The Marine-derived, Multi-mineral formula, Aquamin, Enhances Mineralisation of Osteoblast Cells *In Vitro*

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Osteoporosis is a global health problem characterized by low bone mass and an increase in bone fragility. It is now well accepted that dietary factors play a central role in bone development and health. Diet that lacks adequate minerals is considered to be a risk factor for osteoporosis. The food supplement, Aquamin, is a natural, multi-mineral derived from the red algae *Lithothamnion corallioides*, rich in calcium, magnesium and 72 other trace minerals. The aim of this study was to evaluate the effect of Aquamin on osteoblastic behaviour and mineralisation in a pre-osteoblastic cell line. Cell number and metabolic activity were assessed using Hoescht DNA and AlamarBlue assays respectively. Osteogenic differentiation was measured using an alkaline phosphatase assay while mineralisation was determined using von Kossa and alizarin red staining. It is reported here that Aquamin promotes increased mineralisation in osteoblast cell culture. These data suggest that the nutritional supplement Aquamin plays an important role in promoting bone formation and may be useful in treating bone diseases such as osteoporosis. Copyright © 2011 John Wiley & Sons, Ltd.

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INTRODUCTION

Osteoporosis is a condition characterized by low bone mass, low mineral content and microarchitectural deterioration leading to enhanced bone fragility and consequent increase in risk of bone fracture (Raisz, 2005). Dietary factors are known to play an important role in bone health determining final bone mineral density and the extent of age-related bone-loss in later life (Palacios, 2006). Diets lacking adequate inorganic minerals, especially calcium, have been identified as risk factors for osteoporosis (Peacock *et al.*, 2000). Although calcium is considered to be the most important mineral in bone, several other minerals are also known to play a major role in bone health, including magnesium, manganese, copper, zinc and selenium (Nieves, 2005; Palacios, 2006). It is now accepted that many individuals who do not obtain the recommended daily amount (RDA) of these minerals from dietary sources depend on calcium and multi-mineral supplements.

Aquamin is a natural, marine-derived, multi-mineral rich in calcium and magnesium as well as measurable levels of 72 other minerals including manganese, selenium, copper and zinc. It is harvested from the skeletal remains of the red marine algae *Lithothamnion corallioides* from the cold Atlantic waters off the coasts of Ireland and Iceland.

Poor diets, especially those that contain a high proportion of fat, have been identified as risk factors

for osteoporosis (Wohl *et al.*, 1998). In a recent 15 month study, mice were fed normal chow, a high fat western style diet (HFWD), or HFWD plus Aquamin. Results showed that HFWD mice lost a significant amount of bone compared with control mice, and that Aquamin reversed the negative effects of the HFWD (Aslam *et al.*, 2010a). The authors were unable to identify why exactly Aquamin preserved bone structure and function in the mice other than stating that many of the individual components of Aquamin have a direct effect on bone structure and function. However, they hypothesized that the positive effects of Aquamin on bone may also be a secondary response to the control of systemic inflammation by Aquamin.

Whether a direct result or as a secondary response Aquamin has also been shown to alter markers of bone metabolism in yearling horses (Nielsen *et al.*, 2010). Treatment with Aquamin resulted in an increase in osteocalcin, a marker of bone formation. In conjunction with this was an increase in C-telopeptide crosslaps of type I collagen, a bone resorption marker. This enhanced bone turnover may provide a means to remove and repair old or damaged bone, which is essential in maintaining bone health. As calcium is the main constituent of Aquamin it is possible that the calcium present stimulates the production of bone morphogenic proteins which increase proliferation of osteoblasts (Nakade *et al.*, 2001).

Aquamin has also been shown to be of benefit in joint health (Frestedt *et al.*, 2008; Frestedt *et al.*, 2009). Two randomized, double-blind, placebo-controlled studies using Aquamin showed reduction of osteoarthritic symptoms and increased activity scores in subjects with moderate to severe osteoarthritis. It was proposed

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that this was mediated through tumor necrosis factor alpha, a cytokine involved in systemic inflammation, and interleukin 1β, a cytokine involved in the inflammatory response (Ryan *et al.*, 2011). Finally, in digestive health a recent *in vitro* study described the growth inhibitory effects of Aquamin on calcium-sensitive and calcium-resistant colon carcinoma cells (Aslam *et al.*, 2009). Another study showed how Aquamin inhibits polyp formation and inflammation in the gastrointestinal tract of mice on a HFWD diet (Aslam *et al.*, 2010b).

