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The effect of a United Fisheries Limited MCHC fish bone powder on osteoblast function and osteoclast-precursor differentiation

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Lay Summary

This study was designed to test the effect of a United Fisheries Limited “MCHC fish bone powder” on:

Aims:

The proliferation and differentiation of osteoblast-like cells.

What effect does the United Fisheries MCHC fish bone powder have on the osteoblast? Osteoblasts are the cells which build bone. Does this powder cause the cells to grow and multiply (proliferation) and develop the characteristics of cells which could make bone (differentiate) or does the powder prevent this from happening?

The differentiation of osteoclast precursors into osteoclasts.

What effect does the United Fisheries MCHC fish bone powder have on osteoclast precursor cells? Osteoclasts are the cells that break down bone, these cells are absolutely necessary for healthy bones, but if their activity exceeds the amount of bone made by the osteoblast then we can lose bone mass (eg. osteopenia or worse osteoporosis). “Osteoclast precursor cells” are the “early stage” version of the osteoclast, they are like the hull of a boat, but without further work they can’t do anything useful. A mature osteoclast which can resorb (breakdown) bone doesn’t form from the precursor until “called into duty” by a range of stimuli. In this study we want to test if the United Fisheries powder encourage or inhibit this process.

What we did:

We used osteoblast and osteoclast precursor cells grown in the lab. It is accepted practice to use mammal cells, in this case mouse cells. We used a mouse osteoblast cell called MC3T3-E1 subclone 4, and a mouse osteoclast precursor cell called the RAW 264.7 macrophage. Both cells, or cell lines, are well established models used to demonstrate osteoblast and osteoclast functions and activities. We treated these cells with the MCHC powder to test the above aims. This is a similar process as would be used with the first steps of testing factors or drugs that might have a positive effect on bone growth or a protective effect from bone breakdown.

What we found:

The MCHC fish bone powder had a significant effect on bone cells in the lab, indicating potential positive effects on bone cell function.

The MCHC fish bone powder increased osteoblast growth and differentiation, this means that the powder causes bone-making osteoblast cells to grow and show characteristics of cells that could produce bone matrix. This also means that the powder is not toxic to bone cells at the concentrations it was tested at.

The fish bone powder reduced and inhibited the formation of osteoclast cells.

The MCHC fish bone powder had to be dissolved into solutions before cells were treated. The powder did not dissolve completely in the cell growth solutions.

Recommendations:

Recommendation 1:

The powder didn't dissolve completely in the cell culture media which the cells were grown in (i.e. part of the powder remained as a solid powder and therefore couldn't be put on the cells). Because of this, we recommend that we repeat the process of dissolving the powder in cell culture media and then submit the dissolved portion for chemical analysis for

- i. Protein
- ii. Calcium
- iii. Magnesium
- iv. Phosphorus
- v. Collagen
- vi. Glycosaminoglycan
- vii. Chondroitin sulphate
- viii. Fat

There will be two samples total, the United Fisheries MCHC fish bone powder solution, and a cell culture solution by itself.

Why do this? This will tell us what proportions of each of the above analysed compounds were dissolved and are present in the solutions which the cells were treated with.

Recommendation 2

Test to see if the United Fisheries powder is able to enhance osteoblast mineralization. The development of the osteoblast bone cell is a three step process, proliferation followed by differentiation, and then final the production of mineral. The current study has looked at the first two steps but not the last which is a more prolonged experiment. Treating the cells with the powder(s) will test to see if they enhance the production of mineral by the osteoblasts.

Recommendation 3

The United Fisheries powder was partially soluble in cell culture media (a balanced salt solution compatible with cells). Powders such as this are likely to be dissolved or digested better in the stomach as there are digestive factors in the digestive tract that break down products better.

To replicate the form that this powder might take when they have been through the stomach, it is recommended that the powder is subjected to a simulated gastric digestion in the lab and then treating the cells with this digested form of the powder. Using a digestion method compatible with the osteoblast and osteoclast models, this could completely digest the entire powder sample (instead of the soluble part only) in a form similar to what would be presented to the gut. This may give you a better representation of the form that the powder might take when they enter the bloodstream when absorbed from the gut.

