# Short-courses of dexamethasone abolish bisphosphonate-induced reductions in bone toughness.

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**ABSTRACT**

Atypical femoral fractures, which display characteristics of brittle material failure, have been associated with potent remodeling suppression drugs. Given the millions of individuals treated with this class of drugs it is likely that other factors play a role in these fractures. Some evidence suggests concomitant use of corticosteroids may contribute to the pathogenesis although data in this area is lacking. The goal of this study was to assess the combined role of bisphosphonates and dexamethasone on bone mechanical properties. Skeletally mature beagle dogs were either untreated controls, or treated with zoledronic acid (ZOL), dexamethasone (DEX), or ZOL + DEX. Zoledronic acid (0.06 mg/kg) was given monthly via IV infusion for 9 months. DEX (5 mg) was administered daily for one week during each of the last three months of the 9 month experiment. Ribs were harvested and assessed for bone geometry, mechanical properties, and remodeling rate (n=3-6 specimens per group). DEX significantly suppressed intracortical remodeling compared to vehicle controls while both ZOL and the combination of DEX+ZOL nearly abolished intracortical remodeling. ZOL treatment resulted in significantly lower bone toughness, determined from 3-point bending tests, compared to all other treatment groups while the toughness in ZOL+DEX animals was identical to those of untreated controls. These findings suggest not only that short-courses of dexamethasone do not adversely affect toughness in the setting of bisphosphonates, they actually reverse the adverse effects of its treatment. Understanding the mechanism for this tissue-level effect could lead to novels approaches for reducing the risk of atypical femoral fractures.

**Key words:** zoledronic acid, mechanical properties, toughness, remodeling suppression

## INTRODUCTION

Atypical femoral fractures have been associated with potent remodeling suppression pharmaceutical drugs such as bisphosphonates [1]. Although a definitive causal link between the pharmaceutical agents and these fractures does not exist, several reports provide intriguing data regarding the proposed association [2-7]. The mechanism underlying these atypical femoral fractures remains unclear although if anti-remodeling agents do in fact play a role it is likely that tissue-level changes related to low bone remodeling contribute. What is clear about atypical femoral fractures is that they are catastrophic and debilitating [1].

Given the millions of individuals treated with anti-remodeling agents, the rarity of atypical femoral fractures suggests they are multi-factorial. The 2010 American Society for Bone and Mineral Research task force report on fractures identified glucocorticoid treatment as one potential co-factor [1]. Chronic high dose glucocorticoid treatment has well-established negative effects including increased osteoclast and decreased osteoblast activity, induction of osteocyte apoptosis, loss of bone mass, and reductions in mechanical properties [8]. Although bisphosphonates are approved for reducing fracture risk associated with glucocorticoid-induced osteoporosis [9], they produce their beneficial effect in this setting by suppressing bone loss and thus maintaining bone mass. The effects of bisphosphonates on material properties, those independent of bone mass, in the setting of concomitant glucocorticoid treatment have not been extensively studied [10].

Pre-clinical data demonstrate bisphosphonates reduce bone toughness, the ability of the bone material to absorb energy prior to fracture [11-16]. Reduced toughness is analogous to increased brittleness and thus these data are consistent with the fracture characteristics of atypical femoral fractures. Recently, our laboratory undertook a study focused on a condition known as osteonecrosis of the jaw in which we

treated animals with a combined of bisphosphonates and glucocorticoids [17]. Using material saved from this experiment, the aim of this study was to test the hypothesis that the combination of zoledronic acid and dexamethasone would significantly reduce bone toughness more than either treatment alone.

## MATERIALS AND METHODS

### *Animals*

Twenty-four skeletally mature female beagles (~ 1-2 years old) were purchased from Marshall Farms USA (North Rose, NY) and housed throughout the experiment in environmentally controlled rooms at Indiana University School of Medicine's AAALAC accredited facility. All animal procedures were approved prior to the study by the IU School of Medicine Animal Care and Use Committee.

### *Experimental Design*

Following two weeks of acclimatization, animals were assigned to untreated control (CON; n=6), zoledronic acid (ZOL; n=6), dexamethasone (DEX; n=6) or zoledronic acid plus dexamethasone (ZOL+DEX; n=6) treatment groups. The primary goal of this study was to investigate the combined effects of ZOL and DEX on oral wound healing [17], thus ZOL was administered via IV infusion at a dose of 0.06 mg/kg (40 mL total volume over 15 minutes), which corresponds to the dose used in cancer patients, adjusted on a mg/kg basis [18]. ZOL was infused every 2 weeks, roughly twice as frequently as used clinically, in order to maximize drug exposure during the experimental period. Dexamethasone was given via daily oral dosing (5 mg) for the first seven days of the $7^{th}$, $8^{th}$, and $9^{th}$ months of the experiment. This dosing was based on a modified version of a low-dose protocol used clinically in multiple myeloma pateints [19].

