J Musculoskelet Neuronal Interact 2013; 13(2):185-194

Original Article

Hylonome

Mineralization- and remodeling-unrelated improvement of the post-yield properties of rat cortical bone by high doses of olpadronate

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Abstract

Some pharmacologic effects on bone modeling may not be evident in studies of remodeling skeletons. This study analyzes some effects of olpadronate on cortical bone modeling and post-yield properties in femurs diaphyses (virtually only-modeling bones) of young rats by mid-diaphyseal pQCT scans and bending tests. We studied 20/22 male/female animals traetad orally with olpadronate (45-90 mg/kg/d, 3 months) and 8/9 untreated controls. Both OPD doses enhanced diaphyseal cross-sectional moments of inertia (CSMI) with no change in cortical vBMD and elastic modulus. Yield stiffness and strength were mildly increased. Post-yield strength, deflection and energy absorption were strikingly enhanced. Ultimate strength was enhanced mainly because of effects on bone mass/geometry and post-yield properties. The large improvement of post-yield properties could be explained by improvements in bone geometry. Improvements in bone mass/geometry over weight-bearing needs suggest an enhanced modeling-related response to mechanical stimuli. Effects on tissue microstructural factors (not measured) could not be excluded. Results reveal novel olpadronate effects on bone strength and toughness unrelated to tissue mineralization and stiffness, even at high doses. Further studies could establish whether this could also occur in modeling-remodeling skeletons. If so, they could counteract the negative impact of anti-remodeling effects of bisphosphonates on bone strength.

Keywords: Bisphosphonates, Olpadronate, Bone Biomechanics, Peripheral QCT (pQCT), Bone Toughness, Post-yield Bone Strength

Introduction

The impact of any pharmacological intervention on whole-bone biomechanics should be analyzed as a function of the changes induced in: a. bone material properties (tissue stiffness and toughness, mostly determined by genetic factors, bone formation and bone remodeling); b. the molecular or modeling-dependent adaptations of bone structure to mechanical stimuli (bone design, or geometric properties) and c. the impact of all those changes on the structural stiffness, toughness and strength of the bones (Figure 1). These 3 pharmacological fields of action are all especially important concerning bisphosphonate (BP) effects on bones.

Strikingly, the effects of BPs and other bone-seeking agents on post-yield bone properties have been generally overlooked in skeletal studies. We have already reported effects of cortisol excess[1], hypophysectomy, and pharmacological doses of alendronate[2] on the post-yield behavior of bones, and independent effects of pamidronate on bone stiffness and ultimate strength[3] which could have been exerted separately on the elastic and plastic properties of bones. In this study we show a more direct evidence of some novel, positive effects of a bisphosphonate on post-yield bone properties.

Currently available data are inconclusive in establishing which aspects of bone strength are affected *in vivo* by different BPs at variable dose-time schedules[4]. Even the earliest review on BP effects on bone biomechanics[5] had already shown a no-

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Edited by: F. Rauch
Accepted 27 March 2013

