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Olpadronate Prevents the Bone Loss Induced by Cyclosporine in the Rat

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Abstract. The aim of the present *in vivo* experimental study was to investigate changes in bone turnover and bone mineral density (BMD) induced by cyclosporine (CsA) administration. The effectiveness of olpadronate (OPD) in preventing bone loss associated with CsA treatment was also evaluated. Forty male Sprague-Dawley rats (approximately 5 months old) were treated as follows: Group I: CsA + OPD vehicles (control); Group II: CsA 15 mg/kg + OPD vehicle; Group III: CsA 15 mg/kg + 4 ug OPD/100g rat; Group IV: CsA 15 mg/kg + 8 ug OPD/100g rat; Group V: CsA 15 mg/kg + 16 ug OPD/100g rat. CsA was administered by daily oral gavage and OPD by intraperitoneal injection once a week. Serum bone-alkaline phosphatase (b-ALP) and urinary deoxypyridinoline (DPyr) were measured on days 0, 14 and 30. Total skeleton, femur, lumbar spine, proximal, and middle tibia BMDs were measured on days 0 and 30. No significant differences were found between the CsA and the control groups as regards serum bALP levels, on days 14 and 30. CsA + OPD treated rats presented a transient increment in serum b-ALP on day 14 and a significantly lower level on day 30 compared to the control and CsA groups ($P < 0.05$). On days 14 and 30, DPyr excretion increased in the CsA group compared to control animals ($P < 0.05$). The three studied doses of OPD induced a significant decrease in DPyr excretion in the CsA group on days 14 and 30 ($P < 0.05$). Group V (receiving the highest dose of OPD) presented a significantly lower level of DPyr compared to the other two OPD-treated groups ($P < 0.05$). On day 30, the CsA group presented a significant reduction in proximal tibia, spine and whole femur BMDs ($P < 0.05$) compared to controls. On day 30, OPD treatment increased BMD of all the studied areas in CsA rats. Proximal tibia BMD of group V reached significantly higher values than the other studied OPD groups ($P < 0.05$). In summary, this study suggests that CsA-induced high bone resorption and trabecular bone loss is prevented by cotreatment with OPD. Moreover, it encourages the possible use of OPD to treat patients receiving CsA as immunosuppressive therapy.

Key words: BMD — Cyclosporine — Deoxypyridinoline — Olpadronate — Rats.

The use of cyclosporine (CsA) as a therapeutic agent has improved the effectiveness of organ transplantation despite its side effects, which include osteopenia and even osteoporotic fractures which have a negative effect on the quality of life [1]. CsA may affect the immune system, inhibiting production and release of cytokines, which could affect bone metabolism and remodeling at a local level [2]. Several authors have reported that CsA accelerates bone resorption and bone formation, inducing a high-turnover osteopenic state [3–6]. Indeed, bone disease is common in patients awaiting or during the first months after organ transplantation. Then, safer therapies are needed for the prevention and treatment of immunosuppressant-induced osteoporosis.

Bisphosphonates (BPs) are useful drugs that effectively inhibit resorption in several bone diseases characterized by high bone turnover and in osteoporosis [7]. Although it is necessary to use BPs with caution in certain clinical situations, they are promising drugs for preventing bone loss in organ transplantation. Olpadronate (OPD), an aminobisphosphonate, has been shown to have a great antiresorptive potency. In a previous experimental study we demonstrated the effectiveness of OPD in preventing bone loss associated with ovariectomy and/or hyperthyroidism [8].

Some histomorphometric and biochemical studies [3–5] have shown the high-turnover bone remodeling that follows CsA administration as well as the preventive action of the alendronate [6]. However, according to our knowledge there are no longitudinal experimental studies showing changes in bone mass *in vivo*. Dual energy X-ray bone densitometry has proven useful in

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studies with small animals. Based on the above, the present study sought to investigate changes in bone markers and bone mineral density (BMD) induced by CsA administration using an *in vivo* experimental model. The possible beneficial effects of OPD on bone loss in CsA-treated rats was also evaluated.

Materials and Methods

Animals

A total of Forty male Sprague-Dawley rats (250–300 g), approximately 5 months old were housed at room temperature (21 ± 1°C), 55 ± 10% humidity in 12- hour light/dark cycles. They were fed a commercial formula (Purina Lab, Buenos Aires) containing 1.2% calcium, 0.9% phosphate and 200 IU of vitamin D%, and tap water *ad libitum*. The National Institutes of Health Guide for the Care and Use of Laboratory Animals was observed.