Cons: The technique is more complicated and timeconsuming, but could give you more meaningful results.

Scientific Summary

This study was designed to assess the effect of a United Fisheries MCHC fish bone powder on osteoblast function and the differentiation of osteoclast precursors into osteoclasts.

The study used the mouse MC3T3-E1 cell line as an *in vitro* osteoblast model, and the mouse RAW 264.7 macrophage cell line as a model of an osteoclast precursor cell. Both cell lines are well established models used to demonstrate osteoblast and osteoclast phenotypic activities respectively.

1. The United Fisheries MCHC fish bone powder increased MC3T3-E1 osteoblast cell proliferation and differentiation indicating an osteogenic effect (anabolic) of the soluble component of the powder *in vitro*.
2. The fish bone powder inhibited osteoclast formation in RANKL-stimulated RAW 264.7 cells indicating a possible role for controlling bone resorption *in vitro*.
3. While the powder had a significant effect on bone cells *in vitro*, the powder was only partially soluble in solution (i.e. there was a significant amount of powder which was insoluble).
4. The soluble portion of the powder had some effect on some of the bone cell function models used indicating bioactivity of some of the soluble components.
5. To further investigate the effects of this powder, it is recommended that the powder solubilisation process is replicated and samples of the soluble fractions are submitted for chemical analysis for (i) protein, (ii) calcium, (iii) magnesium, (iv) phosphorus, (v) collagen, (vi) glycosaminoglycan, (vii) chondroitin sulphate, and (viii) fat. Including respective blank controls for each of the cell culture medias used.
6. Subject the powder to a simulated gastric digest *in vitro*. Using a method compatible with the osteoblast and osteoclast models, this could completely digest the entire powder sample (instead of the soluble part only) in a form similar to what would be presented to the gut.

1. Introduction

1.1 Aims

To test the effect of a United Fisheries MCHC fish bone powder on:

- Osteoblast cell proliferation.
Is this powder toxic to the cells or does it promote cell growth and numbers?
- Osteoblast differentiation.
Does this powder stimulate osteoblast cells to develop and exhibit features expected in a mature osteoblast that might synthesise and lay down components of bone matrix?
- Osteoclast differentiation.
Does this powder prevent the normal process of osteoclast formation from precursor cells?

1.2 Definitions

Bone is a dynamic and living tissue that grows, repairs and degenerates through the activity of two main cells. **Osteoblasts** are cells which synthesize the organic (protein based connective tissue in a variety of forms) and lay down an inorganic matrix (largely calcium and phosphate-based compounds) that bone is made up of. Osteoblasts are important for laying down or apposition of bone matrix and their activity has been shown to be influenced by factors and compounds that reach them from the extra-cellular environment (the fluid around them).

Osteoclasts on the other hand are a specialised type of motile cell which breaks down the bone matrix (i.e. does the opposite of the osteoblast). This process is done by osteoclasts moving to or being formed in an area where bone needs to be remodelled (for growth or repair) and releasing compounds and chemicals which digest the bone. Their activity is usually followed by the apposition of new bone by osteoblasts.

In the body, osteoblasts and osteoclasts work in concert so that bone can grow (osteoblast activity greater than osteoclast activity), repair, or degenerates (osteoblast activity less than osteoclast activity), the latter occurs in diseased state or in the latter stages of the life cycle. Two important phases in the human lifecycle with respect to bone health and nutrition are the bone growth stage, where good “bone” nutrition can increase a person’s maximum possible skeletal health or peak bone mass, i.e. maximising the “osteoblast activity > osteoclast activity” balance . The second phase is during the latter life when “osteoblast activity < osteoclast activity”, where pharmaceutical agents and now increasing nutrition are being examined as ways to reduce osteoclast activity and therefore reduce the rate of bone degeneration.

