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Vitamin D insufficiency reduces the protective effect of bisphosphonate on ovariectomy-induced bone loss in rats

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Abstract

The present study was carried out to obtain an experimental model of vitamin D (vit D) insufficiency and established osteopenia (experiment 1) to then investigate whether vit D status, i.e. normal or insufficient, interferes with bone mass recovery resulting from bisphosphonate therapy (experiment 2). Rats ($n = 40$) underwent OVX ($n = 32$) or a sham operation ($n = 8$). The first 15 days post-surgery, all groups were kept under fluorescent tube lighting and fed a diet containing 200 IU% vit D (+D). They were then assigned during an additional 45 days to receive either +D or a diet lacking vit D (−D) and kept under 12 h light/dark cycles using fluorescent or red lighting. Serum 25HOD was significantly lower in −D rats ($P < 0.0001$). The type of lighting did not induce differences in 25OHD, calcium (sCa), phosphorus (sP), bone alkaline phosphatase (b-AL), CTX, bone density or histology. No osteoid was observed in undecalcified bone sections. Experiment 2 (105 days): rats were fed either +D or −D according to experiment 1 and were treated with either placebo or 16 μg olpadronate (OPD)/100 g rat/week during the last 45 days. Whereas 25HOD was significantly lower ($P < 0.0001$) in −D/OPD than in +D/OPD rats, no significant differences in sCa, sP, b-AL or CTX were observed. OPD prevented the loss of lumbar spine (LS) and proximal tibia (PT) BMD and the decrease in bone volume (BV/TV) ($P < 0.05$) and in the number of trabeculae observed in untreated rats. However, +D/OPD animals presented significantly higher values of LS BMD, PT BMD and BV/TV than −D/OPD rats ($P < 0.05$). No osteoid was observed in undecalcified sections of bone. In summary, this is the first experimental study to provide evidence that differences in vit D status may affect the anticatabolic response to bisphosphonate treatment. However, the molecular mechanism through which vit D insufficiency reduces the effect of the aminobisphosphonate remains to be defined.

Keywords: Rats; Osteopenia; Vitamin D insufficiency; Bone markers; Bisphosphonates

Introduction

It is now clear that vitamin D (vit D) deficiency is deleterious to bone and other body tissues [1,2]. Indeed, since hypovitaminosis D influences calcium-regulating hormones, insufficient status of vitamin D has negative effects on skeletal metabolism. A decrease in serum concentrations of 25hydroxyvitamin D (25OHD) stimulates parathyroid hormone (PTH) secretion [3], leading to an increment in bone remodeling, particularly osteoclastic bone resorption, which increases bone loss and consequently fracture risk [4,5].

For most mammals, including humans and rats, the skin is a physiological route of entry of vitamin D (vit D). Haddad et al. [6] showed that orally acquired vit D is absorbed with chylomicrons and taken up quickly by liver metabolism, while dermally acquired vit D is bound to vit D binding protein and metabolized gradually [6]. Because most people mainly obtain vit D from sun exposure [7] and only a small amount is obtained from food and supplements, subclinical hypovitaminosis D is a frequent phenomenon [8], even among middle-aged persons [9]. Serum 25HOD closely reflects the status of vit D [10] and is the best indicator to define vit D deficiency, insufficiency,

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sufficiency and toxicity [11]. The levels of 25OHD decline with age [12,13], and although osteomalacia is uncommon, vit D deficiency may play a role in the pathogenesis of osteoporosis in the elderly [5].

It is known that estrogen deficiency is characterized by high bone remodeling. Bone resorption is greater than bone formation, increasing bone loss [14,15]. As a result of low bone mass, fracture risk increases and treatment with anticatabolic agents is required. Ovariectomized (OVX) rats are often used as an experimental model of estrogen (Eo) deficiency [16] to test the efficacy of different therapies in preventing bone loss.

There is some evidence from clinical experience for a link between insufficiency of vit D levels and low bone mineral recovery after anticatabolic treatment with bisphosphonates. Koster et al. [17] studied the effects of intermittent cyclical etidronate therapy in postmenopausal women with and without pre-existing vit D deficiency. They found that bone mass in the lumbar spine and femoral neck was significantly lower in vit-D-deficient patients. Yamanaka et al. [18] studied the relationship between vit D status and the effect of alendronate treatment in osteoporotic postmenopausal Japanese women and concluded that adequate status of vit D is essential to the effectiveness of alendronate for the treatment of osteoporosis. Bisphosphonates are useful anticatabolic agents in several bone diseases including primary osteoporosis [19]. However, to our knowledge, there is no experimental report in the literature on the possible effects of vitamin D status on the effectiveness of bisphosphonate treatment.