Osteoblasts play an essential role in bone metabolism and new bone formation (Parfitt 1984). Osteoblasts differentiate from mesenchymal cells and are responsible for the synthesis of bone matrix and bone mineralisation, synthesis of growth factors and hormones and also for the regulation of osteoclastogenesis and bone resorption. The present study was conducted to examine the effect of Aquamin on osteoblastic behaviour and mineralisation in the mouse calvarial pre-osteoblastic MC3T3-E1 cell line. This cell line has the ability to differentiate into mature osteoblast phenotype and to mineralise in the presence of β-glycerophosphate and ascorbic acid (Menard *et al.*, 2000).

In this study we test the hypothesis that Aquamin directly enhances osteogenesis by providing not only an extra source of calcium but also other essential bone supporting minerals necessary for the mineralisation of extracellular matrix deposited by these cells.

MATERIALS AND METHODS

Aquamin. This study employed the soluble form of Aquamin containing 12% calcium and 1% magnesium as well as trace quantities of other bone-supporting minerals including manganese, selenium, copper and zinc as described recently (Aslam *et al.*, 2010a). The mineralised fronds of the red marine algae *Lithothamnion corallioides* are prepared under ISO and HACCP certification and sold as Aquamin® (EU and FDA approved, GRAS 000028) (Marigot Ltd, Cork, Ireland).

The experimental dose for Aquamin (1 mg/mL) was obtained from preliminary experiments that examined the pH of dose media, cell metabolic activity and cell number/viability in MC3T3-E1 cells (data not shown). Similar concentrations were used in previous experiments using low passage human dermal fibroblasts, colon carcinoma cell lines (Aslam *et al.*, 2009) and cortical glial-enriched primary cultures (Ryan *et al.*, 2011).

Unless stated otherwise, all consumables and chemicals were purchased from Sigma-Aldrich (Germany).

Cell culture. This study was carried out in 2D using six-well plates and 22 mm discs. MC3T3-E1 murine calvarial osteoblasts were maintained in α-modified minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 1% L-glutamine and 2% penicillin/streptomycin. The media was replaced every 3–4 days. Cells were detached using trypsin-EDTA (Ethylenediaminetetraacetic acid), centrifuged at $500\,g$ for 5 min at 20 °C and resuspended to obtain a seeding density of 50000 cells per disc. Osteogenic media were prepared with the addition of ascorbic acid (50 μg/mL), β-glycerophosphate (10 mM), dexamethasone (100 nM) and Aquamin (Marigot Ltd, Cork, Ireland) at a dose of

1 mg/mL to standard medium. An osteogenic medium without the addition of Aquamin was used as a control.

Metabolic activity. Osteoblast activity was evaluated using AlamarBlue (Invitrogen) as described previously (O'Brien *et al.*, 2000). Four milliliters of supplemented α-MEM media containing 10% AlamarBlue solution were placed into each well and placed in a 37 °C incubator with 5% CO_2. After 2 h the plates were removed from the incubator and 100 μL of the reduced AlamarBlue supernatant was plated out in triplicate into a 96-well plate and absorbance of the samples was read on a spectrometer (Titertrek Multiscan, MSC, Ireland) at 540 nm and 620 nm. Percentage reduction of the AlamarBlue solution was determined according to the manufacturer's specifications. This method was repeated at every time point up to 28 days.

Cell number. Cell number was determined using a DNA assay, Hoechst 33258, according to a previously published protocol (Kim *et al.*, 1988). Cells were detached using trypsin-EDTA. Three millilitres of media were added to each well and the cell suspension centrifuged at $500\,g$ for 5 min. The cell pellet was then resuspended in 400 μL papain buffer solution (100 mL Dulbecco's phosphate buffered saline (DPBS), 1 mL 0.5 M EDTA (pH 8.0), 79 mL cysteine-HCl; 10 mg papain per 10 mL buffer), transferred to eppendorfs and incubated at 60 °C for 40 min. A 10× solution of Hoechst buffer was made: 10 mM Tris, 10 mM Na$_2$EDTA, 1 M NaCl; pH 7.4 and filter sterilized. A working dye concentration was freshly made 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 and protected from light. Once cell suspension had been fully digested, 30 μL of each sample was mixed with 600 μL of working dye solution and vortexed briefly. The resulting solution was plated out in triplicate and read at an excitation of 355 nm and an emission of 460 nm (Wallac Victor, PerkinElmer Life Sciences). Readings were converted to cell number by standard curve. Controls were measured as blank scaffold samples digested in papain. These experiments were repeated to provide a sample of $n = 3$ for each time point for 28 days.

