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Orlaith Brennan
Royal College of Surgeons in Ireland

Joseph Sweeney
Royal College of Surgeons in Ireland

Brian O'Meara
Royal College of Surgeons in Ireland

See next page for additional authors

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A natural, calcium-rich marine multi-mineral complex preserves bone structure, composition and strength in an ovariectomized rat model of osteoporosis

Orlaith Brennan[1,2,3,4 *], Joseph Sweeney[2], Brian O'Meara[2], Amro Widaa[2,3], Franck Bonnier[5], Hugh J Byrne[6], Denise M O'Gorman[7] and Fergal J O'Brien[2,3,4]

1. Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin 2, Ireland
2. Tissue Engineering Research Group, Royal College of Surgeons in Ireland, Dublin 2, Ireland
3. Trinity Centre for Bioengineering, Trinity College Dublin, Dublin 2, Ireland
4. Advanced Materials and BioEngineering Research Centre (AMBER), Trinity College Dublin and Royal College of Surgeons in Ireland, Dublin 2, Ireland
5. Université François-Rabelais de Tours, UFR Sciences Pharmaceutiques, EA 6295 Nanomédicaments et Nanosondes, 31 avenue Monge, 37200 Tours, France
6. FOCAS Research Institute, Dublin Institute of Technology, Kevin Street, Dublin 8, Ireland
7. Marigot Ltd, Strand Farm, Currabinny, Carrigaline, Co. Cork, Ireland

* Corresponding Author:

Dr Orlaith Brennan

Dept. of Physiology and Medical Physics,

123, St Stephen's Green,

Dublin 2

Ireland

E-mail: obrennan1@rcsi.ie

Phone: +353 1 402 2375

Fax: +353 1 4022355

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Abstract

Purpose: Calcium supplements, typically calcium carbonate based, are prescribed for postmenopausal women as an aid to prevent osteoporotic fractures. Recent literature suggests these calcium carbonate based supplements in addition to having a low bioavailability, can have adverse side effects including increased risk of heart attack. This study used a calcium–rich, marine multi-mineral complex (Aquamin) in an ovariectomised rat model of osteoporosis to demonstrate its efficacy in comparison to calcium carbonate.

Methods: Animals were randomly assigned to either non-ovariectomy control (Control), ovariectomy plus calcium carbonate (OVX), ovariectomy plus Aqaumin (Aquamin) or ovariectomy plus Aquamin delay where treatment started at week 8 (Aquamin delay). At the end of the twenty week study, the trabecular architecture was measured using micro computed tomography, composition was assessed using Fourier transform infrared spectroscopy and the mechanical properties were determined using nanoindentation and three point bend testing.

Results: The study demonstrates that oral ingestion of Aquamin has the ability to preserve trabecular bone structure, mineral composition, tissue level biomechanical properties and whole bone strength in the proximal tibia of rats following ovariectomy better than calcium carbonate.

Conclusion: This study supports the use of Aquamin as a supplement to aid in the prevention of bone loss in post-menopausal women.

Mini-Abstract

Calcium carbonate supplements are prescribed to prevent osteoporotic fractures. However, they are linked to increased risk of heart attack. We examined a marine source of calcium, Aquamin, and determined that it preserved bone structure better than calcium carbonate. Aquamin could be used in the prevention of bone loss in post-menopausal women.

Introduction

Osteoporosis is a disease that degrades bone mass and architecture, and impairs the ability of the skeleton to perform fundamental mechanical functions. Physiologically, osteoporosis occurs due to an imbalance in bone cell activity by which excessive resorption occurs without adequate new bone formation, thereby reducing total bone mass. As a consequence, bone strength is reduced and typically fractures of the vertebrae, hip or wrist occur potentially leading to deformity, severe pain and in some cases death. Osteoporosis causes more than 8.9 million fractures annually worldwide, or one every three seconds, approximately half of which occur in Europe, corresponding with one fracture every 8 seconds [1]. These 4 million osteoporotic fractures are estimated to cost the EU in the region of €31.7 billion, a figure which is expected to increase to €76.7 billion in 2050 based on the anticipated changes in the demography of Europe [2].

Calcium is a main constituent of the hydroxyapatite mineral which provides mechanical strength to the skeleton. In addition to the skeletons' roles of providing support, protection and enabling movement, it also acts as a calcium reserve for the body. Therefore, when calcium levels elsewhere in the body are low, this triggers the parathyroid hormone (PTH) to release calcium from bone by stimulating bone breakdown [3]. To prevent this from occurring, many pre- and post-menopausal women are prescribed calcium supplements in a bid to avert bone loss and prevent a fracture from occurring [4,5]. The majority of these calcium supplements are calcium carbonate based, and have been linked to myocardial infarction, angina, transient ischaemic attack or stroke [6,7]. A study published in the British Medical Journal stated that a reassessment of the role of calcium supplements in the management of osteoporosis was warranted [8].

