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The Osteogenic Potential of the Marine-Derived Multi-Mineral Formula Aquamin Is Enhanced by the Presence of Vitamin D

A. Widaa,[1,2,3] O. Brennan,[1,2,3] D.M. O'Gorman[4] and F.J. O'Brien[1,2,3]*

[1]Tissue Engineering Research Group (TERG), Dept. of Anatomy, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland
[2]Trinity Centre for Bioengineering, Trinity College Dublin, Dublin 2, Ireland
[3]Advanced Materials and Bioengineering Research (AMBER) Centre, RCSI & TCD, Ireland
[4]Marigot Ltd., Strand Farm, Carrigaline, Cork, Ireland

Bone degenerative diseases are on the increase globally and are often problematic to treat. This has led to a demand to identify supplements that aid bone growth and formation. Aquamin is a natural multi-mineral food supplement, derived from the red algae *Lithothamnion* species which contains calcium, magnesium and 72 other trace minerals. It has been previously reported to increase bone formation and mineralisation. This study aimed to investigate the 28 day *in vitro* osteogenic response of Aquamin supplemented with Vitamin D. The osteogenic potential of MC3T3-E1 osteoblast-like cells was analysed in standard osteogenic medium supplemented with Aquamin +/− Vitamin D3, and the controls consisted of osteogenic medium, +/− Vitamin D3. Proliferation of osteoblasts, metabolic activity and cell viability did not differ between Aquamin and the osteogenic control groups. Alkaline phosphatase (ALP) levels and mineralisation were increased by the supplementation of Aquamin, and the addition of Vitamin D3 increased mineralisation for all groups. The combination of Aquamin and Vitamin D3 yielded a significant increase in ALP and mineralisation over Aquamin alone and the standard osteogenic control +/− Vitamin D3. This study demonstrates that Aquamin aids osteogenesis, and that its osteogenic response can be enhanced by combining Aquamin with Vitamin D3. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: Aquamin; Vitamin D3; osteoblast; bone; osteogenesis; nutritional supplements.

INTRODUCTION

The World Health Organisation in 2000 established the 'Bone and Joint Decade' as a preventative measure to address an increase in degenerative bone diseases (Woolf and Pfleger, 2003). Bone is a dynamic organ and is constantly being remodelled in order to facilitate growth and repair. This process requires the involvement of osteoblast cells, which function in generating and mineralising bone giving strength and rigidity to the skeletal system (Parfitt, 1984). Osteoporosis is one disease which results from an imbalance in remodelling and is characterised by low bone mass, micro architectural deterioration and bone composition changes ultimately leading to fracture (Raisz, 2005). Bone strength is reduced to such a degree that fractures can occur with minimal trauma, often involving loads no more than typically experienced in routine daily activity (Morgan, 2008). Current data suggest that as many as one in three women and one in five men over the age of 50 are at risk of an osteoporotic fracture (International Osteoporosis Foundation 2013). Diet supplementation may assist in the prevention of osteoporosis in individuals whose daily mineral intake falls below that recommended by the Food and Drug Administration and equivalent bodies. Although there are multiple causes of bone loss, two main factors that are associated with bone loss are insufficient calcium and Vitamin D (Peacock *et al*., 2000). The supplementation of diets with calcium and Vitamin D has also been of significant interest for the prevention of fractures in adults in the United States (Moyer, 2013).

Aquamin is a commercially available food supplement which is derived from the calcified skeletal remains of the red marine algae species *Lithothamnion*. It is rich in calcium as well as containing 72 other trace minerals such as magnesium, manganese, iron, copper and zinc (Ryan *et al*., 2011). Aquamin has been identified in possessing anti-inflammatory properties by inhibiting nuclear factor kappa B (NFκB) signalling (O'Gorman *et al*., 2012). NFκB transcription factor plays a major role in bone pathophysiology, resulting in osteoblast cell death, inhibition of proliferation, pro-inflammation and increased bone resorption cytokine signalling (Kwan Tat *et al*., 2004; Claro *et al*., 2013). These anti-inflammatory properties have suggested Aquamin as beneficial for the treatment of osteoarthritis and improves clinical scores (Frestedt *et al*., 2008; Frestedt *et al*., 2009). Aquamin has also been shown to increase bone metabolism and mineralisation in yearling horses when compared with a limestone supplement containing equivalent levels of calcium (Peacock *et al*., 2000). Mice which were fed a high-fat western diet supplemented with Aquamin were found to have significantly more bone than mice which were not supplemented with Aquamin (Moyer, 2013).