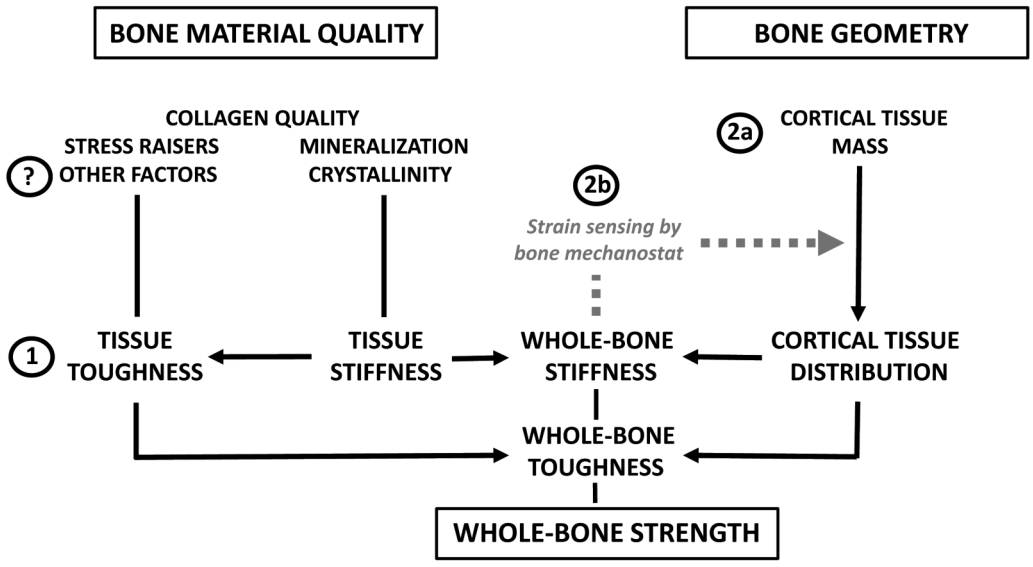

Figure 1. Schematic representation of the biological determination of the mechanical quality of cortical bone tissue (left column) and the spatial distribution of that tissue (right column) to comform the structural striffness, toughness and strength of long-bone diaphyses as combined outputs (bottom). The thick, grey dasehd arrows indicate the regulation loop which would control the whole-bone stiffness according to the *mechanostat* theory. Circled numbers indicate the possible points involved in the OPD effects as described and discussed in this paper.

table sensitivity to species, gender, age, body habitus, bone compartment, metabolic-endocrine status, and physical activity. Furthermore, the strong inhibitory BP effects on bone remodeling in relatively large, long-living mammals and humans may blunt to a large extent the biomechanical impact of other BP actions which can, independently, impair or improve bone strength.

The study of BP effects on diaphyseal bone in growing rodents (which shows virtually no remodeling[6]) presents obvious limitations, but it can reveal a number of effects wich may not be evident otherwise, especially when high-dose, medium-term protocols are implemented. These effects can be positive on cortical tissue microstructure, mass and distribution, and tend somehow to neutralize or even overpass the negative impact of any remodeling blockage. Hence, their investigation offers some pharmacological interest.

Olpadronate (dimethyl-pamidronate, OPD[7,8]) is an N-containing BP currently under development. Either *in vitro* or *in vivo*, OPD has been shown to produce both anti-osteoclast and pro-osteocyte/osteoblast effects[9,10] of varying magnitudes within a wide range of doses. Such effects may induce unpredictable outcomes when produced in cortical tissue of non-remodeling bones[10]. Since OPD has a higher ED50/LD50 safety margin than other BPs, it is possible to study its impact on bone with doses close to saturation of the skeleton[7]. As part of a 3-month preliminary carcinogenetic study with high OPD doses in young intact rats, this report reveals some novel, positive effects of high doses of OPD on tomographic and mechanical indicators of bone stiffness and toughness, with a strikingly high impact on bone post-yield and ultimate strength, that were unrelated to bone mineralization.

Materials and methods

Animals and treatments

Fifty-nine Wistar rats (28 male, 31 female) 4-5 weeks old were studied. The animals were kept in group cages with water and food (standard chow diet with 1.0% Ca and 0.6% P contents) *ad libitum* in a temperature-controlled room (23ºC) with natural luminous cycle. Eight male and 9 female animals selected randomly remained untreated and were considered the control groups for each sex. Twenty male and 22 female animals were treated orally with high doses (45 or 90 mg/kg/d, 10 male and 11 female rats per dose) of OPD (dimethyl-pamidronate, *IG-8801*, crystal form A, Gador SA, Buenos Aires)[7,8] with the drinking water. Animals were weighed weekly to re-calculate the administered dose. This treatment was a preliminary test for carcinogenicity, performed following GLP rules. After 3 months, the rats were sacrificed by ether overdose and their femurs were dissected avoiding periosteal lesion.

Bone tomographic determinations

The fresh, excised femurs were scanned at the mid-diaphysis with a pQCT machine (*XCT-2000* with special software for small animals; *Stratec, Pforzheim, Germany*) set-up at a 0.09-mm voxel size and a 0.9 cm^{-1} attenuation threshold for the determination of "cortical" bone[11]. Two-millimeter thick slices were analyzed and the following variables were determined:

– *Mineral content of cortical bone* (*BMC* in the cortical region of the slice, expressed in mg/cm of slice thickness). This is an indicator of bone mineral mass.
– *Volumetric mineral density of cortical bone* (cortical *vBMD*,

expressed in mg/cm^3 of the "cortical" region of the slice volume). This is an indicator of bone mineralization, proportional to the apparent mineral density of bone tissue, which is regarded as a partial determinant of its intrinsic stiffness[12-15].

