

# Influencing Effect of Heat Therapy on Osteoblasts Growth and Differentiation Following Treatment with Bone Antiresorptive Drugs- An *in vitro* Study

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## ABSTRACT

**Objectives:** To find out the effect of mild heat shock on osteoblast proliferation and mineralisation following treatment with bisphosphonates.

**Materials and Methods:** Cell culture of MC3T3 cells was performed in alpha minimum essential medium supplemented with 10% Foetal Calf Serum (FCS), 2 mmol L-glutamine and 100U/ml each of penicillin/streptomycin and were incubated at 37°C in humidified 5% CO<sub>2</sub>. The selected bisphosphonate drugs were administered, following which heat therapy was given by placing the flasks in a temperature-regulated water bath. The proliferation of osteoblast progenitors was measured using the methylene blue staining technique. **Results:** The results showed that mild heat-shock for 2 min at 42°C can stimulate proliferation of osteoblast progenitors, as well as bone nodule formation. In addition, mild heat-shock can reverse

the action of bisphosphonate by stimulation of osteoblast proliferation and mineralisation. **Conclusion:** Mild heat-shock enhances cell differentiation and osteoblasts mineralisation following bisphosphonate treatment.

**Key words:** Bisphosphonates, Heat-shock, Osteoblast, Osteoclast.

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## INTRODUCTION

Bone along with the cartilage constitutes the body's skeleton.<sup>1</sup> Bone remodelling is the process by which bone is turned over and it maintains the integrity of the skeleton.<sup>2</sup> Initially there will be activation of osteoclasts by osteoblasts, the osteoblasts get recruited to the desorption site and then they proliferate and differentiate, resulting in new bone formation.<sup>3</sup> The whole process is regulated by local and systemic hormonal factors. Any disturbance in this process leads to pathological conditions like periodontal disease, osteoporosis or osteonecrosis etc.<sup>2,3</sup>

The aetiology of Osteonecrosis of the Jaw (ONJ) is infection, trauma, medication and radiotherapy.<sup>4</sup> ONJ is primarily associated with radiation therapy, as a correlation between bisphosphonate and ONJ was reported.<sup>4,5</sup> Bisphosphonates are given to patients with osteoporosis, malignant bone disease and hypercalcaemia of malignancy and one of its complication is bisphosphonate induced osteonecrosis of the jaws (BRONJ) or chemo-osteonecrosis of the jaw, caused by binding of the drug to the bone matrix.<sup>6</sup> In adults with cancer-related bone metastases, use of zoledronic acid, a bisphosphonate caused ONJ in 1.3% of patients. Bisphosphonates inactivate osteoclasts and osteoblasts by inhibiting proliferation, differentiation and mineralisation.<sup>6,7</sup>

The commonly used drugs for osteoporosis are bisphosphonates. These drugs have a strong affinity for calcium of hydroxyapatite and also affect osteoclast enzyme farnesyl-diphosphate synthase in the HMG-CoA reductase pathway.<sup>8</sup> They also inhibit osteoblast proliferation, differentiation and function, which, in turn leads to osteonecrosis.<sup>9</sup> In such cases there is a need for stimulation of bone remodelling and angiogenesis.

The two major factors that have a stimulatory effect on the cellular growth process are oxygen supply and temperature. It has been shown

that elevated temperature above the physiological normal range has a stimulatory effect on bone growth, via stimulation of cell proliferation and differentiation and also affecting the bone microenvironment by stimulating growth factors. Several studies reported a positive effect by thermotherapy in the treatment of various clinical diseases like bone malignancies, inflammatory osteoarticular disorders and bone metastasis.<sup>10,11</sup> But still the underlying mechanism by which temperature interacts with bone cells remains controversial. We carried this study to examine the effect of mild heat-shock on osteoblast proliferation and mineralization, following treatment with bisphosphonates.

## MATERIALS AND METHODS

### Cell culture

MC3T3 cells are new-born mouse calvarial-derived cells, which have been characterised for their osteoblast precursor-like properties. All cell culture procedures were performed in a sterile air-flow chamber. Cells were cultured in alpha minimum essential medium supplemented with 10% Foetal Calf Serum (FCS), 2 mmol L-glutamine and 100U/ml each of penicillin/streptomycin and were incubated at 37°C in humidified 5% CO<sub>2</sub>.