* Correspondence to: F.J. O'Brien, Department of Anatomy, Tissue Engineering Research Group (TERG), Advanced Materials and Bioengineering Research Centre (AMBER), Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland.
E-mail: fjobrien@rcsi.ie

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A recent study from our laboratory published in *Phytotherapy Research* demonstrated that Aquamin can increase the osteogenic potential of bone-forming osteoblastic cells *in vitro* (O'Gorman *et al.*, 2012). We demonstrated that Aquamin increases alkaline phosphatase (ALP) levels and, therefore, *in vitro* bone formation, and also results in higher mineralisation (O'Gorman *et al.*, 2012). Vitamin D has also been identified as playing a role in osteogenesis. Vitamin D3 readily binds to the osteoblast Vitamin D receptor and initiates pro-osteogenic transcription and subsequent osteogenesis (Arriagada *et al.*, 2010; Pike, 2011). Previously, it has been identified that Vitamin D3, including different metabolite forms, can stimulate ALP production from osteoblast cells (Haneji *et al.*, 1983; Kurihara *et al.*, 1986). Moreover, Vitamin D3 is known to increase *in vitro* mineralisation of osteoblasts (Matsumoto *et al.*, 1991). Therefore, the aim of this study was, building on our earlier knowledge, to determine if the combination of Vitamin D3 and Aquamin has an additive increase on the osteogenic potential of pre-osteoblastic cells in comparison to Aquamin alone. Furthermore, we wanted to investigate if Aquamin, with/without Vitamin D3, has an effect on osteoblast cell viability after 28 days.

MATERIALS AND METHODS

Reagents. All reagents were obtained from Sigma Aldrich (Wexford, Ireland), unless stated otherwise.

Tissue culture conditions. The mouse clonal MC3T3-E1 pre-osteoblastic cell line (ATCC, Middlesex, United Kingdom) was used for all experiments. This is a common cell line used routinely for investigating osteogenesis. The cells were cultured in standard T175 tissue culture flasks (Sarstedt, Ireland) containing α-minimum essential medium supplemented with 10% foetal bovine serum (Biosera Ltd., United Kingdom), 2% penicillin–streptomycin solution and 1% L-glutamine. The cells were incubated at 37°C, 5% CO_2 and 95% humidity. The media was replaced every 3–4 days, and after confluency cells were harvested using trypsin-EDTA and re-suspended in standard medium. MC3T3-E1 cells were seeded at a concentration of 5×10^5 cells/well in six-well adherent plates for 24 h before differentiating in standard osteogenic media. MC3T3-E1 pre-osteoblastic cells were differentiated to matrix secreting mature osteoblasts using the standard osteogenic cocktail over 28 days, by the supplementation of dexamethasone (100 nM), ascorbic acid (50 μg/mL) and β-glycercolpentahydrate (10 mM).

Preparation of supplements. Aquamin Soluble (Marigot Ltd., Cork, Ireland) was prepared by dissolving in H_2O: HCL (19:1) at a final concentration of 1 mg/mL. This experimental concentration was chosen as optimal due to pH of the media and MC3T3-E1 cell viability, as previously identified (O'Gorman *et al.*, 2012). This concentration of Aquamin equates to ~12% calcium (Aslam *et al.*, 2010). The equivalent to physiological levels of extracellular calcium is ~0.5 mg/mL Aquamin (O'Gorman *et al.*, 2012). Vitamin D3 was diluted in 100% EtOH and stored at 2-8°C. Final experimental concentration was adjusted to 5 nM.

Statistical analysis. Statistical analyses were performed using a One-Way ANOVA per time point utilising the statistical software IBM SPSS Statistics Version 20. Data were represented as the mean value ± the standard error of the mean. Values were considered significant at $P < 0.05$.

