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The effects of estrogen deficiency and bisphosphonate treatment on tissue mineralisation and stiffness in an ovine model of osteoporosis.

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1 The Effects of Estrogen Deficiency and Bisphosphonate Treatment on Tissue Mineralisation
2 and Modulus in Trabecular Bone from an Ovine Model of Osteoporosis

3

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26 **Abstract**

27 While much research has been dedicated to understanding osteoporosis, the nature of mineral
28 distribution and the mechanical property variation in diseased bone is poorly understood. The
29 current study aimed to determine the effect of estrogen deficiency and bisphosphonate
30 therapy on bone tissue properties using an ovine model of osteoporosis. Skeletally mature
31 animals (4+ years) were divided into an ovariectomy group (ovx, n=20) and a non treatment
32 control group (control, n=20). A zoledronic acid treated group was also included in which
33 animals were estrogen deficient for 20 months prior to receiving treatment (Zol, n=4). Half of
34 the control and ovx groups were euthanized 12 or 31 months post-operatively and all Zol
35 animals were euthanised at 31 months. Individual trabeculae were removed from the
36 proximal femur and at specific locations across the width of the trabeculae. The mineral
37 content was measured using quantitative backscatter electron imaging and the modulus was
38 measured using nanoindentation.

39 The spatial distribution of tissue modulus and mineral content in bone from ovariectomised
40 animals was similar to control. However, ovariectomy significantly reduced the overall
41 mineral content and tissue modulus relative to the control group after 12 months.
42 Interestingly, significant differences were not maintained 31 months post-OVX. Treatment
43 with zoledronic acid increased the mineral content and tissue modulus relative to both the
44 ovariectomised and control groups. Zoledronic acid was also found to alter the mineral and
45 modulus gradients normally associated with healthy bone tissue. The current study provides
46 evidence that both estrogen deficiency and zoledronic acid therapy significantly alter mineral
47 content and the mechanical properties of trabecular tissue.

48

49

50

51 **Introduction**

52 Trabecular bone has a complex lamellar structure, and the degree of lamellar mineralisation
53 increases with the distance from the trabecular surface to their centres (Renders et al., 2006).
54 The distribution of mineral provides an inhomogeneous mechanical structure with the elastic
55 modulus of trabecular bone increasing towards the centre of trabeculae (Brennan et al., 2009)
56 along the same path as the degree of mineralisation (Mulder et al., 2007).

57

58 Osteoporosis is a skeletal disease characterised by an imbalance between bone resorption and
59 formation, which results in bone loss and deterioration of the trabecular microarchitecture
60 and leads to an increased risk of fracture. Osteoporotic bone is believed to be less mineralised
61 than healthy bone due to the increase in bone turnover seen following estrogen deficiency
62 (Type I osteoporosis) and also the imbalance between resorption and formation that occurs
63 with age (Type II osteoporosis). Consistent with this belief, mineral content is reduced in
64 bone tissue from primate models of postmenopausal osteoporosis (Type I) and
65 ovariectomised rats (Gadeleta et al., 2000; Cheng et al., 2009). However, others have found
66 no change in mineral content (Li and Aspden 1997) or an increase in the mineral content
67 following estrogen deficiency (Dickenson et al., 1981; Boyde et al., 1998; Ciarelli et al.,
68 2003; McNamara et al., 2006). Trabecular mineralisation also increases with age (Grynpas
69 1993). While overall bone mass and bone mineral density is reduced during estrogen
70 deficiency, the yield strength and elastic modulus of the remaining tissue increased by 40–
71 90% relative to controls in an ovariectomized rat model of osteoporosis (McNamara et al.,
72 2006). While variations in experimental methods, animal model or anatomical location might
73 explain the discrepancies seen previously, it is still unclear how bone tissue mineral content
74 and mechanical properties are altered during osteoporosis. Cellular processes and molecular
75 signalling pathways governing pathological bone resorption have been identified to a certain

76 extent; however, further research is required to comprehensively characterise the events that
77 lead to bone fracture (McNamara 2010).