All animals were administered calcein (5 mg/kg, intravenous) using a 2-12-2-5 schedule, meaning it was administered on two consecutive days, 12 days were allowed to pass, injected for another two consecutive days, and then 5 days passed prior to euthanasia. After 9 months animals were euthanized by intravenous administration of sodium pentobarbital and the right and left $9^{th}$ rib were dissected free and placed in 70% ethanol and frozen PBS soaked gauze, respectively. Bones from all animals were available for histology (n=6/group) but bones for mechanical testing were only saved for a subset (n=3) of both control and dexamethasone groups. The rib was chosen because it has traditionally shown consistent alterations in mechanical properties associated with bisphosphonate-treatment [11,15].

*Peripheral Quantitative Computed Tomography (pQCT)*

Volumetric bone density and geometry were quantified using a Norland Stratec XCT Research SA+ pQCT (Stratec Electronics, Birkenfeld, Germany). Specimens were cut to 40 mm in length and a single scan at the mid-point was conducted using a voxel size of 0.07 x 0.07 x 0.50 mm. Total bone mineral content (BMC, mg/mm), total volumetric bone mineral density (vBMD, mg/ $cm^3$), bone area (BA, $mm^2$), periosteal circumference, cortical thickness, and cross-sectional moment of inertia (CSMI, $mm^4$) were obtained using standard scanner software and segmentation algorithms (Cortbd mode with a threshold of 710 mg/$cm^3$) to separate cortical bone from marrow. Bone diameter was measured using digital calipers. Diameter and CSMI values were calculated in the plane perpendicular to the axis of three-point bending.

*Biomechanical Testing*

Three-point bending was conducted in accordance with our previously described method [11]. Briefly, bones were thawed to room temperature in saline and then placed on a

three-point bending fixture (bottom support span = 25 mm) with the convex rib surface facing up. Specimens were loaded to failure at a displacement rate of 20 mm/minute, and data were collected at 10 Hz. Structural mechanical properties, ultimate load, stiffness, displacement (pre-yield, post-yield, and total), and energy absorption (pre-yield, post-yield, and total) were determined from the load–deformation curves using standard definitions [20]. Material-level properties, ultimate stress, modulus, and toughness (pre-yield, post-yield, and total), were estimated by normalizing the structural parameters using standard equations that include bone diameter and CSMI [20].

*Histology*

Ribs were processed for assessment of fluorochrome labels using standard methods of undecalcified histology [21]. Two serial semi-thin sections (80–100 μm) were cut approximately 5 mm apart using a diamond wire saw. Fluorochrome labels were assessed using an analysis system (Bioquant OSTEO 7.20.10; Bioquant Image Analysis, Nashville, TN) attached to a microscope equipped with an ultraviolet light source. Dynamic histomorphometric measures of the intracortical bone envelope was made on one section per animal using methods previously published and in accordance with recommended standards [22,23].

*Statistics*

Statistical tests were performed using SAS software (SAS Institute, Inc.). Histological parameters were compared among groups using one-way ANOVA with Fisher's protected least significant difference (pLSD) post-hoc tests. Due to unequal and small sample sizes among groups for geometry and mechanical testing parameters, data were assessed using Kruskal Wallis non-parametric tests followed by Mann-Whitney tests to

determine group differences. For all tests, $p < 0.05$ was considered to be statistically significant.

**RESULTS**

Material-level estimates of toughness, the ability of the material to absorb energy, were significantly lower in ZOL-treated animals compared to vehicle controls (Figure 1). These effects were driven by significant differences in post-yield toughness with no significant difference among groups in pre-yield toughness. The negative effects of ZOL on total and post-yield toughness were abolished in animals treated with both ZOL+DEX, which had levels of toughness significantly higher than ZOL and statistically similar to VEH animals (Figure 1). There was no significant difference among groups for structural-level energy to fracture (Figure 1) indicating that the alterations in geometry with ZOL were sufficient to maintain whole-bone energy absorption.

There was no significant treatment effect on whole bone stiffness or material-level modulus (Table 1). While there was no difference among groups in ultimate load, the material-level strength (ultimate stress) was significantly higher in both ZOL treated groups compared to DEX-treatment alone (Table 1). Pre-yield displacement was similar among groups while post-yield displacement was significantly lower in ZOL compared to both VEH and DEX; the combination of ZOL+DEX returned post-yield displacement to VEH-level values.