### Mild heat-shock treatment

MC3T3 cells were seeded in 25 cm<sup>2</sup> flasks and incubated for 24 hr at 37°C, in 5% CO<sub>2</sub> and 95% air. Heat shock was administered by placing the flasks in a temperature-regulated water bath. The temperature was maintained within the error limits of ±0.1°C. The flasks were immersed in the water bath at various predetermined temperatures of 32°C, 42°C

and 47°C for 2 and 5 min. One group of cells was left untreated in the incubator at 37°C. All the flasks were incubated at 37°C for a further 24 hr to allow for the expression of heat-shock proteins.

### Bisphosphonate drugs

The selected BP drugs have an established route of administration and mechanism of action. Pamidronate was administered intravenously, whereas alendronate was orally administered. The concentration range of the tested bisphosphonates was based on the information provided in the literature and on preliminary experiments, which allowed us to exclude the cytotoxic dose.

### Proliferation

Proliferation was measured using the methylene blue staining technique. At the end of the experimental period, the cells were washed and fixed with methanol for 2 min, after which 0.1% methylene blue in 0.01 M borate buffer, pH 8.5, was added to each well and left for 30 min. The excess dye was removed by thoroughly washing with borate buffer then leaving to dry. The dye from the stained cells was eluted using 100 µl of 0.1 N HCl with 2% ethanol. The absorbance of each well was measured on an automated spectrophotometer at 650 nm.

### Bone nodule formation

For the mineralised nodule formation assay, the mineralised matrices were analysed using alizarin red staining. In brief, the cell cultures were twice washed with Phosphate Buffered Saline (PBS) and fixed with 2.5% glutaraldehyde for 3 min. Next, the cells were washed three times with 70% ethanol, left to dry for 30 min, treated with a 2% alizarin red S solution for 20 min and washed three times to remove the remaining staining. The stains were then eluted with 500 µl of 10% cetylpyridinium and transferred to a 96-well micro plate in 100µl amounts. The plate was then read by automated spectrophotometer at OD405 values.

### Alkaline phosphates activity

The cells were seeded in a 24-well tissue culture plate at a density of 10x10<sup>4</sup> cells per well. By end of the experiment, 0.5% Triton in PBS lysis buffer was added to each well, following removal of the old medium and washing with PBS. The cells were then transferred to micro tubes and stored at -20°C. An Alkaline Phosphatase Assay Kit (Bio Vision®) was used according to the manufacturer's instructions. The reaction mixtures were transferred to a 96-well micro plate and absorbance was read at 405nm using a spectrophotometer.

### Statistical analysis

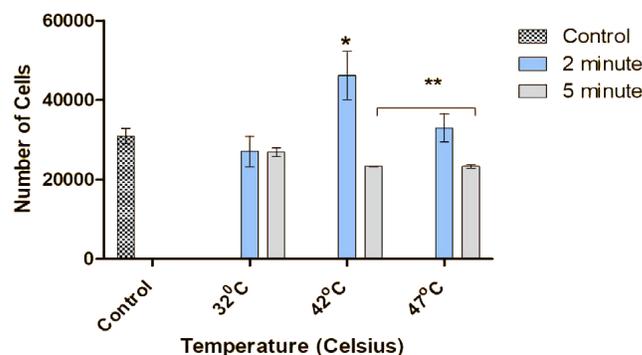
Statistical analysis was carried out using the student *t*-test in GraphPad Prism software (v5.04), with  $p < 0.05$  considered to be statistically significant.