78 A highly successful treatment for reducing the occurrence of osteoporotic fractures is the
79 family of anti-resorptive drugs known as bisphosphonates which are potent inhibitors of
80 osteoclast activity and thus bone resorption. Mineralisation is increased following treatment
81 with bisphosphonates such as pamidronate (Grynpas et al., 1992), risedronate and alendronate
82 (Burr et al., 2003; Day et al., 2004; Spadaro et al., 2006; Yao et al., 2006). In humans, the
83 reduction of bone turnover by risedronate increased the mineral content in the iliac crest
84 (Borah et al., 2005). The newest generation and most potent bisphosphonate, zoledronic acid,
85 has also proven successful at preventing loss of bone structure and mechanical strength in
86 vertebral and long bones of ovariectomised rats (Hornby et al., 2003) and preventing bone
87 loss in postmenopausal women (Reid et al., 2002).

88

89 In the current study we test the hypothesis that estrogen deficiency and zoledronic acid
90 therapy alter bone tissue mineral content and microstructural modulus in an ovine
91 ovariectomised model. Scanning Electron Microscopy with quantitative backscatter electron
92 imaging (qBEI) and nanoindentation were carried out across the width of individual
93 trabeculae from femoral bone of ovariectomized (OVX) sheep, OVX sheep treated with
94 zoledronic acid and control animals to determine bone tissue mineral content and
95 microstructural stiffness. Furthermore the results were compared with a previously published
96 study from this group which examined the tissue modulus at an earlier time point (Brennan et
97 al., 2009).

98

99

100 **Materials and Methods**

101 Forty four skeletally mature (aged 4-5 years) mixed breed ewes were randomly assigned into
102 one of two groups, ovariectomy (OVX, n=24) or control (control, n=20) on which no
103 operative procedure was carried out. All surgery was performed under an animal licence
104 granted by the Irish Department of Health and subject to ethical approval. Animals were
105 maintained at pasture and feeding and activity levels were the same for both groups. Twelve
106 months post ovariectomy, half of each group was sacrificed at which point the animals were
107 aged 5-6 years. Twenty months post-OVX, four OVX animals were randomly selected to
108 serve as a bisphosphonate treated group, OVX plus zoledronic acid (Zol; Novartis Pharma,
109 Basel, Switzerland). Each animal received a 5mg dose of zoledronic acid in 100mls of saline
110 infused over 30 minutes via an indwelling jugular catheter. This procedure was repeated for a
111 further four weeks, giving each animal a supra-pharmacological 25mg dose. All remaining
112 animals were sacrificed 31 months post-OVX by which stage animals were 7-8 years of age.
113 All bones were harvested and frozen at -20°C. Individual trabeculae were randomly selected
114 and excised from the anteromedial region of the medullary cavity of the left proximal femur
115 using a scalpel blade and forceps under 30X magnification as was described previously
116 (Brennan et al., 2009).

117

118 **Quantitative Backscatter Electron Imaging (QBEI)**

119 Five trabeculae from each animal were assessed. Individual trabeculae were embedded in
120 polymethylmethacrylate (PMMA) and planoparallel cuts were made to reveal a cross section
121 of the trabeculae which was polished. Samples were mounted on individual scanning electron
122 microscopy mounting stubs and were sputter-coated with carbon using a Bio Rad
123 (Microscience Division) Carbon Coater, Model TB500. Quantitative backscatter electron

124 imaging (QBEI) was carried out using a JEOL JSM-5410 LV Scanning Electron Microscope
125 (SEM). A conventional backscattered electron detector (paired semiconductor type; JEOL),
126 as supplied with the JEOL JSM-5410 LV microscope, was employed. An operating voltage
127 of 15kV and a working distance of 10mm were employed during scanning. The grey-level
128 was calibrated according to Roschger *et al* (Roschger et al., 1998). This method involves
129 using two different reference materials, carbon (C) and aluminium (Al), and altering the
130 brightness (offset voltage) and contrast (gain) conditions of the backscattered electron
131 detector amplifier. Prior to testing of the bone samples, the brightness and contrast of the two
132 standards were adjusted to give a grey-level index value of 25 ± 1 and 225 ± 1 for C and Al
133 respectively. Roschger determined that using the calibration technique the % weight calcium
134 within a given sample could be evaluated using Equation 1 where x is the grey-level.

135 Equation 1: $\% \text{ Weight Calcium} = -4.332 + 0.1733x$

136 Bone samples were mounted on stubs in the same sample holders as the standards and
137 scanned by qBEI. Measurements of gray level were taken across the width of the trabeculae:
138 in an outer ring (Superficial), half way towards the centre of the trabeculae (Intermediate) and
139 in the centre of the trabeculae (Centre) (Figure 1).