It is known that: (1) a reduction in one or both sources would unavoidably induce vit D insufficiency and (2) OVX rats lose approximately 20% of their total body bone mineral content (BMC) becoming osteopenic 40 days after surgery. Based on the above, the present study was carried out to obtain an experimental model of vit D insufficiency and established osteopenia to then investigate whether vit D status, i.e. normal or insufficient, interferes with the bone mass recovery resulting from bisphosphonate therapy.

Materials and methods

A total of 72 female adult Wistar rats (250–300 g) were housed at room temperature (21 ± 1°C), 55 ± 10% humidity and under 12-h light/dark cycles using fluorescent tube lighting and allowed access to deionized water ad libitum. Body weight was recorded weekly. The rats were maintained in keeping with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Animal models and experimental design

The experimental design consisted of two non-concurring protocols. The first protocol was designed to obtain a model of vit D insufficiency and established osteopenia. The second experiment was designed to evaluate vit D status and bone mass recovery after bisphosphonate treatment.

Experiment 1

After a 1-week acclimatization period, 40 rats were assigned to undergo bilateral OVX by a dorsal approach (n = 32) and the remaining 8 rats were subjected to a sham operation. Surgery was performed under anesthesia (0.1 mg/100 g body weight (bw) ketamine hydrochloride and 0.1 mg/100 g bw acepromazine maleate) (Holliday Scott SA, Buenos Aires, Argentina).

The first 15 days post-surgery, all the groups were kept under fluorescent tube lighting and fed a synthetic diet containing 20% protein, 0.9% calcium, 0.4% phosphate and added with 200 IU% vit D. The rats were then assigned to receive one of the following treatments during 45 days in order to complete a 60-day period:

C group: Sham animals were housed under 12 h light/dark cycles with *fluorescent tube* lighting and fed the synthetic diet containing 200 IU% vit D.
+D + Light group: OVX animals were housed under 12 h light/dark cycles with *fluorescent tube* lighting and fed the synthetic diet containing 200 IU% vit D.
−D + Light group: OVX animals were housed under 12 h light/dark cycles with *fluorescent tube* lighting and fed the synthetic diet but with no vit D (0 IU%).
+D − Light group: OVX animals were housed under 12 h light/dark cycles with *red* light and fed the synthetic diet containing 200 IU% vit D.
−D − Light group: OVX animals were housed under 12 h light/dark cycles with *red* light and fed the synthetic diet but with no vit D (0 IU%).

Fasting blood samples were collected and total skeleton was scanned at baseline (t = 0) and at the end of the experience (t = 60). At the time of sacrifice, the right tibiae were resected in order to perform histological studies.

Experiment 2

Olpadronate (OPD), the aminobisphosphonate used in this study, was kindly provided by Gador SA (Buenos Aires), and the used doses were based on previous experimental dose–response studies [20,21].

A total 24 rats were OVX and 8 animals were sham-operated. According to the results of experiment 1, the *absence of* exposure to *fluorescent tube* light did not significantly modify the serum 25HOD levels attained by the dietary intake of vit D. For this reason, the entire experimental period, which lasted 105 days, was carried out using *fluorescent tube* light. The treatments established in Experiment 1 were followed throughout the first 60 days of the experiment. During the remaining 45 days, the animals were treated intraperitoneally (i.p.) with either placebo or OPD, according to the following regimens (Fig. 1)

C group: Sham animals fed the synthetic diet containing 200 IU% vit D throughout the entire experimental period (105 days) and treated with placebo during the last 45 days.
+D group: OVX animals fed the synthetic diet containing 200 IU% vit D throughout the entire experimental period (105 days), treated with placebo during the last 45 days.
+D/OPD group: OVX animals fed the synthetic diet containing 200 IU% vit D throughout the entire experimental period (105 days), treated with 16 μg OPD/100 g rat/week during the last 45 days.
−D/OPD group: OVX animals fed the synthetic diet containing 200 IU% vit D during 15 days and with no vit D (0 IU%) for 90 days, treated with 16 μg OPD/100 g rat/week during the last 45 days.

Fasting blood samples were collected and total skeleton was scanned at baseline (t = 0), at treatment onset (t = 60) and at the end of the experimental period (t = 105). The right tibiae were resected at the time of sacrifice in order to perform histological studies.

Biochemical determinations

Blood was collected to determine serum levels of calcium (sCa), phosphate (sP), 25OHD, bone alkaline phosphatase (b-ALP) and carboxy-terminal C-telopeptide cross-links of type I collagen (CTX).

