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The effects of osteoprotegerin on the mechanical properties of rat bone

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Osteoprotegerin (OPG) is a naturally secreted protein that decreases bone resorption by inhibiting osteoclast differentiation and activation while promoting osteoclast apoptosis [8]. In this study, the effects of osteoprotegerin injections on long bone mechanical and material properties were investigated in young male Sprague-Dawley rats. OPG increased fracture strength at the femur mid-diaphysis in three-point bending by 30%, without affecting the elastic or maximum strength. At the femoral neck, OPG significantly increased the elastic (45%), maximum (15%), and fracture (35%) strengths. There was not a difference in microhardness at the femur mid-diaphysis in comparing the placebo and OPG groups. There were, however, significant increases in whole bone dry mass (25%), mineral mass (30%), organic mass (17%), and percent mineralization (4%); percent mineralization at the mid-diaphysis (3%); and percent mineralization at the distal epiphysis (6%) due to the OPG treatment. While OPG decreased endocortical bone formation (52%), total bone area, endocortical bone area, and periosteal bone formation were maintained with OPG treatment. A 30% increase in the X-ray opacity of the bone at the proximal metaphysis of the right tibiae was observed. Overall, OPG increased mineralization and strength indices in the rat femur. Its effects on strength were more pronounced in the femoral neck than at the mid-diaphysis.

1. Introduction

Osteoclasts are the primary cells responsible for the resorption of bone. These cells derive from hematopoietic precursors and differentiate into osteoclasts due to interactions with hormones and their microenvironments. One factor that induces differentiation is osteoprotegerin ligand (OPGL). This member of the tumor necrosis factor (TNF) family binds to the precursor cell and initiates its differentiation into an osteoclast [1, 2]. OPGL also activates mature osteoclasts and promotes their survival. Osteoprotegerin (OPG) is a naturally-secreted glycoprotein that is a member of the TNF receptor family. OPG acts as a decoy receptor for OPGL. The binding of OPG to OPGL prohibits OPGL from binding to the osteoclasts and their progenitors, and thus osteoclast activity and differentiation is inhibited. A reduction in the number of differentiated osteoclasts, coupled with decreased activity of the remaining osteoclasts, decreases the amount of bone resorption. In addition to preventing new osteoclasts from forming, OPG has also been found to increase mature osteoclast apoptosis and thus further decrease bone resorption. OPG's hypocalcemic effect on rats is evidence of a reduction of bone resorption [3]. OPG also prevents the effect of many known bone resorption increasing hormones (interleukin-1β, tumor necrosis factor-α, parathyroid hormone, and 1α,25-dihydroxyvitamin D_3) [4]. Additionally, OPG knockout mice exhibit severe osteoporosis due to increased numbers of osteoclasts [5].

Osteoporosis is a disease characterized by markedly more bone resorption than formation. A major complication of osteoporotic bones is that they fracture under much lower loads than healthy bones. Methods that improve the material and mechanical properties of bones may be useful in the prevention and/or treatment of osteoporosis. Mechanical testing is required to ascertain if OPG's effects extend to the bone's structural properties, which may prevent fractures. Such testing should include the femoral neck due to the frequency and severity of osteoporotic fractures in this region. This study examines the effects that OPG has on the structural, mechanical, and material properties of rat femurs.

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2. Materials and methods

2.1. Animals

Twenty Sprague-Dawley rats were used in this study. The forty-day-old rats were assigned to placebo ($n = 10$) and OPG ($n = 10$) groups. The chimeric form of OPG, where the amino acids 22–194 of human recombinant OPG are fused at the N terminus to the Fc domain of human immunoglobulin (Amgen Inc., Thousand Oaks, CA), was administered (2 mg/kg, i.v.) on days 0, 3, 7, 10, and 14 of the 17 day study [4]. Tetracycline (Sigma, St. Louis, MO, USA) was injected (20 mg/kg, i.p.) on days 1 and 15 as a fluorescent marker for bone mineralization. Both groups were anesthetized (90 mg/kg sodium pentobarbital, i.p.) and sacrificed via cervical dislocation on day 17 of the study. The rats were weighed on day 0 and at sacrifice. The hearts, kidneys, and spleens were also weighed at sacrifice. The Animal Care and Use Committee of the University of Colorado approved the protocol for this study.