Aquamin is a natural, calcium-rich marine multi-mineral complex food supplement which is derived from the calcified skeletal remains of the red marine algae species Lithothamnion, found off the coast of Ireland and Iceland. It is available commercially in a number of forms, which have calcium concentrations ranging up to 31 weight percent (wt %). In addition to calcium, Aquamin also contains 73 other trace minerals including magnesium, strontium, manganese, selenium, copper and zinc, which have been identified as important in bone health [9]. Minerals from sea-water accumulate in the fronds of the algae during their lifetime, until eventually the fronds break off and the calcified remains are harvested and processed. The supplement does not contain any additives; the mineralised fronds are simply sterilised, dried and milled to produce the Aquamin supplements. Unlike calcium carbonate, which is extracted from limestone or rock and is made up entirely of calcite, Aquamin is crafted naturally in the sea, providing a unique porous honeycombed vegetative cell structure with argonite, vaterite and calcite calcium salts present. This structure not only gives a number of significant benefits in its chemical behaviour and its absorption, it also lowers serum lipid levels, a precursor to many cardiovascular events, in postmenopausal women [10].

A number of recent trials have also identified several positive effects of Aquamin on bone health. Two double-blind, placebo-controlled studies measured the bioavailability of Aquamin versus calcium carbonate. These studies found that as early as 60 minutes following treatment, PTH levels were significantly reduced in the Aquamin group compared to calcium carbonate and placebo groups. *In-vitro* studies on osteoblast cells cultured in the presence of Aquamin exhibited early osteogenic potential and produced more mineral than those cultured in its absence [11-13]. An *in-vivo* study in which Aquamin was used as a dietary supplement in mice on a high-

fat Western diet (HFWD) showed that bone structure and function were preserved [14]. Indeed, the bone structure of these mice was superior to that of mice on the standard low-fat diet, thus asserting the osteogenic effect of Aquamin on bone cells. In a study conducted in yearling horses, Aquamin's influence on bone turnover was measured and found to be increased, suggesting that old or damaged bone could be replaced or removed which could, in turn, reduce incidents of clinical bone injury [15]. These results indicate that Aquamin has the potential to act as a supplement to enhance bone formation.

Using an established model of osteoporotic bone loss, the purpose of this study was to determine whether Aquamin has the potential to prevent bone loss and function. A secondary aim was to assess Aquamin's ability to restore bone following significant loss. Specifically bone structure, composition and mechanical properties were assessed in ovarectomised rats and those treated with Aquamin.

Methods

Animals

Eighty-eight female, retired breeder Wistar rats, both ovariectomised and age matched controls, were obtained from Harlan Laboratories (UK) and allowed to acclimatize for 5 days before the start of the experiment (Week 0). The rats were maintained with a cycle of 12 hours light and 12 hours darkness. The experiment was approved by the Animal Ethics Committee of Trinity College Dublin, Ireland and performed under licence issued by the Department of Health. All applicable institutional and national guidelines for the care and use of animals were followed. Two diets were used in this study, a regular murine chow (RM1) and a specially formulated RM1 supplemented with Aquamin (both from Special Diet Services, UK). For the supplemented feed, calcium (in the form of calcium carbonate) was removed from RM1 and Aquamin (31 wt% calcium) was added at a concentration such that calcium levels were equal in both feeds. The rats were divided into four groups: (1) control (n=28; RM1), (2) OVX (n=24, RM1), (3) OVX plus Aquamin (n=24, supplemented RM1) and (4) OVX plus Aquamin delay (n=12; RM1 for 8 weeks followed by supplemented RM1). Rats were ovariectomized one week prior to week 0, during the acclimatisation period all animals were fed RM1. At week 0, Aquamin treatment was started in group 3 while group 4 treatment started at week 8. Animals were allowed to eat and drink ad libitum and routinely weighed before being euthanized at weeks 0, 2, 8, 12 and 20.

Micro Computed Tomography (MicroCT) (Structure and composition)

Following sacrifice, the left tibia was removed for microCT scanning (µCT40, Scanco, Switzerland) at two locations. The midpoint of the tibia was identified and 10% of the tibial length was scanned around this position. From the diaphyseal scans, the hydroxyapatite (HA) content was determined. The metaphyseal trabecular region of the proximal tibia immediately below the epiphyseal growth plate was selected and 100 adjacent slices were scanned. From these scans, the trabecular bone was manually selected and bone structural parameters of bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and the hydroxyapatite content were automatically determined using Scanco software. Microarchitecture was analyzed at a resolution of 8µm, at 70kVp and 114µA and a threshold value of 210.

Fourier Transform Infrared Spectroscopy (FTIR) (Composition)

One-hundred micron thick samples were sectioned from the distal tibia using a diamond saw (Minitom, Struers, Denmark). As water has a strong absorbance in the infrared, tissues are dehydrated before analyses, thus the water spectrum is not observed. The dehydrated samples were placed on a calcium fluoride slide and positioned on the microscope stage of the Spotlight 400 FTIR Imaging system (Perkin Elmer). The system is equipped with an AutoImage microscope system operating with a 40X Cassegrain objective. A visible white-light image was recorded of the sample and the area to be scanned using infra-red was specified. FTIR images were acquired in attenuated total reflectance (ATR) imaging mode. The data were collected over the nominal free-scanning spectral range, 4000 – 600 cm^{-1}. Spectral measurements were acquired with a pixel size of 6.25 μm x 6.25 μm at a spectral resolution of 4 cm^{-1}. Background measurements were acquired on a region with no tissue with 120 scans per pixel, whereas 32 scans per pixel were recorded from the sample.

