

# Changes in bone volume and bone resorption by olpadronate treatment in an experimental model of uremic bone disease

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## Abstract

Thirty male adult Wistar rats (300±10 g body weight) underwent either 5/6 nephrectomy (Nx, n=20) or sham operation (SHAM, n=10) to determine olpadronate effects in an experimental model of uremic bone disease. For a 38-day period, 10 rats received olpadronate (16ug/100g bw) once a week (Nx+OPD) and the other vehicle (Nx). SHAM received vehicle. At baseline, treatment onset (t=7days) and end of study (t=45 days) calcium, phosphorus, creatinine, bone alkaline phosphatase (b-ALP) and deoxypyridinoline crosslinks (DPyr) were determined. At t=0 and t=45 bone mineral density (BMD) was measured by DXA. At t=45 the right tibia was removed for bone histology. There were no differences in serum calcium. Phosphorus increased in Nx and Nx+OPD compared to SHAM (p<0.05). The b-ALP increased from t=0 to t=7 in Nx and Nx+OPD (p<0.05) and decreased thereafter to SHAM levels. DPyr/creat increased in Nx compared to SHAM (p<0.05) and Nx+OPD (p<0.001). Nx+OPD presented lower DPyr excretion than SHAM rats (p<0.01). At t=45, tibia BMD of Nx was 20% and 26% lower than in SHAM (p<0.005) and Nx+OPD, respectively, (p<0.001). BMD was higher Nx+OPD than in SHAM (p<0.05). OPD prevented the loss of bone volume, the increment in osteoid volume and erosion surface observed in Nx group (p<0.05). OPD also prevented the increment in the number of osteoclasts (OC) and the active OC surface and decreases the number of TRAP(+)-OC (p<0.05). In summary, OLP may be beneficial in osteopenia associated to high turnover bone disease of CRF. However, the use of bisphosphonate therapy for renal insufficiency must be further investigated in order to clarify essential aspects of treatment as the patient selection and several aspects of treatment, such as optimum dose, frequency, and safe period of administration.

**Keywords:** Uremic Bone Disease, Olpadronate, High Turnover, DXA, Rats

## Introduction

The kidney plays a critical role in the overall regulation of mineral homeostasis and a progressive decline in glomerular filtration rate (GFR) is associated with marked changes in bone and mineral metabolism<sup>1</sup>. Indeed, renal failure leads to a wide spectrum of bone disorders relating to metabolic and excretory functions that include the regulation of calcium and phosphorus excretion, the target organ for

parathyroidhormone (PTH) or the synthesis of 1,25dihydroxyvitamin D<sup>2</sup>. However, the renal bone disease which develops in chronic renal failure (CRF) is not a uniform disorder and in the context of bone remodeling it ranges from high turnover, the most common disturbance of CRF, to low turnover<sup>3</sup>.

The prevention and treatment of renal osteodystrophy (ROD) continues to be a therapeutic dilemma for the nephrologist. Bisphosphonates (BPs), potent anti-resorptive agents, are increasingly being used to treat a variety of bone diseases characterized by high bone turnover<sup>4</sup>. Theoretically, patients with renal failure and high turnover bone disease could benefit from treatment with BPs.

An animal model is useful to investigate new preventive and therapeutic drugs to avoid the bone and mineral alterations of secondary hyperparathyroidism (2° HPT) in CRF. The rat remnant kidney is one of the most used models to assess bone and mineral changes in experimental uremia