Assessment of cell viability. *Quantification of cell number.* Osteoblasts were washed in warm PBS before detaching cells using a cell scraper. Cells were then pelleted by transferring to a tube and centrifuging at ×500 g for 5 min. Media was then removed, and each pellet was re-suspended using 100 μL of papain buffer solution [100 mL PBS, 1 mL 0.5 M EDTA (pH 8.0), 79 mL cysteine-HCl; 10 mg papain per 10 mL buffer] and was incubated at 60°C overnight. The following day, 30 μL of the sample was added to 600 μL of the Hoechst buffer/working dye solution [10 mM Tris, 10 mM Na_2EDTA, 1 M NaCl; pH 7.4 and filter sterilized with a working dye concentration for each experiment at 0.1 μg per mL (1:10000) dilution – 2 mL 10× Hoechst buffer, 18 mL distilled water, 2 μL Hoechst stock dye]. Controls were measured as blank samples containing papain. This mixture was plated out at 200 μL (in triplicates) in a 96-well plate. Fluorescence was read at an excitation of 365 nm and an emission of 485 nm (Varioskan Flash, Thermo Scientific). Readings were converted to cell number by standard curve.

Metabolic activity. Non-specific metabolic activity was measured using Alamar Blue (Invitrogen, Biosciences, Ireland) as a colour metric assay. Media was removed from the six-well plates and wells were washed using PBS. A 10% solution of Alamar blue was made in the dark using the pre-warmed osteoblast media. Wells with Alamar blue but no cells were used as controls. To each well, 3 mL of the 10% Alamar blue solution was added. The six-well plates were returned to the incubator for 2 h at 37°C, 5% CO_2 and 95% humidity following which 100 μL (in triplicates) from each well was pipetted into a 96-well plate. The plate was placed into a plate reader (Varioskan Flash, Thermo Scientific) and absorbance was measured at wavelengths of 540 nm and 620 nm.

Quantification of dead cells. Live/Dead viability kit (Invitrogen, Biosciences, Ireland) was utilised to quantify the amount of dead osteoblasts. This viability assessment incorporates the use of Ethidium homodimer-1 (EthD-1) as a fluorescence stain, to target the nucleus of dead osteoblasts. At day 28, osteoblasts were examined for cell death, in which the positive control consisted of cells pre-treated with 100% ethanol. Cells were washed with warm PBS before the addition of 500 μL of an 8 μM EthD-1 solution in PBS. The cells were then incubated with the fluorescence dye at 37°C, 5% CO_2 and 95% humidity for 1 h. The plate was then read for fluorescence at 530 nm excitation, 645 nm emission using a plate reader (Varioskan Flash, Thermo Scientific).

Assessment of osteogenesis. *Measurement of ALP activity.* Intracellular levels of ALP, a well-established biochemical marker for bone formation, were evaluated. Osteoblast media was removed, and cells were washed with 1 mL of warm PBS, before subsequent aspiration. Osteoblasts were lysed with 1 mL of lysis buffer containing a substrate for acid phosphatase (0.1 M

Sodium acetate, 2% Triton X-100 and 10 mM para-nitrophenol phosphate [p-npp]) and incubated in the dark for 1 h at 37°C. The reaction was stopped using 0.3 M NaOH. Triplicates (100 μL) of each well were then added to a 96-well plate and absorbance was then read at 405 nm, using a microplate reader (Wallac Victor 2, Turku, Finland).

Von Kossa histological staining for mineralisation. Media was removed from cultured wells and cells were washed with 1 mL of warm PBS, before subsequent aspiration. Cells were fixed in 70% EtOH for 1 h at −20°C and then washed with dH_2O. Cells were stained with 5% silver nitrate for 60 min under a light source and then washed with dH_2O. Afterwards, 5% sodium thiosulphate was added to the wells for 2 min, while stirring the plate. Wells were washed four times with dH_2O to remove any remaining unbound stain. Images were obtained using a bright field inverted microscope (Leica DMIL, Wetzlar, Germany) at x100 magnification and recorded using a camera (Leica DFC 420C, Heerbrugg, Germany).

Alizarin Red histological staining and quantification for mineralisation. Cultured osteoblast cells were fixed as before in ice cold 70% EtOH for 1 h and then washed with dH_2O. Cells were then stained with 2% Alizarin Red S (pH 4.2) for 20 min and then washed four times afterwards with dH_2O, as before, to remove residual stain. Images were obtained using a bright field inverted microscope (Leica DMIL, Wetzlar, Germany) at x100 magnification and a camera (Leica DFC 420C, Heerbrugg, Germany), as above.

Histological staining was quantified by adding 10% cetylpyridinium to each well for 1 h. Triplicates (100 μL) of each well were then added to a 96-well plate and absorbance was read at 540 nm using a microplate reader (Varioskan Flash, Thermo Scientific).