– Indicators of the cross-sectional (geometric) properties such as the *periosteal perimeter (PoPm) and the average thickness, area (CortA)*, and anterior-posterior (A-P) *bending second moment of inertia (xCSMI)* of the cortical bone region. The *xCSMI* was automatically calculated through an integration procedure as *xCSMI* (mm^4)= $\Sigma(A_i\ d_i^2)$, such that A_i is the area of each individual pixel including "cortical" bone tissue in the cross section, in mm^2, and d_i^2 is the squared distance of that pixel to the A-P bending axis (x) of the image, in mm^2 [14,11]. Accordingly with this calculation, the xCSMI grows exponentially with the distance at which the "cortical" tissue is distributed from the bending axis (x) in the cross section. The verified geometrical uniformity of the femur diaphysis cross-sections along the tested portion of the bones (coefficients of variation of bone diameters being always below 6% for samples of values taken at the central point and at the two ends of the tested segment in every rat studied) supported the use of xCSMI values as suitable indicators of the cortical design architectural efficiency as related to the deformation that occurs as a consequence of A-P bending[12].

Bone mechanical testing

Immediately after the pQCT study, the femurs were immersed in water at 36 ºC for 1 hour. Then, their diaphyses were placed horizontally on two supports separated by 13 mm (*L*) with their anterior aspect facing down and bent by a centrally applied load at a low strain rate (0.25 mm/min) until fracture occurred[14]. The force *F* (N, ordinate) exerted by the assayed volume of bone (*vol*=L.CortA, mm^3) as a Newtonian reaction against the applied load, and the displacement (*d*, mm, abscissa) of the bending diaphysis were graphically recorded. The F/d curves (schematic representations of which are shown in Figure 1) showed the sequence of linear (pre-yield) and non-linear (post-yield) behaviors, separated by the *yield point (y)*. The yield point was determined following the "0.2%-offset method": a line, parallel to but offset by a 0.002 (0.2%) strain distance from the linear portion of the F/d curve was constructed and the intersection of this line and the curve denoted the yield point[12]. The F/d curves allowed direct determination of the following mechanical properties of the whole bones.

– *Yield deflection* (*d* at the yield point, *dy*, mm). This is an indicator of how much the bone could be deformed during the whole elastic behavior in the assayed conditions.
– *Yield load or strength* (limit elastic load, or F exerted by the whole-bone at the yield point, *Fy*, N). This is an estimator of the bone's resistance to generation of the first cracks within the "solid" mineralized tissue.
– *Diaphyseal (structural) stiffness* (load/deformation ratio in elastic conditions = *Fy*/*dy*, N/mm).
– *Maximal load or strength* (maximal force exerted by the whole-bone until fracture = *Fmax*, N).
– *Deflection at the maximal load* (*d* value corresponding to Fmax value = d_{Fmax}, mm). This indicator expresses the amount of deflection the bone can stand at the point it is reacting most strongly to the load during the plastic deformation period.
– *Elastic absorption of energy by the deforming bones at yield*, measured as the integrated area under the F/d curve up to dy (*eEa*, N.mm or mJ). This indicator estimates the bone resistance to the production of the first crack (yielding) as a bone structural property.
– *eEa per unit of assayed bone volume = eEa / vol* (mJ/cm^3). This indicator expresses the bone resistance to the production of the first crack standardized per unit of cortical tissue volume as a bone intrinsic property.
– *Post-yield loading ability* (assessed as the difference *Fpy= Fmax-Fy* expressed either in absolute terms (N), or as a percentage of Fmax, *Fpy%=100 Fpy/Fmax*). This indicator is representative of the post-yield fraction of the total load-bearing capacity Fmax of the bones. Hence it estimates the bone's resistance to crack generation and progress, as an expression of bone structural toughness in force units, during the plastic period of the mechanical test.
– *Post-yield (plastic) absorption of energy by the deforming bones (pyEa (mJ)* = integrated area under the F/d curve from the yield point up to Fmax). This indicator estimates the bone resistance to crack generation and progress during the plastic period (since yielding until fracture), i.e. the structural toughness of the bone in energy units, during the post-yield behavior.
– *pyEa per unit of assayed bone volume = pyEa/vol* (mJ/cm^3) This indicator expresses the bone resistance to crack generation and progress (bone toughness in energy units) during the whole plastic period, standardized as a bone intrinsic property.

Indirect calculation of bone tissue's elastic modulus

The intrinsic bending stiffness of the cortical tissue (Young's modulus of elasticity) constitutes a representative indicator of bone material properties and is independent of bone size and shape. It was not directly measured but indirectly calculated from indicators of structural and architectural properties as $E\ (MPa) = Fy\ L^3\ /\ 48\ dy\ xCSMIav$[13,14,16], where *xCSMIav* is the average xCSMI value calculated from the data measured at the center of the bone and at the points corresponding to the positions of each of the two supports during the bone testing. The application of this algorithm has been regarded as a suitable procedure for comparative purposes[17,18].