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	SHAM (n=10)	Nx (n=10)	Nx+OPD (n=10)
<b>Body weight (g)</b>			
<b>Initial</b>	357±10	375±12	361±9
<b>Final</b>	355±9	360±11	352±11
<b>Serum Creatinine (mg/dl)</b>			
<b>Baseline</b>	0.44±0.08	0.44±0.08	0.44±0.08
<b>7 days</b>	0.49±0.13	0.76±0.23*	0.78±0.19*
<b>45 days</b>	0.51±0.11	1.38±0.21*	1.44±0.28*
<b>Serum Calcium (mg/dl)</b>			
<b>Baseline</b>	10.7±0.3	10.7±0.3	10.7±0.3
<b>7 days</b>	10.6±0.4	10.6±0.3	10.9±0.2
<b>45 days</b>	10.5±0.1	11.0±0.5	10.8±0.2
<b>Serum Phosphorus (mg/dl)</b>			
<b>Baseline</b>	5.4±0.4	5.4±0.4	5.4±0.4
<b>7 days</b>	5.6±0.3	5.3±0.5	5.7±0.4
<b>45 days</b>	5.9±0.3	6.8±0.5*	6.3±0.4*
<b>Serum b-ALP (IU/L)</b>			
<b>Baseline</b>	27±6	27±6	27±6
<b>7 days</b>	28±5	45±4*	48±3*
<b>45 days</b>	26±5	28±7	29±2
<b>Urinary Ca/Creat (mg/mg)</b>			
<b>Baseline</b>	0.38±0.03	0.38±0.03	0.38±0.03
<b>7 days</b>	0.35±0.02	0.35±0.02	0.39±0.04
<b>45 days</b>	0.38±0.05	0.32±0.03	0.15±0.02**,#

**Table 1.** Body weight and biochemical data at baseline (t=0), at the onset of treatment (t= day 7) and at the end of treatment (t= day 45) (mean±SD). (\*): p<0.05 compared to SHAM group; (\*\*): p<0.01 compared to Nx group and (#): p<0.01 compared to SHAM group.

since it provides a homogeneous study population relating to the degree of renal function impairment<sup>5</sup>.

The present experimental study evaluated if one powerful aminobisphosphonate, olpadronate (OPD), could prevent bone alterations development in an experimental model of uremic bone disease.

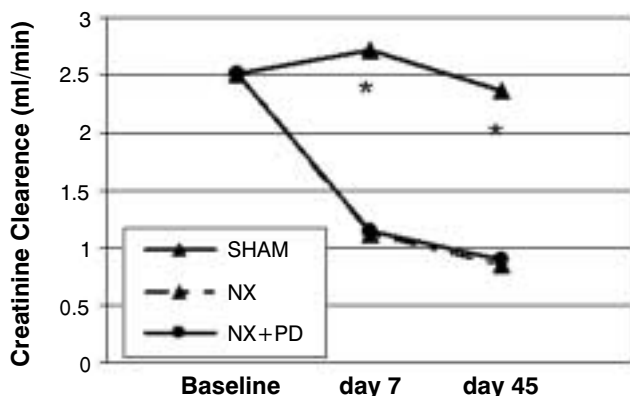
## Materials and methods

### Animals and protocol

Thirty male adult Wistar rats aged 90 days, with a body weight (bw) of 300±10 g were housed at room temperature (21±1°C), 55±10% humidity in 12 hour light/dark cycles. The animals had free access to drinking water and standard rodent diet (Cooperación, Buenos Aires) containing per 100 g of food: 20% protein, 1.0% calcium, 0.6% phosphorus and 200IU% of vitamin D. Body weight was recorded weekly. The National Institutes of Health Guide for the Care and Use of Laboratory Animals was observed.

After one week of acclimatization, rats were allocated to

undergo either 5/6 nephrectomy (Nx, n=20) or sham operation (SHAM, n=10). Surgery was performed under anesthesia (0.1 mg/100gr.bw.ketamine hydrochloride and 0.1mg/100gbw.acepromazine maleate)(Holliday Scott SA, Buenos Aires, Argentina) in a single surgical procedure by the ablation of the upper and lower poles of the left kidney, followed by contralateral nephrectomy. SHAM operation consisted of manipulation of the kidneys without destruction of renal tissue. After 7 days of surgery, Nx rats were divided according to their creatinine clearance to obtain two similar groups. During a 38-day period, one group (n=10) received OPD at a dose of 8ug/100g bw once a week (intraperitoneally) (Nx+OPD) and the other received vehicle (physiologic saline) (Nx). SHAM rats received vehicle. The dose, mode of administration, interval between administration and duration of treatment were based on previous experimental dose-response studies<sup>6,7</sup>. In addition, a SHAM+OPD group was not included in the present report because previously we found that i.p. administration of OPD "per se" in a similar experimental period, reduces phosphorus concentration and bone turnover without changes in serum calcium or body



