Royal College of Surgeons in Ireland

repository@rcsi.com

The effect of bisphosphonate treatment on the biochemical and cellular events during bone remodelling in response to microinjury stimulation.

AUTHOR(S)

Lauren E. Mulcahy, Caroline Curtin, Ryan J. McCoy, Fergal O'Brien, David Taylor, Clive Lee, Garry Duffy

CITATION

Mulcahy, Lauren E.; Curtin, Caroline; McCoy, Ryan J.; O'Brien, Fergal; Taylor, David; Lee, Clive; et al. (2015): The effect of bisphosphonate treatment on the biochemical and cellular events during bone remodelling in response to microinjury stimulation.. Royal College of Surgeons in Ireland. Journal contribution. https://hdl.handle.net/10779/rcsi.10766786.v2

HANDLE

10779/rcsi.10766786.v2

LICENCE

CC BY-NC-SA 4.0

This work is made available under the above open licence by RCSI and has been printed from https://repository.rcsi.com. For more information please contact repository@rcsi.com

URL

https://repository.rcsi.com/articles/journal_contribution/The_effect_of_bisphosphonate_treatment_on_the_biochemical_and_cellular_events_during_bone_remodelling_in_response_to_microinjury_stimulation_/10766786/2

THE EFFECT OF BISPHOSPHONATE TREATMENT ON THE BIOCHEMICAL AND CELLULAR EVENTS DURING BONE REMODELLING IN RESPONSE TO MICROINJURY STIMULATION

L.E. Mulcahy[1,2,†], C.M. Curtin[1,2,3,†], R.J. McCoy[1,2], F.J. O'Brien[1,2,3], D. Taylor[1,3], T. Clive Lee[1,2] and G.P. Duffy[1,2,3,*]

[1]Trinity Centre for Bioengineering, Department of Mechanical Engineering, Dublin, Ireland
[2]Department of Anatomy, Royal College of Surgeons in Ireland, St. Stephen's Green, Dublin, Ireland
[3]Advanced Materials and Bioengineering Research (AMBER) Centre, RCSI & TCD, Dublin, Ireland

[†]Both authors contributed equally to this work

Abstract

Osteoporosis is one of the most prevalent bone diseases worldwide and is characterised by high levels of bone turnover, a marked loss in bone mass and accumulation of microdamage, which leads to an increased fracture incidence that places a huge burden on global health care systems. Bisphosphonates have been used to treat osteoporosis and have shown great success in conserving bone mass and reducing fracture incidence. In spite of the existing knowledge of the *in vivo* responses of bone to bisphosphonates, the cellular responses to these drugs have yet to be fully elucidated. *In vitro* model systems that allow the decoupling of complex highly integrated events, such as bone remodelling, provide a tool whereby these biological processes may be studied in a more simplified context. This study firstly utilised an *in vitro* model system of bone remodelling and comprising all three major cell types of the bone (osteocytes, osteoclasts and osteoblasts), which was representative of the bone's capacity to sense microdamage and subsequently initiate a basic multicellular unit response. Secondly, this system was used to study the effect of two commonly utilised aminobisphosphonate treatments for osteoporosis, alendronate and zoledronate. We demonstrated that microinjury to osteocyte networks being treated with bisphosphonates modulates receptor activator of nuclear factor kappa-B ligand and osteoprotegerin activity, and subsequently osteoclastogenesis. Furthermore, bisphosphonates increased the osteogenic potential following microinjury. Thus, we have shown for the first time that bisphosphonates act at all three stages of bone remodelling, from microinjury to osteoclastogenesis and ultimately osteogenesis.

Key Words: Bone remodelling, basic multicellular unit, microinjury, osteoclastogenesis, osteogenesis, osteoporosis, bisphosphonate treatment.

*Address for correspondence:
Garry P. Duffy, BSc, PhD
Department of Anatomy
Royal College of Surgeons in Ireland
123 St. Stephens Green
Dublin 2, Ireland

Telephone Number: +353 1 4022105
FAX Number: +355 1 4022355
E-mail: garryduffy@rcsi.ie

Introduction

Osteoporosis is one of the most prevalent bone diseases worldwide and is characterised by high levels of bone turnover, a marked loss in bone mass and the accumulation of microdamage. Together, these factors culminate in a reduced bone density and altered osseous microstructure, leading to a decrease in the mechanical competence of bone and an increasing susceptibility to fracture (Parfitt, 2002). In 2000, it was estimated there were approximately 9 million osteoporosis related fractures globally each year (Johnell and Kanis, 2006). In 2005, based on data from the previous 25 years (Chrischilles *et al.*, 1994; Hoerger *et al.*, 1999, Phillips *et al.*, 1988, Ray *et al.*, 1997), it was estimated that there would be over 2 million osteoporosis related fractures annually in the United States at a cost of >$17 billion, which would grow to greater than 3 million fractures *per* annum at a cost of >$25 billion by 2025 (Burge *et al.*, 2007; Roche *et al.*, 2005). Osteoporosis is a common condition observed in both elderly men and women; however, women have a four times greater risk of developing osteoporosis, with 30 % of postmenopausal women worldwide being affected. The discrepancy between sexes is believed to be associated with changes in oestrogen content, whereby oestrogen deficiency has been associated with increased bone resorption due to an increase in activated osteoclasts (Eriksen *et al.*, 1999) but also an increase in bone formation that is unable to compensate for the enhanced resorption (Brennan *et al.*, 2011; Jilka *et al.*, 1998).

Whilst bone remodelling (or bone turnover) may be influenced by systemic hormones, such as oestrogen and parathyroid hormone (Donahue *et al.*, 2006), other factors such as mechanical loading are also known to contribute to osteoporosis as it is a major regulator of bone mass and geometry with the sensing and transduction of strains being essential for the maintenance of healthy bones (Bergmann *et al.*, 2010). Individual bones within the skeleton experience different mechanical loads, not only as a function of their location, but also in response to the level of physical activity (Burr *et al.*, 1996), age (Nordin *et al.*, 1990) or environment (Zerwekh *et al.*, 1998) of an individual. Conditioning of bone to withstand these forces occurs through dynamic remodelling that aims to minimise stress or strain concentrations (Lanyon, 1987) and provide an optimal mechanical structure. Stimulation or activation of bone remodelling has been attributed to factors such as fluid flow (Kufahl and Saha, 1990; Weinbaum *et al.*, 1994) and microdamage (Burr *et al.*, 1985; Lee *et al.*,

2002; Martin and Burr, 1982) within the bone, which is hypothesised to be sensed by osteocytes either through mechanotransduction mechanisms (Turner *et al.*, 1994) or by the shearing of cellular processes (Hazenberg *et al.*, 2009; Taylor *et al.*, 2003). This signalling mechanism, between mechanically stimulated osteocytes and osteoblast progenitors within the bone marrow, is believed to be paracrine in nature as osteocytes are spatially isolated from the marrow cavity (Hoey *et al*., 2011). Moreover, regions of osteocyte apoptosis have been shown to be accompanied by increased local bone turnover and targeted bone resorption and are believed to be involved in osteoclast precursor recruitment and differentiation *in vitro* (Al-Dujaili *et al*., 2011). Subsequently, the receptor activator of nuclear factor kappa B ligand-receptor activator of nuclear factor kappa B- osteoprotegerin (RANKL-RANK-OPG) signalling pathway is activated (Mulcahy *et al.*, 2011), and in turn mediates the initiation of bone remodelling through the synchronous action of the basic multicellular unit (BMU) that consists of the main cell types involved in bone remodelling: osteoblasts, osteocytes and osteoclasts. Accumulation of microdamage within the bone due to excessive overloading (Burr *et al.*, 1998), the impairment of osteocyte functionality, or perturbations in the equilibrium of the resorption and formation arms of the BMU (Mashiba *et al.*, 2000), as in osteoporosis, increases an individual's susceptibility to fragility fractures due to diminished structural integrity or loss of bone mass and thus the mechanical competence of bone.

