanine aminotransferase (AST/GOT and ALT/GPT; procedure no. 505-OP, Sigma Co.), amylase (AMY; procedure no. Amylase-10, Sigma Co.), creatinine (CRE; procedure no. 555-A, Sigma Co.), calcium (CA; procedure no. 587-100P, Sigma Co.), and inorganic phosphorus (IP; procedure no. 363-3-100P, Sigma Co.), were measured using commercially available assay kits. Parathyroid hormone (PTH) content in the serum also was measured using a commercially available assay kits (IMMULITE® 2000 Intact PTH, Diagnostic Products Corporation, Los Angeles, CA).

Statistical analysis

The data obtained were evaluated by an analysis of variances statistical method. The *post hoc* test performed was Bonferroni's test. The level of statistical significance is defined as $p < 0.05$.

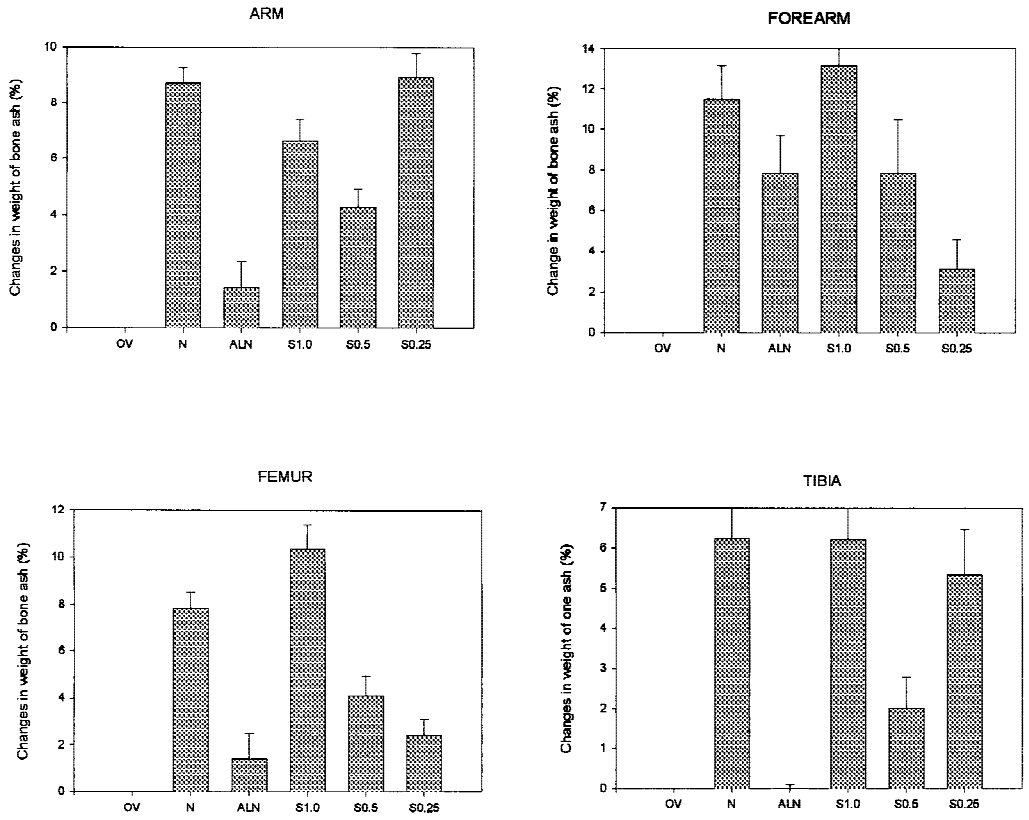

Figure 3. Changes in the weight of the bone ashes after ovariectomy and treatment with alendronate or sintered dicalcium pyrophosphates. Ingestion of 1.0 mg/kg/day of alendronate for 1 month increased the bone ashes 1.44, 7.84, 1.38, and 0.01% for the bone ashes of arm, forearm, femur, and tibia, respectively. Ingestion of 1.0 mg/kg/day of sintered dicalcium pyrophosphate increased the bone ashes 6.65, 13.1, 10.36, and 6.21% for the arm, forearm, femur, and tibia, respectively. Similar results were observed in rats after ingestion of 0.5 or 0.25 mg/kg/day sintered dicalcium pyrophosphate. In the sham-operated normal rats, the changes in the weight of bone ashes were similar to that for the ingestion of 1.0 mg/kg/day of sintered dicalcium pyrophosphate.

