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3 **Raloxifene Enhances Vertebral Mechanical Properties Independent of Bone Density**

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This is the author's manuscript of the article published in final edited form as: Allen M. R., Iwata K., Sato M., Burr D.B. (2006). Raloxifene Enhances Vertebral Mechanical Properties Independent of Bone Density. *Bone*, 29(5): 1130– 5. Available from: http://dx.doi.org/10.1016/j.bone.2006.05.007

Abstract

Anti-remodeling agents produce similar reductions in vertebral fracture risk despite large differences in BMD changes suggesting the mechanism of fracture risk reduction may differ among these agents. Forty-eight intact (non-ovariectomized) skeletally mature female beagle dogs were treated orally for 12 months with clinically-relevant doses of risedronate (RIS, 0.10 mg/kg/day), alendronate (ALN, 0.2 mg/kg/day), raloxifene (RAL, 0.50 mg/kg/day), or saline (VEH, 1 ml/kg/day). After sacrifice, the following measurements were made on vertebral bone: areal (aBMD) and volumetric (vBMD) bone mineral densities, tissue mineralization by ash content, static and dynamic histomorphometric parameters, microdamage, and extrinsic and intrinsic measures of biomechanical strength, stiffness and energy to fracture. At these doses, RAL suppressed bone turnover (-20%) significantly less than the bisphosphonates (-66 and -71%), and did not produce significant differences in aBMD, vBMD, BV/TV or percent ash compared to VEH-treated animals. Microdamage accumulation in RAL-treated animals was not significantly different than VEH; both RIS and ALN had significantly higher crack surface density compared to VEH. Stiffness was significantly higher than VEH in all treatment groups. Ultimate load divided by aBMD, a measure of strength independent of BMD, was significantly higher only in RAL-treated animals compared to VEH (+16%, $p = 0.015$). Based on these data, we conclude that raloxifene produces improvements in bone mechanical properties in ways that do not involve increases in BMD.

Key words: Bisphosphonates – SERMS – Osteoporosis – Microdamage – Biomechanics

Introduction

Contributions to vertebral fracture risk reduction by factors other than bone mineral density (BMD) are suggested by data showing that anti-remodeling therapies reduce vertebral fracture risk by roughly the same degree (33-49% after the first 3 years of treatment) even though there is as much as a six-fold difference in the increase in spine BMD [1-7]. In addition, the risk of fracture to either the forearm [8] or the hip [9, 10] increases with age even at equivalent bone mineral density. At the hip, for instance, the ten year probability of fracture for a woman with a T-score of -2 is about 4-fold greater at the age of 80 than at the age of 50 [9]. These findings, both from non-treated individuals and from those on therapy, demonstrate that bone strength and fracture risk are determined by more than areal bone mineral density (aBMD).

Raloxifene, a selective estrogen receptor modulator (SERM), suppresses bone turnover and increases BMD by about half as much as the bisphosphonates (alendronate, risedronate, zoledronate) but reduces vertebral fracture risk by roughly the same degree. Three years of treatment with raloxifene (60 mg) reduces vertebral fractures in postmenopausal women by 30% [6] compared with 41-49% with risedronate [3, 4] or 44-47% with alendronate [1, 2]. The change in vertebral BMD with raloxifene treatment accounts for only 4% of the reduction in vertebral fracture risk [11], compared to 16-28% by bisphosphonates [12-14]. Together these data suggest some fundamental differences in the way these two classes of anti-remodeling agents reduce vertebral fracture risk. The goal of the current study was to determine if clinically-relevant doses of raloxifene alter properties of canine vertebral bone in ways that differ from the bisphosphonates.

Materials and Methods

Animals

Forty-eight skeletally mature female beagles (average age 1.3 ± 0.2 years) were purchased from Marshall Farms USA (North Rose, NY). Upon arrival, lateral X-rays of all dogs were obtained to confirm skeletal maturity (closed proximal tibia and lumbar vertebra growth plates). Animals were housed two per cage in environmentally controlled rooms at Indiana University School of Medicine's AALAC accredited facility and provided standard dog chow and water. All procedures were approved prior to the study by the Indiana University School of Medicine Animal Care and Use Committee.