Alkaline phosphatase expression. Alkaline phosphatase expression (ALP) was chosen as a marker of early bone formation and measured using a *p*-nitrophenyl phosphate (*p*NPP) assay as per manufacturer's instructions. Two millilitres of ALP lysis buffer (0.1 M sodium acetate anhydrous, 2% triton-X 100®, 10 mM *p*NPP solution) were added to each well and the plates incubated in the dark at 37 °C for 1 h. Two-hundred microlitres of the resulting supernatant were plated in triplicate into a 96-well plate. One-hundred microlitres of 0.3 M NaOH were added to each well to stop the reaction and the plates were read on a spectrometer (Titertrek MultiScan, MSC, Ireland) at 405 nm at each time point and this was repeated to obtain $n = 3$ for all time points. The results are presented with ALP expression normalized to cell number evaluated from the DNA assay,

Mineralisation staining. Mineralisation was detected using von Kossa staining and was quantified with alizarin red absorbance readings. These techniques are used routinely in our laboratory (Gleeson *et al.*, 2010).

Von Kossa staining. Cells were stained for the presence of mineralisation with Von Kossa stain, which is highly specific for calcium/phosphate deposition. Disc sections over the 28 days were prepared for staining and re-hydrated through a series of ethanol/distilled-water baths. Once the sections had been rehydrated, 5% silver nitrate solution was pipetted onto the surface of the disc and left under a lamp source for 1 h. Discs were washed three times in distilled water and incubated with sodium thiosulphate for 2 mins. Discs were again washed and stained with toluidine blue as background staining for cells and dehydrated, air-dried and cover-slipped before being viewed under light microscopy.

Alizarin red S staining. Disc sections were stained with alizarin red S at the specified time intervals over the 28 day culture period. Alizarin red S attaches non-specifically to calcium and is used to observe if bone mineral has been deposited. Sections were rehydrated through a series of ethanol/distilled-water baths. Once sections had been rehydrated, slides were placed into a bath containing 2% alizarin red S and left for 2 mins. Sections were dehydrated back through water/ethanol but were left to dry completely without cover slip addition. A 10% acetylpyridinium chloride solution was made by dissolving the powder in distilled water by heating while stirring. One millilitre of the resulting solution was added to each well. Plates were gently agitated on a rocker for 20 min. One-hundred microlitres of the solution were pipetted in triplicate into a 96-well plate and read at 540 nm. This was repeated for $n = 3$ for all time points up to 28 days.

Statistical analysis. One-way ANOVA was used in all analyses with the Holm–Sidak t-test used for multiple comparisons. Statistical significance was determined at $p < 0.05$.

RESULTS

Cellular metabolic activity increased in both the un-treated and Aquamin-treated groups over the course of the culture period. Statistically higher levels of metabolic activity ($p \leq 0.047$) were seen at days 14, 21 and 28 relative to the earlier time points in both groups (Fig. 1A). No difference in metabolic activity was found between the control and Aquamin-treated groups at any time point.

Statistical analysis indicated ($p \leq 0.042$) that cell number was significantly increased by days 14, 21 and 28 relative to the earlier time points of days 1, 3 and 7 in both groups (Fig. 1B). Cell number at day 21 in the control group exhibited the highest cell number during the culture period and was statistically higher ($p \leq 0.016$) relative to all other groups and time points.