The sCa (mg/dl) was measured by atomic absorption spectrophotometry employing Lanthanum Chloride as interference suppressor, and sP (mg/dl) was measured using colorimetric methods [22,23]. Levels of 25OHD (ng/ml) were measured using radioimmunoassay (RIA) (Diasorin) [22].

Serum CTX (ng/ml) was measured employing immunoassay (ELISA) (Ratlaps. Osteometer BioTech, Herlev, Denmark), with a 6% intra-assay variation coefficient (CV). The b-AL was measured using a colorimetric method

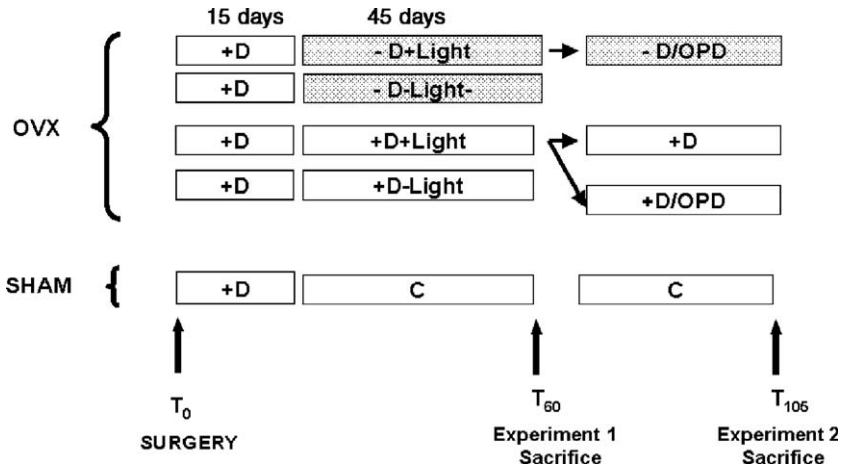

Fig. 1. Summary of the 2 experimental protocols. Surgery was performed at baseline (T_0). All the groups were fed a diet containing 200 IU% of vitamin D (+D) during the first 15 days. During the last 45 days, sham rats were kept on +D and OVX rats were assigned to one of four groups according to the diet and light regimen: diet lacking vitamin D (−D); 12 h light/dark cycles with *fluorescent tube* lighting (+Light), 12 h light/dark cycles with *red* light (−Light). Experiment 1 finished at T_{60}. Experiment 2 continued until day 105 using +Light only. Conditions of Experiment 1 were repeated throughout the first 60 days; rats were treated with either 16 μg olpadronate (OPD)/100 g rat/week or vehicle during the remaining 45 days.

(Boehringer Mannheim, Germany) after bone enzyme isoform precipitation with wheat-germ lectin [22].

DXA measurements

Bone mineral density (BMD) and BMC were determined "in vivo" under light anesthesia using a total body scanner with software designed specifically for small animals (DPX Alpha 8034, Small Animal Softer, Lunar Radiation Corp., Madison, WI) as previously described [21].

All the rats were scanned under light anesthesia using an identical scan procedure. The precision of the software in determining total body BMD was assessed by measuring one rat five times after repositioning between scans both on the same and on different days [21]. The coefficient of variation (CV) was 0.9% for total skeleton BMD and 3.0% for BMC. The different sub-areas were analyzed on the image of the animal on the screen, using an ROI for each segment. The BMD CV for the different studied areas was: 1.8% for lumbar spine (LS) and 3.5% for the proximal tibia (PT). All analyses were carried out by the same technician in order to minimize inter-observer variation.

Histological determinations

At the time of sacrifice, the right tibiae were resected and fixed by immersion in buffered formalin for 48 h, decalcified in 10% ethylene-diamine tetraacetic acid (EDTA) (pH 7) during 25 days and embedded in paraffin. Two 8- to 10-μm-thick longitudinally oriented sections of subchondral bone were obtained at the level of the middle third, including primary and secondary spongiosa. One section was stained with hematoxylin–eosin, and the other was used for histochemical detection of tartrate-resistant acid phosphatase (TRAP) [24]. The sections were microphotographed (AXIOSKOP, Carl Zeiss) to perform histomorphometric measurements on the central area of the metaphyseal bone displayed on the digitalized image. The following static histomorphometric parameters were measured according to Parfitt et al. [25]: Bone Volume (BV/TV) (%): the percentage of cancellous bone within the total measured area; osteoblast surface (Ob.S/BS) (%): the fraction of trabecular bone surface covered with osteoblasts; eroded surface (ES/BS): the fraction of trabecular surface covered with lacunae (including "active" lacunae with osteoclasts and lacunae in reversal phase); osteoclast number (Oc.N/B.TA): the number of osteoclasts in the total studied area; number of TRAP+ osteoclasts in the studied area (Oc.N/B.TA-TRAP+).

