Hyberbaric Oxygen Therapy Suppresses Osteoclast Formation and Bone Resorption

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ABSTRACT: The cellular and molecular mechanism through which hyperbaric oxygen therapy (HBO) improves osteonecrosis (ON) is unclear. The present study therefore examined the effect of HBO pressure and hyperoxia on RANKL-induced osteoclast formation in RAW 264.7 cells and human peripheral blood monocytes (PBMC). Daily exposure to HBO (2.4 ATA, 97% O2, 90 min), hyperbaric pressure (2.4 ATA, 8.8% O2, 90 min) or normobaric hyperoxia (1 ATA, 95% O2, 90 min) significantly decreased RANKL-induced osteoclast formation and bone resorption in normoxic conditions. HBO had a more pronounced anti-osteoclastic effect than hyperoxia or pressure alone and also directly inhibited osteoclast formation and resorption in hypoxic conditions a hallmark of many osteolytic skeletal disorders. The suppressive action of HBO was at least in part mediated through a reduction in RANK, NFATc1, and Dc-STAMP expression and inhibition of hypoxia-induced HIF-1α mRNA and protein expression. This data provides mechanistic evidence supporting the use of HBO as an adjunctive therapy to prevent osteoclast formation and bone loss associated with low oxygen partial pressure.


Keywords: osteoclast; hyperbaric oxygen therapy; osteonecrosis; hypoxia

In healthy bone oxygen levels range from 6% to 9% but this falls to 1–3% within necrotic bone and hypoxia is a hallmark of many skeletal disorders associated with excessive osteoclast formation and bone resorption. Hyperbaric oxygen therapy (HBO) is often used as an adjunctive treatment to improve patient outcomes in necrotic skeletal disorders such as osteomyelitis and osteonecrosis (ON) of the jaw. However, at present there is little mechanistic data regarding the effect of HBO on osteoclast differentiation and activity and it is unclear if HBO is more effective than elevated oxygen (hyperoxia) or pressure alone. Similar to the molecular action of HBO on osteoclast differentiation has also yet to be determined. To address these questions the current study evaluated the effect of HBO, hyperoxia and elevated pressure on RANKL-induced osteoclast differentiation and bone resorption from RAW264.7 and human peripheral blood mononuclear cells (PBMC) in normoxic and hypoxic conditions.

MATERIALS AND METHODS

Cell Culture

RAW 264.7 monocytic cells (ATCC, London, UK) were cultured in DMEM supplemented with 10% fetal calf serum, 100 mg/ml streptomycin, 100 mg/ml penicillin, and 100 mg/ml L-glutamine (Invitrogen, Oxford, UK). Peripheral blood was obtained from healthy volunteers by venipuncture using heparin to prevent coagulation in accordance with preapproved national ethical guidelines. Blood was diluted 1:1 in a nutrient free aMEM blood suspension over 25 ml of Histopaque-1077 (Sigma-Aldrich, Dorset, UK). For induction of osteoclast formation RAW 264.7 cells were cultured at 5 × 10⁴ cells/well and treated with RANKL (30 ng/ml), while 1 × 10⁴ PBMCs/well were cultured with 50 ng/ml M-CSF and 30 ng/ml RANKL. For the assessment of bone resorption cells were cultured on slices of devitalized bovine cortical bone.

For hypoxia (2% O2) and normoxia (21% O2) treatments cells were incubated in airtight chambers, flushed with appropriate gas mixtures for 90 min and then sealed and incubated at 37°C. Chambers were re-gassed daily as above with appropriate O2 concentrations. Cells were exposed to HBO (97.9% O2, 2.1% CO2, 2.4 ATA), pressure (2.4 ATA, 8.8% O2, 2.1% CO2, and 89.1% N2) and hyperoxia (95% O2, 5% CO2, 1 ATA) in stainless steel hyperbaric chambers. Chambers were flushed for 4 min with relevant gas mixtures and then pressurized to 2.4 atmosphere absolute (ATA) over 2 min. Cultures were exposed to HBO, pressure or hyperoxia for 90 min to replicate the duration of treatment received by hyperbaric therapy patients.

RAW 264.7 experiments were stopped after 4 days to assess for osteoclast differentiation by staining for tartrate resistant acid phosphatase (TRAP) and 8 days for resorption. qPCR experiments were stopped after 12 days for TRAP staining and 20 days for assessment of resorption.