Bisphosphonates, synthetic analogues of inorganic pyrophosphate, containing a phosphate-carbon-phosphate backbone, have been used as drugs to treat osteoporosis for the last 30 years (Morris and Einhorn, 2005). They act as potent inhibitors of bone resorption, in an effort to conserve bone mass and thereby effectively reduce the risk of osteoporotic fracture, by the prevention of pre-osteoclast recruitment, fusion and differentiation (Russell *et al.*, 2008). They function by binding tightly to bone mineral, where they are internalised by osteoclasts by the process of endocytosis during bone resorption. Here, they inactivate osteoclast function and induce osteoclast apoptosis. Bisphosphonate-treated bone is characterised by fewer osteoclast cell numbers, most of which are located away from the bone surface, and many of which are undergoing apoptosis (Pivonka *et al*., 2012). Bisphosphonates are not metabolised and therefore are either excreted renally or bind to hydroxyapatite within the bones where they can remain for years (Reginster *et al.*, 2013). This skeletal binding affinity has been shown to increase in rank order through risedronate, ibandronate, alendronate and zoledronate, with higher affinity bisphosphonates becoming rebound more quickly thereby increasing skeletal retention (Russell *et al.*, 2008). Many studies have reported that bisphosphonate treatment increases the degree of mineralisation in bone, by facilitating the binding of hydroxyapatite crystals onto bone surfaces, principally at the sites of bone remodelling (Boivin *et al.*, 2000; Rogers, 2003; Spadaro *et al.*, 2006).

In vitro model systems that allow the decoupling of complex highly integrated events, such as bone remodelling, provide researchers with a tool by which biological processes may be studied in a more simplified context. This will allow researchers to expand the knowledge space pertaining to the interactions between individual components of the biological system and secondly, create a test bed for studying how current treatments exert their observed clinical effects and how potential therapeutic treatments may modulate inherent biological functions. This study aimed to utilise a previously established *in vitro* model system of bone remodelling, that comprises all three major cell types of the bone (osteocytes, osteoclasts and osteoblasts), in which osteocyte networks can be subjected to controlled amounts of microinjury, allowing the investigation of bone's capacity to sense damage and initiate a BMU response (Mulcahy *et al.*, 2011). We have applied this system to assess the effect of alendronate and zoledronate, two aminobisphosphonate treatments for osteoporosis that exhibit the highest affinity for bone mineral, on the osteocytic response to microdamage and the subsequent impact on BMU activation.

Materials and Methods

Cell culture

MLO-Y4 cells, an osteocyte-like cell line originally derived from murine long bones (Kato *et al.*, 1997) were generously provided by Dr. Linda Bonewald (School of Dentistry, University of Missouri, Kansas City, MO, USA), and were maintained in α Modified Eagle's Medium (αMEM; Biosera, Uckfield, UK) supplemented with 5 % foetal bovine serum (FBS; Biosera, East Sussex, UK), 5 % iron supplemented calf serum and 1 % penicillin/streptomycin (P/S; Sigma Aldrich, Dublin, Ireland). Cells were cultured in collagen coated flasks (0.15 mg/mL rat tail collagen type 1; BD Biosciences, Oxford, UK) at 37 °C and 5 % CO_2.

Pre-osteoclasts (RAW 264.7 cells, ATCC, LGC Standards, Teddington, UK) were cultured in high glucose Dulbecco's Modified Eagles Medium (DMEM; Sigma Aldrich) supplemented with 10 % heat inactivated FBS (Biosera) and 1 % P/S.

Pre-osteoblasts (MC3T3-E1 cells, ATCC, LGC Standards, Middlesex, UK) were maintained in αMEM supplemented with 10 % FBS, 1 % L-glutamine (Sigma Aldrich), and 2 % P/S.

All cell lines were used in experiments below passage 20.

Generation of osteocyte microinjury and conditioned medium samples

Conditioned media was obtained following application of microinjury to gel-embedded osteocyte (MLO-Y4) cells, as previously described (Mulcahy *et al.*, 2011). Briefly, constructs were prepared by the addition of 1 volume of Matrigel Basement Membrane Matrix (BD Biosciences) to 1 volume of a collagen solution comprised of 58 % Collagen, 27 % 5×DMEM, 2.5 % FBS, 2.5 % calf serum, 5 % sodium hydroxide (Sigma Aldrich) and 5 % cell suspension. An incubation of 5 d was sufficient to facilitate cell process network formation. Microinjuries were applied through insertion of needles of diameter 160, 300 and 400 μm into the centre of the hydrogel, creating a planar

defect by drawing the needle through the hydrogel at a right angle to the point of insertion to create a defect 7 mm in length and 5 mm in depth. After 72 h of culture, conditioned medium samples were collected. The term microinjury has been applied to the planar defects created in this study, which are intended to be reflective of microdamage, but due to acknowledged differences between the *in vitro* cultures compared to the *in vivo* scenario, such as osteocyte density and process number *per* osteocyte, we have refrained from using the word "microdamage" to represent the defects created, as they are not truly of a length scale equating to microdamage. The individual cell types involved in bone remodelling were spatially decoupled by cultivating them separately. Cellular crosstalk was evaluated through transfer of conditioned medium from the 3D gel-embedded osteocyte (MLO-Y4 cells) network cultures, 72 h post microinjury, to the osteoclast (RAW 264.7 cells) monolayer cultures, which was then in turn serially transferred to pre-osteoblast (MC3T3 cells) monolayer cultures to yield doubly conditioned media. Media were also assessed in all experiments from gel-embedded osteocytes not subjected to injury, termed uninjured cells (*i.e.* the negative control).

For all experiments assessing the effects of bisphosphonate treatment on pre-osteoclasts and pre-osteoblasts, 10^{-5} M concentrations of alendronate or zoledronate were added to the culture medium only at the time of osteocyte seeding on the gels. Conditioned and doubly conditioned media were then taken as described previously for treatment of the pre-osteoclast and pre-osteoblast cells.

Cell migration assays

In order to assess whether osteocyte injury or osteocyte injury/osteoclastogenesis, with and without 10^{-5} M bisphosphonate treatment, stimulates the migration of pre-osteoclast and pre-osteoblast cells respectively towards a site of damage, a cell migration assay was performed. This assay is based on the concept that following a period of serum starvation, cells will migrate through a porous membrane towards a stimulus. Under serum free conditions, 1×10^4 pre-osteoclasts/pre-osteoblasts were cultured on the upper side of 8 μm pore hanging cell culture inserts (Millipore, Cork, Ireland). Following a 2 h incubation, serum free medium was replaced with conditioned/doubly conditioned medium from injury experiments ($n = 3$ *per* injury size). Media were also assessed from gel-embedded osteocytes not subjected to injury, termed uninjured cells. Controls were serum free medium (OptiMEM; Invitrogen, Dublin, Ireland) and cell culture medium containing 10 % FBS. Cells were incubated for 18 h, following which cell-seeded membranes were fixed in 4 % formalin for 10 min, and stained with haematoxylin for 10 min. The membrane was then removed and mounted on a glass slide, bottom side down. Cell migration was quantified by counting the cells on the bottom side of the membrane in 5 random fields ($n = 3$ *per* treatment group).