No ALP expression was found at day 1 while peak expression occurred at day 3 in both groups (Fig. 2) with significantly higher expression found at days 3 and 7 than at days 1, 14, 21 and 28 in both groups ($p \leq 0.005$). Notably, expression of ALP was significantly higher in the Aquamin-treated group compared with the control at day 21 ($p \leq 0.05$) with no difference found at any of the other time points.
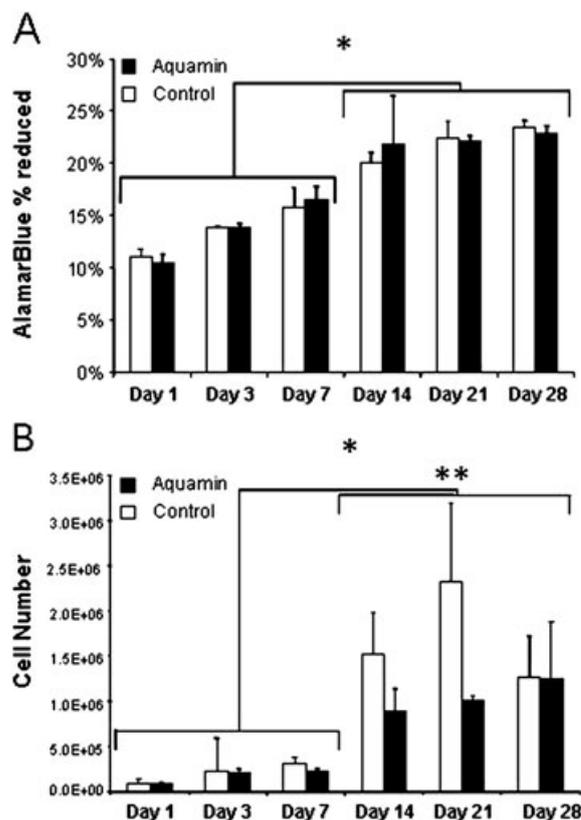

Figure 1. Effect of Aquamin on (A) metabolic activity and (B) cell number over 28 days. Cell metabolic activity was determined at 1, 3, 7, 14, 21 and 28 days using AlamarBlue® assay (A). Results are expressed as % reduction ($n = 6$). Error bars represent SD. *$p \leq 0.047$ relative to days 1, 3 and 7. Cell number was determined using a Hoescht DNA assay (B). Error bars represent SD. $n = 3$ for each time point. *$p \leq 0.042$ relative to days 1, 3 and 7. **$p \leq 0.05$ relative to all other groups and time points.
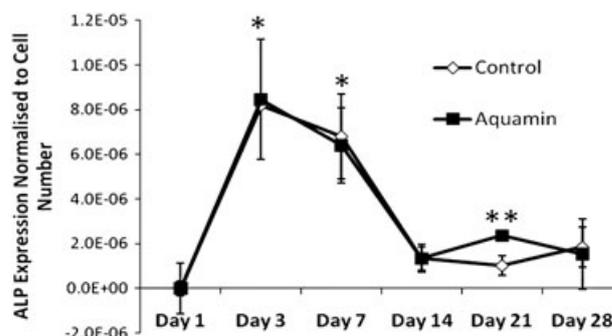

Figure 2. Effect of Aquamin on the expression of alkaline phosphatase. Alkaline phosphatase expression was determined via a pNPP assay and normalized to cell number. Expression was significantly higher in both groups at days 3 and 7 than days 1, 14, 21 and 28. Normalized ALP expression in the Aquamin-treated group was greater than in the control group at day 21. Error bars represent SD. $n = 3$ for each time point. *$p \leq 0.005$. **$p \leq 0.05$.