To evaluate the presence of osteoid, the contralateral distal tibiae were fixed in 10% phosphate-buffered formaldehyde. After dehydration, undecalcified bone samples were embedded in methyl methacrylate and longitudinal sections (5–7 υm) were cut employing a Polycut microtome (Reichert Jung, Heidelberg, Germany). Bone sections were stained using the modified Masson's Trichrome staining technique [26].

Statistical methods

Results were expressed as mean ± standard error (SE). Data were analyzed by one-way analysis of variance (ANOVA), and Bonferroni multiple comparisons test was performed when significant differences were encountered. Similarly, histologic data were analyzed using a single time point factorial analysis. Statistical analyses were performed using SPSS for Windows 11.0 (SPSS, Inc., Chicago, IL). A value of P below 0.05 ($P < 0.05$) was considered significant.

Results

Experiment 1

Fig. 2 shows the results related to vit D status at the end of the experiment (day 60). The type of light did not induce differences in serum 25OHD among the studied groups (+D +Light

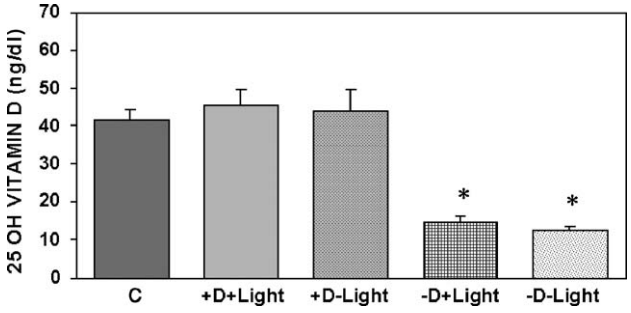

Fig. 2. Vitamin D status of the studied groups. Levels of 25OH vitamin D according to the vitamin D regimen and type of light exposure at the end of Experiment 1 expressed as mean ± SE. Rats fed +D presented significantly higher levels of 25OH vitamin D than rats fed −D. The type of light did not induce differences in serum 25OHD among the studied groups. The asterisk denotes statistically significant differences compared to groups C and +D ($P < 0.0001$).

Table 1
Serum calcium (mg/dl), serum phosphate (mg/dl), bone alkaline phosphatase (IU/l), serum β-CTX (μg/dl), total skeleton BMD (mg/cm^2), bone volume (%), osteoblast surface (%) and osteoclast surface (%) at the end of Experiment 1

Groups	Serum calcium (mg/dl)	Serum phosphate (mg/dl)	Bone alkaline phosphatase (IU/l)	Serum β-CTX (μg/ml)	Total skeleton BMD (mg/cm^2)
C	9.5 ± 0.7a	5.1 ± 0.8a	61 ± 4a	20.7 ± 9.5a	298 ± 8a
+D + Light	9.7 ± 0.2a	6.0 ± 0.9a	60 ± 4a	41.2 ± 9.2b	297 ± 3a
+D − Light	9.5 ± 0.6a	6.1 ± 1.2a	57 ± 8a	42 ± 17.0b	292 ± 8a
−D + Light	9.7 ± 0.3a	6.7 ± 1.1a	52 ± 6a	38.4 ± 11.5b	296 ± 8a
−D − Light	9.8 ± 0.5a	6.6 ± 0.8a	55 ± 5a	39.9 ± 10.3b	299 ± 5a

All values are expressed as mean ± SE.
Different letters indicate statistical significance ($P < 0.05$).

vs. +D − Light and −D + Light vs. −D − Light groups). As expected, serum 25OHD levels in OVX groups receiving no dietary vit D were significantly lower than in those fed a diet containing vit D ($P < 0.0001$).

The results obtained at the end of the experiment showed no differences in sCa among groups (Table 1). The OVX groups, including those receiving and not receiving dietary vit D, exhibited similar sP levels and slightly higher levels compared with C animals (Table 1).

Serum b-AL was lower and CTX was higher in OVX groups compared to the C group, but only differences in CTX reached statistical significance ($P < 0.05$) (Table 1). Moreover, no statistically significant differences in either of these two bone markers were found among the OVX groups, regardless of whether they had received +D or −D (Table 1).