Nanoindentation Material Test (Mechanical properties)

Two-hundred micron thick samples were sectioned from the distal tibia (adjacent to the FTIR samples) using a diamond saw (Minitom, Struers, Denmark) before being polished with a series of graded silica-carbide grinding sheets. A Nano indenter XP system (MTS System Corporation, Oak Ridge, TN) was used to measure tissue level mechanical properties of proximal tibia sections. The Young's modulus (E) and contact hardness (H_c) were assessed using an AccuTip™ diamond Berkovich indenter tip with defined elastic modulus of 1141GPa, a Poisson's ratio equal to 0.07 and a radius of <50 nm. A total of twenty indents were performed on each sample: 10 evenly spaced indents in the cortical shell and a further 10 indents in the trabecular bone, five in an outer ring and five in an inner ring at the centre of the sample. A permanent hardness impression was made by driving the indenter into the sample for 90 s to a maximum load of 20 mN, holding for 120 s and then unloading. This cycle was repeated 3 times at each location and the modulus was determined from the unloading segment of the final indent [16].

Three-Point Bend Test (Mechanical properties)

Following microCT, a standard three-point bending test was applied to the tibia. The tibiae were placed on the lateral surface on two rounded supporting bars with a distance of 12 mm between them. A preload of 1 N was applied (Z020; Zwick, Ulm, Germany) at the medial surface of the diaphysis by lowering a third rounded bar. A constant displacement rate of 5 mm/minute was applied until failure. Displacement was measured from the actuator displacement transducer of the testing machine. Maximum load, maximum displacement and stiffness were calculated for each sample.

Statistics

All results are presented in graphs as the mean ± standard deviation. For statistical analyses, results were tested for normality with the Shapiro-Wilk test and outliers were identified using P-P plots and scatter plots. The analysis was carried out using the SPSS software package (SPSS Inc., Chicago, IL). One-way ANOVA and the Mann-Whitney rank sum tests were used to determine statistical significance. A difference of $p \leq 0.05$ was considered significant.

Results

Structure

Three-dimensional and two-dimensional representative samples from each of the groups are shown in Figure 1. These slices and reconstructions show an intact and well-connected trabecular microstructure in the control samples. A significant loss of trabecular bone structure following 20 weeks of ovariectomy is evident in the centre of the OVX samples. The trabecular structure is preserved when Aquamin administration is begun at week 0. The Aquamin delay group has a structure which appears intermediate between the OVX and OVX+ Aquamin group.

Qualitative analysis shows that as early as week 2, the ovariectomized group displayed large changes in structural parameters indicating the development of osteopenia (Figure 2). Significant losses in bone volume fraction (BV/TV) were observed in the OVX group at week 8 following a strong trend at week 2 ($p = 0.07$). Similarly trabecular separation (Tb.Sp) was significantly increased in the OVX group by week 12 while trabecular number (Tb.N) was significantly reduced in the OVX group by week 20. No significant changes were measured in trabecular thickness (Tr.Th) between groups over the study period.

Treatment with Aquamin resulted in a significant preservation of bone volume fraction by week 20 relative to the OVX group. Although not significant, strong trends were evident by week 20 between OVX and the Aquamin treated group with respect to Tr.N ($p = 0.1$) and Tr.Sp ($p = 0.1$). The Aquamin delay treatment group showed strong trends although no significant improvement in trabecular architecture was measured following 8 weeks of normal chow and a subsequent 12 week Aquamin treatment.

Composition: Hydroxyapatite content

Hydroxyapatite content measured using microCT indicated a significant reduction in the HA content of both trabecular and cortical bone by week 20 as seen in Figure 3. Beginning oral administration of Aquamin at week 0 prevented this loss in both trabecular and cortical bone. Beginning administration of Aquamin 8 weeks following ovariectomy was also sufficient to prevent a significant loss of mineral content from trabecular bone.

Qualitative FTIR

In Figure 4a, the week 20 representative control sample visualised with white light (centre panel) shows bone with a healthy micro architecture and good trabecular interconnectivity. The ATR spectrum which is recorded can be represented as an absorbance spectrum. The same region of bone visualised as an absorbance heat map (left hand panel) displays an abundance of red/orange indicating increased absorption on the surface of the sample. In ATR mode (right hand panel), blue/purple indicates increased reflection at the surface. Visual inspection of the white light and infrared maps for the OVX sample shows significant microarchitecture deterioration and less absorption and reflection than in the control group. Treatment with Aquamin shows increased absorbance represented by the increased presence of red in absorbance and purple/blue in the ATR heat maps. The Aquamin delay group displays absorbance and reflection profiles which are intermediate to the OVX and the Aquamin groups as indicated by red and green in the absorbance and purple and green in the ATR maps.

