Hyperbaric Oxygen Therapy Suppresses Osteoclast Formation and Bone Resorption

Hadil Al Hadi,[1] Gary R. Smerdon,[2] Simon W. Fox[1]

[1]School of Biomedical and Biological Sciences, Plymouth University, Drake Circus, Plymouth, Devon, PL4 8AA, UK, [2]Diving Diseases Research Centre, Plymouth, Devon, PL6 8BU, UK

Received 8 March 2013; accepted 25 June 2013
Published online 22 July 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.22443

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% O_2, 90 min), hyperbaric pressure (2.4 ATA, 8.8% O_2, 90 min) or normobaric hyperoxia (1 ATA, 95% O_2, 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. © 2013 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 31:1839–1844, 2013

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[1] and hypoxia is a hallmark of many skeletal disorders associated with excessive osteoclast formation and bone resorption.[2,3] 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.[4] Similarly 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 un-supplemented media (α-MEM) and PBMC were isolated by centrifuging 15 ml of αMEM blood suspension over 25 ml of Histopaque-1077 (Sigma-Aldrich, Dorset, UK). For induction of osteoclast formation RAW 264.7 cells were cultured at 5×10^4 cells/well and treated with RANKL (30 ng/ml), while 1×10^4 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% O_2) and normoxia (21% O_2) 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 O_2 concentrations. Cells were exposed to HBO (97.9% O_2, 2.1% CO_2, 2.4 ATA), pressure (2.4 ATA, 8.8% O_2, 2.1% CO_2, and 89.1% N_2) and hyperoxia (95% O_2, 5% CO_2, 1 ATA) in stainless steel hyperbaric chambers. Chambers were flushed for 4 min with relevant gas mixes 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)[5] and 8 days for resorption, which was assessed as described previously.[6] PBMC 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 $\triangle\triangle C_T$ methodology. Total RNA was isolated using a Sigma Genelute 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 GCGCGGCTACAGCTTCA/TGGCCGTCAGGCAGCTCGTA; RANK: GGTGGTGTCTGTCAGGGCACG/TCTCCCCCACCTCCAGGGGT; Dc-STAMP: GTTGGCTGCCCTGCACCGAT/TCCCTCATCCTGGGGCTGCC; NFATc1: GGTCTCGAACACTCGCTCTGCC/GCAGTCGGAGACTCGTCCCTGC; HIF1-α: CAGAAATGGCCTTGT/CAGGCTGTGTCGACTGAG. Reaction conditions were 94°C for 2 min,

Grant sponsor: Iraqi Government and the Diving Diseases Research Centre.
*Correspondence to: Simon W. Fox (T: +44-1752-584625; F: +44-1752-584605; E-mail: simon.fox@plymouth.ac.uk)

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 $MgCl_2$, 150 mM $NaCl_2$, 1% Na_2PO_4 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% nonfat 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 0.44/044 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

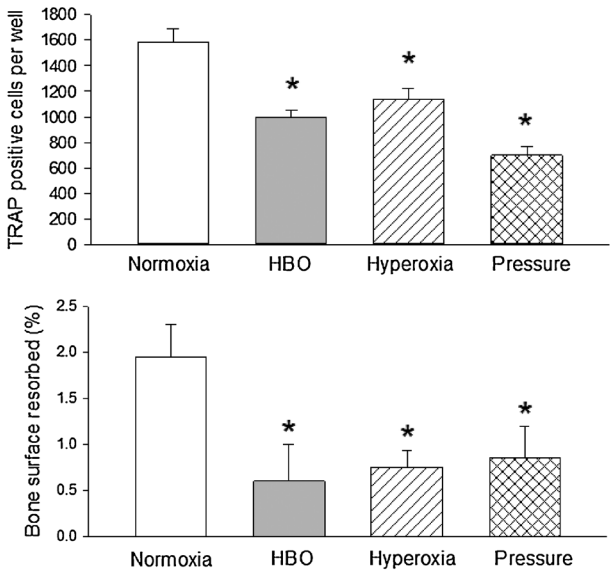

Figure 1. Daily exposure to HBO, hyperoxia or pressure inhibits RANKL-induced osteoclast formation and bone resorption. RAW264.7 cells were treated with RANKL (30 ng/ml) and incubated in normoxic conditions (21% O_2, 1 ATA) or normoxic conditions with 90 min/day exposure to HBO (97.9% O_2, 2.4 ATA), hyperoxia (95% O_2, 1 ATA) or elevated pressure (8.8% O_2, 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.