There have been few studies to test the efficacy fish bone extracts on bone health *in vitro* (in the lab on cells or tissues) or *in vivo* (in animal or clinical studies). Please refer to previously supplied literature review prepared by Dr F. Wolber for a more comprehensive appraisal of this literature.

1.3 Scope

In this study, we use two well established *in vitro* osteoblast and osteoclast models to test the effect of the United Fisheries MCHC fish bone powder on the function and development of these two bone cell types. The mouse MC3T3-E1 cell is an osteoblast cell line. Using this cell line we will test the effect of the powder on cell viability at a range of concentration (i.e. do they kill the cell or do they make them grow?). We will also use this cell to test if this powder encourage or cause MC3T3-E1 cells to develop and show features or markers that are typical of what we would see in a osteoblast they could produce bone matrix.

To test the effect of the powder on osteoclasts, we will use the mouse RAW 264.7 macrophage cell. This cell line can be triggered to form osteoclasts by treating it with a special peptide or cytokine. We will treat the cells with different concentrations of the powder in the presence of the cytokine to see if osteoclast formation is affected.

Both of these models are well characterised and accepted models of osteoblast and osteoclast cells which are commonly used for the screening of compounds for bioactivity.

2. Materials and methods

2.1 Preparation of fish bone (MCHC) powder for *in vitro* testing

Fish bone powder was added to MEM α with 0.1% bovine serum albumin (BSA; osteoblast assays) or DMEM with 10% fetal bovine serum (FBS; osteoclast assays) on a per protein basis (MCHC protein content = 24.75%) to produce a 1 mg/ml stock solution. To maximise the solubilisation of the water soluble content, the powder was added to cell culture media warmed to 37°C, the suspension was then kept at this temperature for 10 min with frequent agitation. After 10 min, the sample was sonicated for 30 seconds at 15 W to break up fine particulates. The suspension was then mixed further by vortexing for 20-30 seconds before being allowed to settle. A fraction of the powder was insoluble, the insoluble fraction was allowed to settle and the soluble fraction was decanted and sterilised by passing it through a 0.20 μ m syringe filter. The sterile stock solution was then used to create test dilutions (0.1, 1.0, 10, 100, 1000 μ g/ml) in MEM α with 0.1% BSA (osteoblasts) or DMEM with 10% FBS (osteoclasts).

2.2 *In vitro* bone models

2.2.1 Osteoblasts

Cell proliferation bioassay (Does the MCHC sample increase osteoblast cell number?)

Murine pre-osteoblast Mc3t3-E1 sub-clone 4 cell line (ATCC® CRL-2593™, Manassas, VA, USA) were used in culture (*in vitro*) to represent osteoblast cells that lay down bone matrix as would be found in the body. These cells were grown in growth media, MEM α cell culture medium (Gibco 12571-063, Life Technologies, NZ) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco 10093-144, Life Technologies, NZ) supplemented with 0.25% gentamicin reagent solution (Invitrogen 15710-064, Life Technologies, NZ).

Cells were seeded in 96-well tissue culture plate (flat bottom) at 100 μ m/well at 0.4×10^5 cells/ml and incubated at 37°C in an atmosphere containing 5% CO₂ for 24 hours. After 24

hours, cell growth was arrested by removing the growth media and replacing it with MEM α containing 0.1% bovine serum albumin (BSA) media. Cells were incubated at 37°C in 5% CO₂ for 24 hours.

To test the effect of the MCHC sample on osteoblast proliferation, the MEM α containing 0.1% bovine serum albumin (BSA) media was removed after 24 hours and replaced with a media only control (0), or the MCHC sample at protein concentrations of 0.1, 1.0, 10, 100, 1000 μ g/ml. Each treatment was repeated in 6 wells, and each plate (experiment; MCHC sample) was repeated on three separate plates. The plates were incubated for 48 hours at 37°C in 5% CO₂.

