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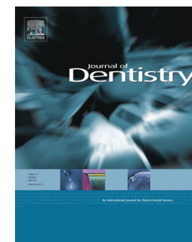

Hyperbaric oxygen therapy accelerates osteoblast differentiation and promotes bone formation

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ABSTRACT

Objectives: Hyperbaric oxygen therapy (HBO) has been used as an adjunctive therapy in the treatment of radiotherapy or bisphosphonate-induced osteonecrosis of the jaw however the effect of HBO on osteoblast formation and mineralisation has not been extensively studied. The current study therefore examined the effects of HBO, elevated pressure or elevated oxygen alone on osteoblast differentiation and bone nodule formation.
Methods: Saos-2 human osteoblast cells were exposed to HBO (2.4 ATA, 97.9% O_2, 90 min per day), elevated pressure alone (2.4 ATA, 8.8% O_2, 90 min per day) or elevated oxygen alone (1 ATA, 95% O_2, 90 min per day) after culturing under normoxic or hypoxic conditions and osteoblast differentiation and bone formation assessed by alkaline phosphatase activity and calcein incorporation. Expression of key regulators of osteoblast differentiation and bone matrix proteins were assessed by quantitative PCR.
Results: Daily exposure to HBO accelerated the rate of osteoblast differentiation as determined by increased alkaline phosphatase activity and expression of type I collagen and Runx-2 mRNA during the early stages of culture. HBO also augmented bone nodule formation in hypoxic conditions. HBO had a more pronounced effect on these key markers of osteoblast differentiation than elevated oxygen or pressure alone.
Conclusions: The data from this study shows that daily HBO treatment accelerated the rate of osteoblast differentiation leading to an increase in bone formation.
Clinical significance: These studies add to our understanding of HBO's reparative action in osteonecrotic bone loss. In addition to stimulating angiogenesis HBO may also improve surgical outcomes through a direct beneficial effect on osteoblast differentiation generating a larger bone mass available for reconstruction.

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1. Introduction

Blood vessel integrity and bone homeostasis are often disrupted in patients receiving high dose bisphosphonates or head and neck radiotherapy. This is associated with the formation of necrotic areas of alveolar bone causing pain and loss of function. Several studies have shown a beneficial effect of hyperbaric oxygen therapy (HBO) on the skeleton[1–5] and HBO has been used to promote healing in osteonecrosis, bone grafts and dental implants.[6–8]

Changes in oxygen partial pressure directly impact on osteoblast function with hypoxia being associated with decreased osteoblast formation and mineralisation *in vitro*.[9]

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HBO rapidly delivers oxygen to areas of ischaemic tissue damage by elevating plasma oxygen concentration.[10] The subsequent increase in oxygen tension is thought to promote tissue regeneration through multiple mechanisms including changes in vascular reactivity, angiogenesis, free radical production, cytokine synthesis and modulation of the immune response.[11] Therefore by promoting capillary proliferation HBO may indirectly help restore osteoblast formation at formerly hypoxic sites in the jaw. In addition to indirectly promoting osteoblast activity it is possible that HBO may also have direct actions on osteoblasts that further enhance HBO's regenerative capacity. However the direct effect of HBO on osteoblast formation and function has not been examined. The aim of this paper is therefore to examine if HBO has a direct effect on markers of osteoblast differentiation and bone nodule formation in normoxic and hypoxic conditions.

2. Media and reagents

2.1. Cell culture

Saos-2 human osteoblast-like cells were obtained from ECACC (Porton Down, UK) (ECACC cat. no.89050205) and cultured in Dulbecco's minimum essential medium supplemented with 10% charcoal stripped foetal calf serum (Autogen Bioclear, UK) 2 mmol/l glutamine, 100 IU/ml benzylpenicillin and 100 mg/ml streptomycin all from Sigma (Poole, Dorset, UK). All incubations were performed at 37 °C in 5% CO_2 or equivalent. Cultures were fed every 2–3 days by replacing half the medium with fresh reagents.