Mid-diaphysis cortical BMC, volumetric BMD cortical bone area, and cortical thickness were all significantly higher in both ZOL-treated groups compared to vehicle control (Table 2). There was no significant difference among groups for periosteal circumference or cross-sectional moment of inertia.

Intracortical labeled osteon number and bone formation rate were both significantly lower in DEX-treated animals compared to control (Table 3). Labeled osteon number in ZOL and ZOL+DEX groups was 98% lower than control. The lack of double labeled osteons negated the calculation of bone formation rate in the ZOL and DEX+ZOL groups.

**DISCUSSION**

The current study provides evidence that bisphosphonate-induced reductions in toughness, which have been consistently shown in multiple studies [11-16], can be overcome with *in vivo* treatment. Using a short-course dexamethasone protocol, one that is consistent with what is used clinically in some situations [19], the reductions in toughness brought about by zoledronate treatment were completely abolished. The effect of dexamethasone in the absence of zoledronate treatment was unimpressive, although the low number of samples (n=3) in this group likely contributed to the lack of statistical significance. However, when combined with zoledronate, dexamethasone raised toughness to values that were in line with untreated controls and significantly different than ZOL-treatment alone. We interpret these data as evidence that dexamethasone is interacting uniquely in bone that has been exposed to bisphosphonate.

The underlying mechanism for dexamethasone's reversal of zoledronate-induced toughness reduction is unclear. Previous work has shown that glucocorticoid treatment significantly reduces the modulus surrounding the osteocyte perilacunar matrix in trabecular bone of ovariectomized rats [10]. This is consistent with our current results in which dexamethasone non-significantly reduces the estimated modulus (calculated from a stress/strain curve) relative to animals treated with zoledronate alone. The perilacunar

matrix plays an important role in determining the tissue-level properties of bone [10,24,25]. Reductions in perilacunar modulus could be expected to lower the strain concentrations around the lacunae and reduce crack propagation through the matrix and effectively toughening the bone.

Glucocorticoids have well-documented adverse effects on bone cells, bone mass, and mechanical properties [8]. The majority of these data are derived from chronic, high-dose treatment. The effects of short-course dosing such as those used in the current experiment have not been explored with respect to skeletal properties yet evidence exists showing dose-dependent effects on osteocytes in cortical bone [26]. It is not clear if the results of the current study are specific to low-dose treatment, or whether even higher doses when combined with the potent anti-remodeling effects of ZOL would produce similar effects. The anti-remodeling effect of zoledronate effectively abolishes the enhanced osteoclast activity normally induced by dexamethasone and is the basis for its efficacy in glucocorticoid-induced bone disease [8,9]. It is assumed that this reduction in bone loss is the basis for reduced fractures with bisphosphonates in glucocorticoid-treated patients. The current data raise the possibility that when combined with bisphosphonates, glucocorticoids exert mechanically-beneficial modifications of material-level properties that are independent of bone mass.

Although glucocorticoid treatment has been suggested as a potential co-factor for atypical femoral fractures [1], data to support such a link remain equivocal. Several reports have documented that between 8 and 30% of patients with atypical sub-trochanteric fractures were either on or had been on glucocorticoid treatment in the past year [2,3,27]. These cross-sectional studies are limited – but at face value certainly do not suggest a strong link. On the other hand, the results of this current study would suggest those on glucocorticoid treatment should actually be protected from these types fractures that have characteristics of brittle material failure. It is important to note that

our data do not address how the duration or dose of bisphosphonate treatment would affect the ability of glucocorticoids to reverse the effect, or if the effects of glucocorticoids are similar at higher doses, such as consistent, high-dose treatment often used clinically.

Previous work has consistently documented reduced bone toughness in bisphosphonate-treated dogs [11-16]. These experiments have utilized oral bisphosphonates at doses at and above those used clinically for treatment of post-menopausal osteoporosis. The current work shows similar results with intravenous zoledronate, albeit at doses that exceed those used even in cancer patients. The proposed mechanisms for reduced toughness with alendronate are related to its suppression of remodeling. Thus the findings in the current study with zoledronate are not unexpected given that IV zoledronate suppresses remodeling more rapidly and dramatically compared to oral alendronate [22].