Control and Aquamin-treated discs were evaluated for calcium deposits using Von Kossa, which labels deposits of calcium with silver nitrate (Fig. 3). Toluidine blue staining was used for background visualization of the cell layer. Red arrows show cell nodule formation occurring in the cell layer. Deposits of calcium were first seen at day 21 in the control and Aquamin-treated groups (Fig. 3, G and H) and continued to day 28. Nodule formation occurred as the culture period increased and osteoblasts tended to layer themselves within these
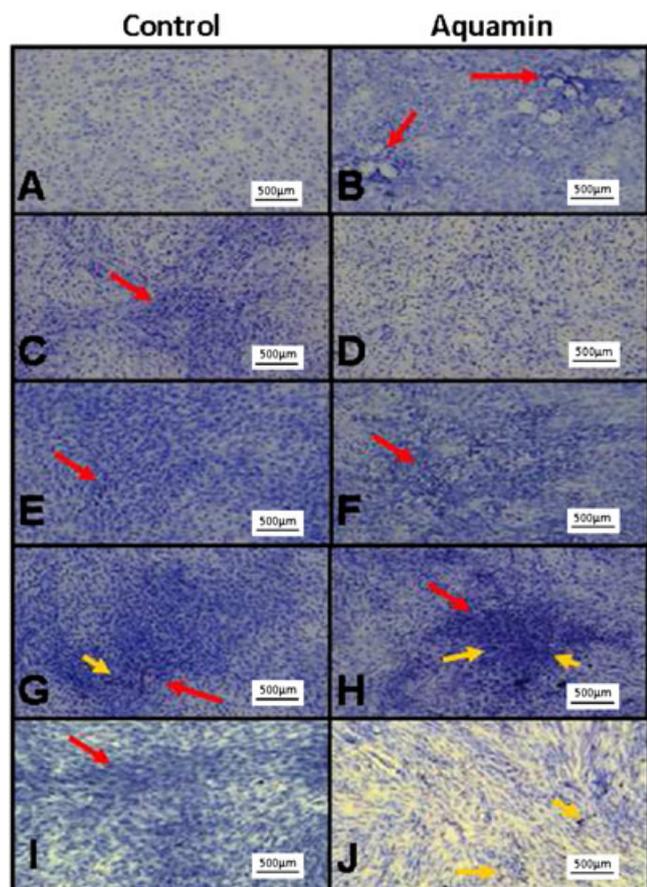
Figure 3. Mineralisation staining with von Kossa. Visualized using 100× magnification. (A) Control day 3. (B) Aquamin day 3. (C) Control day 7. (D) Aquamin day 7. (E) Control day 14. (F) Aquamin day 14. (G) Control day 21. (H) Aquamin day 21. (I) Control day 28. (J) Aquamin day 28. Red arrows indicate cell nodule formation, coupled with dark specks of the black von Kossa stain indicated by the yellow arrows. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr
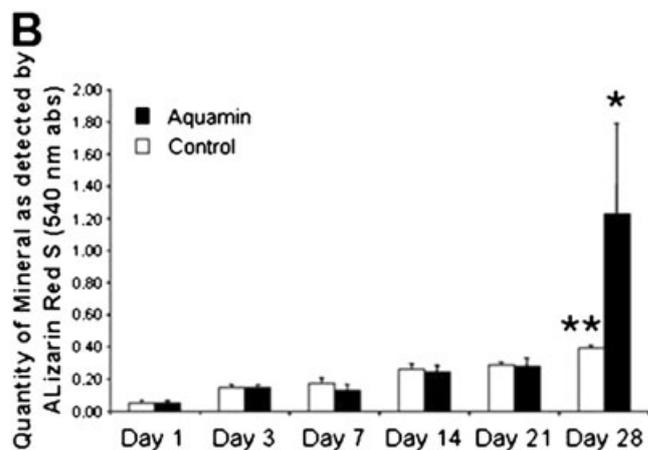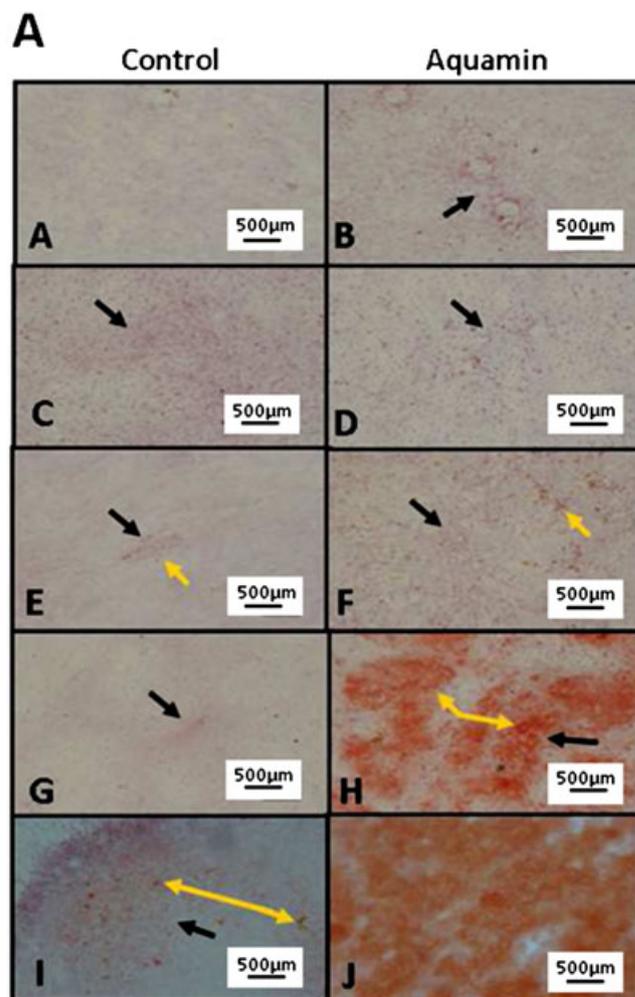

