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

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Short Title: Aquamin-enhanced mineralisation of osteoblasts

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Abstract:

Osteoporosis is a global health problem characterised 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 Alamar Blue 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.

Introduction:

Osteoporosis is a condition characterised 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 to 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 hypothesised 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 randomised, 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 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.*, 2010). 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 mineralization, 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 extra cellular 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 (1mg/ml) was obtained from preliminary experiments which 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.*, 2010).

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

Cell Culture: This study was carried out in 2D using 6-well plates and 22mm discs. MC3T3-E1 murine calvarial osteoblasts were maintained in α-modified 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, centrifuged at 500g for 5 minutes at 20°C and re-suspended to obtain a seeding density of 50,000 cells per disc. Osteogenic media was prepared with the addition of ascorbic acid (50μg/ml), β-glycerophosphate (10mM), dexamethasone (100nM) and Aquamin (Marigot Ltd., Cork, Ireland) at a dose of 1mg/ml to standard medium. An osteogenic media 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). 4mls of supplemented α-MEM media containing 10% alamarBlue solution was placed into each well at 24 hours and placed in a 37°C incubator with 5% CO_2. After 2.5 hours the plates were removed from the incubator and 100μl 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 540nm and 620nm. 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. 3mls of media was added to each well and the cell suspension centrifuged at 500g for 5 minutes. The cell pellet was then re-suspended in 400µl papain buffer solution (100mls DPBS, 1ml 0.5M EDTA (pH 8.0), 79ml Cysteine-HCl; 10mg papain per 10ml buffer), transferred to eppendorfs and incubated at 60°C for 40 minutes. A 10x solution of Hoechst buffer was made: 10mM Tris, 10mM Na_2EDTA, 1M NaCl; pH 7.4 and filter sterilised. A working dye concentration was freshly made for each experiment at 0.1µg per ml (1:10,000) dilution – 2mls 10x Hoechst buffer, 18mls distilled water, 2µl Hoechst stock dye and protected from light. Once cell suspension had been fully digested, 30µl of each sample was mixed with 600µl of working dye solution and vortexed briefly. The resulting solution was plated out in triplicate and read at an excitation of 355nm and an emission of 460nm (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 (ALP): ALP was chosen as a marker of early bone formation and measured using a p-nitrophenyl phosphate (pNPP) assay as per

manufacturer's instructions. 2mls of ALP lysis buffer (0.1M sodium acetate anhydrous, 2% triton-X 100®, 10mM pNPP solution) was added to each well and the plates incubated in the dark at 37°C for 1 hour. 200µl of the resulting supernatant was plated in triplicate into a 96-well plate. 100µl of 0.3M NaOH was added to each well to stop the reaction and the plates were read on a spectrometer (Titertrek MultiScan, MSC, Ireland) at 405nm at each time point and this was repeated to obtain n=3 for all time points. The results are presented with ALP expression normalised 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 re-hydrated, 5% silver nitrate solution was pipetted onto the surface of the disc and left under a lamp source for 1 hour. Discs were washed 3 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 re-

hydrated through a series of ethanol/distilled water baths. Once sections had been re-hydrated, 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. 1ml of the resulting solution was added to each well. Plates were gently agitated on a rocker for 20 minutes. A 100µl of the solution was pipetted in triplicate into a 96-well plate and read at 540nm. 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 Holm-Sidak t-test used for multiple comparisons. Statistical significance was determined at $p<0.05$.

Results:

Cellular metabolic activity increased in both the untreated 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 (Figure 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 (Figure 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 (Figure 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 to the control at day 21 ($p \leq 0.05$) with no difference found at any of the other time points.