Osteoclastogenesis

Pre-osteoclasts were seeded at a density of 4×10^4 cells/well in 12-well adherent plates (Cruinn Diagnostics, Dublin, Ireland) or on poly-D-lysine (1 M)-coated chamber-slides. Pre-osteoclast cell culture medium was replaced with osteocyte conditioned medium, with or without 10^{-5} M bisphosphonate treatment, 24 h after seeding. Conditioned medium was changed every 3 days. After 21 d, tartrate-resistant acid phosphatase (TRAP) activity was quantified ($n = 3$ *per* treatment group) by removing the culture medium from pre-osteoclast cultures and incubating it for 1 h in 1 mM citrate solution at 37 °C. This culture medium solution (20 μL) was then added to 50 μL of a solution containing 100 mmol p-nitrophenyl phosphate (pNPP) (Sigma Aldrich), 200 mmol sodium citrate (Sigma Aldrich, Dublin, Ireland) and 80 mmol/L sodium tartrate (Sigma Aldrich) in a 96-well plate, and incubated for a further 30 min at 37 °C. NaOH (20 μL of 1 M solution) was then added to stop the reaction and absorbance was read at 405 nm by spectroscopy. TRAP staining was performed using a Leukocyte Acid Phosphatase Kit (Sigma Aldrich) in accordance with the manufacturer's instructions. Furthermore, the number of TRAP positive multinucleated cells were quantified, from 3 random fields of view, at 20× magnification, and their frequency expressed at a percentage of cells within the field of view ($n = 3$ *per* treatment group).

Osteoblast differentiation

Pre-osteoblasts were seeded at a density of 4×10^4 cells in 6 well plates. Cells were allowed to attach for 24 h prior to replacing growth medium with a 1:1 ratio of doubly conditioned medium from microinjury/osteoclastogenesis experiments, with or without 10^{-5} M bisphosphonate treatment, combined with either growth or osteogenic medium. Osteogenic medium was prepared by the addition of ascorbic acid (50 μg/mL; Sigma Aldrich), β-glycerophosphate (10 mM; Sigma Aldrich) and dexamethasone (100 nM; Sigma Aldrich) was added to standard αMEM supplemented with 10 % FBS, 1 % L-glutamine and 2 % penicillin/streptomycin. Cells were cultured for 21 d at 5 % CO_2 and 37 °C.

To assess osteogenic differentiation, treatment medium was removed from pre-osteoblasts cultured for 21 d, cells were rinsed in phosphate buffered saline (PBS; Sigma Aldrich) and 500 μL of 1 M HCl added to each well. Cells were manually detached using a cell scraper and the resulting cell suspension placed into a 1.5 mL cryovial. Wells were then washed a second time with an additional 500 μL of 1 M HCl and this was added to the initial cryovial. Samples were left overnight at 4 °C. Calcium deposition was quantified using the Calcium Liquicolor Kit (Stanbio Laboratories, Boerne, TX, USA) according to the manufacturer's protocol and calcium concentration was deduced using a standard curve ($n = 3$ *per* treatment group).

Effect of bisphosphonate treatment on RANKL and OPG expression levels after osteocyte microinjury

Studies were performed to determine an optimum dose of bisphosphonate treatment (data not shown). As a result, a 10^{-5} M concentration of each bisphosphonate, consistent with concentrations suggested in the literature (Naidu *et*

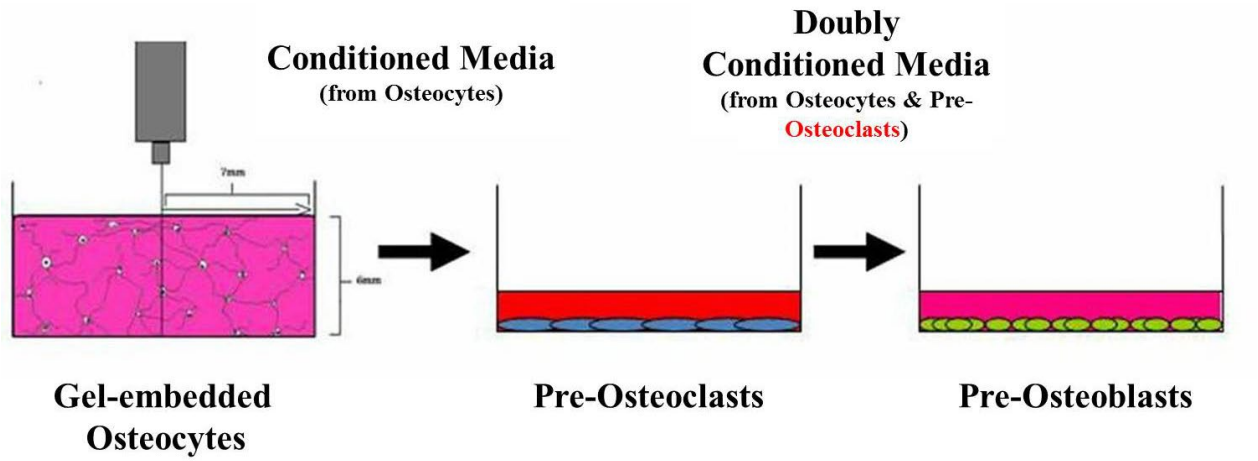


Fig. 1. Diagrammatic representation of the *in vitro* bone remodelling system for studying the relationship between osteocyte signalling in response to microinjury and BMU activation and function. The individual cell types involved in bone remodelling are spatially decoupled by cultivating them separately. Cellular crosstalk is then evaluated through transfer of conditioned medium from the 3D gel embedded osteocyte (MLO-Y4 cells) network cultures, post microinjury, to the pre-osteoclast (RAW 264.7 cells) monolayer cultures, which are then in turn serially transferred to pre-osteoblast (MC3T3 cells) monolayer cultures.

al., 2008), was chosen to be the optimum dose to maintain osteocyte viability whilst still stimulating a temporal pharmacological response.

Gel-embedded osteocytes were incubated using OptiMEM serum-free medium, supplemented with 10^{-5} M concentration of both alendronate and zoledronate. Samples were taken at 24 h intervals over a 72 h time period. RANKL and OPG were quantified by means of single site specific ELISA (Mouse RANKL and OPG ELISA, R&D Systems) under manufacturers' instructions. Cultures (n = 3) were assayed at each injury level, in addition to non-injured controls.

Statistical analysis

All experiments contained n = 3 technical repeats and were performed in triplicate. All data were analysed for significance ($p \leq 0.05$) using one way ANOVA followed by a *post-hoc* Tukey test, to compare means between injured and uninjured groups and between drug treated and non-drug treated groups under the same experimental parameters.

Results

Development of an osteocyte-BMU *in vitro* model

The initial set of studies aimed to identify the required microinjury size needed in the gel-embedded osteocyte networks to yield conditioned medium capable of recruiting and differentiating pre-osteoclasts, which in turn, would be capable of recruiting pre-osteoblasts and yielding increased mineralisation when using doubly conditioned media (media exposed to osteocytes and pre-osteoclasts), thereby mimicking the *in vivo* scenario. Fig. 1 represents a diagrammatic representation depicting the *in vitro* bone remodelling system designed to study the relationship between osteocyte signalling in response to microinjury and BMU activation and function.

The effect of microinjury size to osteocyte networks on pre-osteoclast migration and differentiation

Conditioned media were taken from gel-embedded osteocyte networks, subjected to microinjury widths of 160, 300 or 400 μm in addition to uninjured cells after 72 h, and added to pre-osteoclasts to assess migration and differentiation potential. A significant increase in pre-osteoclast cell migration was observed, in response to the conditioned medium from microinjured osteocyte networks that experienced injury widths of 300 and 400 μm, in comparison to the smaller injury width or the undamaged control (Fig. 2a). Osteoclastogenesis, as measured by TRAP activity, was significantly increased for all injury sizes in comparison to the undamaged control (Fig. 2b). TRAP staining of pre-osteoclasts suggested the presence of larger multinucleated cells was greatest in 300 μm and 400 μm injury widths, when compared to the 160 μm injury width and undamaged control (Fig. 2c), suggesting the larger microinjury widths exerted the most potent effects on pre-osteoclasts.