Figure 4. Mineralisation staining with alizarin red S. (A) Visualized using 100× magnification: A, control day 3; B, aquamin day 3; C, control day 7; D, aquamin day 7; E, control day 14; F, aquamin day 14; G, control day 21; H, aquamin day 21; I, control day 28; J, aquamin day 28. Black arrows indicate cell nodule formation and yellow arrows indicate small mineral deposits. (B) Mineral was quantified using alizarin red S assay. Error bars represent SD. $n = 3$. *$p < 0.001$ relative to all other groups and time points. **$p \leq 0.018$ relative to control and Aquamin at day 1. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

nodules. Mineral deposition was first detected in these cell nodules.

Mineral deposition was also evaluated using alizarin red S at each time point. Small nodules of red-stained mineral appeared on all Aquamin-treated discs at day 14 (Fig. 4A) and continued to increase until staining was completely widespread throughout the cell layer (Fig. 4A, H and J). Small mineral deposits were seen at day 14 in the control group; however, mineral deposition did not increase dramatically during the culture period up to day 28 and remained sparse when compared with the Aquamin-treated group. The cell layer in all groups tended to form cell nodules as the culture period increased and the cells started to layer themselves on each other. It is in these cell nodules that the mineral deposition tends to occur first.

When quantified, mineral deposition was seen to increase linearly over time in both groups with no statistical effect observed between groups until day 28. At this time point statistical analysis indicated that Aquamin resulted in increased mineral deposition ($p < 0.001$) (Fig. 4B).

DISCUSSION

In this study we assessed the effects of the marine-derived, multi-mineral, Aquamin on osteoblast behaviour and mineralisation in the mouse calvarial pre-osteoblastic MC3T3-E1 cell line. Aquamin (EU and FDA approved, GRAS 000028) is sold globally by Marigot Ltd, Ireland following harvesting of *Lithothamnion corallioides*. The mineralised fronds are rich in calcium and magnesium

and other essential minerals required for bone health, including manganese, zinc, copper, selenium, phosphorous and boron (Nieves, 2005). It was hypothesized that Aquamin, with its rich mineral supply, would have a positive osteogenic effect on the cellular behaviour and mineralising capacity of osteoblasts. This study showed that Aquamin does not have any cytotoxic effect and possesses the ability to increase mineralisation by osteoblasts *in vitro*.

Untreated cells proliferated rapidly up until day 14 followed by a period of extracellular matrix development and mineralisation. This is consistent with previous studies (Stein and Lian, 1993). A similar trend was observed in Aquamin-cultured cells whereby cell number was significantly increased by day 14 and this was followed by a period that saw no change in cell proliferation. Interestingly, there were significantly more cells in the control group at 21 days than in the Aquamin- cultured cells. However, by day 28 there was no significant difference in cell number between the two groups, indicating that long-term exposure of osteoblasts to Aquamin does not have a detrimental effect on osteoblast cell number.

Comparison between the treatment and non-treatment groups showed that Aquamin did not significantly affect metabolic activity. However, when taken in the context of cell number, the reduced cell number at day 21 in the Aquamin-treated group implies that these cells were more metabolically active than the untreated cells. As cell number was not significantly increased in the day 28 Aquamin treated group, the results suggest the cells have stopped proliferating and are undergoing differentiation, which is consistent with the increase in ALP expression seen at day 21 in Figure 2.

Differentiation of the pre-osteoblast to a mature phenotype results in the expression of ALP, a bone enzyme which has become a hallmark of osteogenesis and mineralisation (Simao *et al.*, 2007). While it may seem unusual that ALP expression was increased at days 3 and 7 in this study, it is important to remember that MC3T3-E1 cells are a partially differentiated cell line. Therefore the introduction of these cells to osteogenic factors will initiate production of the ALP enzyme. Production of ALP is also increased prior to mineral production by osteoblasts (Stein and Lian, 1993). This is reflected in the increased ALP expression in the Aquamin treated group at day 21 prior to the increased mineralisation that was observed and measured at day 28 in this treatment group.