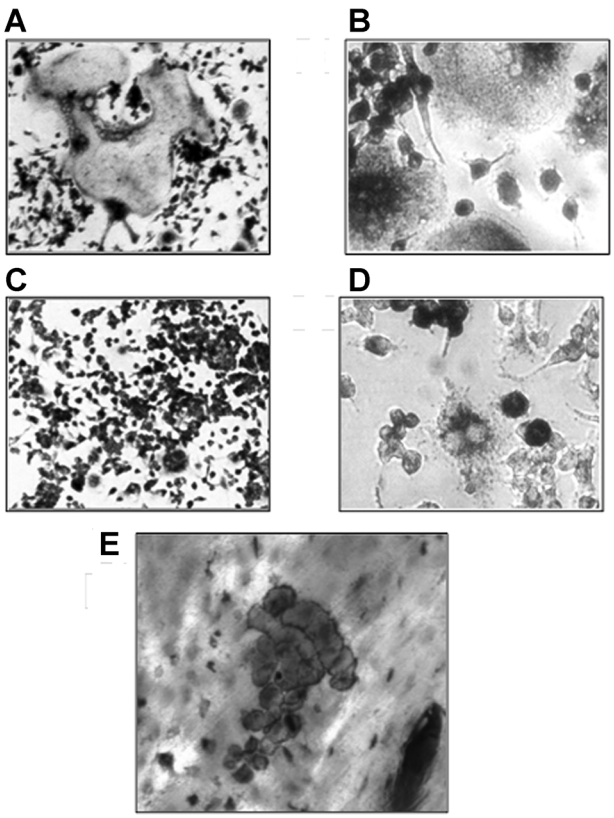

Figure 2. Images of TRAP stained cultures from normoxia (A and B) and HBO (C and D) treated cultures at magnifications of 100× and 400×. Image of representative resorption pits taken by reflected light microscopy at a magnification of 200× from a normoxic culture. Resorption pits appear as darkly stained, clearly marginated areas.

Table 1. The Effect of Treatments on Total Cell Number

	Total Cells Per Well RAW 264.7 Cultures	Total Cells Per Well PBMC Cultures	Total Cells Per Well Hypoxic PBMC Cultures
Normoxia	5,867 ± 390	20,285 ± 2,950	36,867 ± 8,062
Hypoxia	NA	NA	30,478 ± 2,765
HBO	7,093 ± 495	34,352 ± 6,741*	38,451 ± 8,166
Hyperoxia	5,538 ± 448	29,561 ± 3,914*	31,060 ± 4,633
Pressure	5,366 ± 568	18,191 ± 1,848	32,990 ± 5,390