The effect of doubly conditioned medium from microinjury induced osteoclastogenesis on pre-osteoblast migration and differentiation

Doubly conditioned medium (medium exposed to osteocytes and pre-osteoclasts) was then either directly used for pre-osteoblast migration studies or combined in a 1:1 ratio with osteoblast growth or osteogenic medium, to examine the effect on pre-osteoblast mineralisation. Pre-osteoblast migration was proportional to osteocyte network injury width, with doubly conditioned medium from a 400 μm injury size showing significantly increased levels of migration, compared to the undamaged control

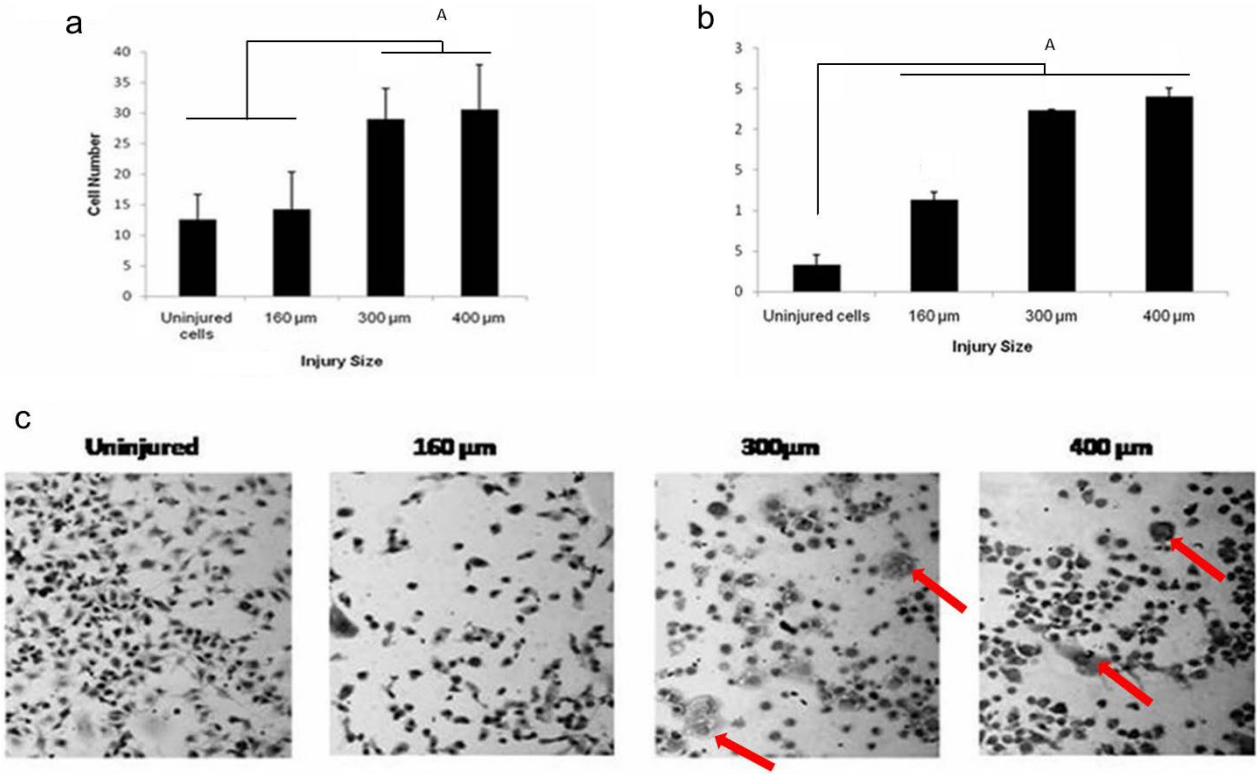


Fig. 2. The effect of injury size within gel embedded osteocyte networks on pre-osteoclast migration and differentiation. Osteocyte cultures were subjected to microinjury having widths of 160, 300 or 400 µm. After 72 h, conditioned medium was collected and used to assess (a) the chemotaxic properties on pre-osteoclasts, (A, $p \leq 0.05$ *versus* 160 µm injuries and undamaged cells), (b) the induction of osteoclastogenesis after 21 d of culture, as quantitatively measured by TRAP activity, (A, $p \leq 0.05$ *versus* uninjured control), or (c) qualitatively measured by TRAP staining, which suggested the presence of large multinucleated cells was greatest in 300 and 400 µm samples. Error bars represent the standard deviation, $n = 3$.

(Fig. 3a). Significantly higher levels of calcification were noted in pre-osteoblasts exposed to doubly conditioned medium from 400 µm injury sizes, in comparison to other injury sizes when cultured in the absence of osteogenic medium. When osteogenic medium was utilised, all injury sizes expressed similar levels of calcification (Fig. 3b). These data suggest that microinjury sizes of 400 µm alone were sufficient to stimulate pre-osteoblast mineralisation.

Using an in vitro osteocyte-BMU assay system to study the effect of bisphosphonate treatment

Based on the studies described above, a 400 µm injury width was the only *in vitro* microinjury size tested capable of inducing cellular crosstalk between the different cell types that replicated the expected *in vivo* response. Thus, further experiments aiming to study the role of bisphosphonates were conducted using this injury size.

The presence of bisphosphonates during osteocyte network microinjury modulates osteoclast migration and differentiation

The treatment of gel-embedded osteocyte networks with alendronate did not reduce migration of pre-osteoclasts in response to a microinjury size of 400 µm, whilst zoledronate surprisingly showed a significant increase in the level of pre-osteoclast migration (Fig. 4a). Interestingly, the treatment of gel-embedded osteocyte networks with alendronate or zoledronate significantly decreased osteoclastogenesis as measured by TRAP activity in undamaged controls. Microinjuries of 400 µm were still capable of inducing osteoclast differentiation in bisphosphonate treated groups; however, the levels of TRAP activity (Fig. 4b) and TRAP positive cells were significantly less than that observed for the non-treated groups post microinjury (Fig. 4c).

Doubly conditioned medium from microinjury induced osteoclastogenesis under bisphosphonate treatment conditions modulates pre-osteoblast migration and differentiation

Gel-embedded osteocyte networks were subjected to a microinjury width of 400 µm in the presence or absence of bisphosphonate treatment. After 72 h, conditioned medium was collected and used in osteoclastogenesis assays (as described previously). This doubly conditioned medium was then either used for pre-osteoblast migration studies or utilised to examine the effect on pre-osteoblast mineralisation. Pre-osteoblast migration significantly increased in all treatment groups in response to microinjury, compared to undamaged controls. Significantly higher levels of cell migration were noted in the zoledronate microinjured group, in comparison to the untreated and alendronate treated microinjured groups (Fig. 5a). Bisphosphonate treatment significantly increased calcium deposition by pre-osteoblasts over a 21 d period, in both the undamaged and microinjured groups, with the