140 Scanning electron microscopy images of a typical trabecular cross section show the regions
141 which were sampled (Figure 1). On the left is a low magnification image where the entire
142 cross section of the individual trabeculae can be visualised. On the right hand side, a section
143 of bone has been magnified and the individual lamellae are evident and can be clearly
144 distinguished from neighbouring lamellae. At each point where a qBEI scan was performed a
145 histogram of gray levels was obtained. The grey-level which corresponded to the maximum
146 pixel count was analysed to calculate the % weight calcium, indicative of mineral content,
147 according to the Roschger equation (Equation 1).

148 Nanoindentation

149 Five trabeculae from each animal were assessed. Individual trabeculae were mounted
150 vertically in non-infiltrating dental stone (Suprastone, Kerr UK Ltd, England) and were cut
151 using a diamond saw to create a cross section of the trabeculum. The surface was polished
152 with a series of graded polishing cloths until finally a 0.25 μ m diamond suspension was used.
153 A Nano Indenter XP (MTS Systems, Oakridge, TN) was used with a load and displacement
154 resolution of 0.05 μ N and 0.01nm respectively. The indenter tip chosen was an AccuTip™
155 Berkovich diamond indenter tip, with defined elastic modulus of 1141GPa, a Poisson's ratio
156 equal to 0.07 and a radius of <50nm. A permanent hardness impression was made by driving
157 the indenter tip into the sample for 90 seconds to a maximum load of 20mN, holding for 120
158 seconds and unloading. This cycle was repeated 3 times at each location and the Young's
159 Modulus (E) was determined on the final unloading segment assuming a Poisson's ratio for
160 bone of 0.3. Indents were made across the individual trabeculae in the regions as described
161 above in QBEL.

162

163 Statistics

164 To test for the magnitude of variance of intra- and inter-specimen differences (between sheep
165 and between regions), a nested ANOVA was performed (Minitab® Statistical Software). This
166 analysis took into account the repeated measures made in each region and the multiple
167 trabeculae analysed per sheep. A posthoc t-test was used to determine significant interactions.
168 A p value of ≤ 0.05 was considered statistically significant.

169 **Results**

170 In the 12 month control group mineral content was significantly greater in the intermediate
171 and centre regions than superficially (Figure 2). Comparison between the control and OVX

172 groups showed a significant reduction in mineral content in the ovariectomy groups at all
173 three locations. When the trabeculae were examined as a whole, a significant reduction in
174 mineral content between the control ($19.9\pm 1.7\%$) and OVX groups ($17.8\pm 1.1\%$, $p<0.001$)
175 was measured.

176

177 At 31 months, mineral content in the control group was significantly increased in the
178 intermediate and centre regions relative to the superficial region (Figure 2). In the OVX
179 group the mineral content was not significantly different between the superficial and either
180 the intermediate or centre regions, albeit that a strong trend ($p=0.09$) was observed along the
181 same pattern as at 12 months. In the zoledronic acid treated group, mineral content in the
182 centre did not differ significantly from the intermediate and superficial regions.

183 In contrast to the 12 month group, comparisons between the control and OVX groups at 31
184 months found no significant difference in the level of mineralisation at any location.
185 Treatment with zoledronic acid resulted in significantly increased mineralisation, in all three
186 regions, relative to both the OVX and control groups ($p<0.01$). At 31 months no significant
187 difference in the overall mineral content between the control ($19.5\pm 0.9\%$) and OVX groups
188 ($18.4\pm 1.6\%$) was observed. As was the case with the individual locations, the overall
189 mineralisation in the Zol group ($21.4\pm 1.8\%$) was significantly greater than both the control
190 and OVX groups ($p<0.005$).

191

192 The nanoindentation experiments showed results consistent with the qBEI study. The results
193 from the 12 month group have been published previously (Brennan et al., 2009). Tissue
194 modulus increased significantly in the intermediate and centre regions relative to the
195 superficial region in trabeculae from both the control, and OVX groups. Comparison between

196 the groups found that tissue modulus was significantly less in all three locations in the OVX
197 group relative to the controls and overall the modulus was significantly less in the OVX
198 group ($17.3\pm 1.3\text{GPa}$, $p<0.005$) than the controls ($20.7\pm 2.4\text{GPa}$). In the current study we
199 found that at 31 months in the control group the tissue modulus was significantly less in the
200 superficial region than the intermediate and centre locations (Figure 3). In the OVX group the
201 modulus was also significantly less between the superficial region and the other two regions.
202 In the zoledronic acid treated group no significant change in modulus was measured between
203 the intermediate or centre regions relative to the superficial region. 31-months post-OVX the
204 modulus did not differ significantly between the control and OVX groups at any location or
205 overall ($18.6\pm 3.1\text{GPa}$ vs. $17.9\pm 2.5\text{GPa}$). However, treatment with zoledronic acid resulted in
206 a significant increase in modulus at the centre and intermediate locations relative to both the
207 control and OVX groups ($p\leq 0.01$). The overall modulus of the trabeculae treated with
208 zoledronic acid ($21.1\pm 3.1\text{GPa}$) was also significantly greater than the ovariectomised group
209 but not the controls.