Quantitative FTIR

The infrared spectrum of bone shows the presence of the major molecular species, phosphate (from the mineral hydroxyapatite), carbonate (from carbonate substitution for hydroxyl and phosphate groups), and amide I, II, and III from the protein constituents of bone (mainly type I collagen) [17]. These peaks are shown in a representative spectrum from a healthy control sample in Figure 4b. The mineral/matrix ratio is linearly related to the mineral content and the carbonate/phosphate ratio is related to the chemically measured carbonate/phosphate content. In the trabecular region from each absorbance spectrum, the mineral-to-matrix ratio was assessed as the ratio of the area of the phosphate band (1170 - 980 cm^{-1}) to the area of the amide I band (1712 - 1592 cm^{-1}). The carbonate/phosphate ratio was assessed as the ratio of the area of the phosphate band to the area of the carbonate band (890-840 cm^{-1}) [17]. Analysis of the ATR spectra (Figure 4c) indicates a significant reduction in the mineral/matrix ratio in the OVX group relative to the control group at week 20. This reduction of mineral/matrix ratio was prevented by starting Aquamin administration at week 0. The late administration of Aquamin did not significantly prevent the change in mineral/matrix ratio. No significant changes were measured in the carbonate/phosphate ratio between groups (Figure 4d).

Biomechanical Properties

Nanoindentation Material Test

The Young's modulus and hardness data are presented in Table 1. In the control group, the value of the Young's modulus is lowest in the cortical shell, increasing significantly in trabeculae near the edge of this cortical shell and increasing again in trabeculae at the core of the sample. A similar pattern is seen for the hardness. In the OVX group, this stepwise increase disappears and there is only a significant difference between the core trabeculae and the edge/cortical shell when the modulus is assessed and there are no differences between locations when hardness is analysed. In the OVX + Aquamin group, trabecular bone moduli and hardness values are significantly greater than the cortical shell values. No significant differences were measured in the OVX + Aquamin delay group.

Analysis between groups in trabecular bone from the core found the modulus for the control group to be significantly higher than the OVX and the OVX + Aquamin delay groups. The OVX + Aquamin group was not significantly different from the control group although it was trending higher than the OVX group. Similar results were observed for hardness. For trabeculae located around the edge of the sample, both the modulus and hardness in the OVX + Aquamin group was significantly greater than that for the OVX group. For cortical bone, both moduli and hardness in the OVX + Aquamin delay group were significantly higher than measured in the control group. Hardness in cortical bone from the OVX + Aquamin group was also higher than the control group.

Three-Point Bend Test

Analysis of the cortical shell by three point bend test found a significant reduction in the maximum load sustained by the OVX group between week 2 and week 20 (Table 4). No significant differences were measured in maximum displacement between any of the groups or over the study duration. A significant increase in stiffness was measured in the OVX + Aquamin group compared to OVX at week 20.

Discussion

In this study we examined the effects of ovariectomy and a natural, calcium-rich marine multi-mineral complex on bone structure, composition and strength in a rat model of osteoporosis. The results of the study indicate that Aquamin has the potential to prevent the loss of trabecular bone in the proximal tibia of ovariectomised rats following 20 weeks of treatment. This protection of structure was accompanied by a preservation of the mineral composition and follows through to maintaining the mechanical integrity. Analysis of the samples from animals subject to the delayed Aquamin treatment was inconclusive for a number of reasons which are discussed in further detail in this discussion. The use of a non-ovariectomy control fed group proved the efficacy of our model as significant losses in bone volume were observed and measured in the ovariectomy group as early as 8 weeks when compared to the non-ovariectomy control. This loss of structure was accompanied by a degradation in the composition and mechanical properties of the remaining bone, typical characteristics of osteoporosis.

In the current study, both the regular chow (RM1) and the Aquamin supplemented chow have equal amounts of calcium. Therefore, the Aquamin treated animals did not receive extra dietary calcium. The significant inhibition of bone loss, measured by week 20 in this study, is therefore not a result of the quantity of calcium ingested. The significant preservation of the trabecular structure in the Aquamin groups is most likely brought about by the unique form in which the calcium is supplied by Aquamin. Since calcium's role in the prevention of osteoporosis in humans was proven some 30 years ago, its bioavailability has been the subject of numerous scientific studies. Calcium carbonate is typically used as the calcium supplement of choice worldwide, however, it is known that its bioavailability is very low, only around 20–30% [18]. The form of dietary calcium is a critical factor in determining the availability of calcium for bone development and maintenance. The structure of the calcium salts in Aquamin dramatically increase the calcium surface area, thus stomach acid can come into greater contact with the calcium making it easy to dissolve into the body and providing greater efficacy. Additionally, calcium carbonate is not easily absorbed as the phosphorus binds tightly to the calcium.

This difference in bioavailability is one of the factors postulated to explain the positive Aquamin results observed in this study. In addition to calcium, Aquamin also contains 73 additional minerals many of which have a proven osteogenic potential through direct or indirect effects on bone cells or bone mineral and collagen. Magnesium is a major component of Aquamin, it is essential for all living cells, including osteoblasts and osteoclasts, as it is fundamental in adenosine triphosphate [19]. However, its role in bone is not confined to simply cell biology, a dietary magnesium restriction has been shown to promote osteoporosis [20]. Potassium is critical for calcium metabolism in many enzymes and potassium is also required for improved bone strength and density [21]. Copper plays a role in collagen crosslinking and fixing calcium within bones [22]. Some elements, such as strontium, are chemically similar to calcium. Strontium has an anabolic activity in bone, and this may have significant beneficial effects on bone balance in normal and osteopenic animals [23]. The osteogenic effect

offered by these elements in combination with a readily available calcium source will likely have a synergistic effect. It is also recognised that calcium bioavailability is influenced and enhanced by the presence of other minerals [24]. Aquamin also contains a unique trace mineral profile gained from its marine source. Alone, these elements may be insignificant, but within a multi-mineral matrix they work synergistically and may give a powerful boost to the action of the calcium.