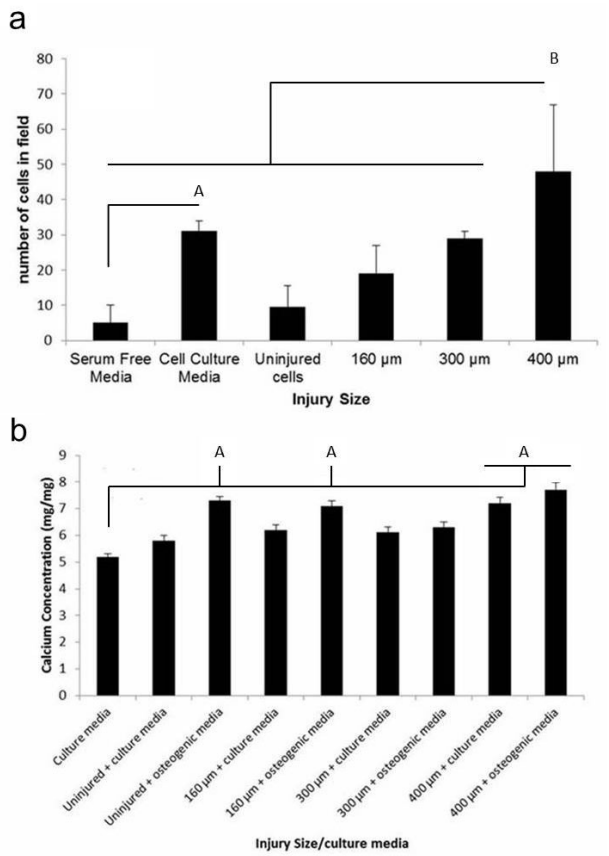


Fig. 3. The effect of microinjury induced osteoclastogenesis on pre-osteoblast migration and differentiation. Gel embedded osteocyte networks were subjected to microinjury having widths of 160, 300 or 400 µm. After 72 h, conditioned medium was collected and used in osteoclastogenesis assays. This doubly conditioned medium was then either directly used to evaluate pre-osteoblast migration (a) (A, $p \leq 0.05$ *versus* serum free control; B, $p \leq 0.05$ *versus* all other groups) or combined in a 1:1 ratio with osteoblast growth or osteogenic medium and used to assess its osteogenic differentiation potential (b) (A, $p \leq 0.05$ *versus* cell culture medium). Error bars represent the standard deviation, $n = 3$.

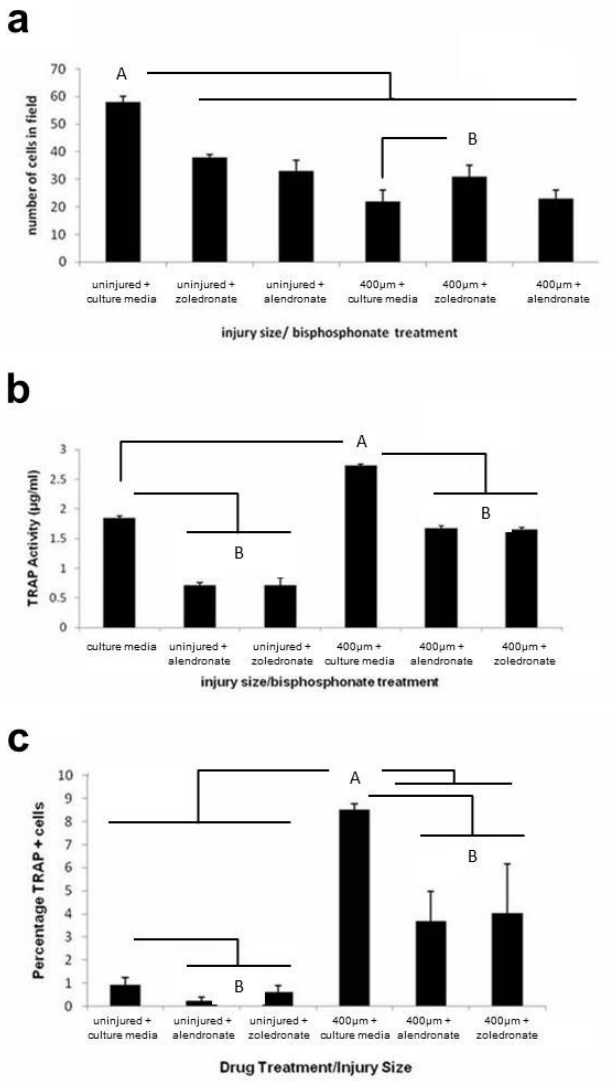


Fig. 4. The effect of microinjury within osteocyte cultures in the presence of bisphosphonates on pre-osteoclast migration and differentiation. Gel embedded osteocyte networks were subject to microinjury having a width of 400 µm in the presence of alendronate or zoledronate. After 72 h, conditioned medium was collected and used to assess either the chemotaxic properties on pre-osteoclasts (a) (A, $p \leq 0.01$ *versus* all other groups; B, $p \leq 0.05$ *versus* 400 µm + culture medium), or the induction of osteoclastogenesis after 21 d of culture, as quantitatively measured by TRAP activity (b) (A, $p \leq 0.05$ *versus* cell culture medium; B, $p \leq 0.05$ decrease *versus* cell culture medium for same injury size) or quantification of TRAP staining (c) (A, $p \leq 0.01$ *versus* all other groups; B, $p \leq 0.05$ decrease *versus* cell culture medium for same injury size). Error bars represent the standard deviation, $n = 3$.

microinjured groups having a further significant increase in calcium deposition levels, suggesting an additive effect of microinjury with bisphosphonate treatment (Fig. 5b).

The effect of bisphosphonate treatment on the RANKL-RANK-OPG pathway

Gel-embedded osteocyte networks were subjected to microinjury having an injury width of 400 µm in the presence or absence of bisphosphonate treatment. Secreted RANKL and OPG protein expression levels were measured by ELISA. Bisphosphonate treatment significantly reduced both cytokines in uninjured and microinjured samples (Fig. 6a and b).

Discussion

It is well known that bone is capable of detecting and repairing microcracks and other forms of microscopic damage and that bone remodelling by BMUs is stimulated by microdamage (Burr *et al.*, 1985; Hazenberg *et al.*, 2009; Martin, 2002; Verborgt *et al.*, 2000). The detection of microdamage and subsequent activation of the BMU to initiate bone remodelling is believed to be facilitated by osteocytes, as a function of their mechanosensory and mechanostimulatory attributes, either through a mechanotransduction mechanism (Turner *et al.*, 1994) or, as hypothesised by our group, by a so called "scissors

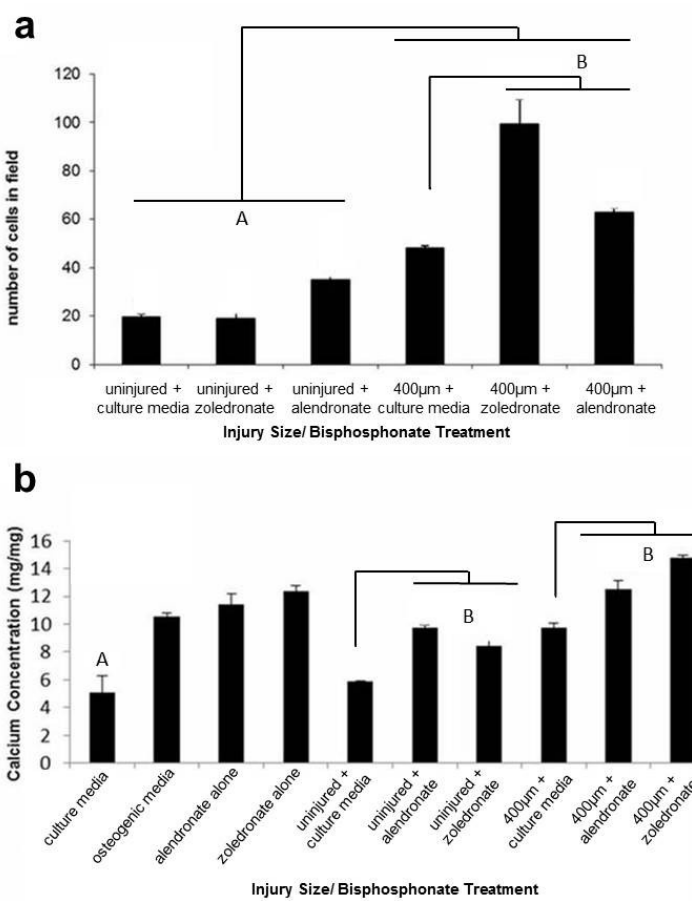


Fig. 5. The effect of bisphosphonate-modulated microinjury-induced osteoclastogenesis on pre-osteoblast migration and differentiation. Pre-osteocyte cultures were subject to microinjury having a width of 400 µm in the presence of alendronate or zoledronate. After 72 h, conditioned medium was collected and used in osteoclastogenesis assays. This doubly-conditioned medium was then either directly used to evaluate pre-osteoblast migration (a) (A, $p \leq 0.05$ decrease *versus* all 400 µm treated groups; B, $p \leq 0.01$ *versus* 400 µm + culture medium) or combined in a 1:1 ratio with osteoblast growth medium and used to assess its osteogenic differentiation potential (b) (A, $p \leq 0.05$ *versus* all other treatment groups except for uninjured + culture medium; B, $p \leq 0.05$ *versus* cell culture medium group for the same injury size). Error bars represent the standard deviation, $n = 3$.