Experimental Design

Following two weeks of acclimatization, animals were assigned to treatment groups (n=12/group) by matching body weights. All dogs were treated daily for 1-year with oral doses of vehicle (1 ml/kg/day saline), raloxifene (0.50 mg/kg/day, Lilly Research Labs, Indianapolis, IN), risedronate sodium (0.10 mg/kg/day, Procter and Gamble Pharmaceuticals, Inc, Cincinnati, OH) or alendronate sodium (0.20 mg/kg/day, Merck and Co., Inc., Rahway, NJ). The bisphosphonate doses were chosen to match those used for treatment of post-menopausal osteoporosis on an mg/kg basis while the raloxifene dose was chosen to produce serum levels equivalent to those documented in post-menopausal women. Both risedronate and alendronate were dissolved in saline and administered to the dogs orally with a syringe. The raloxifene was diluted in 10% hydroxypropyl-β-cyclodextrin made with distilled water and administered orally with a

1 syringe. All drugs were administered in equivalent volumes (1 ml/kg/day) each morning
2 after an overnight fast and at least 2 hours prior to feeding.
3 Prior to necropsy, animals were injected with calcein (0.20 mL/kg, IV) using a 2-12-
4 2-5 labeling schedule (9 animals per group) or a 2-5-2-5 (3 animals per group). The
5 shorter interlabel duration was due to a scheduling error. Animals were euthanized by
6 intravenous administration of sodium pentobarbital (0.22mg/kg Beuthanasia-D Special).
7 After death, thoracic and lumbar vertebrae were dissected and saved for analyses. The
8 ninth thoracic and fourth lumbar vertebrae were separately wrapped in saline-soaked
9 gauze and frozen (-20ºC). Second and third lumbar vertebrae were fixed in 10% neutral
10 buffered formalin.
11 *Densitometry*
12 Areal bone mineral density (aBMD, g/cm^2) of the fourth lumbar vertebra (L4) was
13 quantified using a PIXImus II densitometer (Lunar Corp.). Prior to scanning, the
14 vertebrae were thawed to room temperature. The posterior elements and cranial/caudal
15 endplates were removed using a low speed diamond saw (Labcut 1010, Extec) while
16 under constant irrigation. Endplate removal was done such that surfaces were parallel for
17 mechanical testing. Scanning (0.18x 0.18 mm/pixel) was performed with the vertebral
18 body laying on its medial surface. For each specimen, aBMD of the entire vertebral body
19 was determined.
20 Volumetric bone density and geometry of the L4 vertebra was quantified using a
21 Norland Stratec XCT Research SA+ pQCT (Stratec Electronics). A scout view of each
22 bone was obtained to determine slice locations. One slice (0.07 X 0.07 x 0.50 mm voxel
23 size) was taken at three locations (25, 50 and 75% of total vertebra height). Total and

trabecular volumetric bone mineral density (vBMD, mg/cm^3) and cross-sectional area (CSA, mm^2) were obtained for each slice using contour mode 1, peel mode 2, and a threshold of 710 mg/cm^3. Values from the three slices were averaged together to obtain a single representative value for each parameter for each specimen.

Ash Weight

Percent ash was quantified from the ninth thoracic vertebrae. Vertebrae were thawed to room temperature and a trabecular bone core (4 mm^3) was cut from the mid-cranial metaphysis using a band saw (Marmed Inc.) while under constant irrigation. Trabecular bone specimens were dried using acetone/anhydrous ether and weighed daily until mass was stabilized for two consecutive days (dry weight). Bones were ashed at 800ºC for 12 hours using a 1400 Thermolyne oven (Barnstead). Ashed specimens were allowed to cool and then weighed (ash weight). Percent ash was calculated as ash weight/dry weight * 100.

Histology (Static, dynamic, and microdamage)

Static and dynamic histomorphometric measures of trabecular bone were obtained on second lumbar vertebrae. After 3 days of fixation, bones were transferred to 70% ethanol until processing. Using an automatic tissue processor (Shandon/Lipshaw), specimens were cycled through a graded series of ethanols, cleared using xylene, and infiltrated with methyl methacrylate (MMA; Aldrich). Specimens were transferred to a solution of MMA + 3% dibutyl phthalate (DBP; Sigma-Aldrich) for 3-7 days under vacuum and then embedded using MMA + DBP + 0.25% catalyst (Perkadox 16[3]; Akzo Nobel Chemicals). Mid-sagittal (4 µm) sections were cut using a Reichert-Jung 2050 microtome (Magee Scientific, Inc) and stained with McNeal's tetrachrome for static histomorphometry.