## RESULTS

### The effect of mild heat stress on osteoblast proliferation

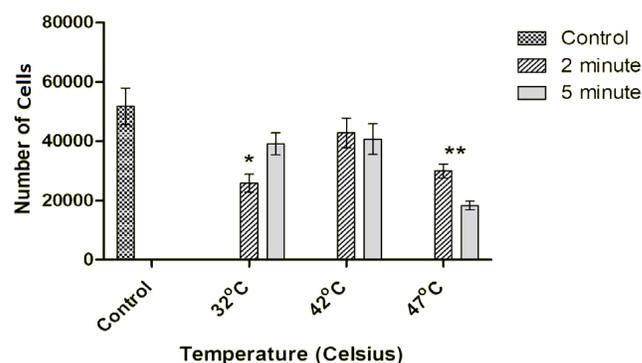
Osteoblast progenitors were exposed to temperatures of 32°C, 37°C, 42°C and 47°C for 2 and 5 min for each and incubated for 24 or 72 hr. The results showed a significant inhibition of osteoblast growth and proliferation at both 24 and 72 hr when exposed to a temperature of 32°C for 2 or 5 min. In contrast, the osteoblasts that had been exposed to a temperature of 42°C for 2 min showed a significant increase in proliferation, as reflected by an increase in cell mass at 24 hr ( $P < 0.003$ ). This effect of stimulation was not seen at 72 hr. Significant inhibition ( $P < 0.005$ ) was seen at exposure to temperatures of both 42°C and 47°C for 5 min (Figure 1). When heat stress for 2 min and incubation for 3 days was tested, a significant inhibition of osteoblast proliferation ( $P < 0.01$ ) was seen in all groups of osteoblasts exposed to temperatures of 32°C and 47°C. Significant inhibition was seen in all the groups of osteoblasts exposed to 47°C ( $P < 0.05$ ) (Figure 2).

### The effect of mild heat stress on osteoblast proliferation 24h



**Figure 1:** The effect of mild heat-stress for 2 min following 24 hr of incubation. Significant stimulation of osteoblasts proliferation can be seen in the osteoblasts treated at 42°C for 2 min ( $*P < 0.003$ ). Significant inhibition ( $**P < 0.005$ ) can be seen at exposure to temperatures of both 42°C and 47°C for 5 min.

### The effect of mild heat stress on osteoblast proliferation 72h



**Figure 2:** The effect of heat stress for 2 min and incubation for 3 days. Significant inhibition of osteoblast proliferation ( $*P < 0.01$ ) can be seen in all groups of osteoblasts exposure to temperatures of 32°C and 47°C. Significant inhibition can be seen in all the groups of osteoblasts exposed to 47°C ( $**P < 0.05$ ).

### The effect of mild heat stress on osteoblast mineralisation

The effect of heat stress on mineralised nodule formation over a 7-day incubation period was examined using Alizarin red-S staining. When the cells were exposed to a temperature of 32°C, nodule formation was slightly inhibited when compared to the corresponding controls. At a temperature of 42°C, nodule formation was significantly stimulated when compared to controls for both 2 and 5 min exposures. There was a significant increase in nodule formation at 42°C in cells exposed for 2 and 5 min ( $P < 0.05$ ) compared to the controls. Heat had no effect on cell mineralisation at a temperature of 47°C (Figure 3).

### The effect of mild heat-stress on alkaline phosphates activity

Cells incubated for 2 min at 42°C and 47°C were stimulated to find out whether they synthesise more alkaline phosphatase compared to the control cultures. However, there was no difference from the control group when incubated for 5 min at 32°C, 42°C and 47°C (Figure 4).

## Experiment to determine if heat stress can reverse the effect of bisphosphonates on MC3T3 mineralisation

In order to investigate whether heat can reverse the action of bisphosphonates, two groups of osteoblasts were seeded in a 24-well plate at a density of 30,000 cells per well and then left to attach overnight. The medium containing 15  $\mu\text{M}$  and 25  $\mu\text{M}$  of drug, was then changed and the cells were incubated for 7 days. At the end of day 7, the medium was changed and group 1 was treated with heat for 2 min at 42°C, while the other group was treated with growth media. The results showed that heat reversed the inhibitory effect of the bisphosphonates compared to treatment with oestrogenic media. The results showed that heat stress significantly stimulated osteoblast progenitor mineralisation following drug treatment for 7 days compared to the group that was treated with oestrogenic media alone. ( $P < 0.04$ ,  $P < 0.009$ ). These Alizarin red-S stain assays showed that a high temperature of 42°C for a short period induced the rate of mineralisation of the MC3T3 cell line following treatment with bisphosphonates (Figure 5).