Quantitative RT-PCR

qPCR was used to detect gene expression of key regulators of osteoclast differentiation using the ∆∆CT methodology. Total RNA was isolated using a Sigma GeneRuler RNA isolation kit and cDNA produced using the ImPromII Reverse Transcription System (Promega, Southampton, UK). qPCR was performed on a StepOne PCR system (Applied Biosystems, Paisley, UK) using SYBR green for detection of PCR product. The forward and reverse primer sets used were as follows: β-actin GCGCGGGCTACAGCATCCA/TGGCGGTCAGGCACTGTGA; RANK: GGGTTGGTCTGTGACGGCCGCAG-TCTGCCACCTCCAGGGGT; NFATc1: GTGTCTGGCATCTCGACTGTCC; Dc-STAMP: GTTGGGCCGCCCTGAGCGCATGGGCACGTCTG; HIF1-α: CAGAATGCGCCATTGAGGCTTG; RANKL: ACGCCACCTCAGCAGCAGTCCTG. Reaction conditions were 94°C for 2 min,
followed by forty cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Gene expression was normalized to β-actin and expressed relative to the reference control group.

**Western Blotting**
Lysates form PBMC cultures were prepared by washing cells in PBS at room temperature then incubating in a buffer consisting of 0.1% triton X-100, tris-glycine pH 10.3, 2.5 mM MgCl₂, 150 mM NaCl, 1% Na₂PO₄ and protease inhibitor. Protein was separated on 10% SDS–PAGE gel at 200 V for 45 min then transferred to PVDF membranes (GE Healthcare, Uppsala, Sweden). Membranes were blocked in 5% non-fat milk/PBS at room temperature for 1 h, washed three times in PBS 0.05% Tween-20, and then incubated overnight at 4°C with mouse anti HIF1α monoclonal diluted 1:1,000 in buffer (Abcam, Cambridge, UK). Blots were washed three times in PBS 0.05% Tween-20 and incubated with an anti-mouse HRP conjugated secondary antibody diluted according to manufacturer’s instructions for 45 min at room temperature. Blots were washed three times in PBS 0.05% Tween-20 and incubated in ECL solution (Millipore, Abingdon, UK). Blots were stripped and re-probed for GAPDH (Abcam). Densitometry was performed using Image J analysis software (NCBI, Bethesda, MD). Results were normalized to GAPDH levels and expressed as the optical density relative to normoxia.

**Statistical Analysis**
Statistical analysis was performed using one-way ANOVA for comparison between groups. All results are expressed as mean ± SEM and significance was considered at \( p < 0.05 \). The analysis was performed using Statview statistical software (Abacus Concepts, Berkeley, CA).

**RESULTS**
RANKL directly stimulated the formation of TRAP positive osteoclast within 4 days in normoxic conditions, which similar to previous findings readily formed resorption pits on bone slices (Figs. 1 and 2). Exposure to HBO, pressure or hyperoxia for 90 min daily significantly reduced the number of TRAP positive osteoclasts and bone resorption in normoxic conditions, with HBO and pressure having a greater effect compared to hyperoxia (Fig. 1). Osteoclast formation and resorption in HBO, hyperoxia and pressure treated cultures was 0.62/0.36, 0.73/0.38 and 0.44/0.44 of normoxic control (Fig. 1). Total cell number was not affected by any treatment (Table 1).

To determine the response of human cells PBMC were exposed for 90 min daily to HBO, hyperoxia or pressure. HBO and hyperoxia significantly reduced the number of RANKL-induced TRAP positive mononuclear and multinuclear osteoclast forming under normoxic conditions (Fig. 3). HBO had a significantly greater inhibitory action on osteoclast differentiation than hyperoxia or pressure alone, with pressure having no effect. Mononuclear and multinuclear osteoclast formation was 0.27/0.40, 0.46/0.75 and 1.12/1.12 of control in HBO, hyperoxia and pressure treated cultures. RANKL-induced bone resorption was also significantly decreased by HBO and hyperoxia, with HBO having a more pronounced effect than pressure

**Figure 1.** Daily exposure to HBO, hyperoxia or pressure inhibits RANKL-induced osteoclast formation and bone resorption. RAW264.7 cells were treated with RANKL (30 ng/ml) and incubated in normoxic conditions (21% O₂, 1 ATA) or normoxic conditions with 90 min/day exposure to HBO (97.9% O₂, 2.4 ATA), hyperoxia (95% O₂, 1 ATA) or elevated pressure (8.8% O₂, 2.4 ATA). Results are expressed as the mean ± SEM of three separate experiments each consisting of eight replicate wells per group. (A) Number of TRAP positive cells per well, (B) percentage of bone surface displaying resorption pits. *\( p < 0.05 \) versus normoxia.