Drugs

CsA, in a solution containing 100 mg CsA/ml and 10% alcohol by volume in olive oil, and an alcohol olive oil vehicle, were kindly provided by Novartis, Inc (Buenos Aires, Argentina). The CsA solution was diluted in the vehicle to obtain a concentration of 15 mg/ml. A dose of 15 mg/kg has been shown to reliably cause high turnover osteopenia [6].

Gador Pharmaceutical Laboratory (Buenos Aires, Argentina) kindly provided the OPD which was dissolved in normal saline prior to administration.

Experimental Protocol

The animals were allowed one week to adapt to their new environment, after which they were randomly divided into five groups and received one of the following treatments during a 30-day period:

Group I: CsA vehicle + OPD vehicle (control)
Group II: CsA 15 mg/kg rat + OPD vehicle
Group III: CsA 15 mg/kg rat + 4 ug OPD/100 g rat
Group IV: CsA 15 mg/kg rat + 8 ug OPD/100 g rat
Group V: CsA 15 mg/kg rat + 16 ug OPD/100 g rat.

CsA was administered by daily oral gavage and OPD was intraperitoneally injected once a week. Body weight (BW) was recorded throughout the study.

Biochemical Parameters

Blood and urine were collected on days 0, 14, and 30. Blood was collected in the morning under anesthesia (0.1 mg/100 g of BW of ketamine hydrochloride plus 0.1 mg/100 g BW of acepromazine maleate) (Holliday-Scott SA, Buenos Aires, Argentina). Urine collection was carried out by placing each rat in an individual metabolic cage. Blood and serum samples were stored at −20°C until tested.

Serum calcium (Ca), phosphorus (P), bone alkaline phosphatase (b-ALP), and serum and urinary creatinine were measured as previously described [8, 9]. The intra- and interassay coefficients of variation (CVs) of serum bALP were 3.8–7.6% and 5.8–7.8%, respectively. Urinary deoxypyridinoline (Dpyr) was analyzed by ELISA using a commercially available kit (Pyrilinks®-D) (Metra Biosystems Inc., Palo Alto, CA) [10]. Dpyr intra- and interassay CVs were: 3.7–8.0% and 5.8–10.3%, respectively. The value of Dpyr in urine samples was expressed as a ratio of creatinine concentration.

Skeletal measurements

Total BMD was assessed *in vivo* on days 0 and 30 using a total body scanner with a software that was specifically designed for small animals (DPX Alpha 8034, Small Animal Software, Lunar Radiation Corp. Madison WI), as described elsewhere [8]. All rats were scanned under light anesthesia using an identical scan procedure as mentioned above. Precision of the software on total body was assessed by measuring one rat five times with repositioning of the animals between scans on the same and on different days [8]. The different subareas were analyzed on the image of the animal on the screen using a ROI for each segment. CVs were 0.9% for total skeleton, 0.8% for femur, 1.8% for lumbar spine, and 0.8%, 3.5%, and 2.7% for whole, proximal, and middle tibia, respectively.

Statistical Methods

Data were expressed as mean ± standard error (SEM). Statistical analyses were performed using the statistics package SPSS (SPSS Inc. Chicago, IL). Differences between means of control and CsA-treated rats were evaluated using the unpaired Student's *t*-test. DPyr, b-ALP, and BMDs data were analyzed by one factor ANOVA with the Dunnet post hoc test to make comparisons among groups at the different experimental times. $P < 0.05$ was considered a significant difference.

Results

Body Weight

There were no significant differences in BW among the studied groups on days 0, 14 and 30.

Biochemical Parameters

On days 0, 14, and 30 there were no significant differences in serum Ca and P levels among groups (Table 1). Serum creatinine levels increased by approximately 12% in the CsA-treated group until 15% in CsA + OPD-treated rats compared to control group ($P < 0.05$) on days 14 and 30, although no significant differences within groups II, III, IV, and V were found (Table 1).

In all studied groups, serum b-ALP levels were lower on day 30 compared with day 14. This difference was statistically significant in all groups except controls. Serum b-ALP levels in the CsA-treated group were similar to the controls on days 14 and 30. All CsA + OPD-treated rats presented a transient increase in serum b-ALP on day 14 compared with controls, this increase reached statistical significance in groups IV and V ($P < 0.05$). On day 30, OPD-treated groups presented significantly lower serum b-ALP than the control and CsA-treated groups ($P < 0.05$) (Table 1).