Control and Aquamin-treated discs were evaluated for calcium deposits using Von Kossa which labels deposits of calcium with silver nitrate (Figure 3). Toluidine blue staining was

used for background visualisation of the cell layer. Red arrows show cell nodule formation occurring among the cell layer. Deposits of calcium were first seen at Day 21 in the control and Aquamin-treated group (Figure 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 nodules. Mineral 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 (Figure. 4A, F) and continued to increase until staining was completely wide-spread throughout the cell layer (Figure 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 to 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 ($p < 0.001$) (Figure 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 which 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. ALP production 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 which 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 3 fold increase in mineralisation when compared to cells which were cultured in the absence of Aquamin.

The lack of strong differences in Von Kossa staining at days 21 and day 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 hypothesised 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 proves 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 immortalised 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 hypothesised 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.

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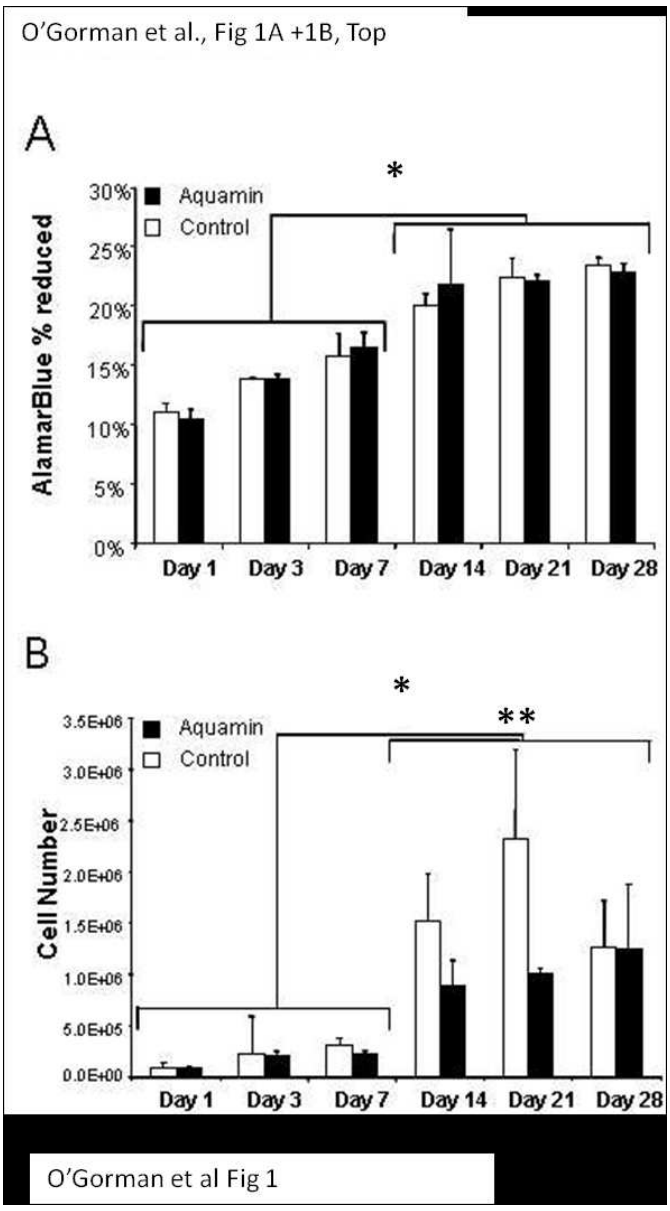
Figure 1: Effect of Aquamin on metabolic activity (A) and cell number (B) over 28 days. Cell metabolic activity was determined at 1, 3, 7, 14, 21 and 28 days using alamarBlue™ assay (1A). Results are expressed as % reduction (n=6). Error bars represent S.D. * = p≤0.047 relative to Days 1, 3 and 7. Cell number was determined using a Hoescht DNA assay (1B). Error bars represent S.D. n=3 for each time point. * = p≤0.042 relative to days 1, 3 and 7. ** = p≤ 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 normalised to cell number. Expression was significantly higher in both groups at days 3 and 7 than days 1, 14, 21 and 28. Normalised ALP expression in the Aquamin treated group was greater than in the control group at day 21. Error bars represent S.D. n=3 for each time point. * = p≤0.005 ** = p≤0.05.