**Figure 1.** Creatinine clearance (ml/min) in operated (SHAM), nephrectomized (Nx) and nephrectomized plus olpadronate (Nx+OPD) rats: baseline, onset and end of the study. (\*):  $p < 0.001$  compared to Nx and Nx+OPD groups.

weight and lead to a non-significant increment in bone mineral density in several areas of the total skeleton<sup>8</sup>.

At baseline ( $t=0$ ), at treatment onset ( $t=\text{day 7}$ ) and at the end of the study ( $t=\text{day 45}$ ) blood was collected to determine serum levels of calcium, phosphorus, creatinine, and bone alkaline phosphatase (b-ALP). The rats were also placed in metabolic cages for 24 hours' urine collection to determine urinary excretion of creatinine (creat) and deoxypridinoline crosslinks (DPyr).

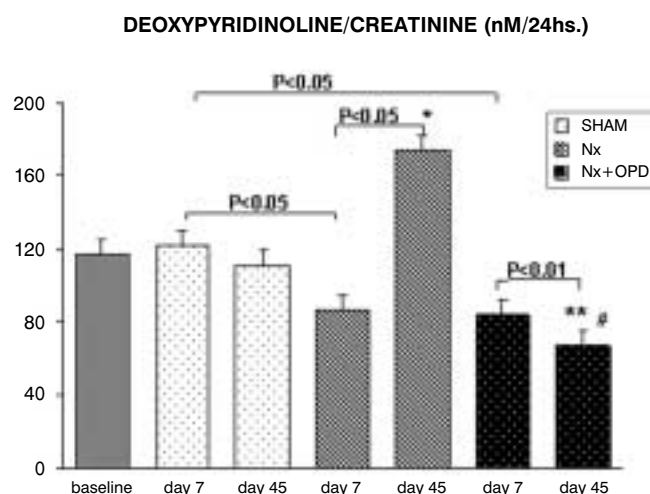
At baseline and at the end of the study, bone mineral density (BMD) was measured under light anesthesia by DXA (Holologic 1000 equipment, Small Animal Software) in an ultra-high-resolution mode and tibia BMD was determined using ROI on the image of the animal on the screen as previously described<sup>9</sup>. At the end of the study, right tibiae were removed for bone histology.

## Analytical methods

Serum calcium, phosphorus and creatinine as well as urinary calcium and creatinine excretions were measured as previously described<sup>10,11</sup>. Urinary DPyr was analyzed by ELISA using a commercially available kit (Pyrilinks-D<sup>®</sup> Metra Biosystems Inc., Palo Alto, CA)<sup>9</sup>. DPyr intra and interassay co-efficients of variation were: 3.7-8.0% and 5.8-10.3%, respectively. Urinary DPyr excretion was expressed as a ratio of creatinine concentration.

## Tissue preparation

For every rat, coronal sections of remnant renal and liver tissue were obtained at the time of sacrifice. One fragment was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. Different sections of each kidney were analyzed to evaluate histologic damage.