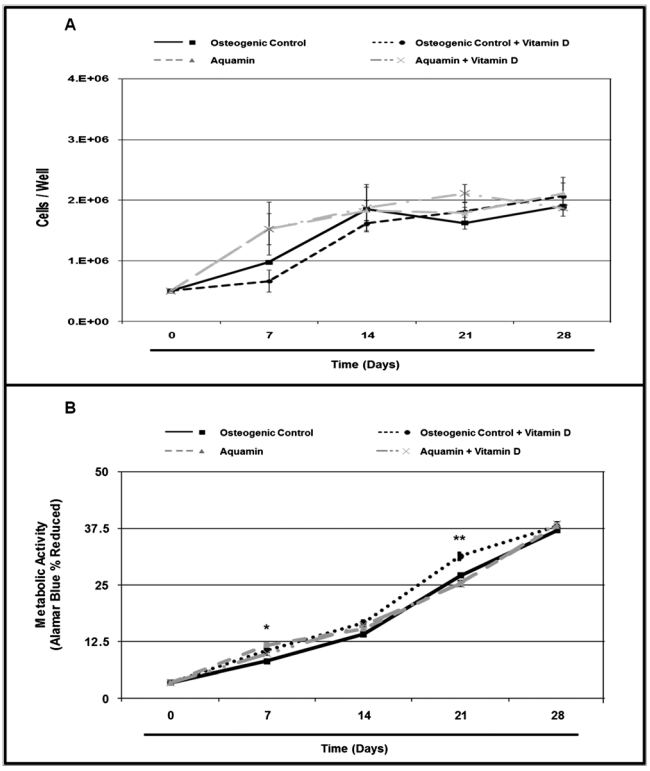

Figure 1. A: Cell number was investigated over 28 days using a Hoechst DNA assay. No differences were observed between Aquamin +/− Vitamin D3 and the osteogenic control +/− Vitamin D3 over the 28 days. No increase in proliferation was observed by the addition of Vitamin D3 to each of the perspective groups. n = 3. **1B**: Cell metabolic activity was determined by Alamar Blue assay over 28 days. The addition of Aquamin elicited an increased response in metabolic activity over the osteogenic control at day 7, and the addition of Vitamin D3 to the osteogenic control at day 21 above all groups, respectively. Overall, Aquamin +/− Vitamin D3 and the osteogenic control +/− Vitamin D3 were similar in metabolic activity after 28 days. *$P < 0.001$, **$P < 0.001$, n = 4.

RESULTS

The effect of Aquamin and Vitamin D on cell viability

The effect of Aquamin +/− Vitamin D3 on osteoblast cell number was investigated by quantifying DNA, using the Hoechst DNA method (Fig. 1A). The addition of Vitamin D3 to the osteogenic control or Aquamin did not affect cell proliferation and no differences were observed at any of the time points. All groups increased in cell number over 28 days, and Aquamin +/− Vitamin D3 treatment demonstrated similar proliferative levels of osteoblast cell growth when compared to the osteogenic control +/− Vitamin D3 (Fig. 1A).

Osteoblasts were assessed for metabolic activity which was examined by the Alamar Blue assay (Fig. 1B). All groups demonstrated an increase in metabolic activity over the 28 days of culture. Aquamin produced higher levels of metabolic activity than the osteogenic control alone on day 7 ($P < 0.01$), but resulted in similar levels over 14, 21 and 28 days (Fig. 1B). The addition of Vitamin D3 to the osteogenic control increased the metabolic activity at day 7 ($P < 0.05$), 14 ($P < 0.05$) and 21 ($P < 0.001$) relative to the osteogenic control alone (Fig. 1B). At day 21, the osteogenic control + Vitamin D3 resulted in the highest metabolic activity above all groups (Fig. 1B, $P < 0.001$), but at day 28 no differences were observed. The supplementation of Vitamin D3 did not increase metabolic activity when compared to Aquamin alone, at each of the time points (Fig. 1B).

Furthermore, osteoblast cell death was examined at day 28 using EthD-1 as a fluorescence stain, to target the nucleus of dead osteoblasts (Fig.2). This investigated the effect of Aquamin and Vitamin D3 on cell viability after 28 days of culture. Aquamin +/− Vitamin D3 treatment of osteoblasts did not increase cell death, when compared to the osteogenic control +/− Vitamin D3 (Fig.2).