Starting oral administration of Aquamin immediately following ovariectomy demonstrated the capacity to prevent trabecular bone loss in the proximal tibia of rats associated with ovariectomy. Although only bone volume fraction was significantly altered by Aquamin, it is important to note that trabecular number, separation and thickness are all incorporated into the software calculation of the trabecular bone volume fraction. Therefore, while individually these parameters were unchanged, collectively they resulted in the overall reduction in bone loss which can be seen visually in the microCT 2-dimensional slices and the 3-dimensional reconstructions. It is important to note that Aquamin is a neutraceutical and is not as potent as pharmaceuticals. Therefore, to achieve a significant preservation of trabecular architecture after only 20 weeks by simply substituting calcium carbonate for Aquamin is a major outcome.

Bone is a composite material consisting of a mineral phase (hydroxyapatite), collagen, non-collagenous proteins, lipids, and water [25]. Fourier transform infrared spectroscopy can show the presence of the major molecular species, phosphate (from the mineral hydroxyapatite), carbonate (from carbonate substitution for hydroxyl and phosphate groups), and amide I, II, and III from the protein constituents of bone (mainly type I collagen). In the current study there was a significant reduction in the mineral-matrix ratio measured in trabecular bone from the ovariectomised group at week 20. This is consistent with previous findings which found a significant reduction in the mineral-matrix ratio in normal vs. osteoporotic bone [17]. This data is supported by the significant reduction in hydroxyapatite measured in both the trabecular and compact bone from the ovariectomised animals. The FTIR mapping of the control samples was also indicative of a high mineral content. In contrast the ovariectomy group showed reduced absorption indicating a reduced mineral level. Those animals which started Aquamin treatment immediately after ovariectomy were found to have a mineral-matrix ratio significantly greater than the ovariectomy only group indicating a preservation of the healthy composition. Again this is borne out in the HA data from microCT analysis where HA content was higher in the Aquamin treated group than OVX. FTIR maps of the Aquamin treated group were more akin to the control group signifying the preservation of mineral. The carbonate-phosphate ratio indicates the level of carbonate substitution in the HA crystal, a decrease in this ratio reflecting an increased phosphate content. However, no significant difference was seen in the carbonate-phosphate ratio in the current study.

In tandem with the significant changes in composition measured using FTIR and microCT were changes in the tissue level mechanical properties as measured using nanoindentation. The biomechanical parameters of trabecular bone from slices adjacent to those used in FTIR revealed unsurprising parallels. The Young's modulus of trabecular bone from ovariectomised animals was significantly less than the controls. This correlates with data from previous studies examining the tissue level mechanical properties of ovariectomised bone compared to healthy samples [16,26]. Trabeculae from animals who received Aquamin had a significantly higher modulus and hardness than from ovariectomised animals. While this effect has not been demonstrated previously, it is in keeping with the preservation of composition by Aquamin shown in the study.

The nanoindentation results did yield some interesting data in terms of the cortical bone. Material strength assessment showed that both modulus and hardness in the Aquamin delay group were significantly greater than the control group. Although not significant, both modulus and hardness were higher in the cortical shell of ovariectomised animals and the Aquamin treated group than of controls. Supporting this was the hydroxyapatite content which was not significantly different between the control and either Aquamin groups. Micro-CT data indicated a sustained loss of trabecular bone volume following ovariectomy over the 20 week study. However, when the trabecular thickness was examined it was found to decrease until week 8 at which point it began to increase again. This data supports the theory of bone compensation whereby new bone is laid down on surviving trabeculae to compensate for the loss of other trabeculae [27,28]. In addition to laying down new bone in trabeculae, new bone may also be laid down in the cortical shell [29]. However, as many studies have shown a thinning of the cortical shell this is probably not the case and the current study showed an overall reduction in the maximum load sustained by the tibial shaft from ovariectomised animals between weeks 2 and 20. It is possible that secondary mineralisation is increased in the cortical bone. In conjunction with the availability of Aquamin this could account for the higher tissue level mechanical values measured. Mechanical testing of the whole tibial shaft did go some way towards supporting this theory. The stiffness measured in the Aquamin group was significantly greater than the OVX. Although not significant, a higher maximum load was measured in the Aquamin (132 ± 13 N) and Aquamin delay (136 ± 12 N) than in the control (121 ± 10 N). It is not immediately clear what influence Aquamin in combination with ovariectomy had on compact bone and further investigation of this is warranted.

To help with those assessments, a longer term study would advance our understanding of how Aquamin behaves. An extended duration would likely have yielded additional significant changes due to Aquamin and also the response to a delayed Aquamin therapy. Further study is also required to fully ascertain the timeframes involved for a delayed treatment regime to be effective. It would also be interesting to measure bone formation and resorption rates in further studies. This could be conducted by either looking at serum biomarkers or through the use of fluorochrome markers to determine where bone formation is taking place. Clinically, calcium is prescribed in combination with vitamin D. Therefore an assessment of the combined influence of Aquamin and vitamin D would be merited.