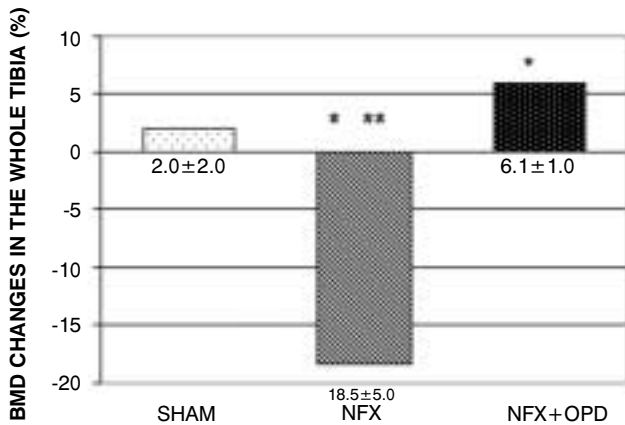


**Figure 2.** Urinary deoxypridinoline excretion/creatinine (nM/24hs creatinine) in SHAM-operated (SHAM), nephrectomized (Nx) and nephrectomized plus olpadronate (Nx+OPD) rats: baseline, onset and end of the study (mean $\pm$ SE). Among groups: At day 7 the significant differences were:  $p < 0.05$  for SHAM vs. Nx group and SHAM vs. Nx+OPD, respectively. At day 45 the significance were: (\*):  $p < 0.05$  compared to SHAM; (\*\*):  $p < 0.001$  compared to Nx and (#):  $p < 0.01$  compared to SHAM group. For the same group there were significant differences between day 7 and 45 in Nx ( $p < 0.05$ ) and in Nx+OPD groups ( $p < 0.01$ ).

## Histologic techniques

At the time of sacrifice, the right tibiae were resected and cross-sectioned through the center. Tibiae were fixed by immersion in buffered formalin for 48 hours, decalcified in 10% ethylene-diamine tetraacetic acid (EDTA) (pH 7) during 25 days and embedded in paraffin. Two 8 to 10 $\mu$ m thick longitudinally oriented sections were obtained from each tibia at the level of the proximal third of the tibial metaphysis. One section was stained with hematoxylin-eosin and the other was used for histochemical detection of tartrate resistant acid phosphatase (TRAP)<sup>13</sup>. 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.<sup>14</sup>:

- 1) (BV/TV)(%) Bone Volume: The percentage of cancellous bone within the total measured area.
- 2) (Ob.S/BS) (%) Osteoblast surface as the fraction of trabecular bone surface covered with osteoblasts;
- 3) ES (OC+)/BS (%): Active erosive surface (with osteoclasts) as the fraction of trabecular bone surface.
- 4) ES (OC-)/BS (%): Inactive erosive surface (without osteoclasts) as the fraction of trabecular bone surface.
- 5) (ES/BS) Eroded surface: the fraction of trabecular surface covered by lacunae (including "active" lacunae with



**Figure 3.** Changes in whole tibia bone mineral density from baseline to the end of the study in operated (SHAM), nephrectomized (Nx) and nephrectomized plus olpadronate (Nx+OPD) rats (mean±SE). The significant differences were: (\*):  $p < 0.005$  compared to SHAM; (\*\*):  $p < 0.001$  compared to Nx and (#):  $p < 0.05$  compared to SHAM group.

osteoclasts and lacunae in reversal phase);

6) (Oc.N/TA) Osteoclast number: the number of osteoclasts per  $\text{mm}^2$  of cancellous bone;

7) (Oc.N/TA-TRAP+): The number of TRAP+ Osteoclasts in the studied area.

To evaluate osteoid, some contralateral distal tibiae were fixed in 10% phosphate-buffered formaldehyde. After dehydration, bone samples were embedded undecalcified in methyl methacrylate and longitudinal sections (5 - 7  $\mu\text{m}$ ) were cut with a Polycut microtome (Reichert Jung, Heidelberg, Germany). Bone sections were stained using the modified Masson's Trichrome stain technique<sup>15</sup>.