The effect of Aquamin and Vitamin D on osteogenesis

ALP is a catalytic enzyme that is understood to play a role in mineralisation. It is routinely utilised as a marker of osteogenesis. ALP was normalised to osteoblasts cell number, in order to rule out discrepancies attributed by minimal differences in cell number. Aquamin alone had lower ALP levels than the osteogenic control at 7 days (Fig.3, $P < 0.05$). Aquamin then demonstrated higher expression of ALP at 14 days (Fig.3, $P < 0.01$) and comparable levels at the remaining time points when compared to the osteogenic control. ALP levels did not

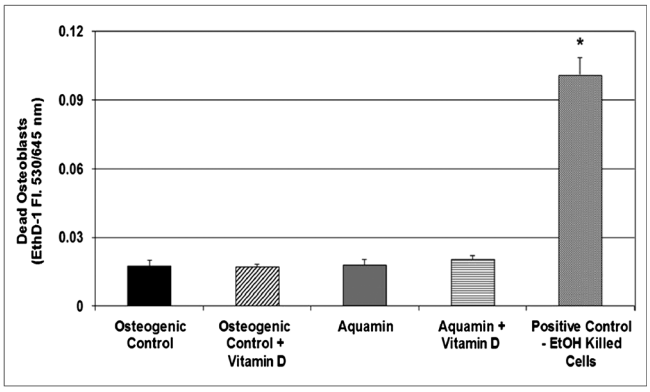

Figure 2. Dead cells were quantified by fluorescence after 28 days and the positive control consisted of treating osteoblasts with ethanol, to induce cell death. Aquamin +/− Vitamin D3 and the osteogenic control +/− Vitamin D3 were comparable in the amount of dead osteoblasts, while the positive control had significantly higher cell death. *$P < 0.0001$, n = 4.

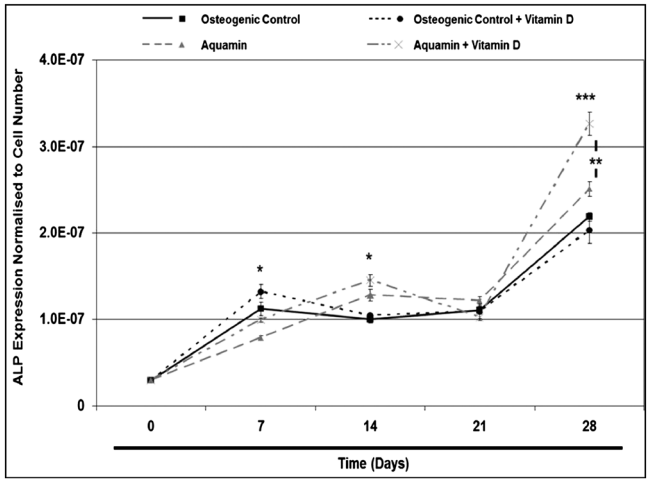

Figure 3. Normalised expression of alkaline phosphatase (ALP) levels was investigated over 28 days. Aquamin +/− Vitamin D3 had higher levels of ALP than the osteogenic control +/− Vitamin D3 on days 14 and 28. Aquamin + Vitamin D3 demonstrated the highest ALP levels on day 28, surpassing Aquamin alone. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$, n = 4.

increase with the addition of Vitamin D3 to the osteogenic control. However, Vitamin D3 supplementation significantly increased ALP levels over Aquamin alone as early as 7 days (Fig.3, $P < 0.0001$). By day 28, Aquamin + Vitamin D3 displayed the highest levels of ALP when compared to Aquamin alone (Fig. 3, P 0.001) and the osteogenic control +/− Vitamin D3 (Fig.3, $P < 0.0001$).

Osteoblast cells produce a matrix that subsequently becomes mineralised, contributing to bone strength. Mineralisation was investigated by histological staining, followed by quantification of the mineralised stain as a direct measure of the degree of mineralisation. Von Kossa and Alizarin Red staining label mineral deposits a brown/black or red colour, respectively. Aquamin alone produced more mineralised nodules than the osteogenic control at day 21, which was further increased by day 28 (Fig.4A and Fig.4B). Nodules were evident at day 21 for all groups except for the osteogenic control. However, by day 28, mineralised nodules were observed in all groups. The addition of Vitamin D3, to Aquamin and the osteogenic control, resulted in early and visibly more mineralised nodules by day 21 (Fig.4A and Fig.4B). Aquamin + Vitamin D3 displayed the highest quantity of mineralised nodules at 21 and 28 days, above all groups (Fig.4A and Fig.4B).