2.2. Assays

Non-osseous tissue was cleared from the left femurs. The femurs were then measured using vernier calipers and air dried for 48 h. They were rehydrated in phosphate buffered saline (PBS) for 90 min prior to mechanical testing in three point bending [6]. The femurs were tested to failure at a rate of 5 mm/min using a span length of 15 mm (Instron 1331, Canton, MA). The force-deflection curves were analyzed to determine the strengths, deflections, and energies at the elastic, maximum, and failure limits. Stiffness was calculated by dividing elastic strength by elastic deflection.

Compositional analysis of the fractured femurs was performed, separately, on the distal epiphysis, head, and diaphysis. The dry mass (Dry-M) was measured after oven-heating the bones for 24 h at 105 °C. The mineral mass (Min-M) was measured after the bones had been ashed by heating for another 24 h at 800 °C. Organic mass is the difference between the dry mass and the mineral mass (Org-M = Dry-M − Min-M). Percent mineralization was calculated by the formula % Min = (Min-M/Dry-M) × 100%.

The right femurs were cleared of all non-osseous tissue and sectioned at the distal end of the tertiary trochanter. The distal surface of the femurs at this cross-section was prepared for quantitative histomorphometry and microhardness testing. These sections were placed in neutral buffered 10% formalin for 48 h. The bones were then rinsed in distilled water before they were placed in 70% ethanol for 7 days and air dried for 3 days. The bones were then embedded in non-infiltrating Epo-Kwick epoxy (Buehler, Lake Bluff, IL). The bone and epoxy were sectioned at the mid-diaphysis with a low speed saw (Buehler, 300 μm diamond blade) and polished (ultimately with a 6 μm diamond paste). Photographs taken under a far blue light (400 nm) at 100 × magnification distinguished the fluorescent labels, bone, and background.

Quantitative histomorphometric analysis of the cross sections was performed using SigmaScan Pro (SPSS, San Rafael, CA). The periosteal and endocortical perimeters (Ps.Pm and Ec.Pm) were measured. The total bone cross sectional area (Tt.B.Ar) and the endocortical area (Ec.Ar), as determined by the software, summed the number of pixels inside these perimeters. The cortical area was calculated from the bone and endocortical areas (Ct.Ar = Tt.B.Ar − Ec.Ar).

Cortical thickness was measured at the medial, lateral, anterior, and posterior cortex and then averaged (Mean Ct.Th). Bone formation areas were measured for the periosteal (Ps.BF.Ar) and endocortical (Ec.BF.Ar) perimeters by tracing the tetracycline labels and summing the pixels between them. Total bone formation area (Tt.BF.Ar) was calculated by adding Ps.BF.Ar and Ec.BF.Ar. Bone formation rates (Ps.BFR, Ec.BFR, Tt.BFR) were found by dividing the corresponding bone formation area by the time between tetracycline injections (14 days). The active mineralizing perimeters (Ec.AMPm, Ps.AMPm, Tt.AMPm) were determined by measuring the length of the perimeter undergoing formation. The mineral apposition rate was determined by dividing the bone formation rate by the active mineralizing perimeter (Ec.MAR = Ec.BFR/Ec.AMPm, Ps.MAR = Ps.BFR/Ps.AMPm, Tt.MAR = [(Ec.MAR × Ec.BFR) + (Ps.MAR × Ps.BFR)]/Tt.BFR) (7).

Microhardness testing was also performed on the distal mid-diaphyseal cross-section after completion of the quantitative histomorphometry. Microhardness is a measure of the quality of the bone at a microscopic level [8, 9]. It is a good predictor of bone mineralization, Young's modulus of elasticity, and yield stress [8, 9]. Microhardness was calculated using a Tukon model MO microhardness tester (Wilson Bridgeport, CT) with a 136° pyramid-shaped Vickers diamond indenter [9]. Three indents were made and measured in both the existing and newly formed bone. These areas were distinguished using the UV microscope photographs described earlier.

Mechanical testing of the femoral neck was conducted on the proximal portion of the right femurs. Specimens were embedded vertically in non-infiltrating Epo-Kwick epoxy (Buehler, Lake Bluff, IL) from the mid-diaphysis sections to 2 mm distal to the base of the femoral neck. These specimens were soaked in PBS for 90 min prior to testing [2] and the cartilage surrounding the femoral head was removed and discarded. The epoxy discs were then held in the Instron 1331 (Canton, MA) with the force being placed on the head of the femur at a rate of 5 mm/min (Fig. 1). The force deflection curves were recorded until failure. Every specimen failed in shear at the femoral neck. The strengths, deflections, and energies at the elastic, maximum, and failure limits were analyzed as described earlier.