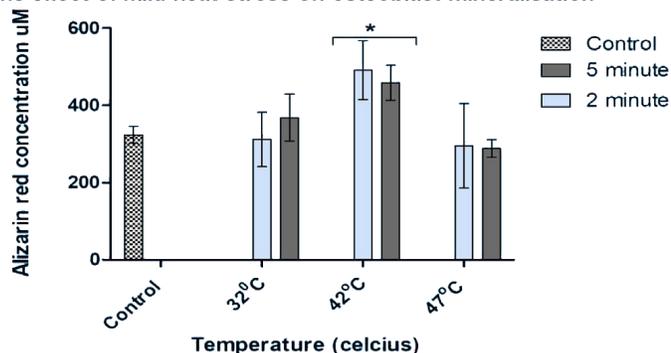
## Experiment to determine if heat stress can reverse the effect of bisphosphonates on MC3T3 proliferation

Osteoblast proliferation was investigated to observe whether heat stress of 42°C for 2 min, following treatment with pamidronate and alendronate for 3 days, could reverse the action of both drugs. The cells were divided into two groups and both were treated with different concentrations of drugs. On Day 3, the medium was changed and group 1 was exposed to heat stress at 42°C for 2 min. The other group of cells was incubated with growth media. The results showed that heat treatment stimulated cell proliferation following drug treatment for 7 days compared to treatment with growth media only. The results showed that heat stress treatment can stimulate osteoblast proliferation following treatment with these drugs for 7 days, compared to treatment with growth media only (Figure 6).

## DISCUSSION

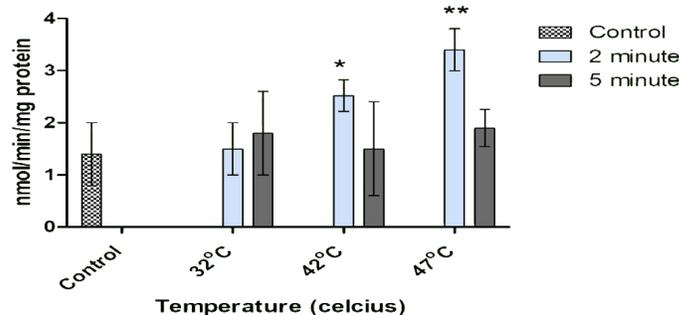
In the present study, we examined the effect of heat on osteoblast precursors following bisphosphonate treatment. Our results suggest that even an exposure of 47°C for 5 min caused cell death and so prevented cells from proliferating as the cell number was reduced. Previous similar suggested that 47°C is the threshold temperature at which bone-cell damage occurs.<sup>12</sup> At the same time bone-cell necrosis and subsequent apoptosis without signs of angiogenesis recovery occurs when bone is

### The effect of mild heat stress on osteoblast mineralisation



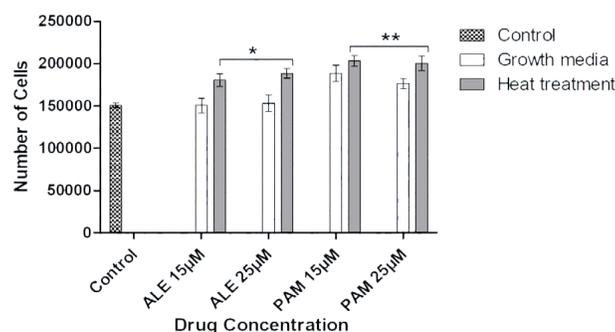
**Figure 3:** The effect of mild heat stress for 2 and 5 min after 10 days of incubation. There was a significant increase in nodule formation at 42°C in cells exposed for 2 min and 5 min ( $*P < 0.05$ ) compared to the controls.

### The effect of mild heat stress on ALP activity



**Figure 4:** Cells incubated for 2 min at temperatures of 42°C and 47°C were stimulated to synthesise significantly more alkaline phosphatase compared to the control cultures ( $p < 0.01$ ). There was no difference from the control group when incubated for 5 min at 32°C, 42°C and 47°C.