No differences in total skeleton BMD were observed among groups at the end of the study (Table 1). Fig. 3 shows that LS BMD was significantly lower in the OVX groups compared to C rats ($P < 0.05$); however, regardless of the type of light supplied, no differences among OVX animals receiving or not dietary vit D were found.

Regardless of the vit D regimen, the histologic findings in OVX compared to the control group showed a reduction in the number of trabeculae (data not shown), a decrease in BV/TV (+D + Light: 24 ± 2; −D − Light: 20 ± 2 vs. C: 32 ± 4, $P < 0.05$) and an increase in the ES/BS (+D + Light: 30 ± 3; −D − Light: 32 ± 4 vs.

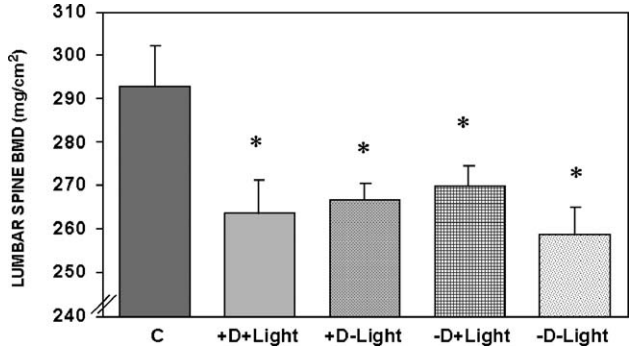

Fig. 3. Lumbar spine BMD (mg/cm^2) at the end of Experiment 1. Data were expressed as mean ± SE. Note that ovariectomized rats presented a diminution in bone density, independently of the vitamin D regimen and type of light. An asterisk denotes statistically significant differences as compared with the sham group ($P < 0.05$).

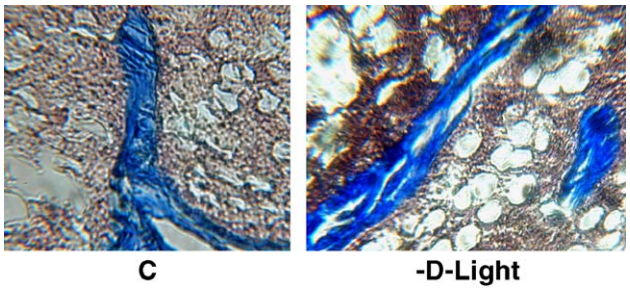

Fig. 4. Histology of undecalcified sections of groups C and −D−Light at the end of Experiment 1. Modified Masson's Trichrome staining technique shows bone-specific collagen I stained in dark blue. Note that no osteoid was observed in the −D−Light group (stained red) ×200. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

C: 11 ± 2, $P < 0.01$) and in Ob.S/BS (+D + Light: 27 ± 8; −D − Light: 32 ± 4 vs. C: 23 ± 7, $P < 0.05$); however, no differences were observed among the OVX groups. Fig. 4 shows the histology of undecalcified sections of groups C and −D − Light. No osteoid was found in animals lacking exogenous and endogenous vit D.

Experiment 2

Table 2 shows the biochemical results obtained at the end of treatment (day 105). The 25OHD levels were significantly lower

Table 2
Serum calcium (mg/dl), serum phosphate (mg/dl), bone alkaline phosphatase (IU/l), serum β-CTX (μg/dl), 25OD vitamin D (ng/ml), total skeleton BMD (mg/cm^2), bone volume (%), osteoblast surface (%) and osteoclast surface (%) at the end of Experiment 2

	C group	+D/OPD group	−D/OPD group	+D group
Serum calcium (mg/dl)	9.6 ± 0.3a	9.7 ± 0.4a	9.4 ± 0.3a	8.9 ± 0.3b
Serum phosphate (mg/dl)	4.2 ± 1.1a	3.8 ± 1.3a	4.5 ± 0.7a	5.4 ± 1.0b
25OH vitamin D (ng/ml)	35.0 ± 4.3a	44.1 ± 8.1a	9.2 ± 2.3b	37.1 ± 4.5a
Bone alkaline phosphatase (IU/L)	57 ± 7a	58 ± 6a	51 ± 10a	21 ± 6b
Serum β-CTX (ng/ml)	21.5 ± 4.7a	28.2 ± 8.9a	30.6 ± 7.8a	22.1 ± 1.0a
Total skeleton BMD (mg/cm^2)	298 ± 12a	301 ± 12a	303 ± 8a	287 ± 7b
Bone volume (BV/TV) (%)	28 ± 4a	30 ± 3a	15 ± 2b	12 ± 2c
Osteoblast Surface (Ob.S/BS) (%)	33 ± 4a, b	39 ± 6b	30 ± 2a	23 ± 6c
Edored surface (ES/BS) (%)	20 ± 4a	20 ± 3a	27 ± 2b	30 ± 3b
Total osteoclast number (Oc.N/B.TA)	33 ± 9a	23 ± 6b	21 ± 4b	28 ± 4b
TRAP + osteoclast number (Oc.N-TRAP+/B.TA)	66 ± 7a	3 ± 2b	1 ± 1b	6 ± 4b

Different letters indicate statistical significance ($P < 0.05$).