RESULTS

After ingestion of either alendronate or sintered dicalcium pyrophosphate for 1 month, most of the biochemical parameters in serum including aspartate aminotransferase and alanine aminotransferase (GOT/GPT), amylase, creatinine, calcium, and inorganic phosphorus did not show any significant change. The markers of bone metabolism, including alkaline phosphatase and parathyroid hormone, did show statistically significant changes (Fig. 1). After ovariectomy, the alkaline phosphatase titer in the serum increased from 167.5 U/L in the normal rats to 355.0 U/L in the ovariectomized rats; ingestion of alendronate did not affect the increase in alkaline phosphatase titer in ovariectomized rats, but the ingestion of sintered pyrophosphate did decrease the alkaline phosphatase titer in the ovariectomized rats (Fig. 1). After ovariectomy, the parathyroid hormone in the serum did not show any statistical change compared to that of the normal rats; ingestion of either alendronate or sintered pyrophosphate increased the parathyroid hormone titer in the ovariectomized rats (Fig. 1).

After ovariectomy, the bone ashes of long bones in the limbs decreased significantly (Fig. 2). Compared with the ovariectomized rats, ingestion of 1.0 mg/kg/day of alendronate increased the bone ashes of arm, forearm, femur, and tibia by 1.44, 7.84, 1.38, and 0.01%, respectively, while ingestion of 1.0 mg/kg/day of sintered dicalcium pyrophosphate increased the bone ashes of arm, forearm, femur and tibia by 6.65, 13.1, 10.36, and 6.21%, respectively. Similar results were observed in the rats after ingestion of 0.5 or 0.25 mg/kg/day of sintered dicalcium pyrophosphate (Fig. 3). In the sham-operated normal rats, the changes in the weight of the bone ashes were similar with ingestion of 1.0 mg/kg/day of sintered dicalcium pyrophosphate (Fig. 3).

In the histologic study of the lumbar vertebrae in the sham-operated normal rat, the cancellous bone of the vertebrae body showed intervening trabecular bone with connectivity of the trabeculae elements. After ovariectomy, there was significant thinning and disconnection of trabeculae in the lumbar vertebrae compared with the sham-operated normal control. The porosity of bony trabeculae also increased significantly in the ovariectomized rats (Fig. 4). After ingestion of the alendronate or sintered dicalcium pyrophosphate, the increases in porosity of bony trabeculae of the ovariectomized rats were reversed and trabeculation of lumbar vertebrae showed a significant increase compared with ovariectomized osteoporosis controls (Fig. 4). In the histologic examination, the lumbar vertebrae showed thickening of the trabeculae with restoration of interconnection (Fig. 5).

DISCUSSION

For the evaluation of agents intended to prevent or treat postmenopausal osteoporosis, the adult ovariectomized rat is a convenient and reliable experimental mode.[1] Like humans, rats have cancellous bone that undergoes remodeling once longitudinal growth has essentially ceased.[2] The clinically useful bisphosphonates are synthetic analogs of inorganic pyrophosphate, an endogenous regulator of bone turnover that inhibits bone resorption and mineralization *in vitro*.[5] All bisphosphonates have a high affinity for hydroxyapatite, but unlike pyrophosphate, they are resistant to metabolism by endogenous phosphatases.[5,12] Treatment with alendronate specifically inhibits the increase in bone resorption connected with osteoporosis and thereby normalizes the rate of bone turnover.[13]

Preclinical evaluations of alendronate in animals with osteoporosis have documented that there is a greater correlation of bone strength through increased bone mass for alendronate-treated bone.[14,15] In this study, we chose ovariectomized rats as the experimental mode.[1] The specific purpose of this study was to compare the effect of sintered dicalcium pyrophosphate on ovariectomized rats with the currently used alendronate preparations and with normal controls.

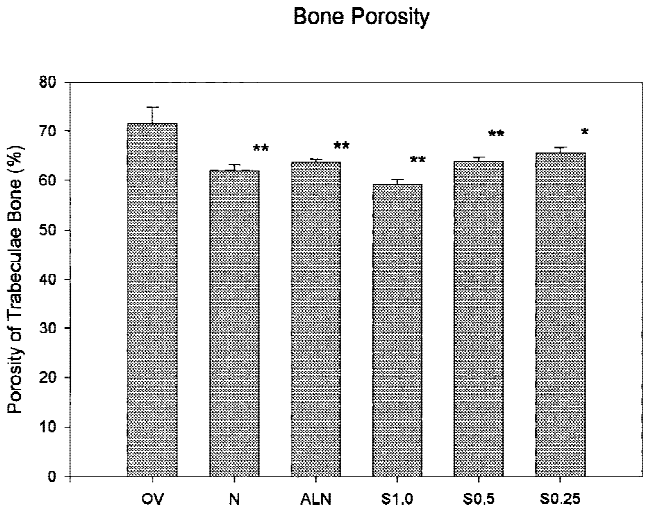

Figure 4. Study of the porosity of the bony trabeculation of the lumbar vertebrae after ovariectomy and treatments with alendronate or sintered dicalcium pyrophosphates. One month after ovariectomy, the porosity of the bony trabeculae is significantly increased compared with the sham-operated normal control. After the ingestion of the alendronate or sintered dicalcium pyrophosphate, porosity of bony trabeculae of the lumbar vertebrae is decreased significantly compared with ovariectomized osteoporosis control. OV: ovariectomy; N: sham-operated normal control; ALN: alendronate, 1.0 mg/kg/day; S 1.0: sintered dicalcium pyrophosphate, 1.0 mg/kg/day; S 0.5: sintered dicalcium pyrophosphate, 0.5 mg/kg/day; S 0.25: sintered dicalcium pyrophosphate, 0.25 mg/kg/day. *$P < 0.05$; **$P < 0.005$ compared with ovariectomy group.