Statistical analyses

Averages of the data per rat and group were calculated. As long as no dose-related effects on any of the variables studied were detected by one-way ANOVA inter-group comparisons (always $p>0.05$), all treated animals were pooled into *male* (n=20) and *female* (n=22) *treated groups* regardless of the administered dose for further analytical purposes. Bone PoPm, CortA, xCSMI and Fmax data, which were found significantly correlated with body weight of the animals, were statistically adjusted to this variable following the describing equations of the corresponding relationships where required in order to deal with any size- or growth-related effect. One-way ANOVA and

	Female rats			Male rats		
	Control	**Treated**	**% difference**	**Control**	**Treated**	**% difference**
Allometric variables						
Body weight (bw), g	295 (26)	285 (23)	- 3.4	517 (39)^{sss}	490 (49)^{sss}	- 5.2
Periosteal perimeter (PoPm), mm	10.7 (0.55)	11.3 (0.40)	+ 5.6***	12.6 (0.35)^{ss}	13.5 (0.78)^{ss}	+ 7.1***
Average cortical thikness, mm	0.734 (0.021)	0.739 (0.044)	+ 0.7	0.811 (0.051)^{sss}	0.920 (0.036)^{ss}	+ 13.4***
Cortical bone area (CortA), mm^2	6.37 (0.38)	6.84 (0.49)	+ 7.4*	8.39 (0.49)^{sss}	9.70 (0.53)^{sss}	+ 20.7***
Bw-Adjusted CortA, mm^2	5.56 (0.38)	6.21 (0.43)	+ 11.7**	5.67 (0.45)	7.45 (0.43)^{sss}	+ 31.4***
Moment of inertia (xCSMI), mm^4	5.33 (0.81)	6.14 (0.88)	+ 15.2*	9.28 (0.97)^{sss}	12.19 (1.35)^{ss}	+ 31.4***
Bw-Adjusted xCSMI, mm^4	4.51 (0.76)	5.52 (0.80)	+ 22.4*	4.47 (1.38)	7.86 (1.50)^{sss}	+ 75.8***
BMC of cortical bone, mg	8.23 (0.42)	8.82 (0.67)	+ 7.2*	11.17 (0.73)^{sss}	13.48 (0.73)^{ss}	+ 20.7***
Bone material properties						
vBMD of cortical bone, mg/cm^3	1302 (27)	1299 (30)	- 0.02	1326 (21)	1325 (23)	0.1
Elastic modulus E, MPa	1352 (304)	1335 (299)	- 1.26	1295 (287)	1274 (265)	- 1.23
Pre-yield behavior of bones						
Yield deflection, dy, mm	0.64 (0.15)	0.62 (0.16)	- 0.4	0.77 (0.10)	0.78 (0.14)	+ 1.3
Yield load, Fy, N	104.2 (7.6)	116.3 (22.0)	+ 11.6*	137.3 (7.9)^{sss}	158.6 (19.9)^{sss}	+ 15.5*
Bw-adjusted Fy, N	97.4 (6.4)	111.1 (9.2)	+ 14.1*	106.4 (7.1)^{s}	122.5 (8.5)^{ss}	+ 15.1*
Diaphyseal stiffness, Fy/dy, N/mm	162.7 (25.7)	188.8 (29.7)	+ 16.0*	178.3 (24.2)	202.1 (27.0)	+ 13.3*
Bw-adjusted Fy/dy, N/mm	151.3 (23.8)	172.5 (38.6)	+ 14.0*	159.3 (30.7)	178.0 (49.9)	+ 11.7*
Elastic energy absorpt., eEa, mJ	33.34 (4.9)	36.1 (14.1)	+ 8.1	52.8 (17.0)^{sss}	61.9 (21.1)^{sss}	+ 17.2
eEa/vol, mJ/cm^3	403 (35)	406 (39)	+ 0.8	484 (43)	491 (38)	+ 1.4
Post-yield behavior of bones						
Ultimate load, Fmax, N	122.4 (10.2)	149.0 (23.5)	+ 21.7***	158.6 (22.1)^{ss}	205.2 (36.2)^{sss}	+ 29.3***
bw-Adjusted Fmax, N	117.9 (10.4)	145.6 (22.6)	+ 23.5**	131.8 (23.1)^{s}	181.2 (34.7)^{sss}	+ 37.5***
Post-yield load Fpy = Fmax - Fy, N	18.2 (5.1)	32.7 (11.5)	+ 79.6***	21.3 (4.2)	46.6 (14.5)^{s}	+ 118.0***
Fpy fraction Fpy% = 100 Fpy/Fmax	14.9	21.9	-	13.4	22.7	-
Deflection at Fmax, d_{Fmax}, mm	1.06 (0.11)	1.22 (0.10)	+ 15.1*	1.18 (0.12)	1.56 (0.14)	+ 32.2 **
Plastic energy absorpt., pEa, mJ	47,46 (4.38)	79,50 (5.21)	+ 67.5**	62.73 (7.15)	141.96 11.27)	+ 126.3***
pEa/vol, mJ/cm^3	573 (65)	894 (72)	+ 56.0***	575 (63)	1125 (92)	+ 95,7***