210

211 **Discussion**

212

213 The current study found that healthy bone tissue has mineral and modulus gradients that
214 increase from the surface of trabeculae to the centre. A similar pattern was observed in the
215 mineral and modulus profiles of trabeculae from ovariectomised animals. Estrogen deficiency
216 reduced mineral content and tissue modulus across the width of the trabecula relative to the
217 controls. Interestingly, after 31 months of estrogen deficiency the deterioration in material
218 properties was not evident compared to 31 month controls. Zoledronic acid treatment
219 significantly increased mineral content and tissue modulus relative to both OVX and control
220 groups.

221 Qualitative backscatter electron microscopy revealed that mineral content increased
222 significantly towards the centre of healthy trabeculae at both time points. This result is not
223 surprising as at any one time in normal bone about 20% of the trabecular surfaces are
224 undergoing remodelling resulting in the formation of new, less mineralised bone along the
225 surface (Eriksen et al., 1994; Ott 1996). This results in a gradual increase in mineral towards
226 the centre of the trabeculae. A similar pattern was observed with the nanoindentation results
227 where bone modulus increased towards the centre of the trabeculae. These results correspond
228 as expected, as bone mineralisation and modulus are positively correlated (Choi et al., 1990;
229 Follet et al., 2004; Silva et al., 2004; Mulder et al., 2007).

230

231 In the OVX group, QBEI showed a significant increase in mineral from the superficial region
232 to the centre of trabeculae from animals 12 months post surgery. However, 31 months post-
233 OVX no statistical increase in mineral content towards the centre of trabeculae was observed.
234 However, with the mineral content increasing from $17.7\pm 1.7\%$ in the superficial region to
235 $19.3\pm 1.6\%$ in the centre ($p=0.09$), there is a trend towards increased mineral content in the
236 centre of the trabeculae from ovariectomised animals. These results suggest that estrogen
237 deficiency does not alter the mineralisation gradient across the trabeculae as a significant
238 difference in the level of mineral content from the edge of the trabeculae to the centre
239 remains. The nanoindentation results support these results as significant increases in tissue
240 modulus are also measured towards the centre of trabeculae at both time points.

241

242 One of the most interesting results from this study was found by examining the intergroup
243 variations over time. At 12 months a significant reduction in mineral content and modulus in
244 the OVX group relative to the controls was measured. However, at 31 months there was no
245 significant difference in mineral content or modulus between the OVX group and the controls

246 suggesting that some change may take place between 12 and 31 months. Estrogens inhibit
247 bone resorption by decreasing both osteoclast numbers and activity (Krassas and
248 Papadopoulou 2001). Following ovariectomy a reduction in the levels of circulating
249 hormones and an increase in bone resorption are expected. In a related study on the same
250 animals, 12 month post-OVX a significant reduction in the level of circulating 17- β estradiol
251 was measured in these animals immediately prior to sacrifice, which was accompanied by an
252 increase in bone turnover and porosity and a reduction in bone strength (Kennedy et al.,
253 2009). These results corroborate the reduction in mineral content and tissue modulus seen
254 after 12 months. In another related study on the 31 month group animals, bone turnover
255 continued to be elevated in the OVX group relative to the controls (Healy et al., 2010).
256 However, the final bone turnover was lower in the 31 month OVX group than the 12 month
257 OVX group while turnover in the controls was the same at 12 and 31 months. This result is
258 consistent with the current study which did not find a significant reduction in mineral content
259 after 31 months in the OVX group relative to the controls. It is also possible that fundamental
260 changes to the secondary mineralisation process occur following long term estrogen
261 deficiency, which may be a compensatory mechanism by the remaining tissue to return
262 mineralisation to normal levels. This study has highlighted the importance of study duration
263 when determining the effects of estrogen deficiency. These results may help to decipher the
264 apparent discrepancies in mineral content following estrogen deficiency seen by other
265 researchers.