Conclusion

This study has demonstrated that providing calcium in the form of a seaweed derived mineral supplement, namely Aquamin, offers significant advantages over calcium carbonate. Trabecular architecture is significantly preserved when using Aquamin as compared to calcium carbonate. This is supported by a preservation of the mineral phase of bone which is mirrored by the improved material properties of the bone. Overall, this study supports the use of Aquamin as a supplement to aid in the prevention of bone loss in post-menopausal women.

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Conflicts of interest

Dr O'Gorman is an employee of Marigot Ltd. Prof O'Brien and Dr Brennan have received funding from Marigot Ltd. to conduct research.

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Table 1: The Young's Modulus and hardness measured in the cortical shell and in trabecular bone from the core and the edge of the proximal tibia at week 20.

Location	Modulus (GPa)			
	Control	**OVX**	**OVX + Aquamin**	**OVX + Aquamin Delay**
Core	7.61 ± 3.67 [a]	5.44 ± 2.53 [a b] *	5.99 ± 1.20 [b]	5.13 ± 0.97 *
Edge	5.56 ± 3.87 [b]	3.71 ± 1.94	5.48 ± 0.95 [b #]	3.91 ± 0.71
Cortical Shell	3.35 ± 0.51	3.86 ± 2.55	4.10 ± 0.55	4.70 ± 0.67 **
Location	**Hardness (GPa)**			
	Control	**OVX**	**OVX + Aquamin**	**OVX + Aquamin Delay**
Core	0.43 ± 0.10 [a]	0.37 ± 0.07	0.42 ± 0.08 [b]	0.29 ± 0.05 * [&]
Edge	0.37 ± 0.08 [b #]	0.28 ± 0.06	0.38 ± 0.07 [b # %]	0.24 ± 0.04
Cortical Shell	0.24 ± 0.04	0.25 ± 0.03	0.27 ± 0.04 ^	0.27 ± 0.04 ^

Mean ± standard deviation. Significant difference of $p \leq 0.05$ between locations: [a] > edge, [b] > cortical shell. Between groups: * < control, # > OVX, ** > control, & < OVX + Aquamin, % > OVX + Aquamin delay, ^ > control.

Table 2: Maximum load, maximum displacement and stiffness measured using three point bend testing of the tibia.

Mechanical Property	Group	Week 0	Week 2	Week 20
Maximum Load (N)	**Control**	*129.62 ± 2.02*	*128.03 ± 20.19*	*121.12 ± 10.20*
	OVX	-	*141.80 ± 9.41*	*122.23 ± 12.10* *
	OVX + Aquamin	-	*132.26 ± 19.36*	*131.85 ± 13.96*
	OVX + Aquamin Delay	-	-	*136.80 ± 12.11*
Maximum Displacement (mm)	**Control**	*0.55 ± 0.14*	*0.54 ± 0.09*	*0.55 ± 0.13*
	OVX	-	*0.53 ± 0.15*	*0.54 ± 0.14*
	OVX + Aquamin	-	*0.54 ± 0.24*	*0.51 ± 0.2*
	OVX + Aquamin Delay	-	-	*0.47 ± 0.06*
Stiffness (N/mm)	**Control**	*244.39 ± 59.76*	*247.86 ± 72.58*	*292.93 ± 66.37*
	OVX	-	*297.35 ± 128.59*	*257.95 ± 67.10*
	OVX + Aquamin	-	*296.39 ± 140.21*	*368.25 ± 86.11*[#]
	OVX + Aquamin Delay	-	-	*293.19 ± 51.24*

Significant difference of $p \leq 0.05$: Within the OVX group, * > week 2. Between groups, # > OVX.