1 Mid-sagittal (8 µm) sections were cut and left unstained for dynamic histomorphometry
2 and wall thickness measures.
3 Third lumbar vertebrae were processed for microdamage assessment by bulk staining
4 in basic fuchsin [15]. Using 1% basic fuchsin dissolved in increasing concentrations of
5 ethanol, specimens were stained according to the following schedule: 4 hours 80%, 4
6 hours in new 80%, 4 hours in 95%, overnight in new 95%, 4 hours in 100%, 4 hours in
7 new 100%. Bones were placed under vacuum (20 in Hg) for all stages during the day and
8 left on the bench top overnight. Following staining, bones were washed 2x in 100%
9 ethanol (five minutes each), placed in 100% MMA under vacuum for 4 hours, and then
10 transferred to MMA + DBP for 3 days. Samples were embedded in MMA + DBP +
11 0.25% catalyst. Mid-sagittal (80-100 µm) sections were cut using a diamond wire saw
12 (Histosaw; Delaware Diamond Knives).
13 Histological measurements were made using a semiautomatic analysis system
14 (Bioquant OSTEO 7.20.10, Bioquant Image Analysis Co.) attached to a microscope
15 equipped with an ultraviolet light source (Nikon Optiphot 2 microscope, Nikon).
16 Measurements were carried out on one stained (static), one unstained (dynamic), and two
17 bulk stained (microdamage) sections per animal. Analysis of a single stained and
18 unstained section has been previously shown to be sufficient to detect significant
19 differences in this animal model [16] while two sections were measured for microdamage
20 variables to reduce the probability of crackless specimens [17]. A 5 x 5 mm region of
21 interest, located 1 mm below the cranial plateau, was used for sampling. Static and
22 dynamic variables were measured and calculated in accordance with ASBMR
23 recommended standards [18]. Microdamage was assessed using UV fluorescence as

1 previously described [19]. Cracks were identified by their typical linear shape, relative
2 size (greater than canaliculi, smaller than vascular channels), and positive fluorescence
3 (due to diffusion of stain into the crack wall). Microcracks were identified at 10x
4 magnification and their lengths measured at 20x magnification. Measurements included
5 crack length (Cr.Le, μm) and crack number (Cr.N, #), with calculations of crack density
6 (Cr.Dn, #/mm^2; Cr.N / bone area) and crack surface density (Cr.S.Dn, μm/mm^2; Cr.N *
7 Cr.Le / bone area).
8 *Biomechanical Testing*
9 The biomechanical properties of fourth lumbar vertebrae were quantified using a
10 servohydraulic testing system (MTS 810, MTS Corporation). Following densitometry,
11 vertebral height was measured using digital calipers (Starrett #721; L.S. Starrett Co).
12 Compression to failure was carried out on saline soaked specimens with displacement
13 control mode (20 mm/min). Load versus displacement curves were recorded using a HP-
14 7090 plotting system. Plots were analyzed for determination of ultimate force (maximum
15 force obtained during test) and stiffness (slope of the linear portion of load/displacement
16 curve). Work to ultimate force (area under the load/displacement curve before ultimate
17 force) was measured by digitizing plots and analyzing the area using standard imaging
18 software (Scion Image; Scion Corp.). Ultimate stress (σ_{ult}), elastic modulus (E), and
19 toughness (U) were estimated using the following equations:
20 $$\sigma_{ult} = (\text{ultimate force} / \text{CSA}) / \text{BV/TV}$$
21 $$E = (\text{stiffness} * (\text{height} / \text{CSA})) / \text{BV/TV}$$
22 $$U = (\text{work to ultimate force} / (\text{height} * \text{CSA})) / \text{BV/TV}$$

where CSA is cross sectional area from pQCT measures of the same vertebrae (L4), height was that measured with digital calipers, and BV/TV was from histomorphometry of L2 histomorphometry.

Statistics

All statistical tests were performed using SAS software (SAS Institute, Inc.). Differences among treatment groups were evaluated using a one-way analysis of variance (ANOVA). When a significant overall F value ($p < 0.05$) was present, differences between individual group means were tested using Fisher's protected least-significant difference (PLSD) post-hoc test. For all tests, $p \leq 0.05$ was considered significant. All data are presented as mean ± standard error.