266

267 Zoledronic acid is a potent antiresorptive agent which prevents osteoclast activity. Under
268 normal circumstances, bone remodelling occurs primarily on the trabecular surface. In this
269 study neither mineral content nor tissue modulus increased significantly towards the centre of
270 the trabeculae from animals which were treated with zoledronic acid. Thus, indications exist

271 that trabecular bone is losing the significant mineral and modulus gradients that are
272 associated with healthy trabecular bone. This result is consistent with a recent study which
273 found that bisphosphonate treatment leads to homogeneous mineral distribution in cancellous
274 bone and also that tissue homogenization may negatively impact bone quality (Gourion-
275 Arsiquaud et al., 2010). Furthermore, computational modeling has predicted that inhibition of
276 resorption (for e.g. by antiresorptive agents) can lead to a less heterogenous mineral
277 distribution (Ruffoni et al., 2008).

278

279 In addition, this study showed that mineral content and tissue modulus were significantly
280 increased following bisphosphonate treatment. Recently alendronate treatment has been
281 shown to increase mineral content in ovariectomised rats (Bitto et al., 2008; Anumula et al.,
282 2010). Ibandronate and risedronate also increased the degree of mineralised bone and
283 indentation modulus in ovariectomised rats (Shahnazari et al., 2010). Similarly zoledronic
284 acid increased the bone mineralisation in ovariectomised rats (Cheng et al., 2009). However,
285 in the current study treatment with zoledronic acid not only restored mineral content and
286 modulus to that of control but actually surpassed them. As bisphosphonates suppress bone
287 remodelling, this allows more time for secondary mineralisation to proceed (Boivin and
288 Meunier 2002). Previous work has suggested that the main mechanism of action of nitrogen
289 containing bisphosphonates, of which zoledronic acid is one, is via inhibition of the
290 mevalonate pathway in osteoclasts (Amin et al., 1992; Crick et al., 1997; Fisher et al., 2000)
291 which ultimately prevents osteoclasts from attaching to the bone surface and thus resorbing
292 bone. However, evidence also suggests that bisphosphonates have a direct action on
293 osteoblasts. In vitro studies on human osteoblasts found that zoledronic acid directly affected
294 the proliferation and differentiation of these cells and thereby enhanced their bone forming
295 potential (Reinholz et al., 2000; Pan et al., 2004). Although the exact mechanism by which

296 bisphosphonates affect osteoblasts is unclear, zoledronic acid has also been shown to induce
297 human osteoblast differentiation via inhibition of the mevalonate pathway (Reinholz et al.,
298 2002). In the current study zoledronic acid likely increased tissue mineral content and
299 modulus above control levels either by reducing osteoclast activity to such a level as to allow
300 increased secondary mineralisation to occur or by directly stimulating osteoblasts to produce
301 more mineral. However, the reality is most probably a combination of both mechanisms.

302

303 This study has a number of limitations, one of which is that due to ethical institution
304 guidelines, a sham operated group was not used and rather a non-operated control was
305 included. This was unlikely to have significantly impacted on this study, in particular limb
306 loading, as those animals which underwent ovariectomy were only subject to anaesthesia for
307 a short period of time (<30mins) and once the anaesthesia had worn off, the animals were
308 immediately mobile. The total number of samples tested per group did differ due to the small
309 sample size of the zoledronic acid treated group. While this is a limitation, the strong
310 statistical significance seen would likely only be improved using larger sample sizes. A visual
311 determination of the points for nanoindentation and also QBEI was made rather than
312 employing a computational method. This allowed selection of the location which best
313 represented the area of interest (i.e. superficial, intermediate or centre) whilst avoiding any
314 holes or other artefacts (e.g. lacunae) in the bone. Whilst this does introduce an element of
315 human error, different trabeculae were assessed in the nanoindentation study and the QBEI
316 study, thus the two techniques are not being carried out on the same location. Another
317 limitation of this study was the lack of any hormone analysis after 31 months. However,
318 hormone analysis was carried out after 12 months and this showed conclusively significant
319 reductions in the levels of circulating hormones (Kennedy et al., 2009).