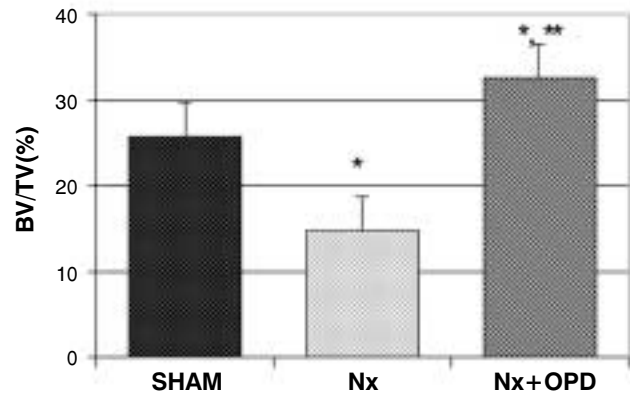
## Statistical methods

Results were expressed as mean±standard error (SEM). Overall significance for body weight and biochemical data was determined by analysis of variance for repeated measures. Differences among groups were calculated using one- and two-way analyses of variance. Similarly, histologic data were analyzed by a single time point factorial analysis. Dunnet's "a posteriori" test was used to determine differences among individual groups. Statistical analyses were performed using SPSS for Windows 6.0 (SPSS, Inc., Chicago, IL). A value of  $p$  below 0.05 ( $p < 0.05$ ) was considered significant.

## Results

### Survival, body weight, and histologic observations:

The survival rate for both Nx groups was 80%. No difference was observed between initial and final body weight in any of the studied groups (Table 1). However mean bw.



**Figure 4.** Bone volume of the tibia metaphysis for SHAM-operated (SHAM), nephrectomized (Nx) and nephrectomized plus olpadronate (Nx+OPD) rats. The significant differences were: (\*):  $p < 0.05$  compared to SHAM and (\*\*):  $p < 0.05$  compared to Nx groups.

change was different in each group: 2g in SHAM, 15g in Nx and 9 g in Nx+OPD rats.

Qualitative histologic observations of the remnant kidney and liver tissues did not show differences between Nx and Nx+OPD groups. However, glomerular hypertrophy was greater in the two groups of rats with reduced renal mass compared to the SHAM group.

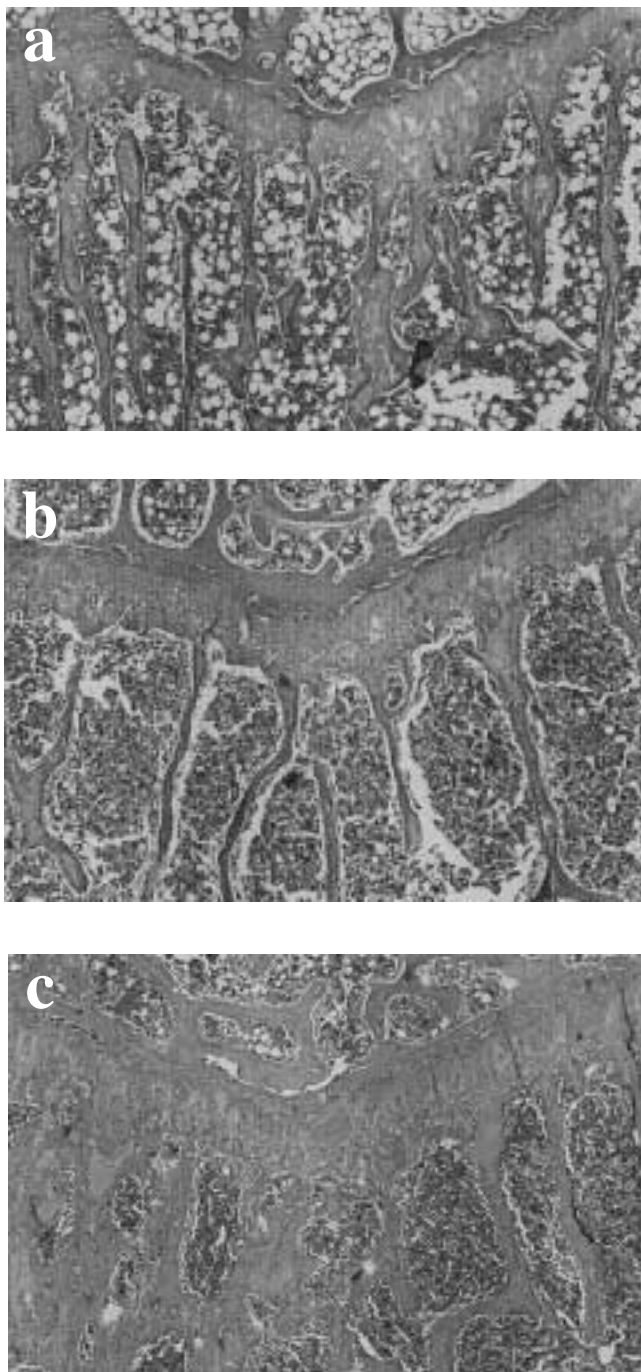
### Serum and urinary parameters:

After 7 days creatinine clearance was significantly lower in Nx groups than in SHAM animals (Figure 1). At the end of the study, Nx rats receiving olpadronate or vehicle had similarly impaired renal function and which was significantly lower in both groups compared to the SHAM group (Figure 1).

There were no differences in serum calcium. At the end of the study, serum phosphorus levels were significantly increased in Nx and in Nx+OPD rats as compared to SHAM ( $p < 0.05$ ) groups. Moreover, serum phosphorus levels were similar in Nx and Nx+OPD groups (Table 1). The b-ALP levels increased significantly from baseline to treatment onset in both Nx and Nx+OPD groups ( $p < 0.05$ ) and decreased significantly thereafter until the end of the study reaching values that were not significantly different from those of the SHAM group (Table 1)

Urinary DPyr/creat excretion increased significantly in Nx from the onset to the end of the study ( $p < 0.05$ ) and at the end of the study was significantly higher than both, SHAM ( $p < 0.05$ ) and Nx+OPD ( $p < 0.001$ ). Moreover, urinary DPyr/creat excretion decreased significantly in Nx+OPD from the onset to the end of the study ( $p < 0.01$ ) and at the end of the study this group presented significantly lower levels than SHAM animals ( $p < 0.01$ ) (Figure 2).

Urinary Ca/creat excretion did not change in SHAM and Nx groups and decreased significantly in Nx+OPD from the onset to the end of the study ( $p < 0.01$ ). Consequently, the



**Figure 5 a, b, c.** Photograph of the histomorphometric measurements on the central area of the metaphyseal bone (a): SHAM, (b): Nx and (c): Nx+OPD.

levels of Ca/creat excretion were significantly lower in Nx+OPD compared to SHAM and Nx groups ( $p < 0.01$ ) (Table 1).

#### **Bone densitometry and histomorphometry:**

At the end of the study, tibia BMD of Nx rats was 20% lower than that of SHAM ( $p < 0.005$ ) animals and 26% lower

than tibia BMD of Nx+OPD rats ( $p < 0.001$ ). OPD administration was associated with a beneficial effect on the decrease in tibia BMD observed in the Nx group when compared to the SHAM rats ( $p < 0.05$ ) (Figure 3).

Bone volume was lower in Nx than in SHAM animals ( $p < 0.05$ ) and higher in rats treated with OPD than in rats given vehicle (Figure 4) ( $p < 0.05$ ). Leaving aside considerations regarding the status of renal function, BMD was higher in rats treated with OPD than in rats given vehicle (figure 4) ( $p < 0.05$ ). The greater bone volume observed in the Nx+OPD group was a result of the increased size and number of trabeculae (figure 5c).

Nx animals presented the typical histologic signs of moderate hyperparathyroid bone disease (Figure 5b and Table 2): increased erosion surface, larger number of osteoclasts and osteoclast surface, larger number of TRAP(+)-osteoclasts, and no significant increase in osteoblast surface compared to SHAM animals ( $p < 0.05$ ). In addition, no paratrabeular fibrosis was observed (Figure 5b). Treatment with OPD prevented the increase in erosive surface, the osteoclast covered surface, and total and TRAP (+) osteoclasts. Consequently, all these parameters were significantly lower than in Nx rats ( $p < 0.05$ ). Moreover, no significant differences were found between Nx+OPD and SHAM animals (Figure 5a and Table 2).