Results

At clinically relevant doses, raloxifene (RAL) had a smaller suppressive effect on bone remodeling in the lumbar vertebra than either of the bisphosphonates, risedronate (RIS) or alendronate (ALN). In this intact dog model, RAL suppressed activation frequency (Ac.f) by 20% compared to VEH-treated controls ($p = 0.10$), whereas RIS and ALN suppressed Ac.f by 66% and 71% respectively ($p < 0.0001$ versus both VEH and RAL) (Figure 1A). RIS and ALN each suppressed turnover by significantly reducing MS/BS (-50% versus VEH and RAL) and MAR (-25% versus VEH) (Table 1). Neither MS/BS nor MAR was significantly reduced with RAL-treatment compared to VEH.

Only ALN-treated animals had significantly higher aBMD ($p = 0.005$) and total vBMD ($p = 0.015$) (Table 2) of the vertebra compared to VEH. Higher BMD in ALN, compared to VEH, occurred through a combination of higher percent ash (+ 3.6%, $p = 0.037$) and trabecular bone volume (+ 20%, $p = 0.015$) (Tables 1 and 2). Compared to

VEH, neither percent ash nor bone volume were significantly different with RAL and in the RIS dogs only percent ash was significantly higher (Table 2).

Even with a relatively low level of remodeling suppression, RAL-treated animals tended to have higher crack surface density (Cr.S.Dn) (2-fold higher vs VEH; $p = 0.14$). Cr.S.Dn was significantly higher than VEH in both RIS (+2.9-fold) and ALN (+3.7-fold) groups; ALN-treated animals had significantly higher Cr.S.Dn compared to RAL (Figure 1B, Table 3). The non-significantly higher Cr.S.Dn with RAL was contrasted by a significantly higher mean crack length ($p < 0.05$ versus VEH; Figure 2, Table 3), whereas the significantly higher Cr.S.Dn in ALN and RIS groups was the result of a greater number of cracks (Figure 2, Table 3).

Stiffness was significantly higher in all groups compared to VEH while there was no significant difference among groups for ultimate load or energy to ultimate load (Table 4). Normalization of ultimate load by aBMD, a measure of strength independent of bone density, revealed a significant increase in RAL-treated animals (+16%; $p= 0.015$) compared to VEH and ALN (Figure 1D). There was no significant difference in UL/aBMD for either ALN- or RIS-treated animals compared to VEH. Vertebral toughness in ALN-treated dogs was significantly less than in RAL-treated dogs (-24%, $p = 0.0007$) and tended to be less than in VEH-treated dogs (-17%, $p = 0.057$) (Figure 1C). There was no significant difference among groups for other estimated material properties (ultimate stress or apparent modulus).

Discussion

Although routinely used as a surrogate of fracture risk, it is well accepted that bone density accounts for only a portion of bone strength [11-14]. In this study we attempted

to separate out the effects of these density-dependent parameters on vertebral bone strength. We found that raloxifene-treated animals had significantly higher vertebral strength (ultimate load) per unit aBMD compared to both vehicle- and alendronate-treated animals. This is surprising in light of the fact that raloxifene treatment suppressed turnover and increased BMD less than the bisphosphonates. These observations are relevant as vertebral fracture risk reduction is often estimated based on changes in bone turnover rate or BMD following treatment, and suggests that raloxifene provides enhanced strength independent of bone volume or mineralization of the matrix.

Bone strength is determined by multiple factors. At the material level, bone strength, stiffness, and energy absorption are influenced by mineral, collagen, and microdamage. Raloxifene-treated animals did not differ significantly from either bisphosphonate-treated group with respect to mineralization (percent ash). Microdamage accumulation (Cr.S.Dn) was significantly higher in raloxifene-treated animals compared to vehicle, and lower than alendronate-treated animals. However, mean crack length was significantly higher with raloxifene treatment compared to all other groups. Despite these differences, select mechanical properties of raloxifene-treated animals were significantly higher compared to alendronate (both toughness and UL/aBMD) and vehicle (UL/aBMD). Given these differences, we suggest that bisphosphonates and raloxifene may differentially alter the collagen component of bone tissue.