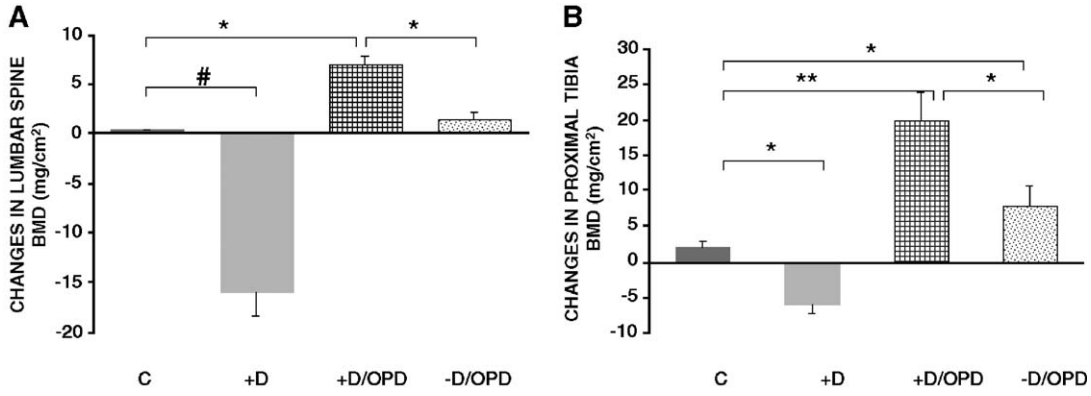

Fig. 5. Percentage of change in (A) lumbar spine (LS) and (B) proximal tibia (PT) BMD from baseline to the end of Experiment 2 ($BMD_{T105} - BMD_{T0}$). Data were expressed as mean ± SE. Untreated rats (+D) exhibited lower LS (#$P < 0.001$) and PT (*$P < 0.05$) BMDs compared to C. OPD treatment prevented this decrease (*$P < 0.05$ for LS; **$P < 0.01$ for PT when compared to C) and had a high effect in the +D groups (*$P < 0.05$ for both areas when compared +D/OPD vs. −D/OPD).

($P < 0.0001$) in rats receiving no dietary vit D. Independent of the vit D regimen, there were no significant differences in sCa, sP, b-AL and CTX between OPD-treated rats. In addition, whereas neither of these two groups showed differences compared to C, sCa and b-AL were significantly higher and sP was significantly lower when compared to +D untreated rats.

Although no differences in total skeleton BMD were observed between C and −D/OPD and +D/OPD at the end of the study, +D rats had the lowest significant level (Table 2). Figs. 5A and B show the change observed in LS and PT BMDs throughout the treatment. C rats did not present changes in BMD in either of the studied areas (1.2% and 0.5%, respectively). The +D rats lost approximately 16% and 5% of their LS and PT BMDs, respectively, and these decreases were statistically significant compared to the C group ($P < 0.001$ and $P < 0.05$, respectively). OPD treatment was found to prevent this decrease, which was greater in +D/OPD than in −D/OPD (LS BMD: 7.4% vs. 5.5%, P = ns and PT BMD: 21.5% vs. 7.7%, $P < 0.05$). Furthermore, differences in LS BMD compared to C failed to reach statistical significance in the −D/OPD group only (Fig. 5A).

The diminution in BV/TV (Table 2) and in the number of trabeculae (Fig. 6) observed in the +D untreated group was prevented by OPD treatment in both OVX groups. However, +D/OPD animals presented significantly higher values of BV/TV than rats not receiving dietary vit D ($P < 0.05$) (Table 2). Both −D/OPD and +D/OPD rats also exhibited increased Ob.S/BS and decreased ES/BS compared to +D groups. Once again, a significant increase in osteoblast surfaces and a decrease in ES/BS was observed in +D/OPD compared with −D/OPD (Table 2). Oc.N/TA and Oc.N/B.TA-TRAP+ were significantly reduced in +D, +D/OPD and −D/OPD compared