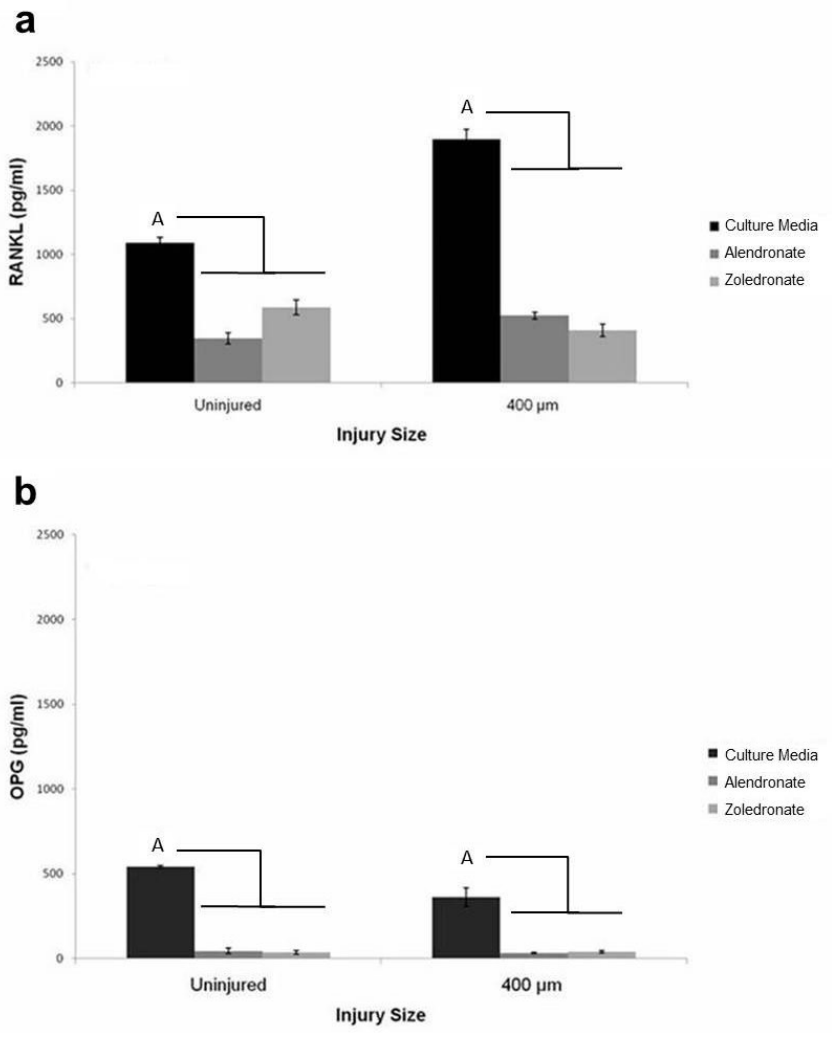


Fig. 6. The effect of bisphosphonate concentration on RANKL and OPG protein expression levels after osteocyte injury. Osteocyte cultures were supplemented with 10^{-5} M alendronate or zoledronate concentrations and subject to microinjury having a width of 400 µm. RANKL (**a**) and OPG (**b**) protein expression levels were quantified by ELISA (A, $p \leq 0.05$ *versus* bisphosphonate-treated groups for the same injury size). Error bars represent the standard deviation, $n = 3$.

model" that involves the shearing of cellular processes (Hazenberg *et al.*, 2009; Taylor *et al.*, 2003). In order to study this behaviour *in vitro,* a model allowing controlled levels of injury and damage to osteocyte networks was developed in-house, in the absence of the bone tissue matrix (Mulcahy *et al.*, 2011). This study showed that this *in vitro* microinjury can modulate the RANKL-RANK-OPG signalling pathway (Mulcahy *et al.*, 2011) in a manner indicative of the *in vivo* response, which is associated with the initiation of bone remodelling through the synchronous action of the BMU. Bone remodelling consists of several stages, which include activation, resorption and formation. Activation refers to the stage by which an influx of osteoclast precursor cells infiltrates the area of osteocyte rupture and fuse to form giant, active osteoclasts. These differentiated osteoclasts then polarise to bone and release lytic enzymes, which break down bone, under the control of RANKL-RANK interaction (Katagiri and Takahashi, 2002). Finally, formation of new bone, by the infiltration of pre-osteoblasts, leads to the secretion of osteoid, which becomes mineralised by calcium and phosphates in peripheral fluids. This occurs following release of OPG, which stops RANKL-RANK interaction, and stimulates the influx of pre-osteoblasts.

This study describes the successful development of an *in vitro* model capable of mimicking this interactive crosstalk between sensory osteocytic networks and the osteoclastogenic and osteogenic activities of the BMU. We showed that in response to microinjury sizes of 400 μm, pre-osteoclast migration was increased, and osteoclast differentiation, as measured by TRAP activity, was significantly upregulated. Furthermore, doubly-conditioned medium was capable of enhancing pre-osteoblast migration and pre-osteoblast differentiation, as measured by the deposition of calcium.

In vitro models, as developed in this study, offer researchers a potential means by which complex systems such as bone remodelling can be decoupled and the effects of pharmaceutical agents on individual components of the system can be analysed. For example, since the early 1990s bisphosphonates have yielded significant potential and have shown great success in the treatment of osteoporosis, as well as other bone diseases, including metastatic bone disease, osteopenia and Paget's disease (Morris and Einhorn, 2005). In spite of the existing knowledge of the *in vivo* responses of bone to bisphosphonates, it is well recognised that the cellular responses to these drugs require further investigation. In this study, an *in vitro* model system was applied to investigate the effect of two aminobisphosphonates, alendronate and zoledronate, on osteocyte stimulation in response to microinjury and the subsequent impact upon the cells constituting the BMU.

The changes observed in the RANKL-RANK-OPG signalling pathway, as a function of bisphosphonate treatment, directly resulted in reduced osteoclast activity as measured by TRAP activity, as observed by other groups previously *in vitro* (Boonekamp *et al.*, 1986; Kimachi *et al.*, 2011; Li *et al.*, 2011), in line with the expected method of action for these drugs *in vivo*. However, previous *in vivo* studies have suggested that alendronate is a less potent inhibitor of osteoclast activity than zoledronate (Watts and Diab, 2010), though the results herein suggest there is no difference between the two bisphosphonates when tested *in vitro*. It is important to point out that the differences observed in inhibition of osteoclast activity *in vivo* relies on a number of factors that primarily includes the method of bisphosphonate administration (*i.e.* intravenous *versus* oral) and also the different bioavailability. For example, oral bisphosphonates have very limited bioavailability due to their poor lipophilicity, with less than 1 % of orally taken bisphosphonates being absorbed in the gut (Ruza *et al*., 2013). As a result, the potency is also different but in a comparable order of magnitude. This was demonstrated in a study by Orwoll *et al*. (2010) that showed similar increased bone density and decreased bone turnover markers when comparing the safety and efficacy of a once-yearly intravenous infusion of 5 mg zoledronic acid *versus* a once-weekly 70 mg oral alendronate in the treatment of male osteoporosis. The effect of bisphosphonate treatment on pre-osteoblast migration and differentiation was subsequently evaluated. Both zoledronate and alendronate significantly increased both pre-osteoblast migration and differentiation in response to microinjury. These findings are in line with previous reports suggesting that aminobisphosphonates, including alendronate and zoledronate, have stimulatory effects on osteoblast differentiation and proliferation (Pan *et al.*, 2004; Reinholz *et al.*, 2000). Although the exact mechanism by which bisphosphonates affect osteoblasts remains elusive, zoledronic acid has been shown to inhibit the mevalonate pathway thereby inducing human osteoblast differentiation (Reinholz *et al.*, 2002). Inhibition of this pathway also prevents osteoclasts from attaching to the bone surface and therefore resorbing bone (Fisher *et al.*, 2000).

