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

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Abstract

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

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

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

Osteoporosis is a skeletal disease characterised by an imbalance between bone resorption and formation, which results in bone loss and deterioration of the trabecular microarchitecture and leads to an increased risk of fracture. Osteoporotic bone is believed to be less mineralised than healthy bone due to the increase in bone turnover seen following estrogen deficiency (Type I osteoporosis) and also the imbalance between resorption and formation that occurs with age (Type II osteoporosis). Consistent with this belief, mineral content is reduced in bone tissue from primate models of postmenopausal osteoporosis (Type I) and ovariectomised rats (Gadeleta et al., 2000; Cheng et al., 2009). However, others have found no change in mineral content (Li and Aspden 1997) or an increase in the mineral content following estrogen deficiency (Dickenson et al., 1981; Boyde et al., 1998; Ciarelli et al., 2003; McNamara et al., 2006). Trabecular mineralisation also increases with age (Grynpas 1993). While overall bone mass and bone mineral density is reduced during estrogen deficiency, the yield strength and elastic modulus of the remaining tissue increased by 40–90% relative to controls in an ovariectomzied rat model of osteoporosis (McNamara et al., 2006). While variations in experimental methods, animal model or anatomical location might explain the discrepancies seen previously, it is still unclear how bone tissue mineral content and mechanical properties are altered during osteoporosis. Cellular processes and molecular signalling pathways governing pathological bone resorption have been identified to a certain
extent; however, further research is required to comprehensively characterise the events that lead to bone fracture (McNamara 2010).

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

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

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

Quantitative Backscatter Electron Imaging (QBEI)

Five trabeculae from each animal were assessed. Individual trabeculae were embedded in polymethylmethacrylate (PMMA) and planoparallel cuts were made to reveal a cross section of the trabeculae which was polished. Samples were mounted on individual scanning electron microscopy mounting stubs and were sputter-coated with carbon using a Bio Rad (Microscience Division) Carbon Coater, Model TB500. Quantitative backscatter electron
imaging (QBEI) was carried out using a JEOL JSM-5410 LV Scanning Electron Microscope (SEM). A conventional backscattered electron detector (paired semiconductor type: JEOL), as supplied with the JEOL JSM-5410 LV microscope, was employed. An operating voltage of 15kV and a working distance of 10mm were employed during scanning. The grey-level was calibrated according to Roschger et al (Roschger et al., 1998). This method involves using two different reference materials, carbon (C) and aluminium (Al), and altering the brightness (offset voltage) and contrast (gain) conditions of the backscattered electron detector amplifier. Prior to testing of the bone samples, the brightness and contrast of the two standards were adjusted to give a grey-level index value of 25±1 and 225±1 for C and Al respectively. Roschger determined that using the calibration technique the % weight calcium within a given sample could be evaluated using Equation 1 where x is the grey-level.

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

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

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

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

Statistics

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

Results

In the 12 month control group mineral content was significantly greater in the intermediate and centre regions than superficially (Figure 2). Comparison between the control and OVX
groups showed a significant reduction in mineral content in the ovariectomy groups at all three locations. When the trabeculae were examined as a whole, a significant reduction in mineral content between the control (19.9±1.7%) and OVX groups (17.8±1.1%, p<0.001) was measured.

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

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

The nanoindentation experiments showed results consistent with the qBEI study. The results from the 12 month group have been published previously (Brennan et al., 2009). Tissue modulus increased significantly in the intermediate and centre regions relative to the superficial region in trabeculae from both the control, and OVX groups. Comparison between
the groups found that tissue modulus was significantly less in all three locations in the OVX group relative to the controls and overall the modulus was significantly less in the OVX group (17.3±1.3GPa, p<0.005) than the controls (20.7±2.4GPa). In the current study we found that at 31 months in the control group the tissue modulus was significantly less in the superficial region than the intermediate and centre locations (Figure 3). In the OVX group the modulus was also significantly less between the superficial region and the other two regions. In the zoledronic acid treated group no significant change in modulus was measured between the intermediate or centre regions relative to the superficial region. 31-months post-O VX the modulus did not differ significantly between the control and OVX groups at any location or overall (18.6±3.1GPa vs. 17.9±2.5GPa). However, treatment with zoledronic acid resulted in a significant increase in modulus at the centre and intermediate locations relative to both the control and OVX groups (p≤0.01). The overall modulus of the trabeculae treated with zoledronic acid (21.1±3.1GPa) was also significantly greater than the ovariectomised group but not the controls.