On days 14 and 30, DPyr excretion increased in CsA-treated rats compared with control animals ($P < 0.05$) (Fig. 1). All OPD-treated groups presented a significant lower DPyr excretion compared with animals treated only with CsA ($P < 0.05$) on days 14 and 30 and compared with the control group ($P < 0.05$) on day 30.

Table 1. Biochemical results (mean ± SE)

	Baseline	Group I (control) (n = 8)	Group II (CsA) (n = 8)	Group III (CsA + 4 ugOPD) (n = 8)	Group IV (CsA + 8 ugOPD) (n = 8)	Group V (CsA + 16 ugOPD) (n = 8)
Serum calcium (mg/dl)	10.2 ± 0.2					
14 days		10.1 ± 0.1	10.0 ± 0.3	10.0 ± 0.2	10.1 ± 0.2	9.8 ± 0.3
30 days		9.7 ± 0.1	9.7 ± 0.1	9.8 ± 0.1	9.8 ± 0.3	9.7 ± 0.2
Serum phosphorus (mg/dl)	6.3 ± 0.4					
14 days		6.7 ± 0.3	6.3 ± 0.4	6.9 ± 0.4	6.8 ± 0.4	6.6 ± 0.3
30 days		6.5 ± 0.3	6.5 ± 0.3	6.3 ± 0.3	6.5 ± 0.2	6.7 ± 0.2
Serum b-ALP (U/L)	30.3 ± 1.4					
14 days		22.8 ± 1.8*	25.7 ± 1.6*	26.3 ± 1.6^{*a}	32.1 ± 1.7^{a,b}	30.8 ± 2.5^{a,b}
30 days		20.2 ± 2.3*	19.7 ± 2.3^{*c}	14.7 ± 0.9^{*a,b,c}	14.9 ± 1.4^{*a,b,c}	16.6 ± 1.1^{*a,b,c}
Serum creatinine (mg/dl)	0.38 ± 0.02					
14 days		0.41 ± 0.02	0.49 ± 0.03^{*a}	0.50 ± 0.02^{*a}	0.52 ± 0.03^{*a}	0.52 ± 0.03^{*a}
30 days		0.44 ± 0.02*	0.50 ± 0.04^{*a}	0.51 ± 0.03^{*a}	0.52 ± 0.02^{*a}	0.53 ± 0.03^{*a}

$^{*}P < 0.05$ compared to baseline; $^{a}P < 0.05$ compared to group I; $^{b}P < 0.05$ compared to group II; $^{c}P < 0.05$ compared to day 14.