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

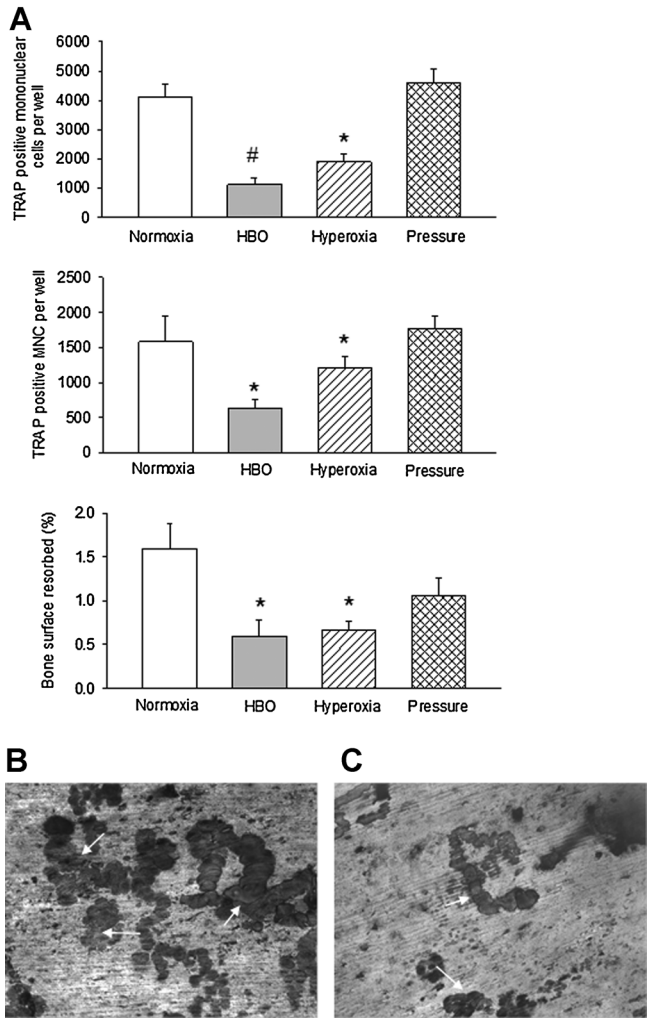

Figure 3. Daily exposure to HBO and hyperoxic conditions inhibit RANKL-induced osteoclast formation and bone resorption. hPBMC were treated with RANKL (30 ng/ml) and M-CSF (30 ng/ml) and incubated continuously in normoxic conditions (21% O_2, 1 ATA) or normoxic conditions with a 90-min daily exposure 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.

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% O_2) 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

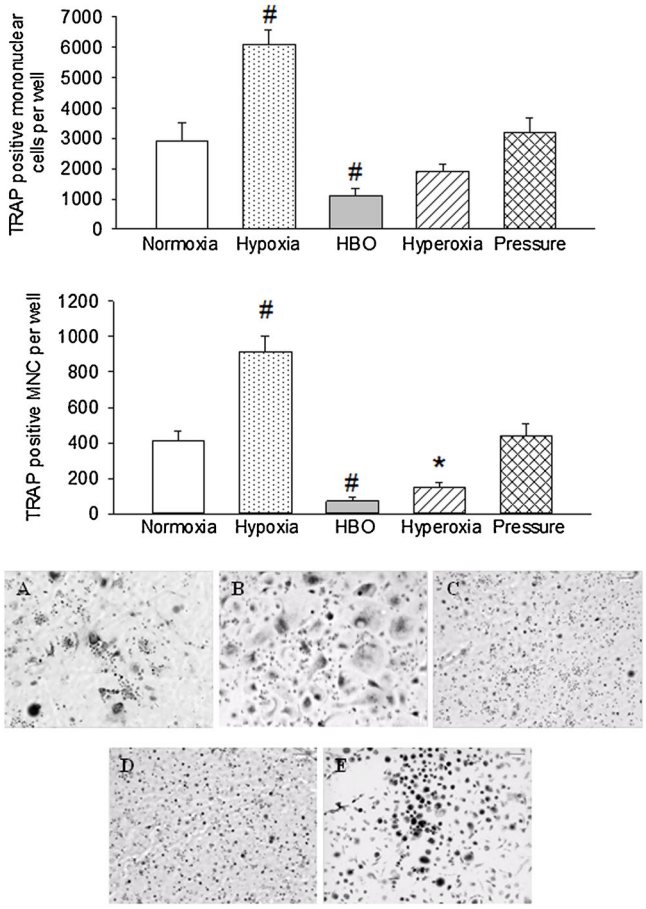

Figure 4. HBO has a greater suppressive effect on hypoxia-induced osteoclast formation than hyperoxia or pressure alone. PBMC were treated with RANKL (30 ng/ml) and M-CSF (30 ng/ml) and incubated continuously in hypoxic conditions (2% O_2, 1 ATA) or hypoxic conditions with a 90-min daily exposure to HBO (97.9% oxygen, 2.4 ATA), hyperoxia (95% oxygen, 1 ATA) or elevated pressure (8.8% oxygen, 2.4 ATA). $^{\#}p < 0.05$ versus all groups, $^{*}p < 0.05$ versus normoxia. Images of TRAP stained cultures (A) normoxia, (B) hypoxia, (C) HBO, hyperoxia, and (D) pressure.

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

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

	NFATc1	DC-STAMP	RANK
Normoxia	1	1	1
Hypoxia	$197.7 \pm 63.7^{\#}$	$19.3 \pm 9.7^{\#}$	$5.57 \pm 1.7^{\#}$
HBO	$0.77 \pm 0.15^{\#}$	$0.53 \pm 0.16^{\#}$	1.10 ± 0.4

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

	HIF-1α mRNA	HIF 1α Relative Protein Expression
Normoxia	1	1
Hypoxia	$1.33 \pm 0.01^{\#}$	$1.50 \pm 0.33^{\#}$
Hyperoxia	$0.35 \pm 0.08^{\#,*}$	$0.75 \pm 0.12^{*}$
Pressure	$0.76 \pm 0.28^{*}$	$0.88 \pm 0.13^{*}$
HBO	$0.14 \pm 0.04^{\#,*}$	$0.59 \pm 0.10^{\#,*}$

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.