**Figure 2.** Images of TRAP stained cultures from normoxia (A and B) and HBO (C and D) treated cultures at magnifications of 100× and 400×. Image of representative resorption pits taken by reflected light microscopy at a magnification of 200× from a normoxic culture. Resorption pits appear as darkly stained, clearly margined areas.
or hyperoxia, whereas pressure had no significant effect on osteoclast activity. While osteoclast number was reduced total cell number was significantly increased by HBO and hyperoxia indicating that this anti-osteoclastic effect was not secondary to a general cytotoxic effect (Table 1).

Necrotic bone is poorly vascularized and as a consequence hypoxic. Hypoxia augments osteoclast formation and HBO is often used as an adjunct to debridement of necrotic bone; with patients receiving HBO for 90 min/day for 30 days before and 10 days after surgery. However the effect of HBO on osteoclast formation in hypoxic conditions has not been studied. We therefore cultured PBMC in hypoxic conditions (2% O2) and exposed them daily to HBO, hyperoxia or elevated pressure for 90 min. Hypoxia significantly augmented RANKL-induced osteoclast formation in PBMC cultures (Fig. 4). Exposure to HBO, hyperoxia or pressure for 90 min prevented the augmentative action of hypoxia on mononuclear and multinuclear osteoclast formation. However, HBO had a significantly greater effect than hyperoxia or pressure alone (Fig. 4). No significant change in total cell number was observed (Table 1).

RANKL-induced osteoclast formation is dependent on a complex network of intracellular signals that promote expression of genes typical of mature osteoclast. RANKL binds to its specific receptor RANK activating downstream signaling components that stimulate nuclear translocation of key osteoclastic transcription factors such as NFATc1, DC-STAMP, and c-fos. Therefore to determine the potential molecular mechanism mediating the suppressive action of HBO we examined RANK, DC-STAMP, and NFATc1 mRNA expression using real time quantitative PCR. Hypoxia stimulated a significant 5.57-fold increase in RANK expression in developing osteoclast, which was prevented by daily HBO (Table 2). Hypoxia also significantly augmented RANKL-induced DC-STAMP and NFATc1 mRNA expression compared to normoxic conditions (Table 3) and this was abolished by HBO. In addition, HBO significantly reduced NFATc1 and DC-STAMP expression below those measured under normoxic conditions.

The enhanced rate of osteoclast formation seen in hypoxic conditions has been attributed at least in part

Table 1. The Effect of Treatments on Total Cell Number

<table>
<thead>
<tr>
<th></th>
<th>RAW 264.7 Cultures</th>
<th>PBMC Cultures</th>
<th>Hypoxic PBMC Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>5,867 ± 390</td>
<td>20,285 ± 2,950</td>
<td>36,867 ± 8,062</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>HBO</td>
<td>7,093 ± 495</td>
<td>34,352 ± 6,741</td>
<td>38,451 ± 8,166</td>
</tr>
<tr>
<td>Hyperoxia</td>
<td>5,538 ± 448</td>
<td>29,561 ± 3,914</td>
<td>31,060 ± 4,633</td>
</tr>
<tr>
<td>Pressure</td>
<td>5,366 ± 568</td>
<td>18,191 ± 1,848</td>
<td>32,990 ± 5,390</td>
</tr>
</tbody>
</table>

NA, not applicable.
*p < 0.05 versus normoxia.

Figure 3. Daily exposure to HBO and hyperoxic conditions inhibit RANKL-induced osteoclast formation and bone resorption. hPBMC were treated with RANKL (30 ng/ml) and M-CSF (30 ng/ml) and incubated continuously in normoxic conditions (21% O2, 1 ATA) or normoxic conditions with a 90-min daily exposure to HBO (97.9% oxygen, 2.4 ATA), hyperoxia (95% oxygen, 1 ATA) or elevated pressure (8.8% oxygen, 2.4 ATA). Results expressed as the mean ± SEM of three repeat experiments each consisting of eight replicate wells. (A) TRAP positive mononuclear cells per well, TRAP positive multinuclear cells per well, percentage of bone surface displaying resorption pits.
*p < 0.05 versus all groups, *p < 0.05 versus normoxia. Images of resorption pits taken by reflected light microscopy from normoxic cultures (B) and HBO treated cultures (C) at 200× magnification.
to changes in HIF expression. Therefore to assess the possibility that the anti-osteoclastic effect of HBO was mediated through an action on HIF expression the effect of HBO on HIF-1α mRNA and protein expression was examined. Hypoxia induced a significant 1.33-fold increase in HIF-1α mRNA and 1.5-fold increase in HIF-1α protein expression (Table 3). This increase was abolished by daily exposure to HBO, hyperoxia or pressure. The suppressive action of HBO was greater than that of hyperoxia, which in turn was significantly greater than that of pressure alone (Table 3).