Discussion

The current study found that healthy bone tissue has mineral and modulus gradients that increase from the surface of trabeculae to the centre. A similar pattern was observed in the mineral and modulus profiles of trabeculae from ovariectomised animals. Estrogen deficiency reduced mineral content and tissue modulus across the width of the trabecula relative to the controls. Interestingly, after 31 months of estrogen deficiency the deterioration in material properties was not evident compared to 31 month controls. Zoledronic acid treatment significantly increased mineral content and tissue modulus relative to both OVX and control groups.
Qualitative backscatter electron microscopy revealed that mineral content increased significantly towards the centre of healthy trabeculae at both time points. This result is not surprising as at any one time in normal bone about 20% of the trabecular surfaces are undergoing remodelling resulting in the formation of new, less mineralised bone along the surface (Eriksen et al., 1994; Ott 1996). This results in a gradual increase in mineral towards the centre of the trabeculae. A similar pattern was observed with the nanoindentation results where bone modulus increased towards the centre of the trabeculae. These results correspond as expected, as bone mineralisation and modulus are positively correlated (Choi et al., 1990; Follet et al., 2004; Silva et al., 2004; Mulder et al., 2007).

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

One of the most interesting results from this study was found by examining the intergroup variations over time. At 12 months a significant reduction in mineral content and modulus in the OVX group relative to the controls was measured. However, at 31 months there was no significant difference in mineral content or modulus between the OVX group and the controls.
suggesting that some change may take place between 12 and 31 months. Estrogens inhibit bone resorption by decreasing both osteoclast numbers and activity (Krassas and Papadopoulou 2001). Following ovariectomy a reduction in the levels of circulating hormones and an increase in bone resorption are expected. In a related study on the same animals, 12 month post-OVX a significant reduction in the level of circulating 17-β estradiol was measured in these animals immediately prior to sacrifice, which was accompanied by an increase in bone turnover and porosity and a reduction in bone strength (Kennedy et al., 2009). These results corroborate the reduction in mineral content and tissue modulus seen after 12 months. In another related study on the 31 month group animals, bone turnover continued to be elevated in the OVX group relative to the controls (Healy et al., 2010). However, the final bone turnover was lower in the 31 month OVX group than the 12 month OVX group while turnover in the controls was the same at 12 and 31 months. This result is consistent with the current study which did not find a significant reduction in mineral content after 31 months in the OVX group relative to the controls. It is also possible that fundamental changes to the secondary mineralisation process occur following long term estrogen deficiency, which may be a compensatory mechanism by the remaining tissue to return mineralisation to normal levels. This study has highlighted the importance of study duration when determining the effects of estrogen deficiency. These results may help to decipher the apparent discrepancies in mineral content following estrogen deficiency seen by other researchers.

Zoledronic acid is a potent antiresorptive agent which prevents osteoclast activity. Under normal circumstances, bone remodelling occurs primarily on the trabecular surface. In this study neither mineral content nor tissue modulus increased significantly towards the centre of the trabeculae from animals which were treated with zoledronic acid. Thus, indications exist
that trabecular bone is losing the significant mineral and modulus gradients that are associated with healthy trabecular bone. This result is consistent with a recent study which found that bisphosphonate treatment leads to homogeneous mineral distribution in cancellous bone and also that tissue homogenization may negatively impact bone quality (Gourion-Arsiquaud et al., 2010). Furthermore, computational modeling has predicted that inhibition of resorption (for e.g. by antiresorptive agents) can lead to a less heterogenous mineral distribution (Ruffoni et al., 2008).