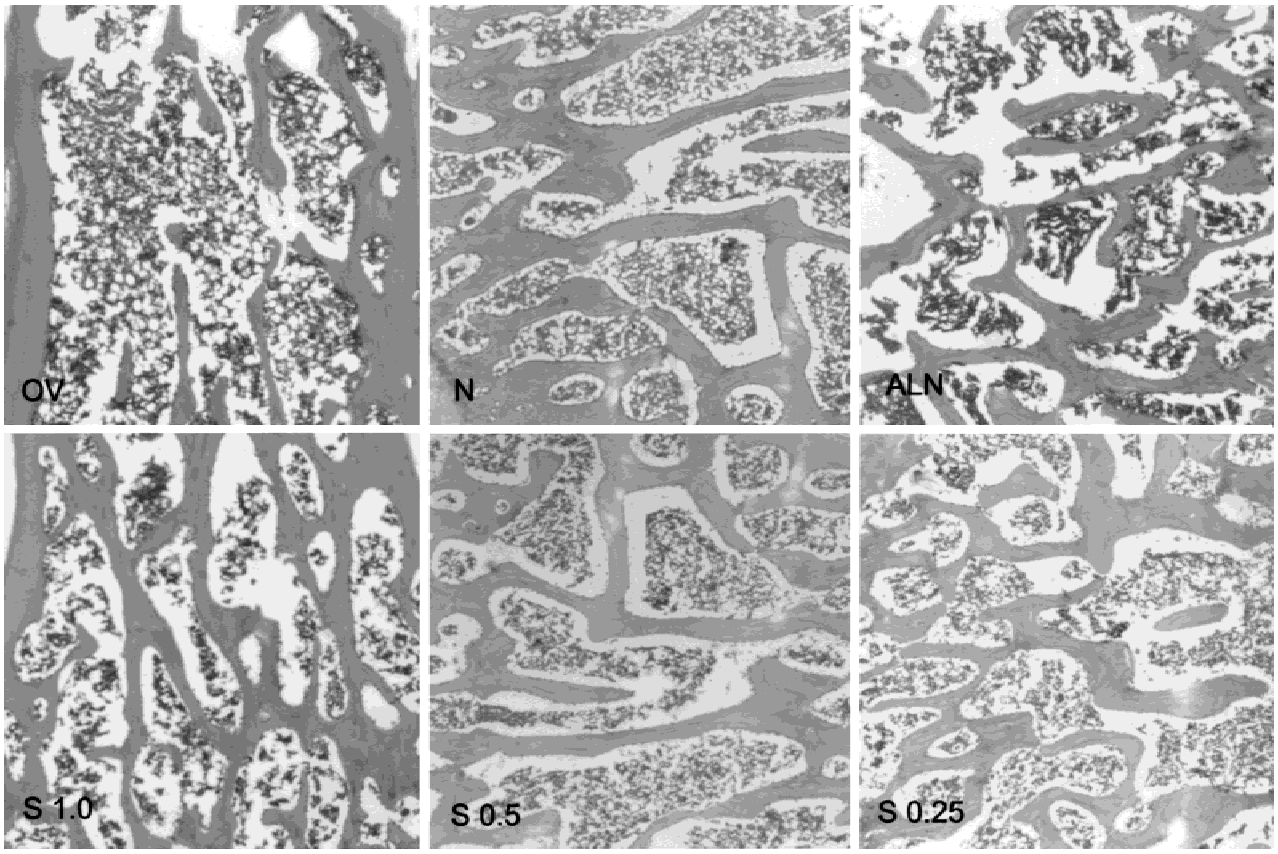

Figure 5. Histologic study of the lumbar vertebrae after ovariectomy and treatments with alendronate or sintered dicalcium pyrophosphates. In the histologic study of lumbar vertebrae in sham-operated normal rat, the cancellous bone of the vertebral body shows intervening trabecular bone with connectivity of the trabeculae elements. In the rats with osteoporosis, 1 month after ovariectomy there is significant thinning and loss of trabeculae, accompanied by a disconnection of trabeculae, compared with the sham-operated normal control. After the ingestion of the alendronate or sintered dicalcium pyrophosphate the lumbar vertebrae show a significant increase in trabeculation compared with ovariectomized osteoporosis controls. The lumbar vertebrae show a thickening of trabeculae with restoration of interconnection. HE stain; bar = 100 μm. OV: ovariectomy; N: sham-operated normal control; ALN: alendronate, 1.0 mg/kg/day; S 1.0: sintered dicalcium pyrophosphate, 1.0 mg/kg/day; S 0.5: sintered dicalcium pyrophosphate, 0.5 mg/kg/day; S 0.25: sintered dicalcium pyrophosphate, 0.25 mg/kg/day.