### DISCUSSION

HBO is employed as an adjunctive therapy in several conditions including refractory osteomyelitis and ON. Previous studies primarily focused on the effect of HBO on osteoblast activity where it was shown to have a positive effect on differentiation, bone nodule formation, and calvarial and mandibular bone regeneration. Excessive resorption is also seen in necrotic bone, but the effect of HBO on osteoclast function is poorly documented. The only previous study examining osteoclastic effects of HBO noted a decrease in 45Ca release from calvarial organ cultures. Organ cultures are heterogeneous in nature so it is unclear if this represents a direct inhibitory effect of HBO on resorption or an indirect action mediated through osteoblasts. Data from the current study using homogenous RAW264.7 cultures suggest that osteoclast precursors are directly sensitive to HBO although this does not preclude additional actions in mixed cell populations.

While there is limited data on HBO and osteoclastogenesis the importance of oxygen in osteoclast function is more widely appreciated. The partial pressure of oxygen in healthy bone is similar to that measured in other tissues (6–9%) but in diseased or necrotic bone partial pressures of 0.5–4% have been recorded. Hypoxia enhances osteoclast differentiation through a direct effect on monocytic precursors and indirectly by promoting the release of cytokines such as VEGF, IGF, and IL-6 that support osteoclast formation. Hypoxia also augmented osteoclast formation in our studies and this was prevented by daily HBO

### Table 2. HBO Suppresses the Stimulatory Effect of Hypoxia on RANKL-Induced NFATc1 and DC-STAMP mRNA Expression in Osteoclast Derived From Human Peripheral Blood Monocytes

<table>
<thead>
<tr>
<th></th>
<th>NFATc1</th>
<th>DC-STAMP</th>
<th>RANK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>197.7 ± 63.7*</td>
<td>19.3 ± 9.7*</td>
<td>5.57 ± 1.7*</td>
</tr>
<tr>
<td>HBO</td>
<td>0.77 ± 0.15*</td>
<td>0.53 ± 0.16*</td>
<td>1.10 ± 0.4</td>
</tr>
</tbody>
</table>

Values are expressed as RQ relative to normoxic conditions using △△CT, all group’s expression are normalised to β-actin. Values are the mean ± SEM of three experiments.*p < 0.05 versus all other groups.

### Table 3. Hypoxia Elevates Whereas HBO Suppresses HIF-1α mRNA and Protein Expression in Osteoclast Derived From Human Peripheral Blood Monocytes

<table>
<thead>
<tr>
<th></th>
<th>HIF-1α mRNA</th>
<th>HIF 1α Relative Protein Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>1.33 ± 0.01*</td>
<td>1.50 ± 0.33*</td>
</tr>
<tr>
<td>Hyperoxia</td>
<td>0.35 ± 0.08*</td>
<td>0.75 ± 0.12*</td>
</tr>
<tr>
<td>Pressure</td>
<td>0.76 ± 0.28*</td>
<td>0.88 ± 0.13*</td>
</tr>
<tr>
<td>HBO</td>
<td>0.14 ± 0.04*</td>
<td>0.59 ± 0.10*</td>
</tr>
</tbody>
</table>

mRNA values are expressed as RQ relative to normoxic conditions using △△CT, all group’s expression are normalised to β-actin. Protein expression values expressed relative to the optical density of normoxia bands, all group’s values normalised to GAPDH expression. Values are the mean ± SEM of three experiments.*p < 0.05 versus normoxia.##p < 0.05 versus hypoxia.
exposure. This is in keeping with Muzyk and Arnett’s data but contrasts somewhat with Knowles and Leger where hypoxia or continuous HIF-1α expression reduced osteoclast number. The reason for this discrepancy is unclear but may relate to differences in culture methods employed. Our cultures required daily re-gassing, during which short periods of higher O₂ partial pressures may have occurred, whereas Knowles’s cultures were maintained at a constant 2% O₂. However the physiological relevance of a suppressive effect of 2% O₂ is uncertain as increased osteoclast formation and resorption are noted in ischemic models of necrosis and bone resorption is a characteristic of metastatic breast tumors in spite of the low partial pressure within and adjacent to tumors. Oxygen levels also fluctuate during ischemic injury due to the initiation of angiogenesis and other reparative mechanisms and it is therefore possible that transient changes in oxygen partial pressure, as seen during our studies, may represent a more realistic model. Whatever the answer it is clear that HBO suppressed RANKL-induced osteoclast formation and bone resorption in the current study. Our results may also potentially underestimate the inhibitory effect of HBO as the normoxic O₂ partial pressure (21%) is already elevated compared to likely oxygen concentrations in bone (6–9%) and this may therefore partly obscure the true suppressive action of HBO and hyperoxia.