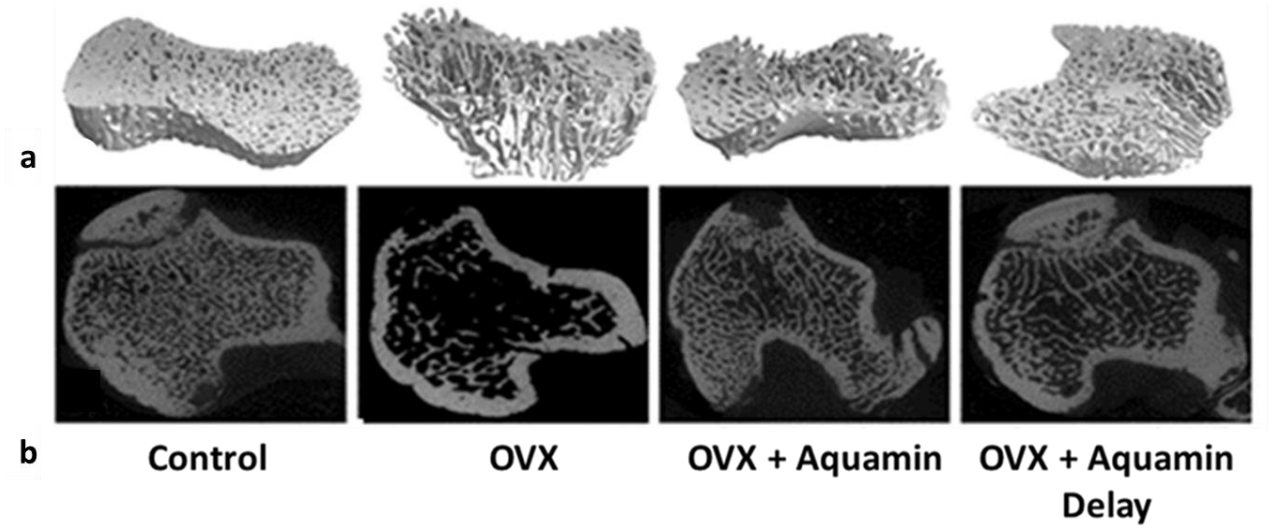

Fig 1: Representative (a) 3-dimensional microCT reconstructions of trabecular bone from the proximal tibia and (b) 2-dimensional slices through the midsection of the proximal tibia of rats at week 20.

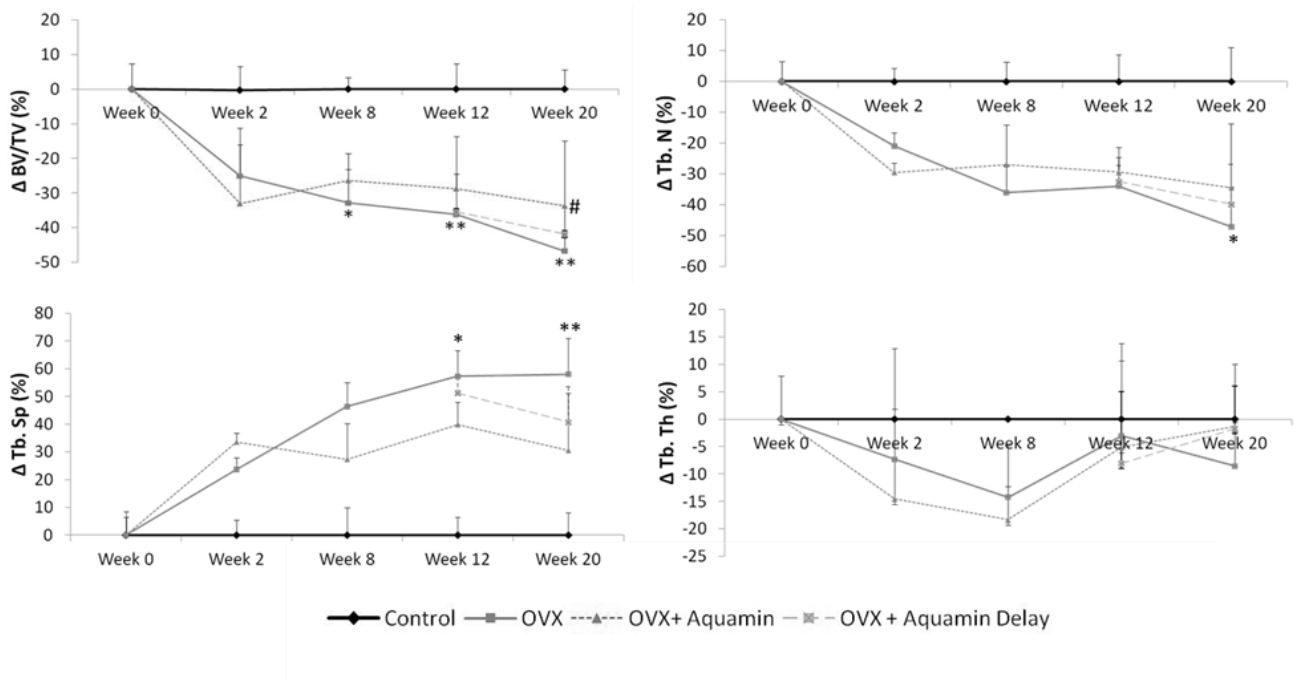

Fig 2: Percentage change in structural parameters in the metaphyseal proximal tibia for all groups over all time points. N = 6 per group (* indicates $p < 0.05$ and ** $p < 0.005$ between control and OVX, # indicates $p < 0.05$ between OVX and OVX + Aquamin).