The hypothesis is that SDCP has a similar effect to that of bisphosphonates in that it can increase the bone mass in ovariectomized rats.

In adult rats, ovariectomy is manifested by an increase in bone turnover associated with bone loss, followed by a permanent deficit in bone mass at several skeletal sites, including the vertebral bodies, the proximal femur, and the metaphyses of long bones, such as the distal femur and proximal tibia.[2] In this study, the increase in bone turnover associated with bone loss was quite obviously indicated by a significant increase in the serum titer of alkaline phosphatase (Fig. 1) and a decrease in bone ashes after ovariectomy (Figs. 2, 3). Following ovariectomy, the porosity of bony trabeculae increased (Fig. 4), and the micro-architectural alterations in cancellous bone were similar to those observed in postmenopausal and age-dependent osteoporosis, which produces architectural discontinuities of cancellous bone (Fig. 5).

Bone provides the strength and rigidity of the skeleton and it acts as a reservoir of calcium and other mineral salts. Parathyroid hormone secretion increases in response to a fall in plasma calcium and acts directly on the kidney to increase tubular calcium resorption. An increase in serum calcium reduces parathyroid hormone secretion.[16] Alendronate is a potent inhibitor of bone resorption through its direct[17] and/or indirect[18] actions on osteoblasts. To maintain calcium and phosphorus homeostasis, ovariectomized rats must secrete more parathyroid hormone. The ingestion of alendronate or sintered pyrophosphate did significantly increase the parathyroid hormone titer in the ovariectomized rats (Fig. 1) as the ability of osteoclasts to mobilize bone was greatly decreased.

Alendronate produces no mineralization impairment at large multiples of the pharmacologically effective dose and has been shown to increase bone mass in animal models of osteoporosis[14,15] and in postmenopausal women.[19,20] This effect also has been observed in this study. After ovariectomy, the inges-

tion of alendronate decreased the porosity of bony trabeculae (Fig. 4) and increased the bone mineral contents in the long bones. The effects were greater after ingestion of sintered dicalcium pyrophosphate (Figs. 2, 3). The beneficial effects of alendronate and sintered dicalcium pyrophosphate were further consolidated by the thickening of trabeculae, with restoration of interconnections demonstrated in the histologic study (Fig. 5).

The ingestion of either alendronate or sintered dicalcium pyrophosphate did not have any deleterious effect on the liver, kidney, or pancreas; that is, there were no significant changes in the biochemical parameters, such as aspartate aminotransferase and alanine aminotransferase, amylase, creatinine, calcium, and inorganic phosphorus (Fig. 1). While the ingestion of alendronate did not cause any change in the serum alkaline phosphatase level, the ingestion of sintered dicalcium pyrophosphate did cause a significant decrease in the serum alkaline phosphatase titer. Unlike bisphosphonates, pyrophosphate can be metabolized only slowly by endogenous phosphatases,[5,11] and sintered dicalcium pyrophosphate has been proved to be a biodegradable bone substitute *in vivo*.[7]

The ingestion of sintered dicalcium pyrophosphate slows down the increase in bone turnover, which is demonstrated by a significant decrease in the serum titer of alkaline phosphatase (Fig. 1) and an increase in bone ashes after ovariectomy (Figs. 2, 3). After ovariectomy, the ingestion of alendronate does not increase bone metabolism, as shown by the lack of any significant change in the alkaline phosphatase titer (Fig. 1). In this study the ingestion of sintered pyrophosphate did reduce the increase in bone metabolism after ovariectomy, demonstrated by a significant decrease in the alkaline phosphatase titer (Fig. 1). As an intermediate product in the biologic mineralization process,[21] it is obvious that sintered dicalcium pyrophosphate is bioabsorbable[7] and causes this decrease in the alkaline phosphatase titer after ovariectomy.

CONCLUSIONS

The ingestion of either alendronate or sintered dicalcium pyrophosphate can increase bone mineral content in the long bones of ovariectomized rats. The mechanism by which sintered dicalcium pyrophosphate acts on bone resorption is still not known. Like alendronate, it may act through either a direct inhibition of the osteoclasts or through an indirect effect on secretion of the soluble osteoclast, activating factors by the osteoblasts.[22] However, this hypothesis needs to be evaluated in further studies.

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