While HBO consistently suppressed osteoclastogenesis human and murine precursors displayed different responses to pressure under normoxic conditions. Similar to Rubin’s studies using mouse marrow cultures elevated pressure had an anti-osteoclastic effect on RAW264.7 cells but had little effect on RANKL-induced osteoclast formation in human PBMC. This may reflect a species difference or could have arisen due to an in-direct action of pressure on lymphocytes present within PBMC cultures. HBO has been shown to induce lymphocyte apoptosis and reduce TNF-α production in human blood cells whereas elevated pressure alone has no effect and as TNF-α augments RANKL-induced osteoclast formation the continued presence of TNF-α producing lymphocytes within pressure treated cultures would be expected to provide a greater stimulus for osteoclast differentiation than in HBO treated cultures. Furthermore despite the increase in osteoclast number in human cultures there was no corresponding increase in pit formation suggesting that pressure may inhibit mature osteoclast resorptive activity.

To assess the mechanism mediating the pro and anti-osteoclastic effect of hypoxia and HBO the expression of key mediators of RANKL-induced osteoclast differentiation was examined. RANKL binds to its membrane bound receptor RANK on monocytic precursors, which via TRAF 2, 3 and 6 activates downstream transcription factors such as NFATc1 that control osteoclastic gene expression. Culturing PBMC in hypoxic conditions significantly elevated RANK expression and HBO prevented this increase, which may in part mediate the augmentative effect of hypoxia and protective effect of HBO on osteoclast differentiation. This is in keeping with the recent studies of Tang which suggest that hypoxia-induced RANK expression in breast cancer cells contributes to metastatic osteolysis, similarly SNPs in RANK are associated with an increased risk of developing bisphosphonate-induced ON suggesting a central role for aberrant RANK signaling in ischemic bone loss. However this is not the only mechanism by which HBO acts as it also suppressed osteoclast formation in normoxic conditions when RANK was not elevated. It is likely that suppression of NFATc1 expression or activity also contributes to the anti-osteoclastic action. This assertion is strengthened by the significant decrease in NFATc1 and NFAT dependent DC-STAMP mRNA expression in HBO treated cultures. The mechanism through which HBO suppresses NFATc1 expression may relate to an effect on upstream transcription factors. The most likely of these candidates is NF-kb as its promoter contains oxygen responsive elements and NF-kb expression is acutely sensitive to changes in oxygen, with hypoxia elevating and HBO suppressing levels and activity. In contrast it is unlikely that c-fos represents the primary target for HBO as the c-fos promoter lacks oxygen responsive elements.

The ability of osteoclast to sense changes in oxygen is dependent on the HIF pathway which regulates the cellular response to hypoxia in many tissues. Under normoxic conditions HIF is rapidly degraded following hydroxylation of conserved proline residues by PHD. These hydroxylated residues are targeted by the ubiquitin ligase VHL. During ischemia PHD activity is suppressed allowing the accumulation of HIF heterodimers that drive expression of oxygen sensitive genes such as NF-kb. Hypoxia increases osteoclast HIF-1α activity leading to the expression of hypoxic responsive genes such as VEGF and osteoclast lacking HIF-1α do not respond to changes in oxygen partial pressure whereas inducers of HIF-1α stimulate resorption. In keeping with this hypoxia increased HIF-1α expression in human PBMC in our studies, which was reversed by HBO with HIF-1α mRNA and protein expression falling markedly below that of normoxic controls. This was a more marked suppressive effect than that induced by hypoxia which also reduced HIF expression but not below that of normoxic controls. This suggests that at least in part the anti-osteoclastic action of HBO is mediated through a reduction in HIF-1α activity, which would be expected to decrease expression of hypoxic responsive genes such as NF-kb and thereby limit NFATc1 expression and osteoclast formation.

The data from this study shows that HBO has a direct suppressive effect on osteoclast differentiation and activity in normoxic and hypoxic conditions. This would appear to be associated with a reduced response...
to RANKL secondary to changes in HIF, RANK, and NFATc1 expression. Thus, the beneficial effect of adjuncive HBO on necrotic bone may occur in part due to a reduction in aberrant osteoclast activity. This provides evidence supporting the use of HBO as an adjuncive therapy to prevent osteoclast formation in a range of skeletal disorders associated with hypoxia.

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REFERENCES