When mineralisation labelled by Alizarin Red was quantified, it corroborated these observations and demonstrated that Aquamin alone has higher mineralisation than the osteogenic control +/− Vitamin D3 on day 21 (Fig. 4C, $P < 0.001$) and 28 (Fig. 4C, $P < 0.0001$). The addition of Vitamin D3 to Aquamin and the osteogenic control increases early mineralisation at days 7 (Fig. 4C, $P < 0.0001$) and 14 (Fig. 4C, $P < 0.0001$). Aquamin + Vitamin D3 resulted in the highest mineralisation at day 21 when compared to Aquamin alone (Fig. 4C, $P < 0.001$) and the osteogenic control +/− Vitamin D3 (Fig.4C, $P < 0.0001$). Aquamin + Vitamin D3 presented higher mineralisation than the osteogenic control +/− Vitamin D3 at day 28 (Fig. 4C, $P < 0.0001$).

DISCUSSION

In this study, we aimed to build on existing knowledge and determine whether the combination of Vitamin D3 and Aquamin would enhance the osteogenic potential of pre-osteoblastic cells in comparison to Aquamin alone. These data sets demonstrated that Aquamin results in higher ALP levels and mineralisation over the osteogenic control, while maintaining comparable cell numbers and metabolic activity. The addition of Vitamin D3 to Aquamin resulted in earlier mineralisation and higher ALP levels, thus confirming our hypothesis and demonstrating the additive osteogenic effect of Vitamin D3 to Aquamin and adding further evidence as to the potential of Aquamin in treating bone disorders.

It was not expected that Aquamin would increase osteoblast cell number, from our previous findings (O'Gorman *et al.*, 2012), and the addition of Vitamin D3 did not increase cell proliferation. This is most likely due to Vitamin D3's designated primary role in mineralisation rather than proliferation (Atkins *et al.*, 2007). The addition of Vitamin D3 to osteogenic controls increased metabolism which peaked at day 21, corresponding with a positive staining for mineralisation. This increase in metabolism by Vitamin D3 was not observed with Aquamin. However, Aquamin resulted in early signs of cellular metabolic activity, but this increase of metabolism was not sustained over time. We propose this may be due to the initial introduction of the multi-mineral formula Aquamin and an initial response in osteoblast metabolism as a result. The treatment of osteoblasts with Aquamin +/− Vitamin D3 did not affect cell viability after 28 days in culture. Previously, the concentration of Aquamin (1 mg/mL) was determined to be the optimal dose that does not affect osteoblast cell viability (O'Gorman *et al.*, 2012) and has been used with cortical glial-enriched primary cells, dermal fibroblasts and colon carcinoma cell lines (Aslam *et al.*, 2010; Ryan *et al.*, 2011). Furthermore, Aquamin inhibits the NFκB pathway (O'Gorman *et al.*, 2012) which is a well-known inducer of cell death and inhibitor of osteogenic differentiation. The reduced expression of NFκB in osteoblasts