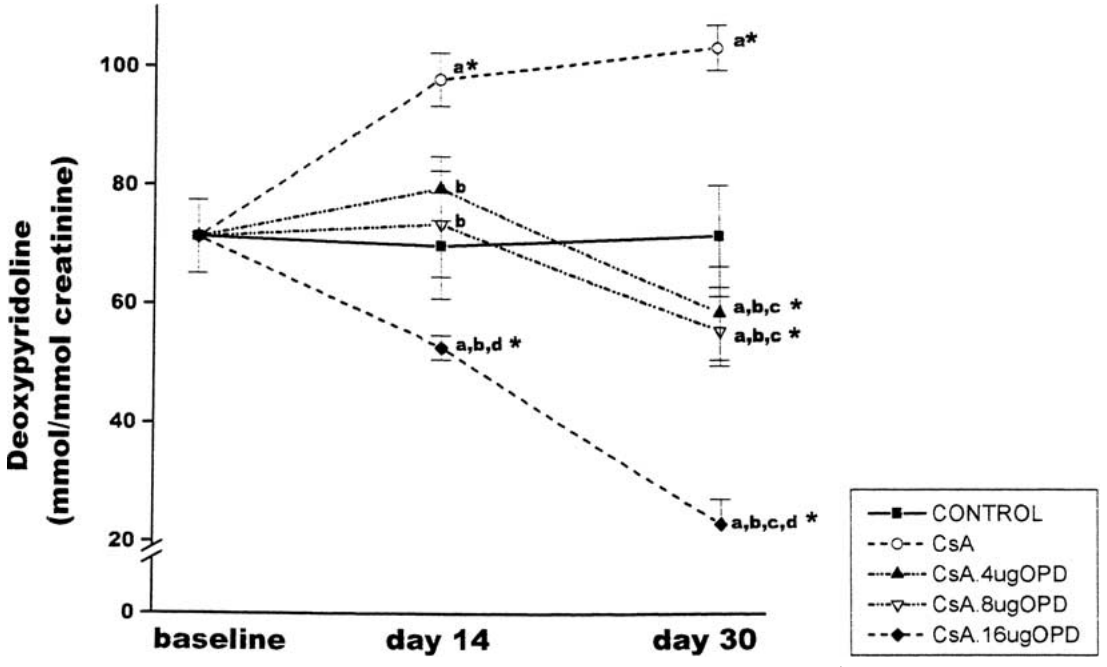

Fig. 1. Deoxypyridinoline (mmol/mmol creatinine) values at baseline and at days 14 and 30 * $P < 0.05$ compared to baseline; ^{a} $P < 0.05$ compared to group I (control); $^{b}P < 0.05$ compared to group II (CsA); $^{c}P < 0.05$ compared to day 14; $^{d}P < 0.05$ compared to the other OPD doses.

As a result, urinary DPyr excretion was significantly lower on day 30 compared to day 14 in each of the OPD-treated groups ($P < 0.05$). It is important to point out that the diminution was greater in group V (receiving the highest OPD dose) than in the other two OPD-treated groups ($P < 0.05$) (Fig. 1)

Skeletal Parameters

CsA treatment: On day 30, CsA treatment did not affect total skeleton or middle tibia BMD compared to controls (Table 2). On day 30, the BMDs in CsA-treated animals were significantly lower at the proximal tibia, lumbar spine, and whole femur regions than in controls ($P < 0.05$) (Figs. 2 and 3 and Table 2, respectively).

OPD + CsA-treated rats: On day 30, except for the middle tibia region, the BMD of the other studied areas was significantly higher in CsA + OPD treated animals than in control and CsA-treated rats ($P < 0.05$) (Fig. 2 and 3 and Table 2, respectively). On day 30, proximal tibia BMD of group V (the highest OPD dose) reached significantly higher values than the other two studied OPD doses ($P < 0.05$) (Figs. 2 and 3).

Table 2. Bone mineral density of the skeleton and the different studied regions (BMD) (mg/cm^2) (mean + SE)

	Baseline	Group I (control) (n = 8)	Group II (Cs A) (n = 8)	Group III (CsA + 4ugOPD) (n = 8)	Group IV (CsA + 8ugOPD) (n = 8)	Group V (CsA + 16ugOPD) (n = 8)
Total skeleton30 days	232 ± 1	$252 \pm 1^{*}$	$252 \pm 1^{*}$	$272 \pm 1^{*a,b}$	$268 \pm 4^{*a,b}$	$266 \pm 2^{*a,b}$
Whole femur 30 days	237 ± 5	$295 \pm 5^{*}$	$264 \pm 5^{*a}$	$311 \pm 7^{*a,b}$	$297 \pm 5^{*b}$	$322 \pm 6^{*a,b,d}$
Middle tibia 30 days	198 ± 3	$226 \pm 3^{*}$	$225 \pm 2^{*}$	$230 \pm 4^{*}$	$229 \pm 7^{*}$	$229 \pm 5^{*}$
Lumbar spine 30 days	203 ± 4	$238 \pm 5^{*}$	$226 \pm 4a^{*}$	$247 \pm 3,b^{*}$	$241 \pm 4^{*b}$	$256 \pm 6^{*a,b,c,d}$
Proximal tibia 30 days	212 ± 3	$240 \pm 8^{*}$	$229 \pm 3^{a*}$	$287 \pm 7^{*a,b}$	$276 \pm 4^{*a,b}$	$303 \pm 5^{*a,b,c,d}$

$^{*}P < 0.05$ compared to baseline value
$^{a}P < 0.05$ compared to group I
$^{b}P < 0.05$ compared to group II
$^{c}P < 0.05$ compared to group III
$^{d}P < 0.05$ compared to group IV