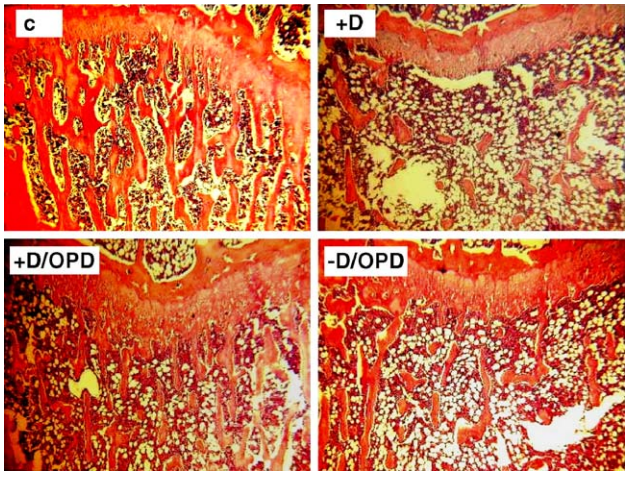

Fig. 6. Histology of decalcified sections of the proximal third of the tibia in all studied groups at the end of experiment 2. Hematoxylin–eosin staining technique shows trabeculae stained in red. Note the decrease in the number and the lack of connectivity of trabeculae in +D group. OPD treatment shows differences between +D/OPD and −D/OPD groups ×50. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

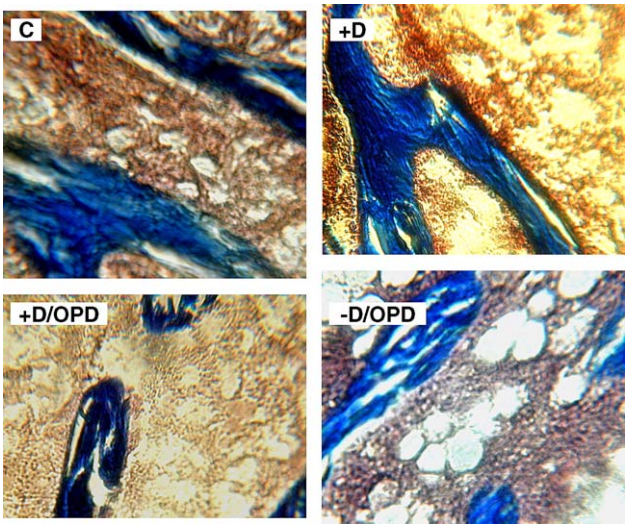

Fig. 7. Histology of undecalcified sections of all studied groups at the end of Experiment 2. Modified Masson's Trichrome staining technique shows bone-specific collagen I stained in dark blue. Note that no osteoid was observed (stained red) ×200. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to C ($P < 0.05$ and $P < 0.001$, respectively) without differences among them (Table 2). None of the studied groups exhibited osteoid, as shown by the histologic study of undecalcified sections (Fig. 7).

Discussion

To our knowledge, this study is the first to characterize an experimental model of insufficient vit D status and established osteopenia without signs of osteomalacia and to document a decrease in the response to bisphosphonate treatment in osteopenic rats presenting vit D insufficiency.

Although estrogen depletion is known to affect bone mass and calciotropic hormones, it has no effect on 25OHD levels. In the present study, serum 25OHD levels of OVX rats receiving dietary vit D were within the range of normal sham animals (34.3 ± 4.3 ng/ml), which in turn is similar to the human normal range. Moreover, exposure or lack of exposure to fluorescent tube light did not modify 25OHD levels in OVX rats.

Circulating 25OHD determines the vit D nutritional status [8]; a concentration below 8–10 ng/ml indicates severe vit D deficiency [27], which leads to rickets and histological signs of osteomalacia [28]. In addition, concentrations between 10 and 16 ng/dl reflect marginal vit D deficiency in both humans [27,29] and rats [30]. A crucial point in the present study was to obtain a vit-D-deficient group presenting a 25HOD concentration of about 16 ng/ml, which corresponds to the average winter value of adults living in western countries. Under the present experimental conditions, the serum concentration of 25OHD in OVX rats receiving no dietary vit D ranged between 9.8 and 16.3 ng/ml and showed no differences when comparing the type of light. Consequently, it could be assumed that the UV radiation in *fluorescent tube* light is not sufficient to induce significant production of vit D in the skin and thus counteract the low 25OHD levels caused by the lack of dietary vit D. In agreement with this conclusion, a number of clinical works reported in the literature have demonstrated a decline in 25OHD under acutely sun-deprived living conditions, as is the case of sailors confined in submarines [28].