### Heat treatment effect on Cells proliferation following Bisphosphonate Treatment

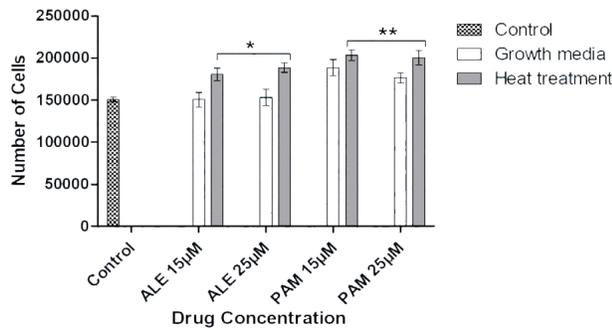


**Figure 5:** Cells were divided into two groups, which were treated with different concentrations of drugs. On Day 7, the medium was changed and group 1 was exposed to heat stress at 42°C for 2 min. The second group was incubated in only osteogenic media. The results showed that heat stress significantly stimulated osteoblast progenitor mineralisation following drug treatment for 7 days compared to the group that was treated with osteogenic media alone. ( $*P < 0.04$ ,  $**P < 0.009$ )

exposed to higher temperatures. This proves that the effect of heat on bone-cell growth and mineralisation depends on the degree of temperature. On contrary, reduced temperature induces osteoblast differentiation and mineralisation.<sup>13</sup> We found that 47°C as the threshold temperature, while 42°C for 5 min enhanced bone regeneration by stimulating osteoblast proliferation and mineralisation. Our findings indicate that application of heat treatment can be used as a new therapeutic tool in management of bone necrosis, such as BRONJ. We used MC3T3 cells, which are new-born mouse calvarial-derived cells characterised for their osteoblast precursor-like properties. Both have shown similar properties to osteoblast precursors and osteoblasts, but the main limitation of these cells is that they grow only as a two-dimensional monolayer in tissue culture flasks. In human bone, they grow in a three-dimensional environment, with the help of bone collagen and the mineral matrix. To sum up the first part of our study, osteoblast proliferation and differentiation are positively sensitive to temperature elevation, if a particular temperature and time are not exceeded.

High doses of pamidronate and alendronate (100  $\mu\text{M}$ ) have shown significant inhibition of osteoblast proliferation and differentiation and will thus interfere with the bone remodelling process. Alendronate is an oral bisphosphonate with a primary indication for osteoporosis and the recommended dose is 10mg/day. Pamidronate was the first intravenous

Heat treatment effect on Cells proliferation following Bisphosphonate Treatment



**Figure 6:** The cells were divided into two groups and both were treated with different concentrations of drugs. On Day 3, the medium was changed and group 1 was exposed to heat stress at 42°C for 2 min. The other group of cells was incubated with growth media. The results showed that heat treatment stimulated cell proliferation following drug treatment for 7 days compared to treatment with growth media only.

BP to be prescribed for bone metastasis and the recommended dose is 90 mg/3 weeks.<sup>14</sup> This is a possible reason why the inhibitory effect of these drugs contributes to the pathogenesis of BRONJ and our study supports this hypothesis. The concentration that we used in the present study covered the physiological range detected in the resorption site and in bone from patients with BRONJ.<sup>15</sup> Furthermore, it has been reported that the main action of bisphosphonates is a toxic effect on osteoclasts and other bone cells, because they have a high affinity to attach to bone hydroxyapatite.<sup>16</sup> In our study, pamidronate and alendronate limited bone mineralisation in two ways: a cytotoxic effect was associated with a high dose and a low dose decreased cell proliferation. The cytotoxic effect of the bisphosphonates can interfere with osteoblast cell metabolism and cause cytoplasmic shrinkage. It is possible that inhibition of osteoblast precursors can reduce the bone remodelling process, thus, the risk of BRONJ will increase. We have also observed that alendronate and pamidronate may stimulate bone-nodule formation at relatively low doses of 100nM and 10nM. The explanation for this finding is that it is possible that the drug acts on different cellular mechanisms of bone cells. We observed that mild heat treatment can reverse the action of bisphosphonates and accelerate osteoblast healing following bisphosphonate treatment.

## CONCLUSION

Temperature facilitates the stimulation of ontogenesis by osteoclasts, so this offers the potential to provide a non-invasive thermotherapy for bone necrosis, for example, BRONJ, or to treat bone defects following bone surgery by providing tissue regeneration. Mild heat-stress or treatment can reverse the action of bisphosphonate in two ways, either by enhancing osteoblast proliferation and differentiation, or by reducing the cytotoxic effect of bisphosphonates. This is could be of considerable therapeutic potential and a promising treatment for BRONJ.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATIONS

**ONJ:** Osteonecrosis of the jaw; **BRONJ:** Bisphosphonate induced osteonecrosis of the jaws; **FCS:** Foetal Calf Serum.

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