This study also showed that the release of two of the principal cytokines involved in bone remodelling, RANKL, which functions by stimulating osteoclastogenesis, and its antagonist, OPG, which sequesters RANKL activity, stimulating osteoblastogenesis (Lacey *et al.*, 1998; Simonet *et al.*, 1997) were significantly reduced in response to bisphosphonate treatments. These results are reflective of the work of others (Giner *et al.*, 2011; Kimachi *et al.*, 2011; Koch *et al.*, 2012), who observed that aminobisphosphonates, including alendronate and zoledronate, posed a modulatory effect on RANKL activity in osteoblastic cells. Considering that in this model the effect of bisphosphonates on an osteocytic network is being assessed, the results may be significant in further demonstrating the biochemical effect of osteoporotic drugs, most notably so in a model of microinjury in bone. However, the reduction in OPG, in both alendronate and zoledronate-treated samples, is something that has not been extensively observed to date and provides further mechanistic information regarding the action of such drugs.

In conclusion, the *in vivo* effects of bisphosphonates are, at present, well documented, and treatment of osteoporosis using bisphosphonates has shown great promise with regard to decreasing osteoclast activity and increasing bone mineral density. However, the cellular response to bisphosphonates, in an environment mimicking microinjury of the osteocyte network, has yet to be clarified. This study, using a novel *in vitro* model of bone remodelling, which

allowed the spatial decoupling of the different cell types involved, has shown for the first time that microinjury, under the treatment of aminobisphosphonates, has a modulatory effect on RANKL and OPG activity, and thus on osteoclastogenesis. Furthermore, the presence of bisphosphonates was shown to increase the osteogenic potential of microinjury. Thus, we have shown that bisphosphonates act at all three stages of bone remodelling, from microinjury to osteoclastogenesis and ultimately osteogenesis.

Acknowledgments

This work was funded by Science Foundation Ireland under the Research Frontiers Programme grant number RFP-ENM-991. The MLO-Y4 cells were generously provided by Dr. Linda Bonewald (School of Dentistry, University of Missouri, Kansas City, MO, USA). Authors' roles: Planning: LM, GD, TCL and DT. Data collection: LM. Data analysis: LM, CC, RM and GD. Manuscript preparation and editing: LM, RM, CC, GD, DT and TCL. GD takes responsibility for the integrity of the data analysis. We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

References

Al-Dujaili SA, Lau E, Al-Dujaili H, Tsang K, Guenther A, You L (2011) Apoptotic osteocytes regulate osteoclast precursor recruitment and differentiation *in vitro*. J Cell Biochem **112:** 2412-2423.

Bergmann P, Body JJ, Boonen S, Boutsen Y, Devogelaer JP, Goemaere S, Kaufman J, Reginster JY, Rozenberg S (2010) Loading and skeletal development and maintenance. J Osteoporos **2011**: 786752.

Boivin GY, Chavassieux PM, Santora AC, Yates J, Meunier PJ (2000) Alendronate increases bone strength by increasing the mean degree of mineralization of bone tissue in osteoporotic women. Bone **27:** 687-694.

Boonekamp PM, van der Wee-Pals LJ, van Wijk-van Lennep MM, Thesing CW, Bijvoet OL (1986) Two modes of action of bisphosphonates on osteoclastic resorption of mineralized matrix. Bone Miner **1:** 27-39.

Brennan O, Kennedy OD, Lee TC, Rackard SM, O'Brien FJ, McNamara LM (2011) The effects of estrogen deficiency and bisphosphonate treatment on tissue mineralisation and stiffness in an ovine model of osteoporosis. J Biomech **44:** 386-390.

Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A (2007) Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. J Bone Miner Res **22:** 465-475.

Burr DB, Martin RB, Schaffler MB, Radin EL (1985) Bone remodeling in response to *in vivo* fatigue microdamage. J Biomech **18:** 189-200.

Burr DB, Milgrom C, Fyhrie D, Forwood M, Nyska M, Finestone A, Hoshaw S, Saiag E, Simkin A (1996) *In vivo* measurement of human tibial strains during vigorous activity. Bone **18:** 405-410.

Burr DB, Turner CH, Naick P, Forwood MR, Ambrosius W, Hasan MS, Pidaparti R (1998) Does microdamage accumulation affect the mechanical properties of bone? J Biomech **31:** 337-345.

Chrischilles E, Shireman T, Wallace R (1994) Costs and health effects of osteoporotic fractures. Bone **15:** 377-386.

Donahue SW, Galley SA, Vaughan MR, Patterson-Buckendahl P, Demers LM, Vance JL, McGee ME (2006) Parathyroid hormone may maintain bone formation in hibernating black bears (*Ursus americanus*) to prevent disuse osteoporosis. J Exp Biol **209:** 1630-1638.

Eriksen EF, Langdahl B, Vesterby A, Rungby J, Kassem M (1999) Hormone replacement therapy prevents osteoclastic hyperactivity: A histomorphometric study in early postmenopausal women. J Bone Miner Res **14:** 1217-1221.

Fisher JE, Rodan GA, Reszka AA (2000) *In vivo* effects of bisphosphonates on the osteoclast mevalonate pathway. Endocrinology **141:** 4793-4796.

Giner M, Rios MJ, Montoya MJ, Vazquez MA, Miranda C, Perez-Cano R (2011) Alendronate and raloxifene affect the osteoprotegerin/RANKL system in human osteoblast primary cultures from patients with osteoporosis and osteoarthritis. Eur J Pharmacol **650:** 682-687.

Hazenberg JG, Hentunen TA, Heino TJ, Kurata K, Lee TC, Taylor D (2009) Microdamage detection and repair in bone: fracture mechanics, histology, cell biology. Technol Health Care **17:** 67-75.

Hoerger TJ, Downs KE, Lakshmanan MC, Lindrooth RC, Plouffe L, Jr., Wendling B, West SL, Ohsfeldt RL (1999) Healthcare use among U.S. women aged 45 and older: total costs and costs for selected postmenopausal health risks. J Womens Health Gend-Based Med **8:** 1077-1089.

Hoey DA, Kelly DJ, Jacobs CR (2011) A role for the primary cilium in paracrine signaling between mechanically stimulated osteocytes and mesenchymal stem cells. Biochem Biophys Res Commun **412:**182-187.

Jilka RL, Takahashi K, Munshi M, Williams DC, Roberson PK, Manolagas SC (1998) Loss of estrogen upregulates osteoblastogenesis in the murine bone marrow. Evidence for autonomy from factors released during bone resorption. J Clin Invest **101:** 1942-1950.

Johnell O, Kanis JA (2006) An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. Osteoporos Int **17:** 1726-1733.

Katagiri T, Takahashi N (2002) Regulatory mechanisms of osteoblast and osteoclast differentiation. Oral Dis **8:** 147-159.

Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF (1997) Establishment of an osteocyte-like cell line, MLO-Y4. J Bone Miner Res **12:** 2014-2023.

Kimachi K, Kajiya H, Nakayama S, Ikebe T, Okabe K (2011) Zoledronic acid inhibits RANK expression and migration of osteoclast precursors during osteoclastogenesis. Naunyn Schmiedebergs Arch Pharmacol **383:** 297-308.

Koch FP, Merkel C, Ziebart T, Smeets R, Walter C, Al-Nawas B (2012) Influence of bisphosphonates on the

osteoblast RANKL and OPG gene expression *in vitro*. Clin Oral Invest **16:** 79-86.

Kufahl RH, Saha S (1990) A theoretical model for stress-generated fluid flow in the canaliculi-lacunae network in bone tissue. J Biomech **23:** 171-180.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell **93:** 165-176.

Lanyon LE (1987) Functional strain in bone tissue as an objective, and controlling stimulus for adaptive bone remodelling. J Biomech **20:** 1083-1093.

Lee TC, Staines A, Taylor D (2002) Bone adaptation to load: microdamage as a stimulus for bone remodelling. J Anat **201:** 437-446.

Li B, Ling Chau JF, Wang X, Leong WF (2011) Bisphosphonates, specific inhibitors of osteoclast function and a class of drugs for osteoporosis therapy. J Cell Biochem **112:** 1229-1242.

Martin RB (2002) Is all cortical bone remodeling initiated by microdamage? Bone **30:** 8-13.

Martin RB, Burr DB (1982) A hypothetical mechanism for the stimulation of osteonal remodelling by fatigue damage. J Biomech **15:** 137-139.

Mashiba T, Hirano T, Turner CH, Forwood MR, Johnston CC, Burr DB (2000) Suppressed bone turnover by bisphosphonates increases microdamage accumulation and reduces some biomechanical properties in dog rib. J Bone Miner Res **15:** 613-620.

Morris CD, Einhorn TA (2005) Bisphosphonates in orthopaedic surgery. J Bone Joint Surg Am **87:** 1609-1618.

Mulcahy LE, Taylor D, Lee TC, Duffy GP (2011) RANKL and OPG activity is regulated by injury size in networks of osteocyte-like cells. Bone **48:** 182-188.

Naidu A, Dechow PC, Spears R, Wright JM, Kessler HP, Opperman LA (2008) The effects of bisphosphonates on osteoblasts *in vitro*. Oral Surg Oral Med Oral Pathol Oral Radiol Endod **106:** 5-13.

Nordin BE, Need AG, Chatterton BE, Horowitz M, Morris HA (1990) The relative contributions of age and years since menopause to postmenopausal bone loss. J Clin Endocrinol Metab **70:** 83-88.

Orwoll ES, Miller PD, Adachi JD, Brown J, Adler RA, Kendler D, Bucci-Rechtweg C, Readie A, Mesenbrink P, Weinstein RS (2010) Efficacy and safety of a once-yearly i.v. Infusion of zoledronic acid 5 mg *versus* a once-weekly 70-mg oral alendronate in the treatment of male osteoporosis: A randomized, multicenter, double-blind, active-controlled study J Bone Miner Res **25:** 2239-2250.

Pan B, To LB, Farrugia AN, Findlay DM, Green J, Gronthos S, Evdokiou A, Lynch K, Atkins GJ, Zannettino AC (2004) The nitrogen-containing bisphosphonate, zoledronic acid, increases mineralisation of human bone-derived cells *in vitro*. Bone **34:** 112-123.

Parfitt AM (2002) High bone turnover is intrinsically harmful: two paths to a similar conclusion. The Parfitt view. J Bone Miner Res **17:** 1558-1559.

Phillips S, Fox N, Jacobs J, Wright WE (1988) The direct medical costs of osteoporosis for American women aged 45 and older, 1986. Bone **9:** 271-279.

Pivonka P, Buenzli PR, Dunstan CR (2012). A systems approach to understanding bone cell interactions in health and disease. In: Gowder S (ed.) Cell Interaction, InTech, Rijeka, Croatia, pp 169-204.

Ray NF, Chan JK, Thamer M, Melton LJ 3rd (1997) Medical expenditures for the treatment of osteoporotic fractures in the United States in 1995: report from the National Osteoporosis Foundation. J Bone Miner Res **12:** 24-35.

Reginster JY, Pelousse F, Bruyere O (2013) Safety concerns with the long-term management of osteoporosis. Expert Opin Drug Saf **12:** 507-522.

Reinholz GG, Getz B, Pederson L, Sanders ES, Subramaniam M, Ingle JN, Spelsberg TC (2000) Bisphosphonates directly regulate cell proliferation, differentiation, and gene expression in human osteoblasts. Cancer Res **60:** 6001-6007.

Reinholz GG, Getz B, Sanders ES, Karpeisky MY, Padyukova N, Mikhailov SN, Ingle JN, Spelsberg TC (2002) Distinct mechanisms of bisphosphonate action between osteoblasts and breast cancer cells: identity of a potent new bisphosphonate analogue. Breast Cancer Res Treat **71:** 257-268.

Roche JJ, Wenn RT, Sahota O, Moran CG (2005) Effect of comorbidities and postoperative complications on mortality after hip fracture in elderly people: prospective observational cohort study. BMJ **331:** 1374.

Rogers MJ (2003) New insights into the molecular mechanisms of action of bisphosphonates. Curr Pharm Des **9:** 2643-2658.

Russell RG, Watts NB, Ebetino FH, Rogers MJ (2008) Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos Int **19:** 733-759.

Ruza I, Mirfakhraee S, Orwoll E, Gruntmanis U (2013) Clinical experience with intravenous zoledronic acid in the treatment of male osteoporosis: evidence and opinions. Ther Adv Musculoskelet Dis **5:** 182-198.

Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R, Boyle WJ (1997) Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell **89:** 309-319.

Spadaro JA, Damron TA, Horton JA, Margulies BS, Murray GM, Clemente DA, Strauss JA (2006) Density and structural changes in the bone of growing rats after weekly alendronate administration with and without a methotrexate challenge. J Orthop Res **24:** 936-944.

Taylor D, Hazenberg JG, Lee TC (2003) The cellular transducer in damage-stimulated bone remodelling: a

theoretical investigation using fracture mechanics. J Theor Biol **225:** 65-75.

Turner CH, Forwood MR, Otter MW (1994) Mechanotransduction in bone: do bone cells act as sensors of fluid flow? FASEB J **8:** 875-878.

Verborgt O, Gibson GJ, Schaffler MB (2000) Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue *in vivo*. J Bone Miner Res **15:** 60-67.

Watts NB, Diab DL (2010) Long-term use of bisphosphonates in osteoporosis. J Clin Endocrinol Metab **95:** 1555-1565.

Weinbaum S, Cowin SC, Zeng Y (1994) A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses. J Biomech **27:** 339-360.

Zerwekh JE, Ruml LA, Gottschalk F, Pak CY (1998) The effects of twelve weeks of bed rest on bone histology, biochemical markers of bone turnover, and calcium homeostasis in eleven normal subjects. J Bone Miner Res **13:** 1594-1601.

Discussion with Reviewer

Reviewer I: For this model, why did the authors not co-culture the osteoblasts and osteoclasts together from the beginning in one dish or plate and then transfer the microdamage conditioned media?

Authors: In order to test this model at the most basic level, we chose to assess the individual cell types to gain a basic understanding of how the individual cell types function during bone remodelling. It was agreed that were a co-culture to be evaluated it would introduce a further level of complexity that was beyond the scope of this particular study but may be of interest in future studies.

Editor's Note: Scientific Editor in charge of the paper: Martin Stoddart.