Estrogen and estrogen-like compounds are known to have an effect on collagen. Estrogens have been shown to inhibit the synthesis of advanced glycation end-products (AGEs) in epithelial tissue, the accumulation of which precipitates formation of non-enzymatic glycation crosslinks [20]. Tamoxifen inhibits ovariectomized-induced

1 increases in trabecular bone collagen glycation in rats [21]. Although hormone
2 replacement therapy increase collagen cross-linking in post-menopausal women, studies
3 have not measured non-enzymatic cross-links [22, 23]. We are not aware of any data
4 regarding bisphosphonates and the formation of non-enzymatic crosslinks, although it is
5 known from both in vitro [24-27] and in vivo [28-30] studies that increased pentosidine
6 and vesperlysine, non-enzymatically-glycated cross-links, are associated with brittleness
7 and reduced work to fracture of bone tissue.
8 Raloxifene-induced changes in bone matrix properties without significantly
9 changing BMD or BV/TV may help to explain its anti-fracture efficacy. In clinical trials,
10 raloxifene induced a significant 2.6% increase in aBMD, but reduced vertebral fractures
11 by 30% over three years [6]. These changes are contrasted by bisphosphoantes which
12 increase spine BMD between 4.6 and 6.2% and reduce vertebral fracture risk by 41-49%
13 [1-4]. The data presented in this paper suggest that raloxifene may alter properties of the
14 bone matrix, and therefore help to prevent fractures by a mechanism that is relatively
15 independent of BMD. This is contrasted by the bisphosphonates which derive a larger
16 portion of their fracture risk reduction through increases in BMD. Indeed, the change in
17 BMD with raloxifene has been shown to account for only 4% of the reduction in
18 vertebral fracture risk [11], compared to 16-28% by bisphosphonates [12-14].
19 These results should be considered within the context of the various limitations of
20 the current study. We used intact, non-ovariectomized beagle dogs and therefore it is
21 unclear if similar changes with raloxifene-treatment would occur in the absence of
22 estrogen. Additionally, for reasons unrelated to this study, serum analyses from three of
23 the raloxifene-treated dogs were conducted after 7 months of treatment and revealed the

1 serum concentration of raloxifene was approximately ½ of what was predicted from the
2 original dosing calculations. However, these levels were still within the range of levels
3 quantified in post-menopausal women receiving the 60 mg/day dose of raloxifene (Lilly
4 data on file). Therefore we are confident that the changes with raloxifene compared to
5 vehicle-treated animals represent changes that are clinically-relevant. As blood was not
6 saved from bisphosphonate dogs we were not able to assess the serum concentration of
7 these agents. So although the *a priori* dosing levels were all chosen to be equivalent to
8 the clinical doses used for post-menopausal osteoporosis, we cannot exclude the
9 possibility that differences between the bisphosphoantes and raloxifene treatments were
10 due to these dosing discrepancies.
11 In conclusion, we show raloxifene significantly improves vertebral bone strength
12 independent of bone mineral density, and that this is a fundamentally different
13 mechanism than occurs with either risedronate or alendronate. 14

1 **Acknowledgements**

2 The authors thank Dr. Keith Condon, Diana Jacob, Mary Hooser, and Lauren Waugh for
3 histological preparation and Dr. Charles Turner for his assistance with mechanical
4 testing. This work was supported by NIH Grants 5R01AR047838-03 and
5 5T32AR007581-09 and research grants from The Alliance for Better Bone Health
6 (Procter & Gamble Pharmaceuticals and sanofi-aventis), and Lilly Research Laboratories.
7 Merck and Co. kindly provided the alendronate. This investigation utilized an animal
8 facility constructed with support from Research Facilities Improvement Program Grant
9 Number C06 RR10601-01 from the National Center for Research Resources, National
10 Institutes of Health.

Figure Legends

Figure 1. Comparison of the effects of three anti-remodeling agents on (A) bone turnover measured by activation frequency, Ac,f; (B) crack surface density (Cr.S.Dn), a measure of microdamage accumulation; (C) toughness, the tissue-level energy absorption to ultimate stress; and (D) ultimate load normalized for areal bone mineral density, UL/aBMD. Data presented as mean ± SE. Numbers within bars represent % difference from VEH. $P < 0.05$ vs [a] VEH or [b] RAL.

Figure 2. Frequency distribution showing that the higher amounts of damage accumulation in bisphosphonate treated animals was due to accumulation of many small cracks, whereas the smaller increase in damage accumulation in raloxifene-treated dogs was primarily the result of fewer but longer cracks. The distribution of crack lengths with raloxifene was similar to vehicle-treated control animals, but skewed to longer cracks than bisphosphonates.