The right tibiae were removed of all non-osseous tissue and allowed to air dry for 24 h. They were then X-rayed using a Model #43855A cabinet X-ray system (Faxitron, Wheeling, IL; 49 s, 60 kV). The X-ray was digitized (ScanJet 6200 scanner and PrecisionScan Pro software; Hewlett-Packard, Palo Alto, CA) at 600 pixels per inch and analyzed with Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). The mean opacity was then measured within a 4 mm square placed at the metaphysis of the tibiae to represent the density of this area. Significant differences in mean opacity indicate differences in bone density for this region.

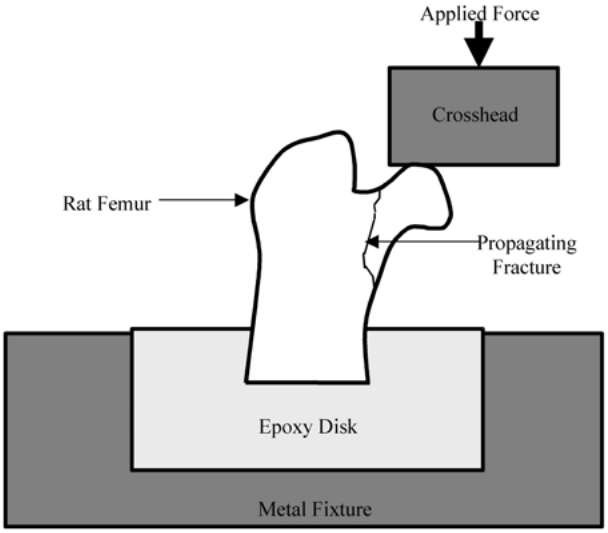

Figure 1 Cross section of the femoral neck testing apparatus.

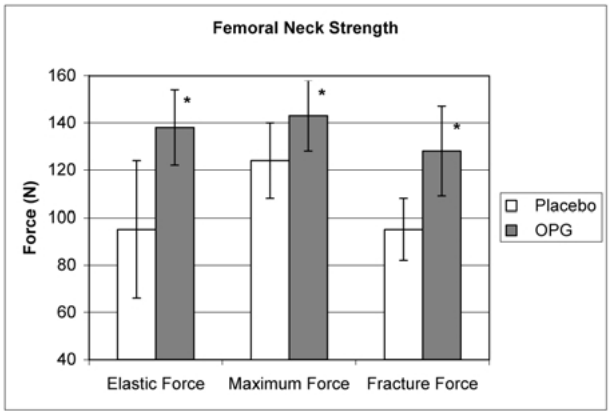

Figure 2 Femoral neck strengths of placebo and OPG treated rats are displayed ± the standard deviation ($n = 10$). *The OPG group is significantly stronger at all three points along the force deflection curve.

Data are presented as mean ± the standard deviation. Statistical analysis was performed with two-tailed *t*-tests. A 95% level of significance was utilized for all tests.

3. Results

OPG treatment resulted in few significant changes in the femur diaphysis mechanical properties (Table I): fracture strength for the OPG group was 30% greater ($p = 0.007$). In comparison, OPG affected most of the femoral neck mechanical properties. The OPG femoral necks were markedly stronger, with a 45% greater elastic strength ($p = 0.02$), 15% greater maximum strength ($p = 0.02$), and 35% greater fracture strength ($p < 0.001$) (Fig. 2). Femoral neck stiffness was not significantly affected by OPG, but the deflection at fracture (42%, $p = 0.005$) and total energy absorbed (46%, $p = 0.005$) were greater for the placebo group.

Compositional analysis was performed to determine how the mass of the bone was apportioned (Table II). The dry mass of the OPG bones was 25% greater; the mineral mass was 30% greater, and the organic mass was 17% greater ($p < 0.001$ for each of these). This corresponds with a 4% increase in percent mineralization ($p < 0.001$). An increase in percent mineralization was found in the diaphysis (3%), distal epiphysis (6%), and head of the femur (6%) ($p < 0.001$). The increase in percent mineralization for the epiphysis is significantly greater than the increase for the diaphysis ($p = 0.007$).