The concentration of serum 25OHD can be altered through a balance between its production and clearance [28]. As regards synthesis, 25OHD diminishes due to the lack of dietary vit D. As to consumption, $1,25(OH)_2D$ production is substrate-dependent in the case of vit D deficiency [5]. Marginal values of 25OHD induce a diminution in calcitriol concentrations [27,28,31], which decrease intestinal Ca absorption [6] and increase PTH synthesis [32]. It is possible that the slight increase in PTH secretion contributes to decreasing the circulating levels of 25HOD by stimulating calcitriol synthesis, due to the lack of negative feedback control on the synthesis and secretion of PTH. Neither serum PTH nor calcitriol was measured in the present report. Nevertheless, it can be assumed that the low levels of 25OHD, the lack of estrogen and the slight increment in sP without changes in sCa stimulated PTH secretion [33–35]. Further studies would be required to establish whether mild hyperparathyroidism is necessary to maintain normocalcemia.

As expected, the lack of estrogen resulted in increased bone resorption without causing significant changes in mineral homeostasis, although a slight non-significant increment in sP was observed. Furthermore, the changes induced by OVX were not modified by the dietary cholecalciferol supply. Indeed, both rats receiving and those not receiving dietary vit D had similar serum mineral concentration and bone turnover, as shown by serum CTX and b-AL levels. It is important to point out that the studied rats were maintained under these vit D nutritional conditions for a total of 45 days. This period would have been long enough to manifest differences in bone markers, if they had existed. Histological studies confirmed the lack of differences in bone turnover when comparing the vit D status of the four studied OVX groups.

Whereas a severely deficient stage of 25OHD is associated with rickets and osteomalacia, mild to moderate degrees of vit D insufficiency have been found to cause neither rickets nor osteomalacia [4]. In the present study, bone and histological findings of the −D rats were comparable to those of the +D animals, and the histomorphometric studies revealed no signs of osteomalacia in the OVX groups receiving no dietary vit D. This result is in agreement with previous studies reporting that, when vit-D-deficient animals are made normocalcemic by experimental or dietary means, no osteomalacia occurs [36] and corroborates that bone in vit-D-deficient rats may be normal when the mineral supply is adequate [36].

In summary, the biochemical, densitometrical and histological findings presented above may suggest that under the present experimental conditions an appropriate model of vit D insufficiency and established osteopenia has been characterized. This model of induced bone loss due to ovarian hormone deficiency with low circulating levels of 25OHD and no signs of osteomalacia was obtained by OVX and lack of dietary vit D. It should result accurate for human studies since it allows mimicking certain characteristics of bone loss, already documented in postmenopausal osteopenic women with vit D deficiency, in one or more respects. In this regard, biochemical indices of bone turnover evidenced increased bone resorption with no changes in bone formation that led to cancellous bone loss, with remains being essentially normal. In addition, marginal deficiency of vit D assessed by levels of 25OHD between 10 and 16 ng/dl was also evidenced. It is necessary to take into account that there is no model that could be considered an exact replica of the human condition. However, the current experimental model would be very useful for future studies involving the relationship between bone loss and insufficient status of vit D, as well as to test the effectiveness of treatment with anticatabolic agents without confounding extrinsic factors.

Bisphosphonates are anticatabolic agents used to prevent bone loss due to osteoporosis [10]. As expected, in the present report, the reduction in bone mass caused by OVX was prevented by the bisphosphonate treatment. However, poor response to bisphosphonate therapy is commonly observed in everyday clinical practice. Although both −D and +D rats were found to present increased trabecular and cortical bone in the present study, OPD treatment seemed to have a less marked effect on −D than on +D animals. Data relating the effectiveness of bisphosphonate

treatment and vit D status are scanty. However, there is some evidence suggesting that the physiological status of vit D may affect response to bisphosphonate treatment showing a link between insufficient vit D levels and low bone mineral recovery after bisphosphonate treatment. In this regard, Koster et al. [17] found a diminished effect of etidronate and Yamanaka et al. [18] observed a diminished effect of alendronate treatment in vit-D-deficient osteopenic postmenopausal women. To our knowledge, the present report is the first experimental study to demonstrate that a deficient vit D status diminishes the effectiveness of aminobisphosphonate treatment. Indeed, bone resorption inhibition, bone volume and positive changes in bone density were higher in a state of vit D sufficiency.

In summary, this experimental study is the first to provide evidence that differences in vit D status may affect the anti-resorptive response to bisphosphonate treatment. However, the molecular mechanism through which vit D insufficiency reduces the effect of the aminobisphosphonate remains to be defined.

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