Asterisks (, **, ***) indicate p<0.05, p<0.01, and p<0.001 significance levels, respectively, of treatment-induced differences in both sexes. ($^{s, ss, sss}$) on the male control and treated groups data indicate p<0.05, p<0.01, and p<0.001 significance levels, respectively, of differences with respect to their homologous, control and treated female groups.*

Table. Means and (SD's) of every studied variable in the different groups of animals.

simple regression tests were employed to evaluate the differences between grouped data and the associations between variables as indicated, aided by Statistica software (*StatSoft, USA*).

Results

General observations

No toxic effects relevant to the skeleton were observed in this short-term study. Adjustment of the allometrically-related variables to bw generally reduced greatly or eliminated the statistical significance of differences between male and female controls, but not between treated and untreated animals.

Data of every variable determined in the four groups studied are shown in the Table, and can be described as follows.

Effects on bone geometry and allometrically-related variables

Despite of their similar age, untreated male animals were about 70% heavier than females. No significant effect of treatment was observed on body weight gain.

All assayed bone geometric or size-related indicators (PoPm, cortical thickness, CortA, xCSMI, cortical BMC) were significantly higher in male compared to female rats, regardless of treatment. However, with the only exception of the xCSMI, these gender-related differences were proportionally smaller than those observed in body weight. Both treated males and females showed higher values of all PoPm, cortical thickness, CortA, xCSMI and cortical BMC than their untreated controls. However, the increases were greater and more significant in males than in females, and proportionally more evident for xCSMI compared to the other indicators. Adjustment of the data to body weight neutralized the gender-related differences in all these variables in control rats (data shown only for CortA and xCSMI). In contrast, this adjustment either had little effect on, or even enhanced the treatment-induced differences within gender for males and females, more evidently for xCSMI than for CortA.