320

321 In conclusion this study has found that both mineral content and tissue modulus increase
322 towards the centre of healthy trabecular bone and also in bone from ovariectomised animals.
323 Ovariectomy was found to significantly reduce mineral content and tissue modulus below
324 that of controls after 12 months. However, after 31 months no significant reductions were
325 found in either mineral content or modulus. This indicates either a reduction in bone turnover
326 between 12 and 31 months or a change in the secondary mineralisation process to return
327 mineral levels to control values and this is worthy of further study. Treatment with zoledronic
328 acid did significantly increase mineral content and tissue modulus relative to both the
329 ovariectomised and control groups. Zoledronic acid treatment also results in a more
330 homogeneous mineral content and modulus across the width of trabeculae. This may have
331 implications for load distribution and fracture resistance at the trabecular level. In conclusion
332 this study provides evidence that estrogen deficiency and zoledronic acid therapy
333 significantly alter the content and distribution of mineral in trabecular tissue.

334

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340 III.

341

342

343 **References**

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488 **Conflict of Interest Statement**

489 None of the authors have any conflict of interests to report.

Figure 1: *Scanning electron microscopy images of cross sections through individual trabeculae. On the left, the entire cross section can be visualised and the sampled regions are depicted. In the image on the right hand side, a higher magnification of the boxed area makes it possible to distinguish the various lamellae of the trabeculae.*

Figure 2: *Bone mineral content across the width of trabeculae from control, ovariectomised and zoledronic acid treated animals at 12 and 31 months post-OVX (* greater than superficial region in that group; # less than control in that region $p \leq 0.05$; ^ greater than control and OVX in that region $p \leq 0.01$).*

Figure 3: *Bone tissue modulus across the width of trabeculae from control, ovariectomised and zoledronic acid treated animals at 12 and 31 months post-OVX (* greater than superficial region in that group; # less than control in that region; ^ greater than control and OVX in that region $p \leq 0.01$).*

Figure 1
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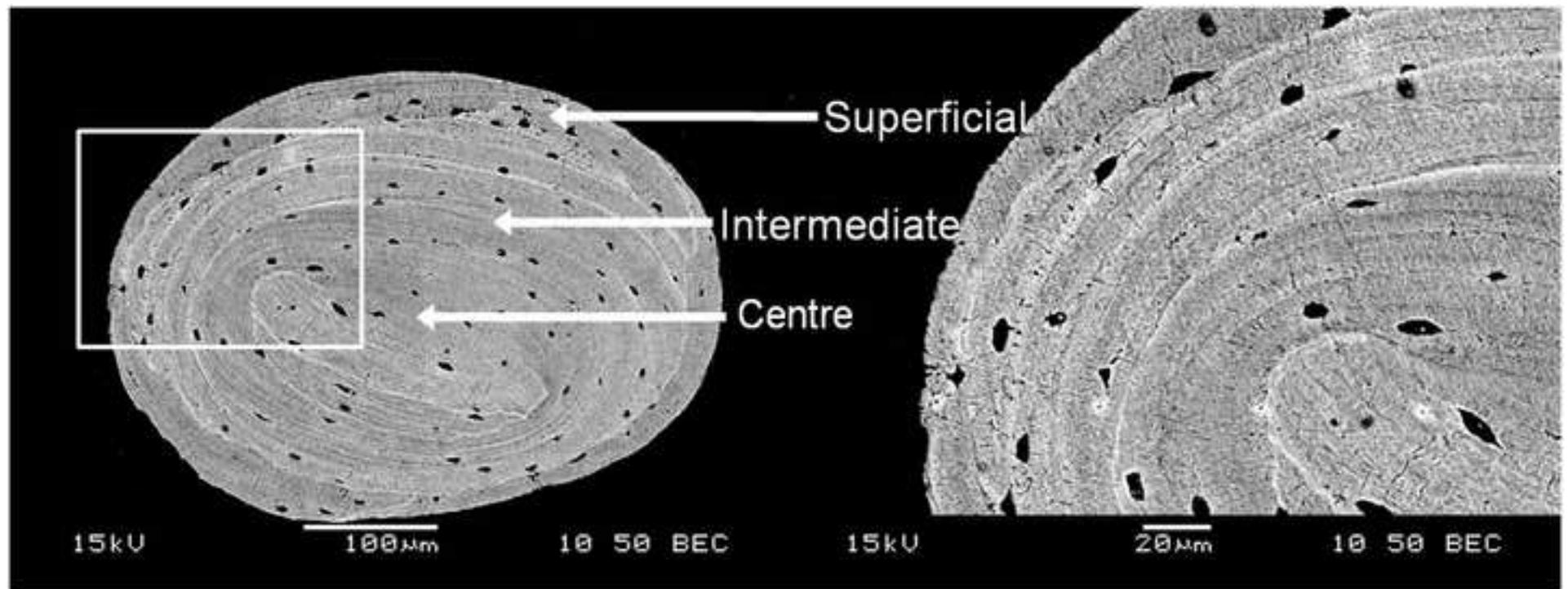