Quantitative histomorphometric analysis of the femur mid-diaphysis (Table III) revealed that OPG had a negative effect on the total bone formation rate (17%, $p = 0.029$). This is due to a 52% decrease in endocortical bone formation ($p < 0.001$) that overshadowed the statistically nonsignificant increase in periosteal bone formation (62%, $p = 0.14$). Consistent with the lower endocortical formation rates for the OPG are the decreased active mineralizing perimeters (30%, $p = 0.002$) and the decreased mineral apposition rates (34%, $p < 0.02$). There was a trend of increased

TABLE I The results of the three point bending tests at the femoral mid-diaphysis and the femoral neck are displayed as the mean ± the standard deviation ($n = 10$). The data shows that strength properties at the mid-diaphysis are relatively unchanged, but at the femoral neck strength is increased dramatically. The deflection and energy data indicate that the femoral neck is more brittle in OPG bones.

Measurement	Placebo	OPG
3-point bending femora mid-diaphysis		
Stiffness (N/mm)	229 ± 49	223 ± 49^{NS}
Elastic strength (N)	85.1 ± 13.4	88.4 ± 14.3^{NS}
Maximum strength (N)	108.5 ± 14.5	110.9 ± 13.9^{NS}
Fracture strength (N)	64.3 ± 10.8	$83.6 \pm 16.7^{p=0.007}$
Elastic deflection (mm)	0.386 ± 0.096	0.409 ± 0.097^{NS}
Deflection at maximum load (mm)	0.623 ± 0.121	0.657 ± 0.116^{NS}
Deflection at fracture (mm)	0.649 ± 0.089	0.669 ± 0.113^{NS}
Elastic energy (mJ)	16.5 ± 5.3	18.4 ± 7.4^{NS}
Energy at maximum load (mJ)	39.7 ± 10.2	42.9 ± 7.5^{NS}
Energy at fracture (mJ)	41.8 ± 7.9	43.7 ± 7.3^{NS}
Femoral neck		
Stiffness (N/mm)	273 ± 124	318 ± 114^{NS}
Elastic deflection (mm)	0.42 ± 0.26	0.47 ± 0.14^{NS}
Deflection at maximum load (mm)	0.62 ± 0.16	$0.48 \pm 0.13^{p=0.072}$
Deflection at fracture (mm)	0.91 ± 0.30	$0.53 \pm 0.14^{p=0.005}$
Elastic energy (mJ)	23 ± 19	32 ± 8^{NS}
Energy at maximum load (mJ)	44 ± 11	$34 \pm 8^{p=0.041}$
Energy at fracture (mJ)	74 ± 29	$40 \pm 8^{p=0.005}$

NS: Difference in the means is not significant (two-tailed *t*-test).

TABLE II Composition analysis of the femur. The dry masses (Dry-M), mineral masses (Min-M), and organic masses (Org-M) of the left femurs are reported as the mean ± standard deviation. Also given are the percent mineralization (% Min) of the whole femur as well as the diaphysis, distal epiphysis, and the head of the femur. OPG altered the composition of the bone extensively as shown by the 25% increase in dry bone mass. The increase in percent mineralization is much more pronounced at the distal epiphysis and head than at the diaphysis.

Measurement	Placebo	OPG
Dry-M (mg)	394 ± 28	$493 \pm 29^{p<0.001}$
Min-M (mg)	243 ± 17	$316 \pm 20^{p<0.001}$
Org-M (mg)	151 ± 12	$177 \pm 9^{p<0.001}$
%Min Whole Femur	61.8 ± 0.6	$64.2 \pm 0.5^{p<0.001}$
%Min Diaphysis	64.4 ± 0.6	$66.4 \pm 0.5^{p<0.001}$
%Min Distal Epiphysis	56.6 ± 0.9	$60.1 \pm 1.2^{p<0.001}$
%Min Head	56.7 ± 1.9	$60.3 \pm 1.1^{p<0.001}$

NS: Difference in the means is not significant (two-tailed *t*-test).