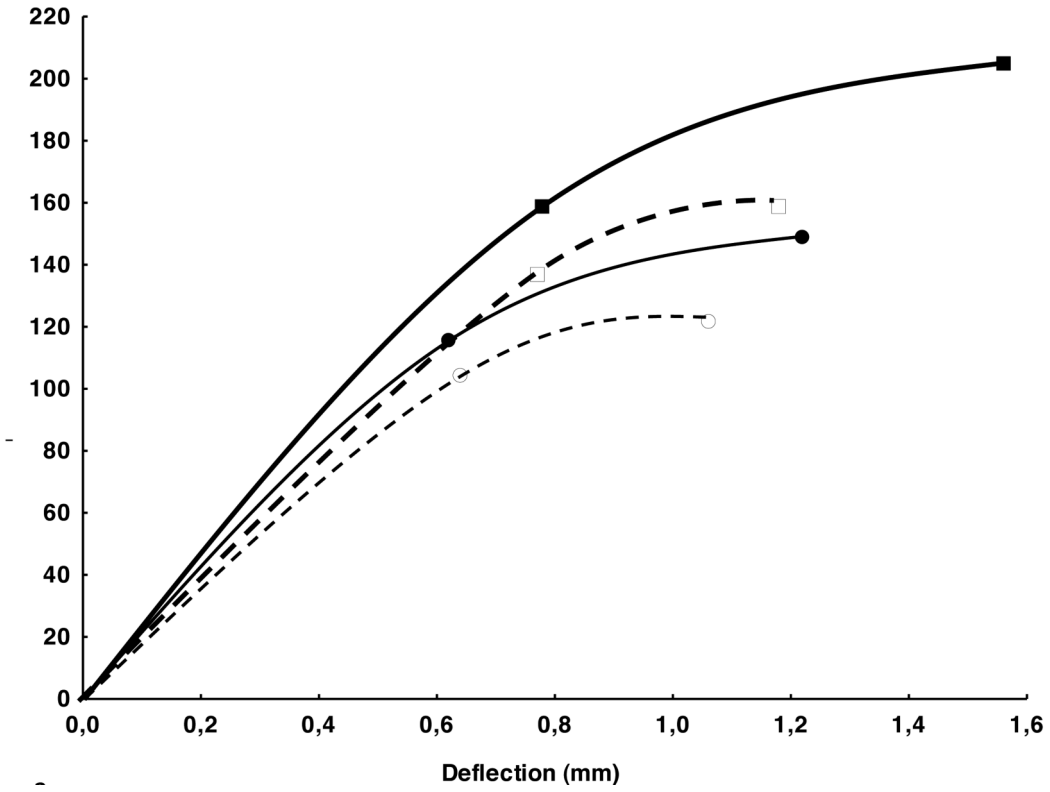

Figure 2. Idealized representation of the W/d curves obtained in the 3-point bending tests of the femurs in the four groups studied, based on the corresponding mean values of dy, d_{Fmax}, Fy and Fmax as shown in the Table. Indicated are the successive elastic and plastic deformation periods, separated by the yield point, in the curves for treated (solid lines, full symbols) and untreated (dashed lines, hollow symbols) male (large squares) and female (small circles) rats. The statistical significance of the inter-group differences in all variables and derived indicators is detailed in the Table.

On analyzing all the animals as a single group, the xCSMI was found closely correlated with the PoPm ($r=0.943$, $p<0.001$) or the cortical thickness ($r=0.961$, $p<0.001$, graphs not shown).

Effects on bone material properties

The cortical BMC data varied proportionally to CortA values in all groups. Accordingly, no inter-group differences were observed in cortical vBMD, yet the values for female bones tended non-significantly to be higher than those obtained in male animals. Treatment was equally ineffective in changing the calculated elastic modulus of cortical bone in both genders.

Effects on the pre-yield behavior of the diaphyses

Figure 2 shows an idealized representation of the W/d curves corresponding to all the groups studied, constructed on the basis of the mean values of the variables dy, Wy, Wmax and d_{FMax} as shown in the Table.

Diaphyseal stiffness tended non-significantly to be higher, and yield load (as assessed by F_y) was significantly higher in male than female animals. Treatment enhanced moderately but significantly the pre-yield diaphyseal stiffness and the yield load of the diaphyses in both males and females, with no significant gender-related differences.

Significant linear and parallel correlations were observed between the yield load (F_y) and the cortical BMC (males, $r=0.702$, $p<0.001$, $R^2=0.493$; females, $r=0.348$, $p=0.047$, $R^2=0.121$) or the xCSMI of the diaphyses (males, $r=0.716$, $R^2=0.513$, $p<0.001$; females, $r=0.339$, $R^2=0.115$, $p<0.05$; graphs not shown). Similar associations were observed between the diaphyseal stiffness (Fy/dy) and the cortical BMC or the xCSMI. The variation in xCSMI values was independent of the calculated E values in all groups.

Effects on the post-yield behavior and the ultimate strength of bones

The maximal load supported by bones (as assessed by Fmax) was significantly enhanced by OPD treatment in both genders (Figure 2). This effect became even more evident after adjusting the data to body weight (Table).

The load-bearing ability of the diaphyses beyond the yield point (as assessed by Fpy) was dramatically improved by treatment in both genders, either in absolute terms or as a percentage of Fmax values. Correlative, significant increases were also induced on the bones' deflection at Fmax and on the ability of bones to absorb energy during the plastic deformation, either in absolute terms (pEa, calculated from the areas under

the F/d curves from the yield point up to Fmax) or related to the assayed bone volume (pEa/vol). The increases in all Fmax, Fpy, d_{Fmax}, pEa and pEa/vol were proportionally larger in male than female animals.

The absolute values of energy absorbed post-yield (pEa) correlated with both cortical BMC and xCSMI only in males (pEa *vs* BMC: r=0.525, p<0.01, R^2=0.276; pEa *vs* xCSMI: r=0.547, p<0.01, R^2=0.299, curves not shown).