Figure 2
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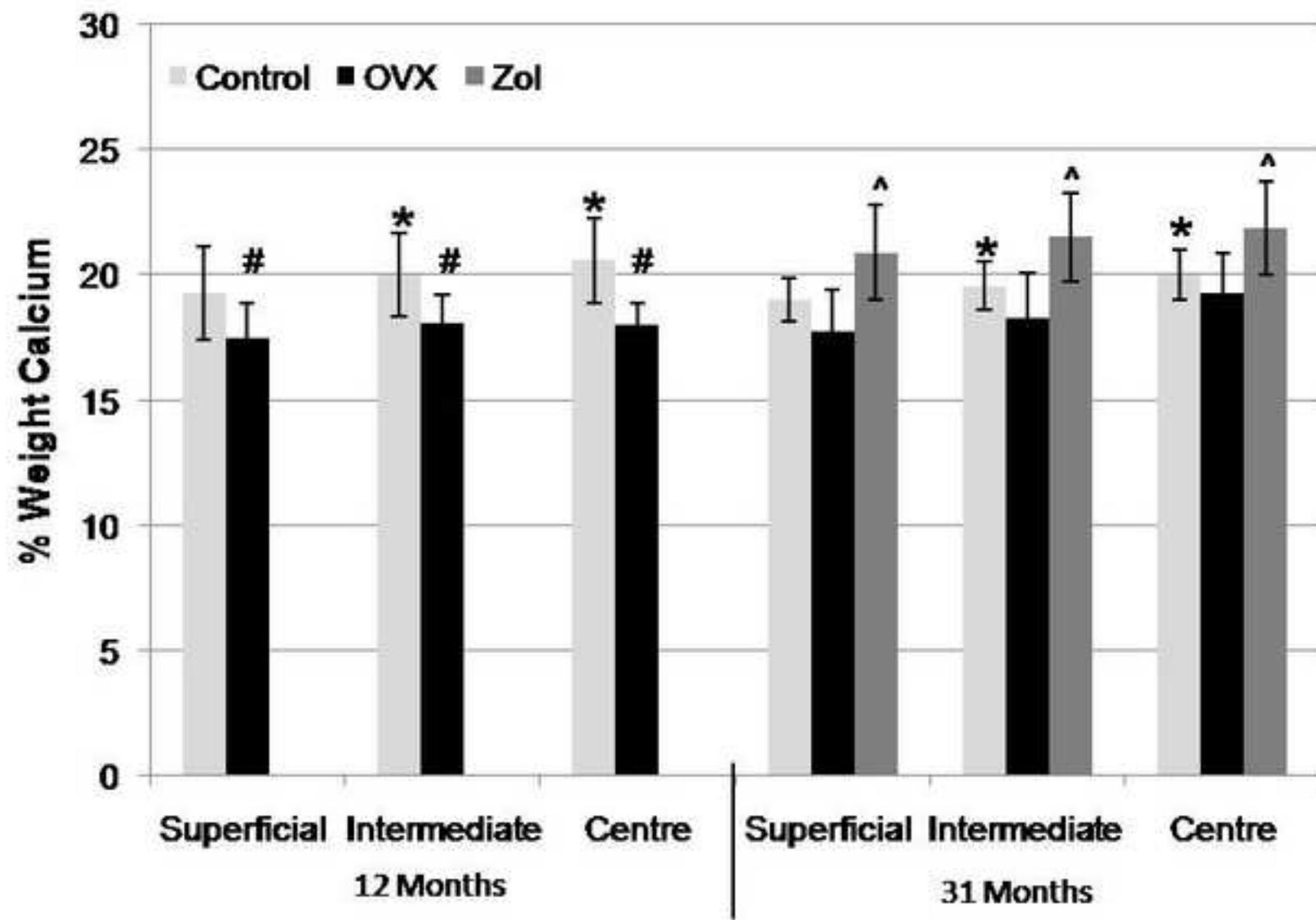
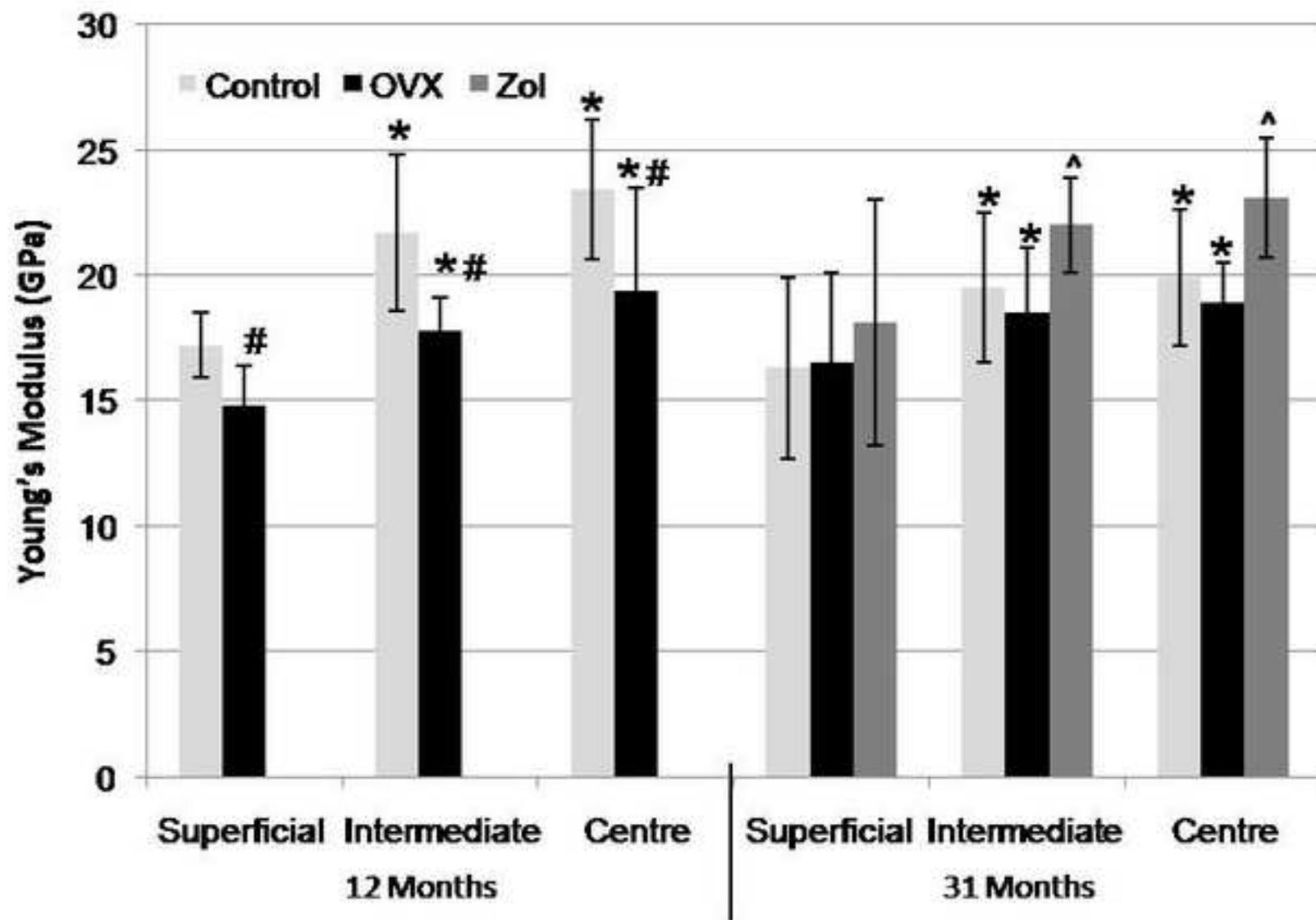


Figure 3
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Conflict of Interest

All authors have no conflicts of interest.

Orlaith Brennan was involved in the conception and design of the ovine model and also the experimental design. She carried out the sample preparation, testing and the analysis and interpretation of data. She drafted the article and approved the final draft.

Oran Kennedy was involved in the conception, design and implementation of the ovine model. He critically revised the manuscript and approved the final draft.

Sue Rackard was involved in the conception and design of the ovine model. She also carried out all procedures on the animals. She critically revised the manuscript and approved the final draft.

Clive Lee was involved in the conception and design of the ovine model and securing funding. He critically revised the manuscript and approved the final draft.

Fergal O'Brien was involved in the conception and design of the ovine model and securing funding. He critically revised the manuscript and approved the final draft.

Laoise McNamara was involved in the conception and design of the SEM study and securing funding. She critically revised the manuscript and approved the final draft.