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

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

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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.

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, on the osteocytic response to microdamage and osteocyte 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 % CO₂.

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 × 10⁴ 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 × 10⁴ 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 × 10⁴ 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% CO₂, 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
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 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.
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 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...
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 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 resoring 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 osteoclastogenesis (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.

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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.