Following 48 hours incubation in each sample, cells were observed by microscopy to assess their morphology. Cell viability or proliferation was assessed using the methyl-thiazolyl tetrazolium (MTT) assay. Briefly, 10 μ l of MTT solution (5 mg/ml in PBS, 0.20 μ m filter sterilised) was added to each well containing cells and incubated in the dark at 37°C under 5% CO₂ for 3-4 hours until the formation of blue/purple crystals was noticeable. Mitochondria of viable cells reduce the yellow soluble MTT solution to a blue/purple amethyst formazan crystal, therefore the more viable cells there are the more blue/purple crystals will be produced. Each tissue culture plate was centrifuged for 10 min and the cell culture media was then carefully aspirated from each well leaving the formazan crystals at the bottom of the well. One hundred μ l of dimethyl sulfoxide was added to each well to solubilise the formazan crystals, each plate was then allowed to sit 5 minutes at room temperature. The optical density (strength of the purple colour solution) was measured at 550 nm using a Elx 808 micro-plate reader (BioTek Instrument Inc, Vermont, USA). The optical density value at 550 nm is proportional to the number of viable osteoblast cells. Results are presented as the mean of each treatment relative to the zero control (treatment/zero) as an average of the three separate experiments.

Osteoblast differentiation bioassay (Does the MCHC sample increase osteoblast cell differentiation?)

Murine osteoblast-like cells, Mc3t3-E1 Clone 4, were cultured as previously described. Cells were seeded in 24-well tissue culture plates at 0.2×10^5 cells/ml (1 ml per well) and incubated at 37°C under 5% CO₂. After 72 hours (day 0) the wells were 80% confluent (80% of the bottom of the well is covered in cells). MCHC powder solutions were made as described in Section 2.1, with the powder made in osteogenic media (MEM α with 10% FBS, 10 mM β -glycerophosphate, and 50 μ g/ml ascorbic acid) at protein concentrations of 0.1, 1.0, 10, 100 μ g/ml. The growth media was replaced with a 0 control (osteogenic media), and MCHC solutions (0.1, 1.0, 10, 100 μ g/ml). Each treatment was repeated in four wells, and each plate (experiment) was repeated three times. Every three days, old media was exchanged for fresh osteogenic media and treatments.

On day 9, all media was removed from the wells of each plate. To measure the amount of alkaline phosphatase produced by differentiated osteoblasts, cells were washed with phosphate-buffered saline (PBS) and the cells fixed with a 1% formaldehyde solution for 15 minutes at room temperature. Cells were then washed again with PBS, and 300 μ l/well of 0.05M p-nitrophenyl phosphate [p-NPP] in Tris-buffed saline (TBS; pH 9.5) was added to each well and incubated at 37°C. After 1 hour, two 100 μ l aliquots were taken from each well and transferred into a 96-well plate. Absorbance was measured 405 nm.

To express the alkaline phosphatase measurements by cell number, the remaining solution p-nitrophenyl phosphate TBS solution in each well was removed and each well gently washed with PBS. 300 μ l of 1% crystal violet solution was added to each well and incubated at 37°C. After 1 hour, excess crystal violet was removed by gently immersing each plate in a tank of running tap water for 15 minutes. All plates were then air dried for 24 hours. 500 μ l/ml of 0.2% Triton-X 100 was added to each well and the crystal violet stain allowed to solubilise for

90 minutes at room temperature. Two 150 µl aliquots were removed from each well to a 96-well plate, and the absorbance of these two aliquots measured at 405 nm with 0.2% Triton-X100 as a blank.

2.2.2 Osteoclasts

Osteoclast differentiation bioassay (Does the MCHC powder prevent RANKL-induced osteoclast differentiation?)

Murine RAW 264.7 cells (ATCC, Manassas, VA, USA) were cultured in 24-well plates at 1.5×10^4 cells/ml in Dulbecco's Modified Eagle Media (DMEM) liquid (Gibco 11995-073, Life Technologies, Auckland, NZ) supplemented with 1% antibiotic-antimycotic (Gibco 15240-062, Life Technologies, Auckland, NZ) and 10% heat-inactivated FBS (Gibco 10093-144, Life Technologies, Auckland, NZ) and incubated at 37°C under 5% CO₂.