TABLE III Quantitative histomorphometry and microhardness testing results. The measurements taken from the UV microscope photograph of the mid-diaphysis cross section of the femur are displayed as the mean ± the standard deviation. Microhardness of the mid-diaphysis for newly formed and extant bone are also reported as the mean ± the standard deviation. Ec = endocortical, Ps = periosteal, Tt = total, V = ventral, D = dorsal, M = medial, L = lateral, BF = bone formation, Ar = area, BFR = bone formation rate, AMPm = active mineralizing perimeter, MAR = mineral apposition rate, and Ct.Th = cortical thickness. OPG has a much larger effect on the endosteal surface. This can be seen by the changes in Ec.BFR, Ec.Ar, Ec.AMPm, and Ec.MAR compared to the corresponding periosteal measurements. OPG did not have an effect on microhardness.

Measurement	Placebo	OPG
Ec.Ar (mm^2)	5.45 ± 0.80	5.93 ± 0.95^{NS}
Ct.Ar (mm^2)	4.46 ± 0.27	4.54 ± 0.33^{NS}
Tt.B.Ar (mm^2)	9.90 ± 1.73	10.5 ± 0.80^{NS}
Ec.BFR (mm^2/day)	0.081 ± 0.027	$0.039 \pm 0.016^{p=0.001}$
Ps.BFR (mm^2/day)	0.035 ± 0.026	0.056 ± 0.035^{NS}
Tt.BFR (mm^2/day)	0.115 ± 0.010	$0.095 \pm 0.025^{p=0.029}$
Ec.AMPm (mm)	7.6 ± 1.2	$5.34 \pm 1.5^{p=0.002}$
Ps.AMPm (mm)	4.08 ± 2.54	5.54 ± 2.58^{NS}
Tt.AMPm (mm)	11.7 ± 2.9	10.9 ± 1.9^{NS}
Ec.MAR (mm/day)	0.0108 ± 0.0042	$0.0071 \pm 0.0019^{p=0.02}$
Ps.MAR (mm/day)	0.0115 ± 0.0068	0.0153 ± 0.0074^{NS}
Tt.MAR (mm/day)	0.031 ± 0.006	0.028 ± 0.006^{NS}
Mean Ct.Th (mm)	0.459 ± 0.058	0.468 ± 0.081^{NS}
Microhardness of newly formed bone (Pa)	54.1 ± 6.0	54.5 ± 5.1^{NS}
Microhardness of existing bone (Pa)	61.4 ± 5.2	62.4 ± 5.8^{NS}

NS: Difference in the means is not significant (two-tailed *t*-test).

endocortical area (9%) and total bone area (6%) for the OPG group, but these differences were not statistically significant.

Microhardness data indicated that OPG did not change the quality of bone that was being formed at the mid-diaphysis (Table III). OPG treatment did not change the microhardness of newly formed bone or existing bone. Although there was not a difference between the OPG and placebo groups, the existing bone was harder than the newly formed bone within both groups (14%, $p < 0.001$), indicating continual mineralization of existing bone. This is consistent with previous findings [9].

X-ray analysis indicated that the OPG bones had greater density around the region of the metaphysis (Fig. 3). This is interpreted from the mean opacity of a 4 mm square consistently placed in this region of the X-ray that was 30% brighter for OPG animals. Individual spicules were very apparent in the placebo group but difficult to distinguish in the OPG group due, presumably, to the increased number of spicules in this region. This increase correlates with the large increase in percent mineralization at the epiphysis found in the compositional analysis.

There was not a significant difference in the lengths of the femurs, average mass of the kidney, spleen, heart,

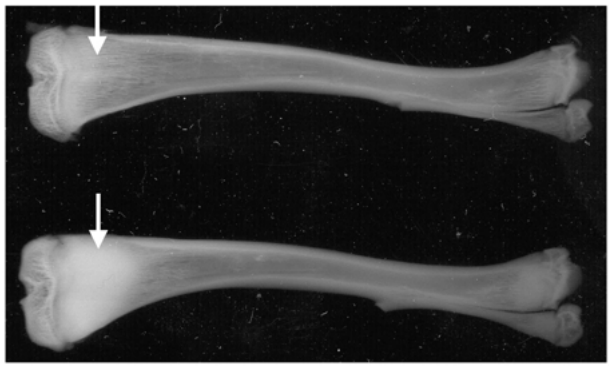

Figure 3 X-ray of a placebo treated rat tibia (top). X-ray of an OPG treated rat tibia (bottom). The bone density is much greater at the secondary spongiosa (arrows) for the OPG treated bone, in which the individual spicules are blurred by their greater number.

whole body mass on day 0, or whole body mass at sacrifice.