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.[7] 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.[8–12] 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 ^{45}Ca release from calvarial organ cultures.[4] 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.[1] Hypoxia enhances osteoclast differentiation through a direct effect on monocytic precursors[13,14] and indirectly by promoting the release of cytokines such as VEGF, IGF, and IL-6 that support osteoclast formation.[15–18] Hypoxia also augmented osteoclast formation in our studies and this was prevented by daily HBO

exposure. This is in keeping with Muzylak and Arnett's data[15,19] but contrasts somewhat with Knowles and Leger where hypoxia or continuous HIF-1α expression reduced osteoclast number.[20,21] 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 O_2 partial pressures may have occurred, whereas Knowles's cultures were maintained at a constant 2% O_2. However the physiological relevance of a suppressive effect of 2% O_2 is uncertain as increased osteoclast formation and resorption are noted in ischemic models of necrosis[22,23] and bone resorption is a characteristic of metastatic breast tumors in spite of the low partial pressure within and adjacent to tumors.[2] 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 O_2 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 (95%).

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[24] 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,[25,26] 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,[27] similarly SNPs in RANK are associated with an increased risk of developing bisphosphonate-induced ON,[28] 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-κb as its promoter contains oxygen responsive elements and NF-κb expression is acutely sensitive to changes in oxygen, with hypoxia elevating and HBO suppressing levels and activity.[29,30] 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 hetrodimers that drive expression of oxygen sensitive genes such as NF-κb. Hypoxia increases osteoclast HIF-1α activity leading to the expression of hypoxic responsive genes such as VEGF,[17] osteoclast lacking HIF-1α do not respond to changes in oxygen partial pressure[13,17] whereas inducers of HIF-1α stimulate resorption.[31] 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 hyperoxia 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 adjunctive 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 adjunctive therapy to prevent osteoclast formation in a range of skeletal disorders associated with hypoxia.

ACKNOWLEDGMENTS

The authors would like to thank the clinical staff at the DDRC for the collection of blood samples. This work was jointly funded by the Iraqi Government and the Diving Diseases Research Centre. Bovine bone slices were a kind gift of Dr Karen Fuller.