MCHC powder solutions were made as described in Section 2.1, with the powder dissolved in DMEM with 10% FBS with recombinant mouse receptor activator of nuclear factor kappa-B ligand (RANKL; R&D 462-TEC-010) to stimulate RAW 264.7 differentiation into osteoclasts. RAW 264.7 cells were seeded (Day 0) in media and 10% FBS, media only (zero control) or with the addition of MCHC at a range of concentrations (three wells per treatment). Each plate included a negative control comprising all treatments without RANKL. Each experiment was conducted in triplicate. Plates were incubated at 37°C under 5% CO₂. After 72 hours, all media and treatments were replaced with fresh media, RANKL, and treatment compounds and then incubated for a further 48 hours. On day 5, a 30 µl of the cell culture media from each well was added to a 96-well plate (duplicate), then 170 µl of tartrate-resistant acid phosphatase (TRAP) stain was added to each well (Sigma 387-A kit, Sydney, Australia). Each plate was incubated in the dark at 37°C. After 1 hour, the optical density of each plate was measured at 550 nm to quantify TRAP levels for each treatment.

Treated RAW 264.7 cells were stained for TRAP, expressed by osteoclast-like multinucleated cells, in the 24-well plates. Briefly, cells were fixed with a fixative solution (Sigma 387-A kit, Sydney, Australia), washed with PBS and then stained with TRAP solution for one hour at 37°C in darkness. After 1 hour, the TRAP stain was removed, the cells washed and then counterstained with haematoxylin for 30 seconds. The plates were then washed gently with tap water and then air-dried before being examined under an inverted microscope. Images from three random fields within each well were taken and the number of multinucleated cells per well (≥ 3 nuclei per cell) were counted.

2.3 Statistical analyses

All results were analysed using Minitab 16 (Pennsylvania, USA). The results were pooled for each treatment for all three plates (experiments) per bioassay. A one-way ANOVA was conducted for all treatments for each of the following: osteoblast cell proliferation and differentiation, osteoclast TRAP levels, and osteoclast number. All graphs and tables show means \pm 95% confidence intervals (CI).

3. Results

3.1 Osteoblast cell proliferation

Fish bone powder (MCHC)

The fish bone powder increased cell proliferation in Mc3t3-E1 subclone 4 cells (Figure 1; $p < 0.0001$). Cell proliferation increased dose dependently and caused a statistically significantly greater than the cell culture media only control at 1 $\mu\text{g/ml}$, increasing to 1.34-fold times that of the cell culture media control (0) at 100 $\mu\text{g/ml}$ before and decreased slightly at the highest tested concentration 1,000 $\mu\text{g/ml}$. The 1,000 $\mu\text{g/ml}$ test concentration is relatively high and it is not unusual that a decrease in cell proliferation was observed.

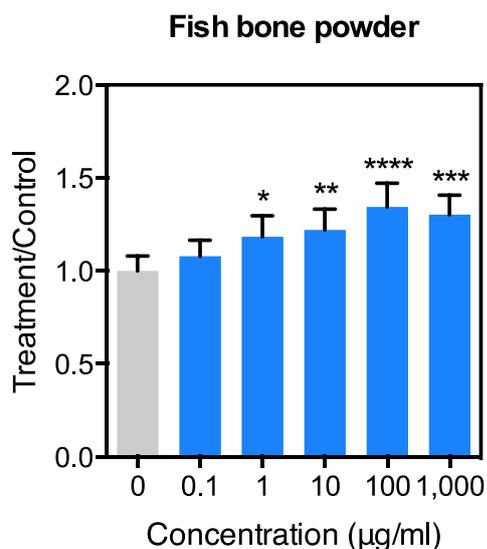


Figure 1. Effect of a MCHC fish bone powder on Mc3t3-E1 subclone 4 osteoblast cell proliferation. Briefly, cells were incubated with solubilised powder samples on a protein concentration basis for 48 hours. Asterisks indicate a statistically significant difference of a treatment from media only (0). Statistical significance is indicated by * $p = 0.01$ to 0.05 , ** $p = 0.001$ to 0.01 , *** $p = 0.0001$ to 0.001 , and **** $p < 0.0001$. Results are shown as the mean \pm 95% CI.