To generate hypoxic (2% O_2) or normoxic (21% O_2) conditions cells were incubated in airtight chambers prepared at the Diving Diseases Research Centre (DDRC, Plymouth, UK). Chambers were flushed with appropriate gas mixtures for 90 min and then sealed. Chambers were re-gassed daily with appropriate O_2 concentrations. Cells were exposed to HBO (97.9% O_2, 2.1% CO_2, 2.4 ATA), elevated pressure alone (2.4 ATA, 8.8% O_2, 2.1% CO_2, and 89.1% N_2) or elevated oxygen alone (95% O_2, 5% CO_2) daily for 90 min to replicate the duration of treatment received by hyperbaric therapy patients. The oxygen and CO_2 concentrations used in the elevated pressure group were designed such that the partial pressures experienced by the cells in this group at 2.4 atmospheres absolute (ATA) were equivalent to 21% O_2 and 5% CO_2 at normal atmospheric pressure.

An overview of the experimental design can be seen in Fig. 1. Exposures were performed in airtight stainless steel culture chambers that were flushed for 4 min with relevant gas mixes and then pressurised to 2.4 ATA over 2 min as needed (Fig. 1). Following treatment cultures were returned to normoxic or hypoxic conditions as necessary.

2.2. Cell proliferation assay

Proliferation was measured using a Cell Titre 96 AQeous non-radioactive cell proliferation assay according to manufacturer's instructions (Promega, UK). Absorbance was measured at 490 nm (Molecular Devices, USA). Standard curves were

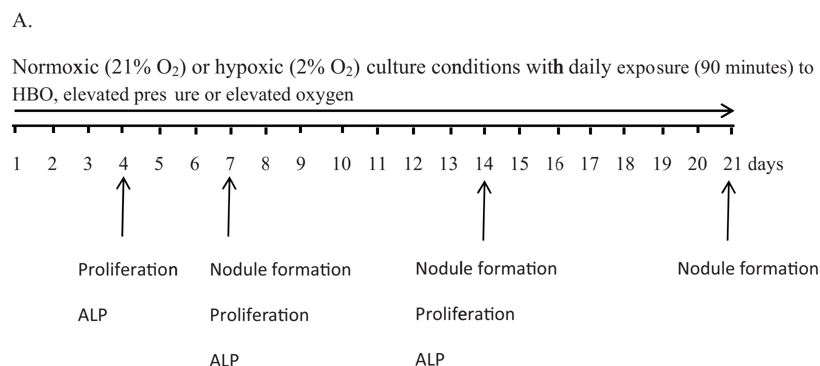

Fig. 1 – Schematic of treatment and assay regimes (A). Image of hyperbaric culture chamber used in HBO exposures (B).

generated from absorbance readings of known cell numbers and these were used to calculate viable cell number in experimental groups.

2.3. Bone nodule formation assay

Bone nodule mineralisation was assessed using a modification of Hale's methodology[12] by measuring calcein incorporation. Saos-2 human osteoblast-like cells were cultured in 96 well plates (5×10^4 cells per well) and treated with beta glycerophosphate (β-GB, 10 mM) and L-ascorbic acid (L-AA, 50 mg/l) to induce osteoblast differentiation and mineralisation. After 7, 14, and 21 days treatment cultures were washed with PBS and incubated in culture medium containing 1 μg/ml calcein for 4 h at 37 °C. Calcein was then removed and cultures washed in PBS four times. The incorporation of calcein into mineralised nodules was then measured with a CytoFluor II fluorescence multi-well plate reader (PerSeptive Biosystems, USA) at 485 nm excitation and 530 nm emission.

2.4. Alkaline phosphatase activity (ALP)

Saos-2 human osteoblast-like cells were cultured in 96 well plates (5×10^4 per well) and treated with β-GB (10 mM) and L-AA (50 mg/l). ALP activity was measured by staining cultures with *p*-nitrophenyl phosphate (1 mg/ml) at 37 °C for 30 min. Absorbance was measured at 405 nm and the results expressed as the amount of ALP required to liberate 1 mmol of p-nitrophenol/min per 10^4 cells.