Nx rats presented an increment in the osteoid volume of the subcondral trabecular bone compared to SHAM and Nx+ODP groups (15-20% versus 1-2% and 2-4%, respectively).

## **Discussion**

**Confirming previous reports**, the present model of surgical renal insufficiency with the ablation of 5/6 of renal mass, reduced the initial renal function by about 2/3 after 6 weeks. In agreement with previous studies<sup>16-17</sup> other features of the present long-term study included an increment of the active bone resorption with no changes in bone formation. These observations may reflect an abnormal bone remodeling that lead to a reduction in the tibia bone density. The administration of the potent aminobisphosphonate, olpadronate inhibited bone resorption and avoided bone loss without inducing changes in the renal function.

After 1 week of surgery, Nx rats showed an increment in the marker of bone formation with slight changes in the marker of bone resorption. Indeed, serum ALP bone isoenzyme levels were elevated suggesting an increment in osteoblastic activity possibly due to changes in PTH production and/or secretion. In this regard, it is important to point out that the half life of b-ALP is very long (1-2 days) and their serum levels are not influenced by a reduction in GRF because it is cleaved from circulation by the liver<sup>18</sup>. In hemodialysis patients, higher bALP are associated with either biological and histological signs of 2°HPT or with high bone turnover<sup>19</sup>. In contrast, during this first period of acute renal failure there was a non-significant reduction in urinary D/Pyr excretion because, due to surgery, Nx rats were still in a recovery/compensatory state and secondary changes that

	Ob.S/BS (%)	ES/BS (%)	ES (OC-)/BS (%)	ES (OC+)/BS (%)	Oc.N/TA (%)	TRAP+ Osteoclast number (1/mm)
SHAM	23.3 ± 4.3 a	10.5 ± 5.7 a	3.4 ± 1.1a	7.1 ± 1.2 a	11.8 ± 2.0 a	13.3 ± 5.6 a,b
Nx	27.0 ± 3.6 a	39.5 ± 1.2 b	5.9 ± 1.1 a	33.7 ± 0.9 b	36.3 ± 5.3 b	25.8 ± 1.5 a
Nx+OPD	38.3 ± 4.3 a	23.9 ± 5.9 a,b	5.2 ± 1.5 a	19.2 ± 4.4 c	21.4 ± 2.9 c	8.5 ± 2.6 b
	NS	<i>P</i> < 0.05	NS	<i>P</i> < 0.05	<i>P</i> < 0.05	<i>P</i> < 0.05

**Table 2.** Histomorphometrical determination of the proximal third of the tibial metaphysis (n=7): (Ob.S/BS) (%) Osteoblast surface, (ES/BS) Eroded surface, ES (OC-)/BS (%) Inactive erosive surface (without osteoclasts), ES (OC+)/BS (%) Active erosive surface (with osteoclasts), (Oc.N/TA) Osteoclast number and (Oc.N/TA-TRAP(+)) number of TRAP(+)-osteoclasts. (\*)  $p < 0.05$  compared to SHAM group and (\*\*):  $p < 0.05$  compared to Nx groups.

affect renal excretion occur. According to previous studies, the acute renal insufficiency could decrease the clearance of the D/Pyr<sup>20</sup> and its urinary variations may not reflect changes in osteoclastic bone resorption, at least in this period.

It is known that the main pathophysiological mechanism of increased bone turnover in this animal model is 2° HPT due to disturbances in calcium and phosphate homeostasis associated with renal impairment. Although serum PTH was not measured in the present study, previous reports demonstrated that this type of surgery induces several fold increase in PTH levels<sup>16-17,21</sup>. In the present report, the increment in both serum phosphorus levels and bone resorption marker, is considered to be suggestive of 2° HPT. Several signs of a high bone remodeling with and uncoupling between bone resorbing and forming parameters were also observed by histology. There was elevated loss of bone volume and osteoid volume, an increment in active erosion surface and in the number of total and TRAP(+)-osteoclasts without significant increment in bone formation parameters or paratrabeular fibrosis. In this regard, there was a trend for osteoblast surface to be higher in Nx than in control rats. Although fibrous tissue occupying the peritrabeular spaces may be found in moderate renal insufficiency, it occurs mainly in severe renal failure<sup>16,19</sup>.