4. Discussion

In this study, the effects of OPG on the structural and physical properties of rat bones were investigated. Only the fracture strength was improved at the femur mid-

diaphysis; however, the elastic strength, maximum strength, and fracture strength increased at the femoral neck. Noting that the trabecular bone content is considerable for the femoral neck and negligible for the mid-diaphysis, these findings suggest a strong effect of OPG on trabecular bone. This is consistent with the short-term nature of the study, the high level of metabolic activity of trabecular bone, and the known trabecular effects of OPG [10].

All the bones fractured across the femoral neck (Fig. 1). In many of the fractures, there was a noticeable area of increased bone fragmentation on the proximal surface that is indicative of a fracture locus beginning here and propagating distally. This is consistent with the expectation that bone would fracture in tension more readily than compression.

The increased size of the elastic region in the femoral neck indicates that the OPG treated bones can withstand more force before permanent damage occurs. In addition, the plastic region of the force-deflection curve is much smaller for OPG treated rats in the femoral neck. This, in combination with the significantly decreased amounts of deflection and energy absorbed at maximum strength and fracture, indicates that the OPG bone is more brittle. The increased strength and brittleness are consistent with the increased mineralization observed.

When considering the increase in fracture strength at the mid-diaphysis, it is also important to note that the deflection at fracture did not change. The increase in fracture strength without a decrease in deflection indicates improved structural properties. If there had been an associated decrease in deflection with the increase in fracture strength, the bone would likely have been failing at an earlier point on the force deflection curve, which would indicate that the cortical bone was more brittle rather than truly stronger.

A limitation of three point bending tests for rodent bones is the large amount of shear stress due to the small span width available on the femur. Considering the 4 mm diameter of the femur diaphysis, a 64 mm span width would be more appropriate to minimize shear stress in the bone (aspect ratio 16 : 1), but the femurs are only 30 mm long [11]. The 15 mm span width utilized in this study increased the amount of deflection due to shear stress to 10–15% of the overall deflection depending on the bone [11]. This produced a loading mechanism that was not pure bending. Since the shear stress is not considered, the strength measurements as presented are likely underestimated and deflection measurements are overestimated.

Although the endosteal bone formation rate was lowered by OPG treatment, the dry mass, mineral mass, and organic mass were all greater than the bone of placebo control rats. The increased mass is therefore attributable to a reduction in bone resorption. The data indicates that the decrease in resorption due to OPG carries with it an osteoclast/osteoblast coupling induced decrease in formation in murines. The inhibition of bone formation at the endocortical surface and preservation of periosteal bone formation is consistent with a previous study by our group on quickly growing mice [12]. An inhibition of bone formation at the endosteal perimeter will reduce the moment of inertia less than an inhibition of bone formation at the periosteal surface, and therefore have a less significant effect on mechanical properties.

The effects of OPG were more pronounced at the proximal and distal epiphyses of the long bones throughout this study. For example, the OPG-induced increase in structural strength was greater at the femoral neck than at the mid-diaphysis and the OPG-induced increase in percent mineralization was greater at the distal epiphysis and head of the femur than the diaphysis. OPG's effects on the epiphyses of the bones, as noted earlier, is likely due to the greater relative amount of trabecular bone and metabolic activity in these regions. It cannot be attributed to changes in normal metaphyseal bone growth because the OPG femurs were the same length as the placebo femurs.

The 3% increase in mineralization at the femoral diaphysis is indicative of a change in material properties and was expected to correlate with an increased microhardness of the bone in this area. The microhardness data did not support this finding in either newly formed or extant bone. This is likely due to a lack of sensitivity of the microhardness to this modest change in mineralization. The actual increase in percent mineralization directly at the mid-diaphysis was probably less than the 3% found for the entire diaphysis because the proximal epiphysis (without the head of the femur) was included in the diaphyseal region. Microhardness at the femoral head was not evaluated in this study, but the large increase in percent mineralization in this area would likely correlate with increased microhardness for OPG treated animals. A previous study demonstrated an OPG induced increase in microhardness for existing bone of tail-suspended mice [12].

While this study was performed on healthy rats and not osteoporotic rats, it did show that bone mass, percent mineralization, and strength in the femoral neck increased due to OPG treatment. Mechanical and material properties were affected to a lesser degree in cortical bone at the mid-diaphysis. It is plausible that OPG will have similar effects on osteoporotic rats and other animals, but this is left as a further area of research.

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