Discussion

Effects on bone material and geometric properties. Bone structural properties are determined by bone material (specific stiffness and toughness) and geometric properties (mass and distribution of the tissue) (Figure 1). The assayed OPD doses in this study did not affect cortical vBMD and E, while they increased largely both cortical mass and geometric properties in close association with the improvements in pre-yield diaphyseal stiffness and yield load, and independently of the animals' body weight. This effect rendered the bones more robust than required to physiologically control the diaphyseal stiffness and strength, and should have been responsible, at least in a good part, for the observed increases in structural stiffness, toughness, and strength.

Bone tissue stiffness and toughness (naturally selected as inversely related bone properties[19]) are determined by different factors. BP effects can involve both bone material and geometric properties in different ways; hence, BP effects on bone tissue toughness could not necessarily correlate with those exerted on bone tissue stiffness, and can also be independent of bone mineralization[20-34]. These effects could be coupled to a remodeling inhibition (in remodeling bones[20,23,28,31,35-45] – a mechanism of action that was avoided in this study). BPs can improve bone geometric properties either as a direct effect on osteoblasts (Figure 1, "2a"), or through an enhancement of the tissue response to mechanical stimuli (in any kind of bones)[46-58]. These effects are involved in the biological control bone stiffness (not toughness – Figure 1, "2b"), as suggested by the bone *mechanostat* theory and by some recent, *in vitro* studies[9,12,22,59,60]. Therefore, the integrated effect of any BP on bone structural toughness and strength are hardly predictable by analyzing only its isolated effects on bone mineralization, geometry, stiffness or toughness (Figure 1, "1", "2a", "2b"), even in only-modeling bones. We have shown that pamidronate reduced cortical bone E and yield stress with a compensatory increase in bone mass and geometry[3], while OPD prevented the OVX-induced reduction of cortical vBMD, E, and yield stress[61]. In contrast, large doses of OPD increased all diaphyseal BMD (DXA), xCSMI, and structural stiffness and strength of rat femurs, yet they did not affect E[62].

General effects on bone structural properties. The relative influence of bone material and geometric properties and the bone pre- or post-yield properties in the determination of bone structural properties is generally difficult to discern. Different studies showed differing effects of BPs on bone material and geometric properties as bone-strengthening determinants. We were among the first to show a dissociation between the effects of wide ranges of doses of pamidronate or OPD on the structural pre-yield stiffness and post-yield strength in rat femurs[3,5,14,62], in different proportions at different doses[3]. In this study, OPD increased mildly the bones' pre-yield stiffness, toughness and strength, while it dramatically enhanced post-yield toughness (pEa, deflection at Fmax) and strength (Fpy%). This suggests that the induced bone strengthening (Fmax) should have reflected predominantly the effects on post-yield, rather than pre-yield properties, and on structural toughness, rather than stiffness.

Effects on structural stiffness. In this study, OPD enhanced mildly the pre-yield structural stiffness of the bones. The lack of OPD effects on bone tissue mineralization or elastic modulus rules out any participation of bone material properties in the determination of that effect. Instead, a large, independent influence of the improved diaphyseal geometry on pre- and chiefly in post-yield bone properties could be detected, especially in bones of the (heavier) male rats. This suggests an involvement of the *mechanostat* system in the induced bone stiffnening, in consonance with the above comments and with some additional evidence. We have also shown that OPD strengthened rat femurs as a function of body weight and bone deformability, parallelly to an improvement in CSMI's over the natural, physiological proportions[62]. A further study in hemi-sciaticectomized rats showed positive effects of OPD on bone geometry and strength, that were larger than expected for the animals' body weight, in direct relationship with bone mechanical stimulation and inversely related to E[5]. Reports by many other authors agree with that idea[49-57].

Effects on bone structural toughness and ultimate strength. In this study, the most striking result was the OPD-inducion of a dramatic increase in the structural pot-yield bone toughness, which on time increased largely the ultimate bone strength. This toughening of the bones resulted from improvements in both, bone material (tissue toughness – Figure 1, "1") and geometric properties (BMC, CSMI's – Figure 1, "2a" & 2b")[24,31]. The involvement of tissue toughness is supported by the volume-adjusted increases in the energy absorbed post-yield by the bones (pEa/Vol). Unfortunately, we were unable to measure any of the micro-structural determinants of tissue toughness (Figure 1, "?") and hence to rule out their participation. The relevance of BMC and CSMI's to bone toughening is revealed by their correlations with pEa as determinant variables, especially in male bones. Others' reports of BP effects on post-yield properties of non-remodeling bones look unconclusive[57,63-69]. Some authors have reported non-significant trends of incadronate and risedronate to increase cortical bone toughness in dogs[38,57] and of OPD to enhance the post-yield strength in rat femurs[62]. High doses of pamidronate prevented significantly the negative effects of arthritis-induced immobilization on bone toughness in rabbits[66], and alendronate prevented the OVX-reduced energy to failure in rat femurs[37]. High doses of ibandronate improved the structural toughness of vertebral bone[44] by enhancing bone mass rather than tissue quality.