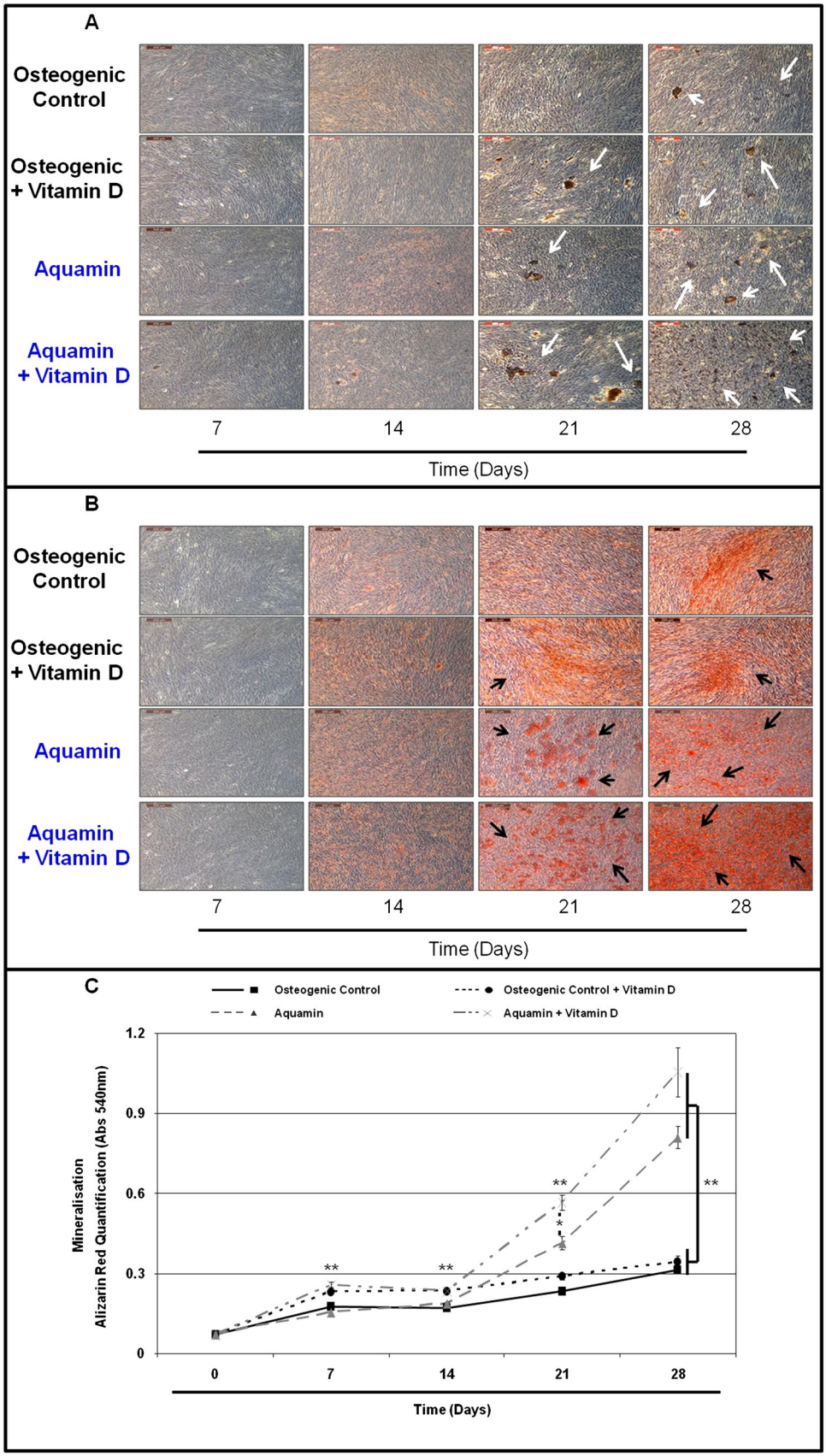

Figure 4. **A**: Von Kossa histological staining which identifies mineralisation by a brown/black stain was examined over 28 days. Aquamin +/− Vitamin D3 displayed more mineralised nodules than the osteogenic control +/− Vitamin D3 on days 21 and 28. Aquamin + Vitamin D3 demonstrated the highest quantity of mineralised nodules on day 28. n = 4. **4B**: Alizarin Red histological staining which identifies mineralisation by a red stain was examined over 28 days. Aquamin +/− Vitamin D3 displayed more mineralised nodules than the osteogenic control +/− Vitamin D3 on days 21 and 28. Aquamin + Vitamin D3 demonstrated the highest quantity of mineralised nodules on day 28, outperforming Aquamin alone. n = 4. **4C**: Alizarin Red histological staining (Fig.4B) was quantified by removing the absorbed red stain and reading absorbance at 540 nm. Vitamin D3 resulted in early mineralisation for both Aquamin and the osteogenic control at days 7 and 14. Aquamin +/− Vitamin D3 displayed higher levels of mineralisation than the osteogenic control +/− Vitamin D3 on days 21 and 28. Aquamin + Vitamin D3 demonstrated the highest levels of mineralisation on day 21, above all treated groups. *$P < 0.001$, **$P < 0.0001$, n = 4. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

results in cell survival and an increase in bone formation (Krum *et al*., 2010).