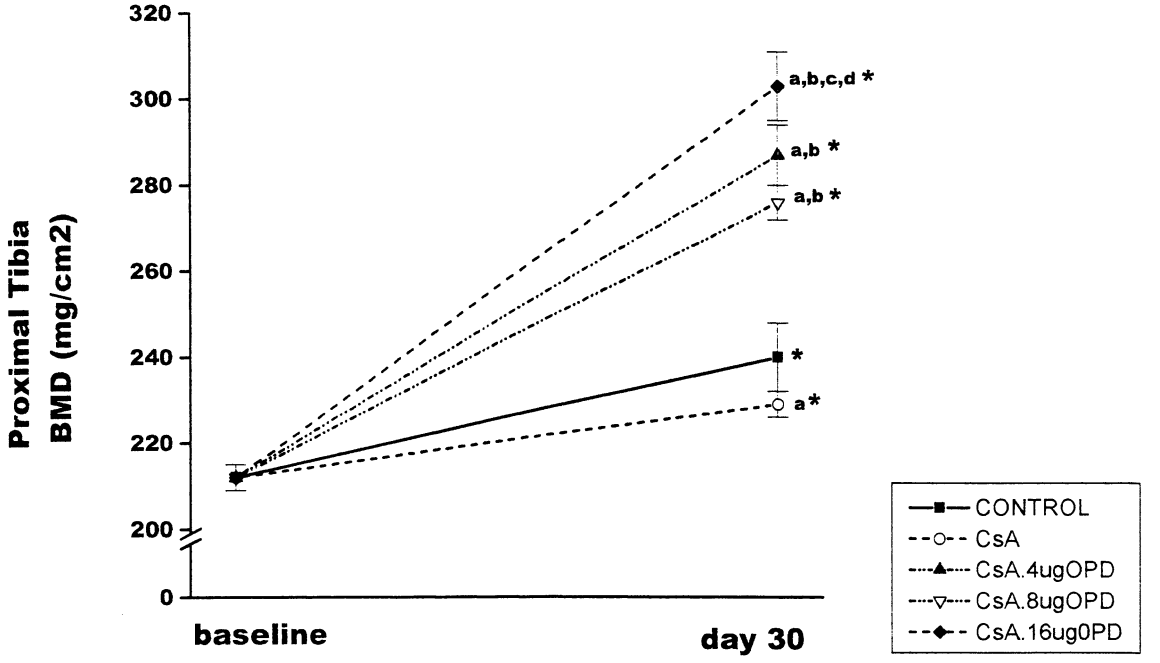

Fig. 2. Proximal tibia BMD (mg/cm^2) values at baseline and at day 30 $^{*}P < 0.05$ compared to baseline; $^{a}P < 0.05$ compared to group I (control); $^{b}P < 0.05$ compared to group II (CsA); $^{c}P < 0.05$ compared to the other OPD doses.

Discussion

Many number of experimental studies reported that CsA treatment produced high bone turnover osteopenia, as evidenced by biochemical and histological techniques [11–17]. In the present *in vivo* study, changes in total skeleton BMD and its different areas by CsA treatment were correlated with changes in biochemical markers. These results indicate that under the experimental conditions stated herein there is an increase in bone resorption without changes in bone formation. This effect induced a deleterious effect on the skeleton and it is reflected in BMD measurements.

In relation to biochemical markers, serum b-ALP showed a transient nonsignificant increase on day 14, although no changes were found on day 30 by CsA treatment. The reason for this difference in pattern on days 14 and 30 is not clear, although a similar increase in osteocalcin levels on day 10 in the absence of a marked renal insufficiency such as in the present study was reported in a previous study [18]. Conversely, urinary DPyr excretion had increased significantly by day 14 and remained at that a level on day 30. The increment observed in DPyr excretion is in agreement with findings reported by other investigators [3, 18] and reflects an increase in bone resorption. Biochemical data suggest an uncoupling condition characterized by an imbalance in bone resorption that exceeded bone formation. Such increment in bone resorption by CsA administration negatively affected bone balance and impaired bone mass in the spine, whole femur, and proximal tibia regions. These results are in agreement with several histomorphometric studies showing tibial trabecular bone loss following administration of CsA (l5 mg/kg) to male