The results of this experiment should be considered within the context of some limitations. First, our study utilized intact female beagles and thus the bisphosphonates were given in an animal with normal remodeling as opposed to estrogen-deficient high bone remodeling. Secondly, we used doses that far-exceeded those used clinically even for cancer patients. As the reductions in toughness in this study are consistent with those from previous studies that used clinically relevant dosing of alendronate [11], it is unlikely that high doses played a major role in the findings. Although the concept of reduced mechanical properties in bisphosphonate treated animals is consistent with atypical femoral fractures, our study utilized ribs while the clinically relevant site is the proximal femur. Whether similar beneficial effects of DEX treatment would be observed at the femur in a similar model is unclear and unfortunately femora from this experiment were not saved. Finally, as mechanical property assessment was not the original focus of the study the sample size was not powered for such analysis with two groups having six specimens and the other two having only three This may have produced

underpowered analyses for some mechanical parameters although the effect on toughness was sufficiently large to allow detection even with this limited sample size.

In conclusion we show that the reductions in toughness brought about by zoledronate treatment in dogs can be completely abolished using short-course dexamethasone treatment. These results could have significant implications given the emergence of atypical femoral fractures as a rare but significant side effect associated with remodeling suppression.

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**Figure Legend**

**Figure 1.** Structural (A-C) and material (D-F) properties of rib as determined by 3 point bending. There was no significant difference among groups for whole bone energy to fracture ($p = 0.09$), pre-yield energy ($p = 0.93$) or post-yield energy ($p = 0.07$). The material-level equivalent, toughness, was significantly different among groups for both total toughness ($p = 0.01$) and post-yield toughness ($p = 0.01$) but not pre-yield toughness ($p = 0.71$). Point plots represent individual test values. $P < 0.05$ versus VEH (*), DEX (^), and ZOL ($).

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**Table 1:** Bone mechanical properties assessed by 3 point bending

| | Control (n=3) | DEX (n=3) | ZOL (n=6) | ZOL + DEX (n=6) | p value |
|---|---|---|---|---|---|
| Ultimate force, N | 133 (83-219) | 192 (168 – 205) | 206 (162 – 249) | 205 (166-254) | 0.487 |
| Stiffness, N/mm | 225 (143-243) | 361 (328 – 367) | 237 (175 – 261) | 219 (93 – 296) | 0.051 |
| Pre-yield displacement, mm | 0.41 (0.36 – 0.48) | 0.36 (0.08 – 0.38) | 0.22 (0.18 – 0.56) | 0.22 (0.13 – 0.53) | 0.719 |
| Post-yield displacement, mm | 5.7 (3.7 – 7.1) | 4.6 (3.4 – 4.7) | 2.7 *^ (1.3 – 3.8) | 3.6 (2.9 – 6.4) | 0.034 |
| Ultimate stress, MPa | 140 (105 – 168) | 144 (143-144) | 200 ^ (149 – 274) | 207 *^ (178 – 239) | 0.012 |
| Modulus, MPa | 4392 (3458 – 4404) | 3975 (3823 – 4808) | 6140 (5152 – 8993) | 5723 (2192 – 6517) | 0.053 |

All data presented as median (minimum – maximum). $p < 0.05$ vs (*) control, (^) DEX.