2.5. Real time quantitative PCR analysis

Quantitative RT-PCR was used to detect osteoblastic gene expression using the $\Delta\Delta C_T$ methodology. SaoS-2 cells (1×10^6 cells per well) were incubated in six well plates while receiving relevant experimental treatments. Total RNA was then isolated using a Sigma GenElute Total RNA isolation kit (Sigma, UK) and used to synthesise cDNA using an ImPromII Reverse Transcription System (Promega, Southampton, UK) according to manufacturer's instructions. Real time PCR was performed on a StepOne PCR system (Applied Biosystems, UK) using the DNA-binding dye SYBR green for detection of PCR product. A total of 2 μl of cDNA was added to a final reaction volume of 25 μl containing 0.05 U/μl Taq, SYBR green and specific primers (0.2 μM) beta-actin F:GCGCGGCTACAGCTTCA, R:TGGCCGTCAGGCAGCTCGTA; Runx-2 F:AGACCCCAGGCAGG-CACAGT, R:GCGCCTAGGCACATCGGTGA, type I collagen F:CCTGGCAGCCCTGGTCCTGA, R:CTTGCCGGGCTCTCCAGCAG. Reaction conditions were 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. PCR amplification was measured by fluorescence emitted from SYBR Green during the extension phase. Gene expression was normalized to β-actin and expressed relative to the reference control group.

2.6. Statistical analysis

Differences between groups were assessed using Fisher's one way analysis of variance (Statview; Abacus Concepts, USA). A difference of $P < 0.05$ was considered statistically significant.

3. Results

3.1. HBO accelerates early stage osteoblast differentiation and prevents the effect of hypoxia

Low oxygen availability in necrotic bone generates a hypoxic environment that influences cellular activity. In this study we generated a hypoxic model by culturing cells in 2% O_2 and treating them daily for 90 min with HBO, elevated pressure or elevated oxygen. Hypoxia decreased osteoblast proliferation at day 4 and 7 of culture but no significant effect was noted at 14 days (Fig. 2). Daily HBO treatment reversed the suppressive effect of hypoxia at day 7 and this proliferative action was more pronounced at 14 days inducing a significant 9.6 fold increase in cell number compared with normoxic control and 11.9 fold increase compared with hypoxia. In contrast daily treatment with elevated oxygen and pressure alone were unable to prevent the anti-proliferative effect of hypoxia (Fig. 2).

Culturing the cells continuously in the hypoxic environment induced a modest increase in ALP activity corrected for cell number at day 4 but decreased ALP activity (−1.8 fold) at 7 and 14 days (Fig. 3). Daily treatment with HBO consistently reversed the suppressive action of hypoxia stimulating an increase in ALP activity at days 4, 7 and 14 (Fig. 3). Daily treatment with elevated pressure alone also enhanced ALP activity at days 4, 7 and 14 but this action was not as pronounced as HBO during the early stages of the experiment (Fig. 3). In contrast treatment with elevated oxygen alone had no effect on ALP activity until day 14 when it enhanced ALP activity compared to normoxic and hypoxic controls (Fig. 3).

3.2. HBO enhances Runx-2 and type I collagen expression

Osteoblast differentiation is regulated by transcription factors that control gene expression. Therefore to determine the