REFERENCES

1. Maurer P, Meyer L, Eckert AW, et al. 2006. Measurement of oxygen partial pressure in the mandibular bone using a polarographic fine needle probe. Int J Oral Maxillofac Surg 35:231–236.
2. Hiraga T, Kizaka-Kondoh S, Hirota K, et al. 2007. Hypoxia and hypoxia-inducible factor-1 expression enhance osteolytic bone metastases of breast cancer. Cancer Res 67:4157–4163.
3. Kurowska-Stolarska M, Distler JHW, Jüngel A, et al. 2009. Inhibitor of DNA binding/differentiation 2 induced by hypoxia promotes synovial fibroblast–dependent osteoclastogenesis. Arthritis Rheum 60:3663–3675.
4. Gray D, Hamblen D. 1976. The effects of hyperoxia upon bone in organ culture. Clin Orthop Relat Res 119:225–230.
5. Burstone MS. 1958. Histochemical demonstration of acid phosphatases with naphthol AS-phosphates. J Natl Cancer I 21:523–539.
6. Karieb S, Fox SW. 2011. Phytoestrogens directly inhibit TNF-alpha-induced bone resorption in RAW264.7 cells by suppressing c-fos-induced NFATc1 expression. J Cell Biochem 112:476–487.
7. Vescovi P, Nammour S. 2010. Bisphosphonate-related osteonecrosis of the Jaw (BRONJ) therapy. A critical review. Minerva Stomatol 59:181–203.
8. Jan AMA, Sándor GKB, Iera D, et al. 2006. Hyperbaric oxygen results in an increase in rabbit calvarial critical sized defects. Oral Surg Oral Med Oral Pathol Oral Radiol Endodontol 101:144–149.
9. Muhonen A, Haaparanta M, Grönroos T, et al. 2004. Osteoblastic activity and neoangiogenesis in distracted bone of irradiated rabbit mandible with or without hyperbaric oxygen treatment. Int J Oral Maxillofac Surg 33:173–178.
10. Wong AK, Schönmeyr BH, Soares MA, et al. 2008. Hyperbaric oxygen inhibits growth but not differentiation of normal and irradiated osteoblasts. J Craniofac Surg 19:757–765. doi: 710.1097/SCS. 1090b1013e31816aac31819
11. Hsieh C-P, Chiou Y-L, Lin C-Y. 2010. Hyperbaric oxygen-stimulated proliferation and growth of osteoblasts may be mediated through the FGF-2/MEK/ERK 1/2/NF-κB and PKC/JNK pathways. Connect Tissue Res 51:497–509.
12. Wu D, Malda J, Crawford R, Xiao Y. 2007. Effects of hyperbaric oxygen on proliferation and differentiation of osteoblasts from human alveolar bone. Connect Tissue Res 48:206–213.
13. Zhao Y, Chen G, Zhang W, et al. 2012. Autophagy regulates hypoxia-induced osteoclastogenesis through the HIF-1α/BNIP3 signaling pathway. J Cell Physiol 227:639–648.
14. Srinivasan S, Avadhani NG. 2007. Hypoxia-mediated mitochondrial stress in RAW264.7 cells induces osteoclast-like TRAP-positive cells. Ann NY Acad Sci 1117:51–61.
15. Arnett TR, Gibbons DC, Utting JC, et al. 2003. Hypoxia is a major stimulator of osteoclast formation and bone resorption. J Cell Physiol 196:2–8.
16. Fukuoka H, Aoyama M, Miyazawa K, et al. 2005. Hypoxic stress enhances osteoclast differentiation via increasing IGF2 production by non-osteoclastic cells. Biochem Biophys Res Commun 328:885–894.
17. Knowles HJ, Athanasou NA. 2008. Hypoxia-inducible factor is expressed in giant cell tumour of bone and mediates paracrine effects of hypoxia on monocyte-osteoclast differentiation via induction of VEGF. J Pathol 215:56–66.
18. Utting JC, Flanagan AM, Brandao-Burch A, et al. 2010. Hypoxia stimulates osteoclast formation from human peripheral blood. Cell Biochem Funct 28:374–380.
19. Muzylak M, Price JS, Horton MA. 2006. Hypoxia induces giant osteoclast formation and extensive bone resorption in the cat. Calcif Tissue Int 79:301–309.
20. Knowles HJ, Athanasou NA. 2009. Acute hypoxia and osteoclast activity: A balance between enhanced resorption and increased apoptosis. J Pathol 218:256–264.
21. Leger A, Altobelli A, Mosquea L, et al. 2010. Inhibition of osteoclastogenesis by prolyl hydroxylase inhibitor dimethyloxallyl glycine. J Bone Miner Metab 28:510–519.
22. Bejar J, Peled E, Boss J. 2005. Vasculature deprivation—Induced osteonecrosis of the rat femoral head as a model for therapeutic trials. Theoret Biol Med Model 2:24.
23. Kim HJ, Chang E-J, Kim H-M, et al. 2006. Antioxidant α-lipoic acid inhibits osteoclast differentiation by reducing nuclear factor-κB DNA binding and prevents in vivo bone resorption induced by receptor activator of nuclear factor-κB ligand and tumor necrosis factor-α. Free Radic Biol Med 40:1483–1493.
24. Rubin J, Biskobing D, Fan X, et al. 1997. Pressure regulates osteoclast formation and MCSF expression in marrow culture. J Cell Physiol 170:81–87.
25. Benson RM, Minter LM, Osborne BA, et al. 2003. Hyperbaric oxygen inhibits stimulus-induced proinflammatory cytokine synthesis by human blood-derived monocyte-macrophages. Clin Exp Immunol 134:57–62.
26. Chen Y-C, Chen S-Y, Ho P-S, et al. 2007. Apoptosis of T-leukemia and B-myeloma cancer cells induced by hyperbaric oxygen increased phosphorylation of p38 MAPK. Leuk Res 31:805–815.
27. Tang Z-N, Zhang F, Tang P, et al. 2011. Hypoxia induces RANK and RANKL expression by activating HIF-1α in breast cancer cells. Biochem Biophys Res Commun 408:411–416.
28. Katz J, Gong Y, Salmasinia D, et al. 2011. Genetic polymorphisms and other risk factors associated with bisphosphonate induced osteonecrosis of the jaw. Int J Oral Maxillofac Surg 40:605–611.
29. Walmsley SR, Print C, Farahi N, et al. 2005. Hypoxia-induced neutrophil survival is mediated by HIF-1α-dependent NF-κB activity. J Exp Med 201:105–115.
30. Yu X, Li YG, He XW, et al. 2009. Hyperbaric oxygen reduces inflammatory response in acute pancreatitis by inhibiting NF-κB activation. Eur Surg Res 42:130–135.
31. Knowles HJ, Cleton-Jansen A-M, Korsching E, et al. 2010. Hypoxia-inducible factor regulates osteoclast-mediated bone resorption: Role of angiopoietin-like 4. FASEB J 24:4648–4659.