In addition, this study showed that mineral content and tissue modulus were significantly increased following bisphosphonate treatment. Recently alendronate treatment has been shown to increase mineral content in ovarietomised rats (Bitto et al., 2008; Anumula et al., 2010). Ibandronate and risedronate also increased the degree of mineralised bone and indentation modulus in ovarietomised rats (Shahnazari et al., 2010). Similarly zoledronic acid increased the bone mineralisation in ovarietomised rats (Cheng et al., 2009). However, in the current study treatment with zoledronic acid not only restored mineral content and modulus to that of control but actually surpassed them. As bisphosphonates suppress bone remodelling, this allows more time for secondary mineralisation to proceed (Boivin and Meunier 2002). Previous work has suggested that the main mechanism of action of nitrogen containing bisphosphonates, of which zoledronic acid is one, is via inhibition of the mevalonate pathway in osteoclasts (Amin et al., 1992; Crick et al., 1997; Fisher et al., 2000) which ultimately prevents osteoclasts from attaching to the bone surface and thus resorbing bone. However, evidence also suggests that bisphosphonates have a direct action on osteoblasts. In vitro studies on human osteoblasts found that zoledronic acid directly affected the proliferation and differentiation of these cells and thereby enhanced their bone forming potential (Reinholz et al., 2000; Pan et al., 2004). Although the exact mechanism by which
Bisphosphonates affect osteoblasts is unclear, zoledronic acid has also been shown to induce human osteoblast differentiation via inhibition of the mevalonate pathway (Reinholz et al., 2002). In the current study zoledronic acid likely increased tissue mineral content and modulus above control levels either by reducing osteoclast activity to such a level as to allow increased secondary mineralisation to occur or by directly stimulating osteoblasts to produce more mineral. However, the reality is most probably a combination of both mechanisms.

This study has a number of limitations, one of which is that due to ethical institution guidelines, a sham operated group was not used and rather a non-operated control was included. This was unlikely to have significantly impacted on this study, in particular limb loading, as those animals which underwent ovariectomy were only subject to anaesthesia for a short period of time (<30mins) and once the anaesthesia had worn off, the animals were immediately mobile. The total number of samples tested per group did differ due to the small sample size of the zoledronic acid treated group. While this is a limitation, the strong statistical significance seen would likely only be improved using larger sample sizes. A visual determination of the points for nanoindentation and also QBEI was made rather than employing a computational method. This allowed selection of the location which best represented the area of interest (i.e. superficial, intermediate or centre) whilst avoiding any holes or other artefacts (e.g. lacunae) in the bone. Whilst this does introduce an element of human error, different trabeculae were assessed in the nanoindentation study and the QBEI study, thus the two techniques are not being carried out on the same location. Another limitation of this study was the lack of any hormone analysis after 31 months. However, hormone analysis was carried out after 12 months and this showed conclusively significant reductions in the levels of circulating hormones (Kennedy et al., 2009).
In conclusion this study has found that both mineral content and tissue modulus increase towards the centre of healthy trabecular bone and also in bone from ovariectomised animals. Ovariectomy was found to significantly reduce mineral content and tissue modulus below that of controls after 12 months. However, after 31 months no significant reductions were found in either mineral content or modulus. This indicates either a reduction in bone turnover between 12 and 31 months or a change in the secondary mineralisation process to return mineral levels to control values and this is worthy of further study. Treatment with zoledronic acid did significantly increase mineral content and tissue modulus relative to both the ovariectomised and control groups. Zoledronic acid treatment also results in a more homogeneous mineral content and modulus across the width of trabeculae. This may have implications for load distribution and fracture resistance at the trabecular level. In conclusion this study provides evidence that estrogen deficiency and zoledronic acid therapy significantly alter the content and distribution of mineral in trabecular tissue.

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References


Conflict of Interest Statement

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 2

Click here to download high resolution image
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.