Figure 3: Mineralisation staining with Von Kossa. Visualised using 100x 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.

Figure 4: Mineralisation staining with Alizarin Red S. (A) Visualised using 100x 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 S.D. n=3. * = $p<0.001$ relative to all other groups and time points. ** = $p\leq0.018$ relative to Control and Aquamin at Day 1.



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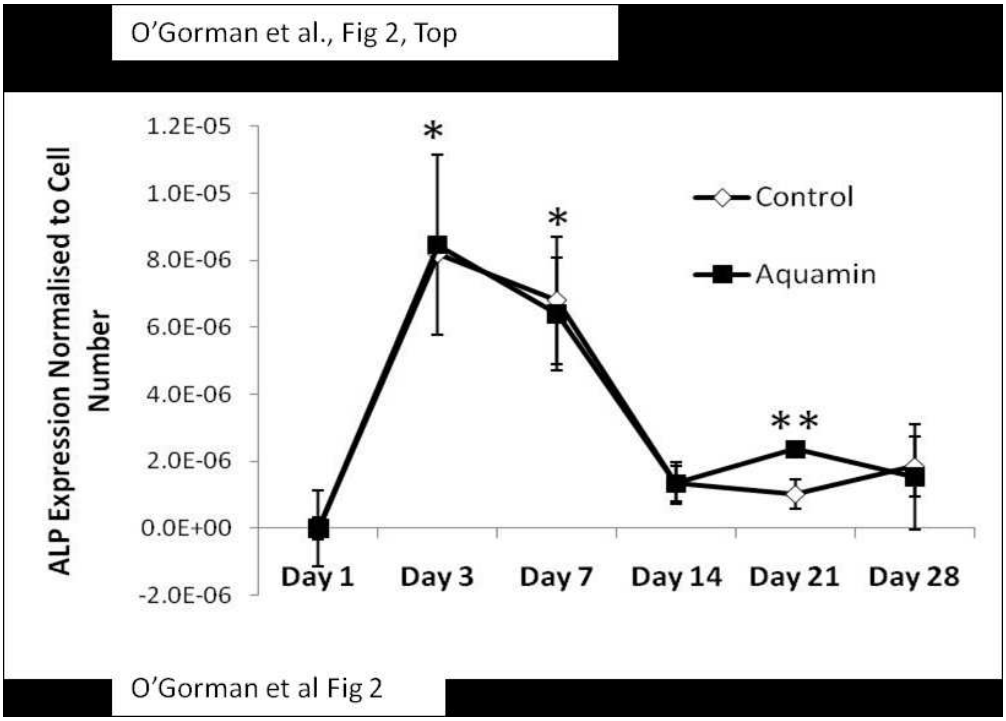