**Table 2:** Rib cortical bone density and geometry

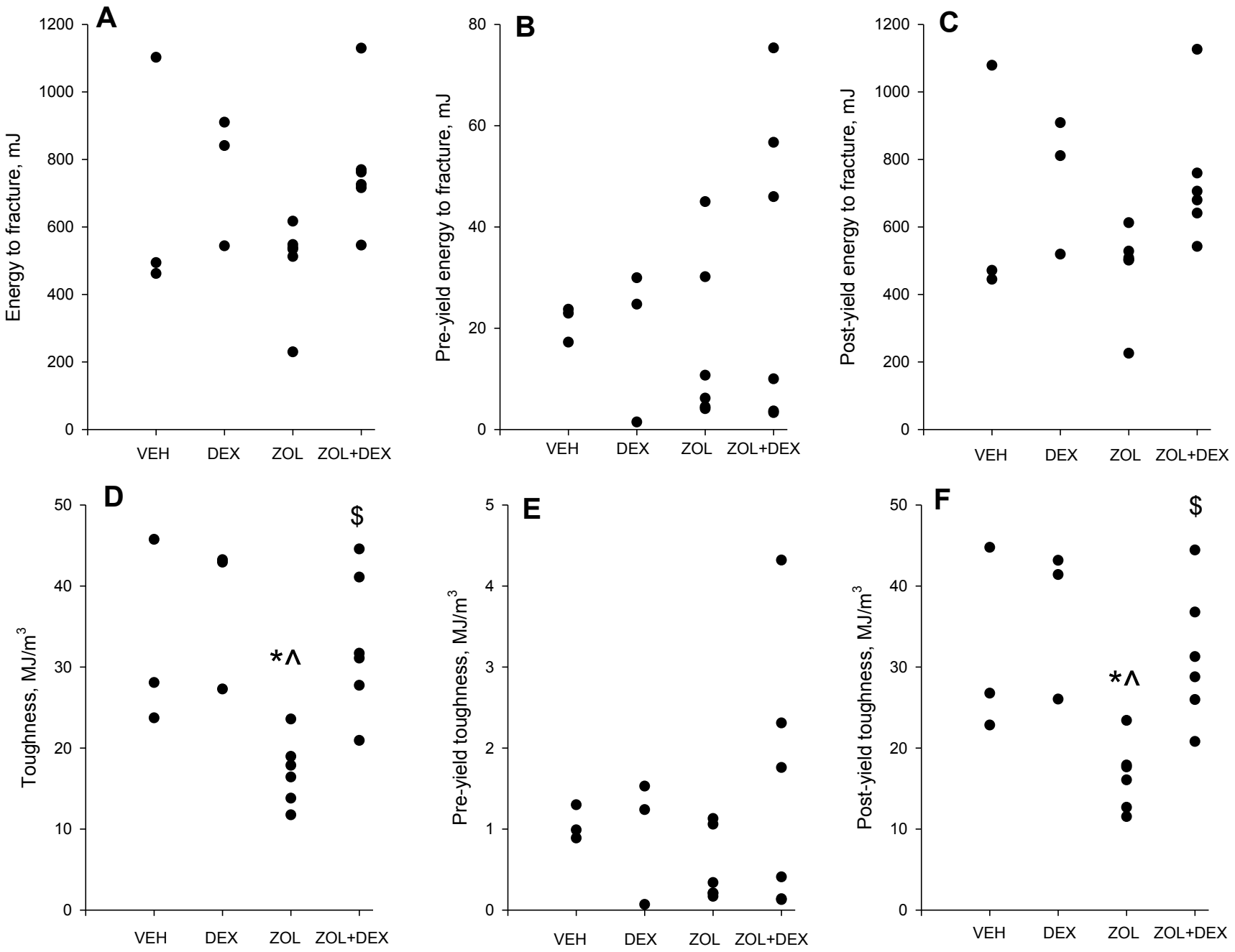
| | Control (n=3) | DEX (n=3) | ZOL (n=6) | ZOL + DEX (n=6) | p value |
|---|---|---|---|---|---|
| vBMD, $mg/cm^3$ | 1105 (1091-1106) | 1105 (1132 – 1195) | 1186 * (1173-1224) | 1174 * (1144 – 1197) | 0.027 |
| BMC, mg/mm | 8.25 (6.49 – 8.28) | 9.5 (9.46-10.29) | 11.46 * (9.65 – 12.68) | 10.52 * (9.86-12.42) | 0.016 |
| Bone area, $mm^2$ | 7.49 (5.88- 7.56) | 8.36 (7.95 – 8.99) | 9.67 * (8.22-10.77) | 9.06 * (8.37 – 10.49) | 0.029 |
| Periosteal circumference, mm | 11.27 (10.29 – 12.6) | 13.16 (12.18 – 13.86) | 11.69 (11.34 – 13.72) | 12.46 (11.9 – 12.88) | 0.129 |
| Cortical thickness, mm | 0.68 (0.66 – 0.76) | 0.73 (0.67 – 0.76) | 0.98 * (0.87 – 1.12) | 0.87 * (0.76 – 1.00) | 0.011 |
| CSMI, $mm^4$ | 17.1 (10.8 – 23.4) | 30.3 (22.7 – 31.9) | 21.2 (18.6 – 32.0) | 26.4 (21.8 – 29.9) | 0.172 |

All data presented as median (minimum – maximum). $p < 0.05$ vs (*) control.

**Table 3:** Dynamic histomorphometry data of intra-cortical rib remodeling

| | Control (n=6) | DEX (n=6) | ZOL (n=6) | ZOL + DEX (n=6) |
|---|---|---|---|---|
| Labeled osteon number, #/mm$^2$ | 2.88 ± 0.51 | 1.26 ± 0.41 * | 0.07 ± 0.02 *^ | 0.05 ± 0.02 *^ |
| Bone formation rate, %/year | 23.7 ± 4.6 | 14.6 ± 3.6 * | NA | NA |

Data presented as mean ± SE. $p < 0.05$ vs (*) control or (^) DEX. NA means there were no double label osteons in any animal and thus bone formation rate could not be calculated.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VEH
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ZOL
ZOL+DEX
B
Pre-yield energy to fracture, mJ
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40
60
80
VEH
DEX
ZOL
ZOL+DEX
C
Post-yield energy to fracture, mJ
0
200
400
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800
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1200
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