3.2 Osteoblast differentiation

Fish bone powder (MCHC)

Osteoblasts treated with the fish bone powder showed higher levels of alkaline phosphatase activity than control cells (0) treated with osteogenic media alone (Figure 2; $p = 0.2041$). However, there was no significant difference between any of the treatments as treatments showed a large amount of variability in alkaline phosphatase activity.

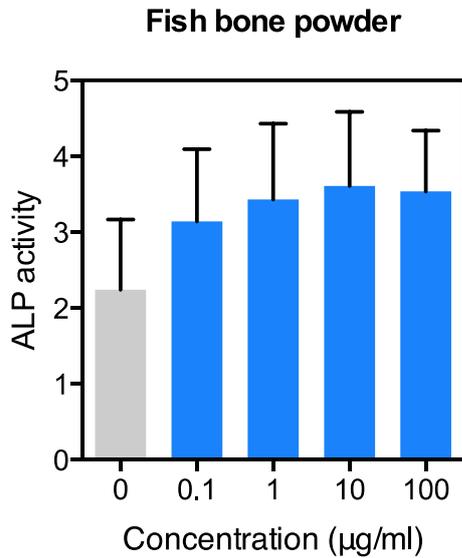


Figure 2. Effect of a fish bone powder (MCHC) on differentiation of Mc3t3-E1 subclone 4 osteoblasts. Briefly, cells were incubated with solubilised powder samples on a protein concentration basis for 9 days in the presence of osteogenic cell culture media. Asterisks indicate a statistically significant difference of a treatment from media only (0). Statistical significance is indicated by * $p = 0.01$ to 0.05 , ** $p = 0.001$ to 0.01 , *** $p = 0.0001$ to 0.001 , and **** $p < 0.0001$. Results are shown as the mean \pm 95% CI.

3.3 RANKL-induced differentiation of monocytes to osteoclasts

Tartrate-resistant acid phosphatase (TRAP) activity

Fish bone powder (MCHC)

The fish bone powder inhibited TRAP activity and multinucleated cell (MNC) numbers in RANKL treated RAW 246.7 macrophages at a protein concentration range of 10-100 $\mu\text{g/ml}$ (Figure 3; both $p < 0.001$). TRAP activity and MNC numbers did not differ from the control (0) at 0.1-1 $\mu\text{g/ml}$, and decreased in a dose dependent manner at 10 and 100 $\mu\text{g/ml}$.

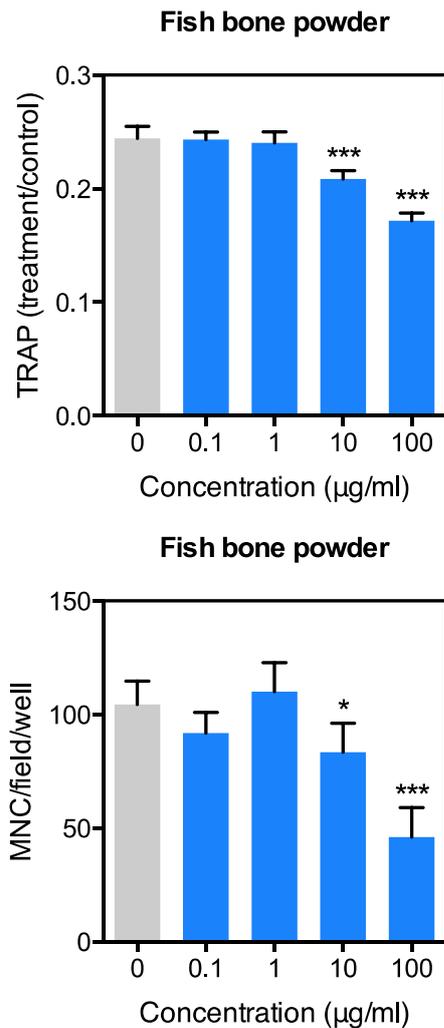


Figure 3. Effect of a fish bone powder (MCHC) on tartrate-resistant acid phosphatase (TRAP) activity and average MNC cell number per photomicrograph field per well in RANKL-treated RAW 264.7 cells. Asterisks indicate a statistically significant difference of a treatment from media only (0). Statistical significance is indicated by * $p = 0.01$ to 0.05 , ** $p = 0.001$ to 0.01 , *** $p = 0.0001$ to 0.001 , and **** $p < 0.0001$. Results are shown as the mean \pm 95% CI.

