Thermally Induced Osteocyte Apoptosis Initiates Pro-osteoclastic And Pro-osteoblastic Signaling Responses

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Introduction: During orthopedic procedures, bone and the surrounding soft tissue can be exposed to elevated temperatures arising from surgical cutting and exothermal reactions from cement and biomaterials. Our recent study has shown that temperatures exceeding 47°C can trigger in vitro cellular responses, such as significant osteocyte apoptosis [1]. Micro-damage induced osteocyte apoptosis in vivo can trigger pro-osteoclastogenic signals that initiate osteoclastic bone resorption [2]. However, whether thermally induced osteocyte apoptosis can initiate expression of osteoclastogenic signals remains to be determined. Furthermore, we have also shown that markers related to osteogenesis (alkaline phosphatase activity (ALP) and calcium deposition) were up-regulated in Mesenchymal Stem Cells (MSCs) when directly exposed to such temperatures [1]. However, it is not yet known whether heat-treated osteocytes can elicit osteoblastic differentiation of neighboring MSCs. The aim of this study is to investigate thermally induced signalling responses by heat-treated osteocyte-like MLO-Y4s, and to examine the influence of these factors on nearby non-heat-treated MLO-Y4s and Balb/c MSCs. This is investigated using a co-culture system, whereby MLO-Y4s (ccMLO-Y4s) and MSCs (ccMSCs) cultured on permeable well inserts are exposed to biochemical molecules produced by heat-treated MLO-Y4s (htMLO-Y4s), while the ccMLO-Y4s and ccMSCs do not undergo heat-treatment themselves.

Methods: Co-culture: MLO-Y4 cells cultured on collagen coated slides were exposed to pre-heated media at either 37°C (control) or 47°C, and maintained on a hot plate at these temperatures for 1 minute. Non-heat-treated Balb/c MSCs (ccMSCs) or MLO-Y4s (ccMLO-Y4s) cultured on permeable inserts (PET 1 μm pores; Millipore, Cork, Ireland) were then added to the wells of the heat-treated MLO-Y4s and co-cultured for up to 21 days. Gene Expression Analysis: mRNA from htMLO-Y4s and ccMLO-Y4s was analysed by real time polymerase chain reaction (RT-PCR) for the expression of key signalling factors related to osteoclast differentiation (Receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG), and osteoblastic differentiation (Cyclooxygenase 2 (COX2)). The RANKL/OPG ratio is an important ratio governing osteoclast activity, an increase promotes osteoclast differentiation while a decrease reduces mature osteoclast concentration [3]. COX2 has been shown to be a vital signalling factor for osteogenic differentiation [4]. Briefly, cells were lysed, the solution was homogenised and the RNA was precipitated. The RNA was washed using an ENZA RNA isolation kit (Omega Bio-tek) and dissolved in 40 μL of RNase free water (Qiagen). RNA quality was measured using a Nanodrop spectrometer (Thermo-scientific) and converted to cDNA using a CDNA synthesis kit (Omega Biosciences) and Gene Amp 9700 A Thermal cycler (Applied Biosystems). RT-PCR was performed on a Step-One plus analyser (Applied Biosystems) using Taqman probes (Applied Biosystems). RT-PCR data was analysed using the 2−ΔΔCt method [5], where GAPDH was used as a housekeeping gene. Osteoblastic Differentiation: ALP activity was measured using cell lysate measured colorimetrically with
p-nitrophenyl phosphate as a substrate (Sigma) for ccMSCs. Mineralization of ccMSCs was quantified by alizarin red staining, whereby the stain was extracted using cethylpyridium chloride and the solution measured at 550nm using a spectrophotometer.

**Results:** Gene Expression by htMLO-Y4s: A significant decrease in RANKL gene expression is seen in MLO-Y4 cells heat-treated to 47°C by days 1 and 7 (p≤0.0011), while OPG expression is significantly increased compared to the control (p=0.0006) at day 7. The RANKL/OPG ratio is therefore consistently down-regulated at days 1 and 7 in htMLO-Y4s compared to the control, significantly so at day 7 (p=0.0073), see Figure (A). A significant increase in COX2 expression is observed at day 7 in MLO-Y4 cells heat-treated to 47°C (p=0.0009), compared to the control, see Figure (A). Gene Expression by ccMLO-Y4s: A significant increase in RANKL gene expression is seen in ccMLO-Y4 (p=0.00248) at day 1, while OPG expression is significantly increased compared to the control (p=0.0066) at day 7. The RANKL/OPG ratio is therefore significantly up-regulated at day 1 (p=0.0385) in ccMLO-Y4s compared to the control, see Figure (B). COX2 gene expression is significantly increased in ccMLO-Y4s by day 7 (p=0.0020), compared to the control, see Figure (B). Osteoblastic Differentiation by ccMSCs: A significant increase in ALP expression is seen in ccMSCs, 7 and 14 days (p≤0.0032) after their addition to heat-treated MLO-Y4s, compared to the control. Calcium deposition is also significantly higher in ccMSCs by day 21 (p=0.0001), compared to the control, see Figure (C).

**Discussion:** This study identifies a link between osteocyte thermal damage and bone remodeling responses in vitro. The results indicate that heat-treated osteocyte-like cells alter the expression of genes associated with both osteoclastogenesis and osteoblastogenesis. We show that osteocyte-like cells directly exposed to 47°C for 1 minute alter the expression of osteogenic genes, whereby the RANKL/OPG ratio is consistently down-regulated, at days 1 and 7 and the pro-osteoblastogenic signaling marker COX2 is significantly up-regulated by day 7. Furthermore, secreted factors from heat-treated MLO-Y4s administered to MSCs using a novel co-culture system are shown to activate MSCs to increase production of the pro-osteoblastic differentiation marker, ALP (day 7, 14), and calcium deposition (day 21). As such, the heat-treated MLO-Y4s produce signals to initiate osteoblastic differentiation. Most interestingly, a different response was demonstrated in nearby non-heat-treated MLO-Y4s (ccMLO-Y4s), whereby an initial pro-osteoclastogenic signaling response (increase RANKL and RANKL/OPG ratio at day 1) followed by later stage pro-osteoblastogenic signaling (down-regulation in RANKL and the RANKL/OPG ratio and an up-regulation in OPG and COX2 by day 7) is observed. Taken together, these results indicate that heat-treated osteocytes can elicit a remodeling response by signaling nearby MLO-Y4s, which in turn signal to activate pre-osteoclasts and pre-osteoblasts, as well as directly signaling to initiate nearby pre-osteoblastic MSCs to undergo osteogenic differentiation.

**Significance:** We elucidate the effect of thermally induced osteocyte apoptosis to the remodeling responses in vitro, and as such provide a novel insight into the mechanisms by which bone cells detect and respond to thermal elevations associated with orthopedic procedures, initiating the healing process.
Figure: The effect of heat-treatment on RANKL/OPG and COX2 gene expression by heat-treated (A) and co-cultured (B) MLO-Y4s. Alkaline phosphatase activity and calcium deposition of MSCs co-cultured with heat treated osteocytes (C). *p<0.05.