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Figure 1

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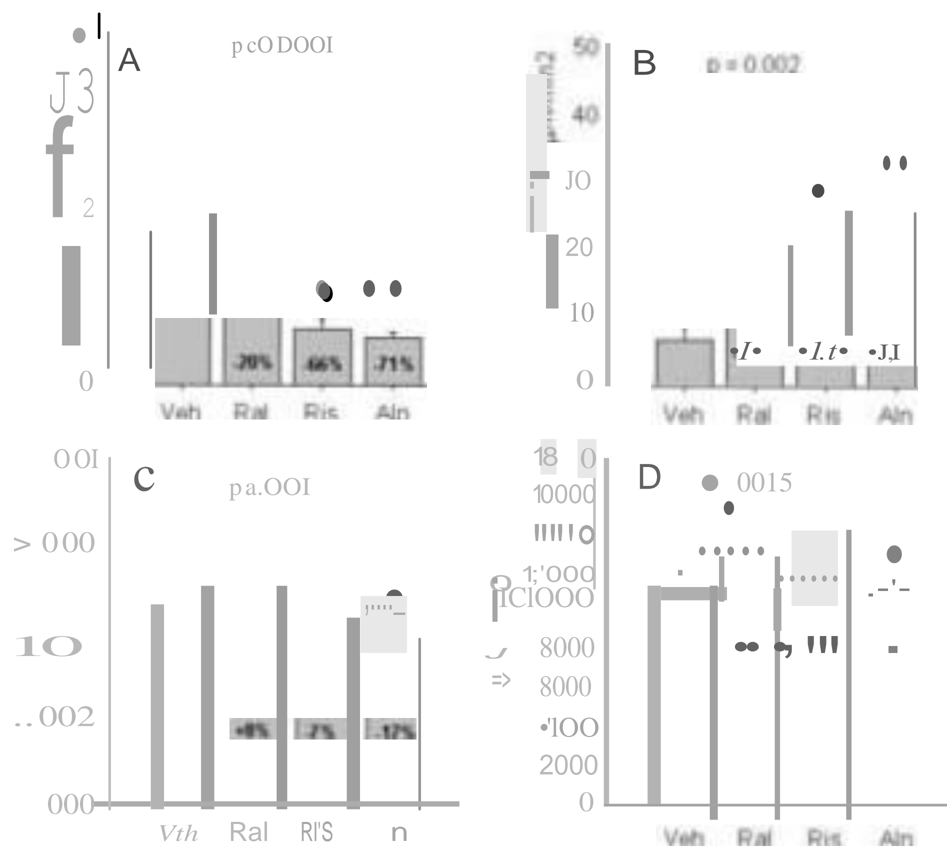

Figure 2

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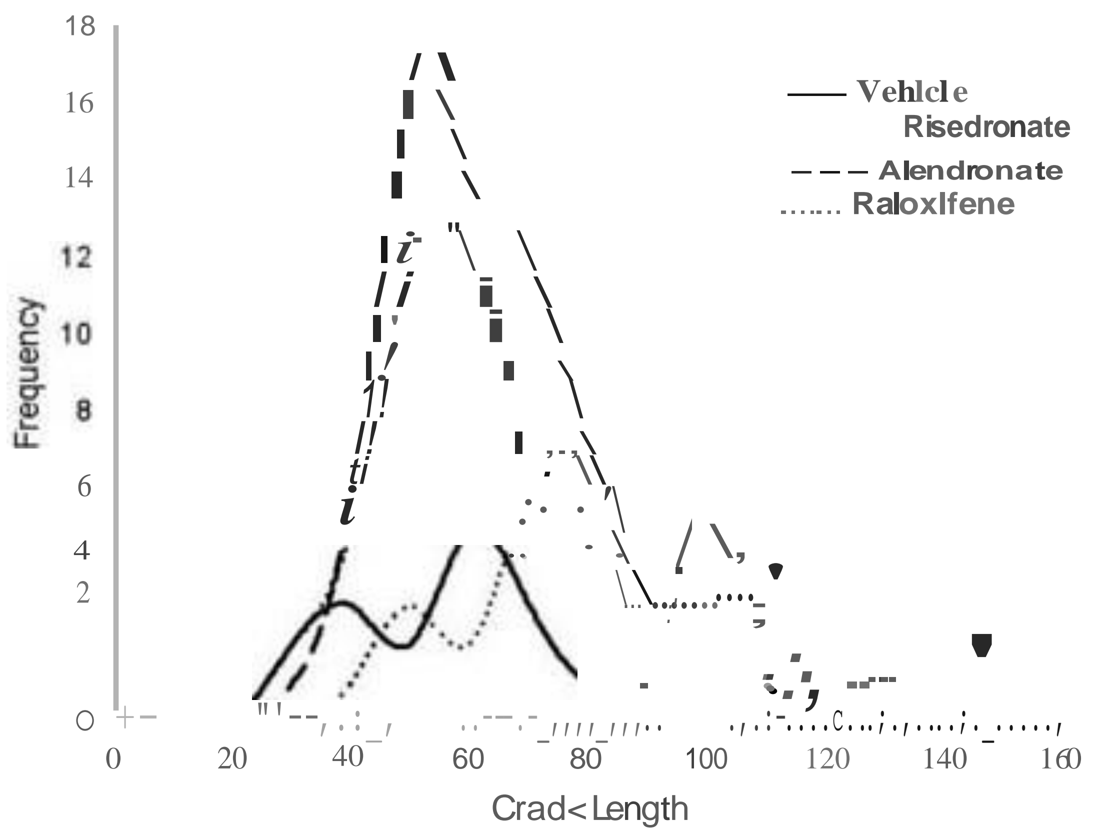