Qualitative analysis of mineralisation using Von Kossa staining showed increased mineralisation in both groups over time. Mineralisation was located around individual cell nodules although no clear differences were seen between groups. Visually, alizarin red S staining was able to detect differences between the individual groups by day 21 with more mineral visible in the Aquamin-treated cells. However, quantitatively no significant differences were measured. Crucially though, Aquamin induced a significant increase in mineralisation by day 28, which was visible and quantifiable. This represented a threefold increase in mineralisation when compared with cells that were cultured in the absence of Aquamin.

The lack of strong differences in Von Kossa staining at days 21 and 28 between groups may seem at odds with the alizarin red staining results. However, von Kossa staining is a highly specific stain for calcium or calcium salt, which primarily stains calcium phosphate and calcium carbonate by binding the positive silver ion with the negative phosphate or carbonate portion. In contrast, the alizarin red S stain is a far broader stain that is not entirely specific for calcium since magnesium, manganese, barium, strontium and iron may be stained during this process (manufacturer's information). Under normal circumstances these elements do not usually occur in sufficient concentrations to affect staining, however, all are present in Aquamin. Therefore, it appears as if these elements are synergistically contributing to the amount of mineral deposition occurring in the cell layer. Despite the apparent discrepancies, both techniques do show increased mineralisation at the later time points and this is in agreement with previous reports where Aquamin was shown to be of benefit in bone health (Aslam *et al.*, 2010a; Nielsen *et al.*, 2010). Other marine calcium carbonate sources such as nacre (mother of pearl) from *Pinctada maxima* also increase mineralisation of MC3T3-E1 mouse pre-osteoblasts (Rousseau *et al.*, 2003).

A number of studies have shown that bone density can be increased with calcium supplementation (Karkkainen *et al.*, 2010; Lips *et al.*, 2010). In the current study Aquamin was found to significantly increase the mineralisation ability of osteoblasts. In an *in vivo* situation this increased mineralisation would be expected to translate to an increase in bone density. Whether this is the case will require further study, however, this study has shown that Aquamin has a direct effect on the mineralisation capabilities of osteoblasts. In previous studies it was hypothesized that Aquamin's positive effects on bone were a secondary response to changes in systemic inflammation. While this may still be the case, the current data prove that Aquamin directly influences the bone-forming cells.

There is no doubt that calcium plays an important role in maintaining bone health. Although recent research has suggested an increased risk of cardiovascular events in people taking high doses of calcium supplements (Bolland *et al.*, 2010). This increased cardiovascular risk does not appear when the calcium is taken in the form of a food supplement (Reid *et al.*, 2011). As Aquamin is a natural calcium-rich food none of the potential risks associated with high doses of calcium supplementation have been reported.

This study has a number of limitations one of which is the use of MC3T3 cells, a murine immortalized cell line. A primary culture of human osteoblasts may provide a better indication of osteoblast cell response. In addition, a more comprehensive analysis of the genes and proteins associated with the process of osteogenesis might help our understanding of the process by which Aquamin enhances osteoblast activity. Although calcium is the most abundant mineral in Aquamin, we do not yet know which of the multiple minerals present contribute to the observed effects. However, it is likely that many of the components synergistically interact to influence bone structure and function and enhance normal mineralisation in osteoblast cells. A potential drawback of this study was the use of toluidine blue as a counterstain in the von Kossa staining rather than nuclear fast red. This dark blue stain makes it difficult to identify the mineralised nodules, which appear as a dark brown/black colour.

Despite these limitations, this study has shown qualitatively that Aquamin can directly influence osteoblast activity. Where previously it was hypothesized that changes in bone metabolism were an indirect response to changes in systemic inflammation, this study has shown that Aquamin can directly influence osteoblasts to produce more mineral. This study does represent the first steps towards understanding how Aquamin improves bone health.

In summary, although it is accepted that further research in both explants and animals is needed, this initial study has demonstrated that Aquamin shows no cytoxicity in osteoblast cell culture and increases mineralisation. It may therefore possess significant potential for treating bone diseases such as osteoporosis.

Acknowledgements

Conflicts of Interest

There are no conflicts of interest.

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