4. Discussion

4.1 The sample

A significant proportion of the fish bone (MCHC) powder was insoluble despite a significant solubilisation protocol involving heating, agitation and sonication. The powder comprises a significant mineral and protein content. It is not clear precisely how much of the protein or mineral component of the powder was solubilised and what remained in solid form. It is recommended that a chemical analysis of the insoluble fraction is conducted to in some way identify what component of the powder was solubilised.

Finding: the powder did not dissolve very easily in an aqueous water-based solution that was compatible with the cells. Approximately ~50% of the powder was dissolvable and therefore could be used to treat the cells. This means that ~50% remained as a solid that couldn't be put on the cells. What is in the 50% that was dissolved and were used to treat the cells?

4.2 Osteoblasts

Fish bone powder (MCHC)

The fish bone powder had no negative effect on osteoblast cell viability. The fish bone powder increased osteoblast cell number in the tested concentration range indicating a possible osteoproliferative effect of some component of the soluble fraction of the fish bone powder, although without a detailed chemical analysis of the soluble phase it is indeterminant as to what would have caused this activity. It is likely that a proportion of the larger components of the powder (i.e. protein, calcium, and phosphorus) would have been solubilised, but which has had a contributory factor in the observed effect can not be determined with the current study. There are next to no published studies describing the effect of a fish bone powder on osteoblast activity *in vitro*. Yamada et al. (2013) described some effect of a fish bone collagen fibre extract on osteoblast differentiation, so it could be possible that the activity may be caused by soluble proteins from the fish powder. Elevated extracellular calcium levels have also been shown to increase cell proliferation and differentiation in MC3T3-E1 cells (Kanatani et al., 1991; Sugimoto et al., 1993). In this study, there was a notable increase in osteoblast differentiation as measured by alkaline phosphatase activity, which is an accepted marker for osteoblast differentiation. This results of this study suggest that the soluble component of the fish bone powder is, *in vitro*, able to stimulate the increase in osteoblast numbers as well as the differentiation of these cells to show functional osteoblast characteristics.

Finding: The portion of MCHC that could be dissolved was not toxic to the osteoblast bone cells. The MCHC treatment enhanced the growth of the osteoblast cells, and under this treatment these cells showed indications of maturing into a form which could produce bone mineral. We know from previous reports that simply increasing the amount of calcium present in the environment that the cells grow in could cause this, so it is important that we know more about what in the MCHC dissolved into the cell culture media.

4.3 Osteoclasts

Fish bone powder (MCHC)

This powder caused a reduction in TRAP activity and multinucleated cell number indicating RANKL-induced osteoclast formation was inhibited. Again, we do not know what components of the powder were dissolved into the cell culture media so it is not possible to identify the likely source of the activity. The size of the effect on both TRAP and MNC numbers is robust making this effect worthy of some more investigation. There has been nothing published on the effect of a fish bone powder on osteoclast formation *in vitro*. A full chemical analysis of the dissolved powder as used *in vitro* would identify likely compounds that might have had this effect. The compositional analysis of the powder provided by United Fisheries Ltd. does not show any lipid component in the powder. It is unlikely that this effect would be mediated by a lipid(s) as their viability and the storage conditions of the powder are likely to make any lipid component inactive for this osteoclast bioassay.

Finding: The MCHC powder reduced the amount of osteoclasts (bone dissolving cells) forming. A chemical analysis of the portion of MCHC that was present in the cell culture media solution that the cells were treated with would help in this interpretation of the likely cause of this effect.

5. References

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