Vitamin D has been identified in inducing the intake of extracellular calcium within seconds, resulting in the synthesis of the pro-osteogenic non-collagenous proteins, osteopontin and osteocalcin (Uchida *et al*., 2010). Moreover, a strong correlation between Vitamin D3 and osteocalcin, that is involved in mineralisation and calcium ion homeostasis, has been demonstrated for ossification *in vivo* (Nakanishi *et al*., 2013). Therefore, we measured early and late markers of osteogenesis, ALP and mineralisation, respectively. The authors chose mineralisation as the most important parameter for analysis as it is end point of osteogenesis. In terms of bone health and osteoporosis prevention and treatment, the ultimate goal is increased mineralisation as brittle bones are associated with diseased skeletal states, consequently leading to pathological fractures (Widaa *et al*., 2012) and patient morbidity.

ALP and mineralisation were increased when introducing Aquamin to the osteoblasts. This is likely due to the presence of calcium in Aquamin, which enhances osteoblast gene expression of bone morphogenetic proteins that are pro-osteogenic (Nakade *et al*., 2001) and is a positive physiological regulator of osteoblast differentiation and survival (Caudarella *et al*., 2011). An additional increase in ALP and mineralisation was observed when supplementing Aquamin with Vitamin D3. Vitamin D3 binding to its receptor on osteoblasts can initiate pro-osteogenic events, which include an increase in ALP, mineralisation (Haneji *et al*., 1983; Kurihara *et al*., 1986; Matsumoto *et al*., 1991; Pike, 2011) and better collagen quality (Nagaoka *et al*., 2008). This suggests that Aquamin and Vitamin D3 complement each other in promoting expression of ALP and increasing mineralisation, which aids bone formation and strength.

There are limitations in this study. For one, utilising alternative cell sources to the immortalised MC3T3-E1 murine osteoblastic cell would be interesting. In particular, it might be of interest to investigate the effects of Aquamin alone and in combination with Vitamin D3 on human primary osteoblast cells or mesenchymal stems cells. In addition to ALP, a more detailed analysis of gene expression might provide further insight into the molecular mechanisms influencing osteogenesis by the presence of Aquamin +/− Vitamin D3. Mineralisation is the final stage osteogenic marker and end point of osteogenesis. A more detailed analysis of osteogenic markers such as collagen type-I, osteopontin and osteocalcin on the mRNA/protein level may provide a more comprehensive analysis into molecular mechanisms involved. In terms of bone health and osteoporosis prevention and treatment, the ultimate goal is increased mineralisation. In this study, our aim was to determine whether mineralisation would be increased with the combination of Aquamin with Vitamin D3 rather than to determine its mode of action specifically. Finally, the effects of Aquamin and Vitamin D3 on osteogenesis should be examined in animal models *in vivo* to provide further information on the potential of the supplement as an anabolic agent in the fight against bone loss.

Skeletal degenerative disease states resulting in bone loss and fractures are on the increase worldwide, and this can be related to an aging population and sedentary life style. Bone loss is associated with bone remodelling, in which the amount of bone being broken down (bone resorption) is less than the amount of bone being produced and results in a net bone loss. This leads to diseased states such as osteoporosis that is characterised by low bone mass and a failure to replace lost bone, consequently resulting in weak bones that are susceptible to fractures (Raisz, 2005). Current understanding suggests that calcium and Vitamin D are key factors that work synergistically and are beneficial as dietary supplements for the prevention of bone loss and fractures (Heaney, 2008; Nowson, 2010; Lips, 2012). Although milk and dairy products offer a source of calcium, an additive non-dairy, natural source of calcium and other minerals is in demand.

Aquamin is a natural food supplement that can provide a source of calcium and 72 other minerals (Ryan *et al*., 2011). The supplementation of Vitamin D3 to Aquamin results in a significant additive effect of pro-osteogenic events and, therefore, may be beneficial when co-administered for maintaining bone health (Peacock *et al*., 2000) and as a possible dietary supplement for treatment of bone loss from infectious diseases (Widaa *et al*., 2012). Most treatments for osteoporosis and bone loss focus on preventing loss through the use of anti-resorptive agents. However, these agents only prevent bone loss and do not encourage new bone formation. The net result of this strategy is old, brittle bones that may actually be more susceptible to fracture. Worryingly, more and more clinical evidence is emerging which is raising clinical concerns involving the long term systemic use of bisphosphonates. The current data suggests the potential of Aquamin in combination with Vitamin D as an anabolic agent in the fight against bone loss.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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