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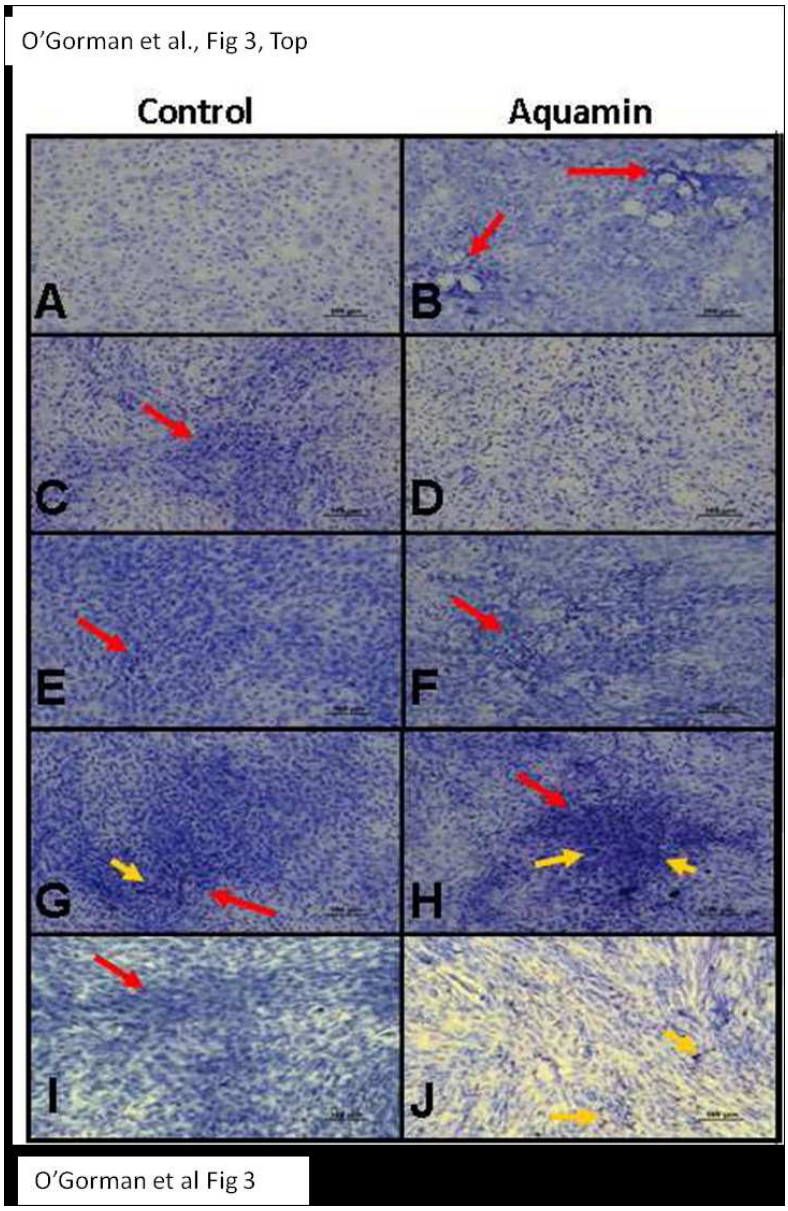

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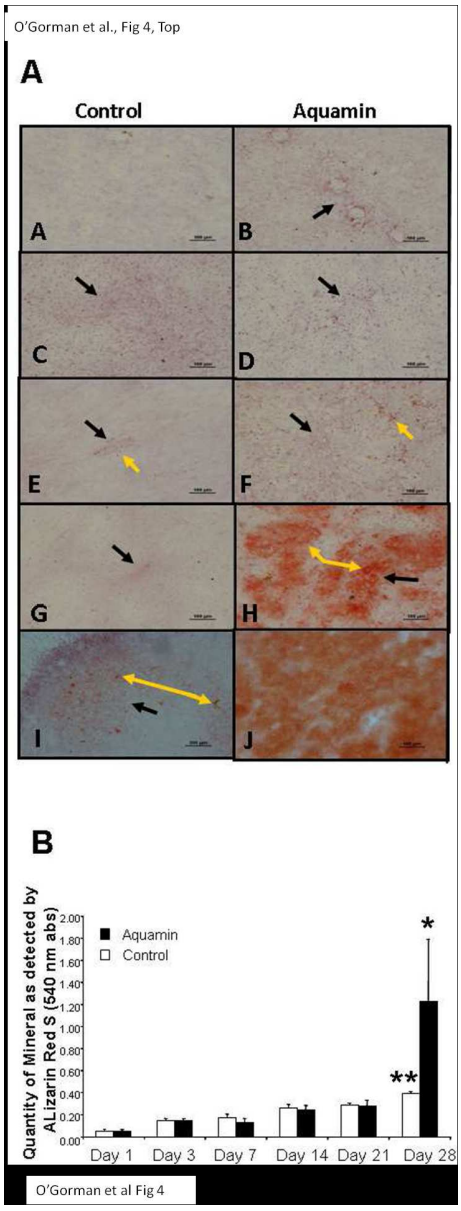

O'Gorman et al Fig 4
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