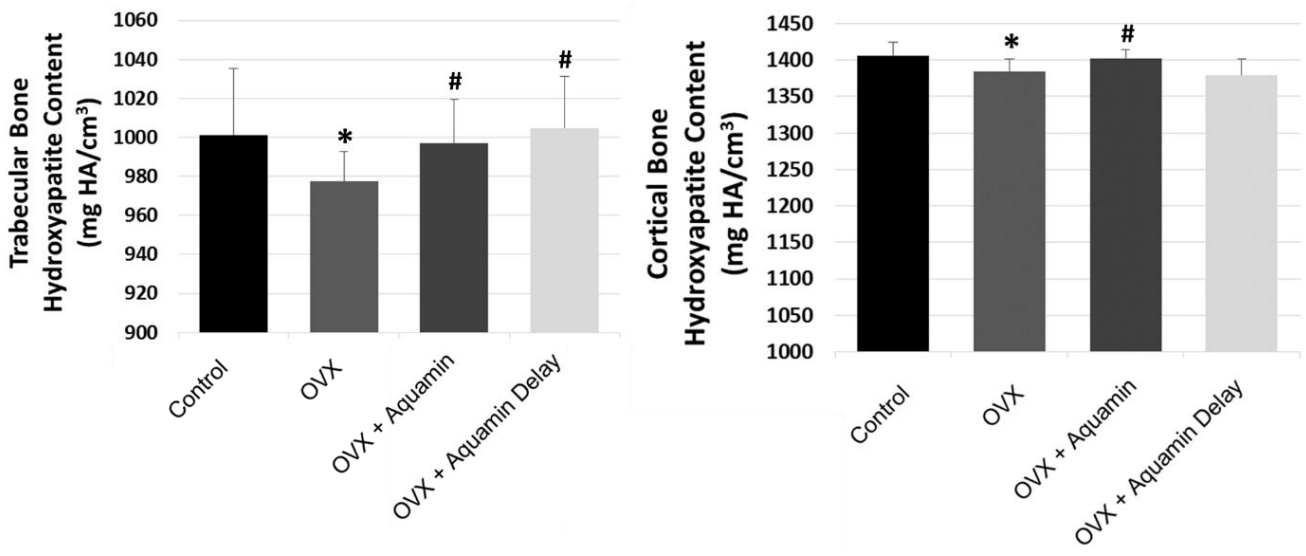

Fig 3: Hydroxyapatite content measured using microCT indicates a significant reduction in HA content in both trabecular and cortical bone following twenty weeks of ovariectomy. Aquamin administration prevented this loss of HA in both bone types and a significant preservation was also seen in the Aquamin delay group (* indicates $p<0.05$ between control, # indicates $p<0.05$ between OVX).

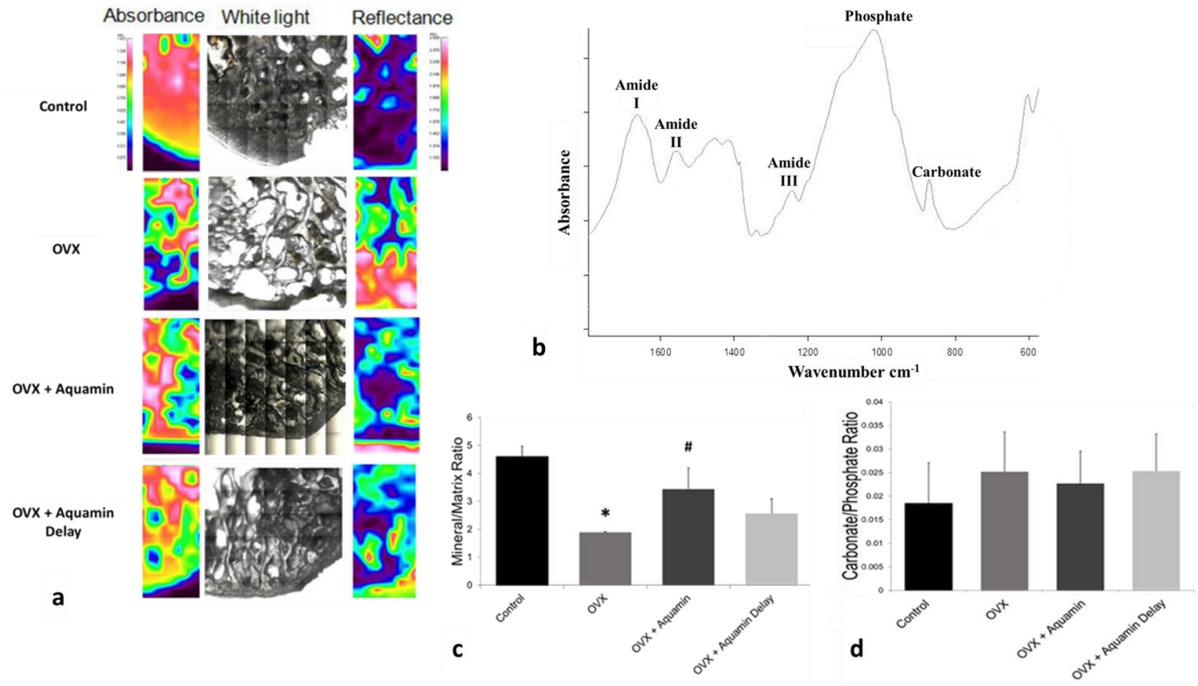

Fig 4: (a) FTIR absorbance (left) and reflection (right) heat maps with white light image (centre) from a representative sample of each group at week 20. In the absorbance maps areas of red indicate increased absorbance (mineralisation) while in infra-red reflection maps, purple indicates increased mineralisation. (b) Typical absorbance spectrum of bone indicating peaks used to calculate

mineral/matrix ratio and carbonate/phosphate ratios. (c) The mineral/matrix ratio was significantly reduced following 20 weeks of estrogen deficiency (* $p<0.05$ relative to control). This loss of mineral was prevented by administering Aquamin from week 0 (# $p<0.05$ relative to OVX). (d) No significant changes were measured in the carbonate/phosphate ratio between groups.