Table 1

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TPkt 1l Trab«:M i.ut morphometf)' or th.e M:colld JuiabM WU1dn

	VtfiiClt	RalOllttnt	RJsteronat	AltnctoNCt	[illegible]
tlVnv,,.	21 Q!09	22 6! 10	228! 1 2	26 4! 10*	0015
TbTl\im	694:. 28	752:.3e	727:.47	779140	0457
Tb N.#	3 17 :009	305!0 13	324!0 14	343!0 11	0 166
0&135,"	454 !100	4 18!091	098!0 16 •	122.!0 23•	<C>000I
	6651:035	645:070	$13:.o eo	583J.0$)	0157
O ''' '''' MAR.""""""	1 56!0 10	139 t0 11	1 1&!0 10•	1 15t009•	0012
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emss...,,..\In',,_	122!t3	99<8	45t 9•	41 t.6 •	<C>000I

l><005 VI Vil\tRtl. fRtl

Table2
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Table 2: Density, geometry, and mineralization of vertebral body

	Vehicle	Ral05	Ris01	"""02	AHOVA
ArN8'C.gem'	033!001	03t .:. 00t	033!001	0.36.1001-	0005
Tccel .mgfcml	572: 15	seo: 1.t	00t .:. 9	6:.9•	0015
Trat>ocul.lr ..eMO.mQ1Cn'	341!1	156 II	36'1.: 4	364!6	0096
Ttllbecutar ashwtlA..	608.:06	622 :.05	630:06•	630:06•	0037

p<005"'•Vtn.'Roi,<Ris

Table3

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Table 3: Trabecular microdamage of the third lumbar vertebra

	Vellcle	Rolo>Qfene	[illegible]	Alt!'OoMle	ANOVA
Cratk nllllber. *li*	; 5,05	2 2'06	4 0 a 0 7 •	50•08•	0002
Ctacklefl91h,.,m	$16:4 5	166:.631Ci11	61.3•56	56S.t.J 4	0001
OOCkOlnll\Y.Mnrr'	0 130!004	0 18Sl.:.OOS	035%006•	045*-0 08•	0001

p t O OS vs 'Ven, "Ral RIS. ALN

Table 4

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Table 4: Biomechanical properties of the fourth lumbar vertebra

	Vtl'IC:lt	RdOlltltnt	sterono:•	• ••	A1¥JVA
IA!m11»load. N	3721!.196	-1097 !. 135	4068 !.274	-4092 16"1	O•n
Stil!ne$$.N'mm	7093.;.530	8798,:.431•	8807 ¿718•	9206:.451•	O"'"
Et*9/ıo l..l.	1742 157	18041 102	1593 140	16441QO	0"3$
l.l>mosi:rm 'evrv	i 481008	16-010	162:.0 10	139:.007	0148
t10!Uus /BVT\l	4f 838	S09!40	1 3!47	44 9:1S	0201

p OOS...s-V611