Gender-related differences. The gender-related differences in OPD effects on bone geometry and post-yield strength in this

study, already reported by us and others employing alendronate in similar, carcinogenetic studies[5,49], could be related to the interaction of body size (larger in males) or bone material's stiffness (normally higher in females, as it tended to be here)[13,68], and/or to the administration of higher effective doses (calculated per body weight, proportionally larger compared to skeletal mass) with a higher availability[69] in males than females.

Limitations of the study

This preliminary, carcinogenicity study was carried out in young rats treated with OPD doses approaching the whole exposition proposed for human osteoporosis treatment, taking profit of the relatively high tolerability of this BP[7,8]. Interestingly, we had shown similar results in ovariectomized rats treated with pharmacological doses of Alendronate[2].

None of our present findings can be extrapolated to pharmacological doses of any BP on similar or different models. Nevertheless, the described effects should be taken into account as possible events taking place together and eventually interacting with those derived from a remodeling blockage.

The elastic modulus of cortical tissue was not directly measured, but calculated from mechanical and tomographic data assuming a geometric shape-size regularity of the diaphyseal samples studied. Obviously, this homogeneity was not absolute, yet the assessment of differences in xCSMI values throughout the bone segments tested yelded relatively low CV's within sexes. This control should also have ruled out any sex-related difference in the mechanical data derived from the unavoidably different relationships between the fixed length (L) of the bone segments and the variable lengths of the bones.

The mechanical tests were performed at a fixed, relatively low strain rate. Thus, results are not extrapolatable to the analysis of high-impact effects on bones[70].

We can only speculate about the possible OPD effects on some of the mineralization-unrelated, microstructural determinants of bone material stiffness and toughness in this study. Therefore, our discussion and conclusions had to be based only on the consistency and coherence of the tomographic and mechanical findings as reported above, as well as on indirect evidences derived from the associations observed between some of the actually measured variables. Further studies with specific designs and employing adequate methodologies are needed to support our present proposals to explain the pathogenesis of the OPD effects observed at the structural level of complexity.

Conclusions

The 3-month treatment with the assayed doses of OPD enhanced rat femur structural bending strength in this model by inducing a significant improvement of the diaphyseal design. This effect, beyond to have had some impact on the pre-yield diaphyseal strength, looks to have largely improved the post-yield toughness and strength of the bones. This striking improvement of the post-yield bone properties was apparently unrelated to the pre-yield behavior, of to the (unchanged) tissue mineralization and stiffness.

The expected effects on bone geometry could have reflected some direct effect on bone formation, but they could also reflect some positive interaction of OPD treatment with the biomechanical control of bone modeling during growth. The apparent sensitivity of OPD effects to the mechanical stimulation of the skeleton is in agreement with previous results from our and others' laboratories, and would support indication of exercise plans in chronic BP treatments.

Effects on post-yield bone behavior could have also involved some mineralization-unrelated microstructural determinants of bone toughness which were out of the scope and technical possibilities of the study.

Our findings describe a positive BP effect on the *mechanism of fracture*, with no evident impairment of bone mineralization and stiffness of the studied (virtually only-modeling) bones at the high doses assayed. Despite the obvious species differences, this might be relevant to studies in older humans, in which cortical bone brittleness has been related to the post-yield rather than the pre-yield properties. That could also help explain why the positive BP effects on DXA-BMD and fracture incidence may not correlate[71].

Pharmacological effects of this and other BPs on the assayed properties in similar or different conditions and in remodeling species remain to be investigated.

Acknowledgements

This work was supported by grants from the Consejo de Investigaciones Científicas y Técnicas (CONICET, PIP 0476) and the National Ministery of Science & Technology (MinCyT, PICT 0695) and by Gador SA (Buenos Aires). Drs. JLF, GRC and RFC are members of the Research Careers, CONICET and Consejo de Investigaciones, Universidad Nacional de Rosario (CIUNR), Argentina. Drs. PSR and LN are fellows of the CONICET and MinCyT, respectively.

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