Our findings are in agreement with previous CRF models with regard to the significant enhancement of bone resorption<sup>16,21-22</sup>. In contrast, the present study differs with regard to the lack of changes in bone formation, the absence of paratrabeular fibrosis or the increment in the active erosion surface and in the number of osteoclasts. These discrepancies may be associated with methodological differences such as the age of the animals or the experimental period which can induce different degrees of renal function impairment.

According to our knowledge there are only few studies in assessing bone density by DXA in Nx rats. In the present report, the observed abnormal bone remodeling with an increase in bone resorption, a transient increment during a short time after surgery with no changes by the end of the

study in bone formation compared to SHAM animals, negatively affected tibia BMD. Several clinical studies have found accelerated bone loss and reduced BMD in CRF patients<sup>23-24</sup>. This reduction in bone mass, besides the abnormalities in bone remodeling, may be an important determining factor of fracture risk. In this regard, and although there is some concern about BP treatment in uremic patients, it is known that bone loss associated with high bone turnover can be minimized by BP therapy.

Previously we report that in normal animals<sup>8</sup> OPD treatment, at the used dose, induced a decrease in D/Pyr excretion with the lack of changes in serum creatinine levels. In the present report, OPD treatment administered to Nx rats also suppressed bone resorption without inducing changes in renal function. In this regard, the bone volume loss induced by nephrectomy was prevented by OPD treatment which also decreased the extent of active erosion surface, the number of total and TRAP positive osteoclasts and the urinary calcium and D/Pyr output. These findings suggest that the aminobisphosphonate ODP, like others, might act *in vivo* by reducing osteoclast lifespan and activity. In addition, osteoid volume was similar in the SHAM and Nx+OPD groups indicating that OPD did not induce osteomalacia. Some of the present findings were previously reported in CRF patients with 2° HPT treated with clodronate<sup>25</sup> or pamidronate<sup>26</sup>. In rats, after ibandronate treatment Geng et al.<sup>17</sup> observed a reduction in DPyr excretion but did not find a decrease in the number of osteoclasts and erosion surface extension, possibly due to differences in the experimental period (6 weeks versus 3 weeks). In addition, the reduce a rate of bone resorption by OPD treatment at the dose used in this study improved tibia BMD when compared to Nx rats.

Our data are in agreement with previous *in vitro* and *in vivo* observations associated with BP treatment such as a decrease in osteoclast recruitment<sup>27</sup> or apoptosis<sup>28</sup> leading to a reduction in the number of osteoclasts and erosion surface extension. However, OPD treatment induced a non-significant mean increment of 42% and 64% in osteoblast surface

compared to Nx and SHAM groups, respectively. In this regard, previous studies had found a stimulatory effect of BPs on osteoblasts. Specifically for OPD Mathot et al.<sup>29</sup> demonstrated in vitro that OPD induced proliferation of rat calvaria-derived osteoblast and Plotkin et al.<sup>30</sup> proposed that OPD may prevent osteoblast apoptosis and indirectly contribute to the relative increase in cell number and activity. Moreover an important issue was the effect of BPs in bone structure or mineralization because they remain for a long time in bone. In this regard, previous studies demonstrated that BPs treatment did not reduce the mechanical properties of bone<sup>31</sup> and/or mineralization<sup>32</sup>.

In summary, olpadronate may be beneficial in osteopenia associated to the high turnover bone disease of CRF. However, the use of bisphosphonate therapy for renal insufficiency must be further investigated in order to clarify essential aspects of the treatment such as optimum dose, frequency and safe period of administration.

#### Acknowledgements

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