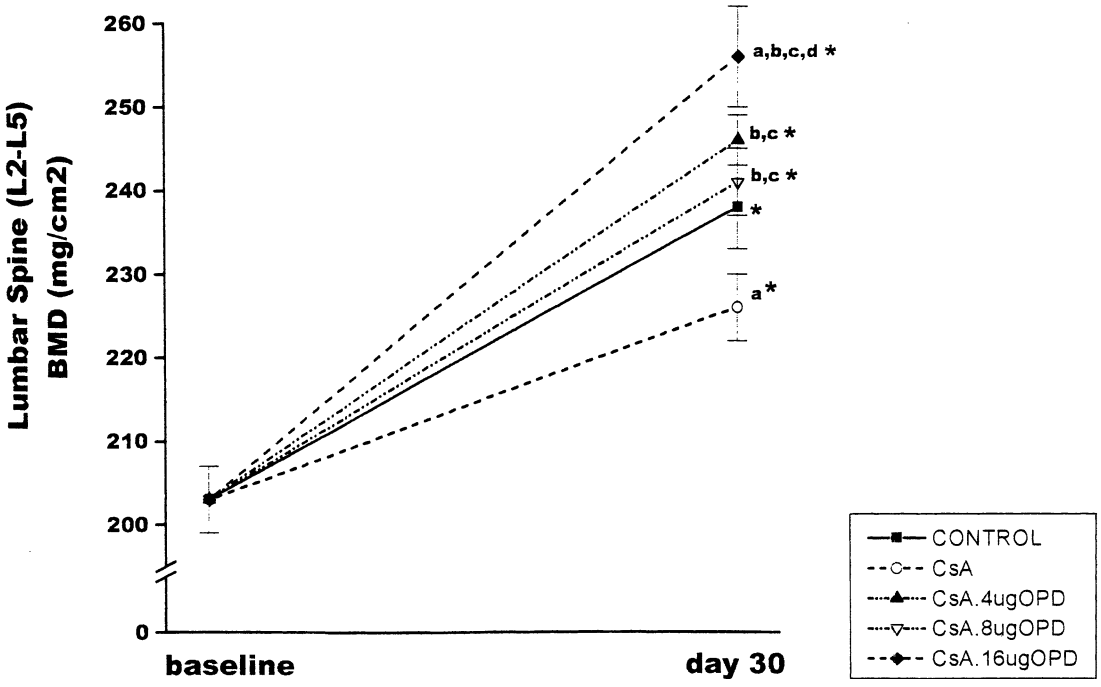

Fig. 3. Lumbar spine BMD (mg/cm^2) values at baseline and at day 30. $^{*}P < 0.05$ compared to baseline; $^{a}P < 0.05$ compared to group I (control); $^{b}P < 0.05$ compared to group II (CsA);

[14, 16, 18] and to OVX rats [19]. Though a similar amount of bone mass impairment was found in the spine and proximal tibial regions (trabecular areas), total skeleton and middle tibia (cortical areas) showed no changes in BMD. These results could reflect that although the osteopenic effect of CsA treatment is similar in the axial and appendicular skeleton, cancellous bone would be more sensitive to bone loss. The differences observed between trabecular and cortical bone agree with previous histomorphometrical studies in which a decrease in trabecular bone volume was found [3, 11, 18, 19, 20]. This different behavior could be partly explained by the fact that trabecular bone is metabolically more active than cortical bone and it would be more susceptible to local factors such as cytokines which are affected by CsA administration [2].

It is important to point out that the effect of CsA on bone mass is not related to abnormal renal function because serum creatinine only showed a small increment of 15% and all mean serum values were within the normal range of 0.2–0.8 mg/dl. Moreover, although immunosuppressive therapy affects weight gain, in the present report no significant differences in body weight were found among the experimental groups (data not shown).

CsA is an established drug in the therapy of patients undergoing organ transplantation [21]. Several experimental reports demonstrated that CsA induces a high turnover osteopenic state in which resorption exceeds formation [11–16], leading to trabecular bone loss [18, 19]. In the present report a significant increment of resorption was found and bone formation evaluated by b-ALP did not change. BPs had proved to be effective enough in preventing high resorption osteopenia [22]. Previous studies using the 2-PEBP [23] and Alendronate [6] prevented the adverse effects of CsA, maintaining trabecular bone volume in male rats. The present report demonstrates that OPD, under our experimental conditions, effectively prevented CsA-induced osteopenia without exacerbating renal insufficiency. Moreover, the small nonsignificant decrease in serum Ca and the lack of change in serum phosphorus might suggest that parathyroid hormone secretion remained unchanged. In fact, OPD treatment was found to suppress bone turnover by decreasing both bone formation (b-ALP −15% to −26%) and bone resorption indices (DPyr −43% to −78%) in CsA-treated rats. In addition, BMD measurements showed that under these experimental conditions OPD not only ameliorated but also completely prevented the adverse effects of CsA administration on bone. Furthermore, the results obtained with the lowest dose of OPD (higher BMD and lower DPyr compared with the control group) suggest that even minor doses might prove sufficient to preserve pretreatment bone mass effectively.

In summary, the present study showed by DXA *in vivo* the changes in bone mass by CsA administration and demonstrates the prevention of CsA-induced high bone resorption and trabecular bone loss by cotreatment with OPD. Moreover, it encourages the possible use of

OPD to treat patients receiving CsA as immunosuppressive therapy.

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