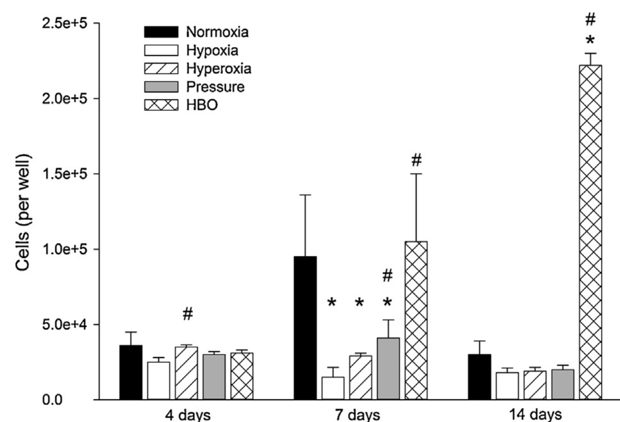

Fig. 2 – The effect of HBO, elevated pressure and elevated oxygen levels on osteoblast number. Saos-2 cells were treated with β-GP (10 mM) and L-ascorbic acid (L-AA 50 mg/l) and cell number assessed using an MTS assay at 4, 7 and 14 days. Values are the mean of three replicates ± S.E.M. Differences between groups were assessed by one-way ANOVA. *Mean values of group were significantly different from normoxic control (*p* < 0.05), #significantly different from hypoxic control (*p* < 0.05).

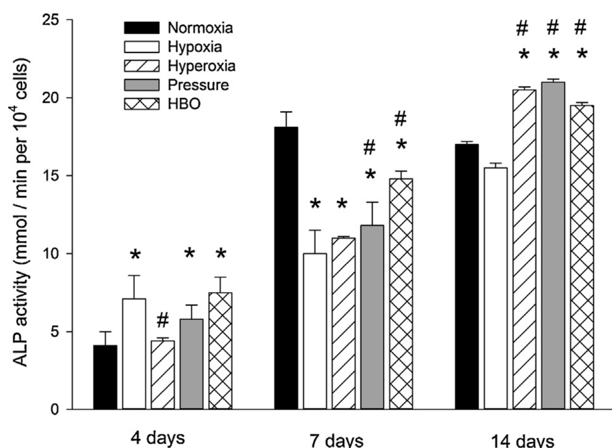

Fig. 3 – HBO reverses the inhibitory effect of hypoxia on ALP activity. Saos-2 cells were treated with β-GP (10 mM) and L-ascorbic acid (L-AA 50 mg/l) and ALP activity assayed after 4, 7 and 14 days. Values are mean of three replicates with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. *Significantly different from normoxic control ($p < 0.05$), #significantly different from hypoxic control ($p < 0.05$).

potential molecular mechanism by which HBO enhances early stage osteoblast differentiation Runx-2 expression was examined using real time quantitative PCR. In normoxic control conditions HBO and elevated oxygen alone significantly increased Runx-2 expression whereas elevated pressure had no effect (Table 1). Hypoxia significantly reduced Runx-2 expression and HBO reversed this effect. In contrast elevated pressure or oxygen alone had no effect on the suppressive effect of hypoxia.

The expression of type I collagen mirrored those observed for Runx-2. Hypoxia decreased type I collagen expression and this was prevented by HBO, which significantly increased collagen expression above that of normoxic controls, whereas treatment with elevated oxygen and pressure alone were unable to prevent this (Table 1).

3.3. *HBO augments bone nodule mineralisation*

The effect of HBO on mineralisation was assessed in cultures of Saos-2 cells incubated in the presence of differentiation factors (L-AA and β-GP) that induced nodule formation (Fig. 4). Hypoxia was shown earlier to inhibit osteoblast differentiation (ALP expression) and unsurprisingly caused a significant 1.9 and 1.2 fold reduction in mineralisation at 14 and 21 days, which was prevented by HBO. HBO prevented hypoxia's effect on mineralisation at day 7 and significantly enhanced mineralisation compared to both hypoxic and normoxic control groups at day 14 and 21 (Fig. 5). Elevated oxygen or pressure alone also had beneficial effects on nodule formation but the effect was not as great as that seen with HBO both being unable to increase nodule formation beyond that seen in normoxic controls (Fig. 5).

4. Discussion

Appropriate bone remodelling is dependent on a delicate balance between osteoblastic bone formation and osteoclastic resorption. Disruption of this balance is often seen in bone diseases such as osteoporosis, metastatic cancer and osteomyelitis.[13] Changes in remodelling activity can also arise as a consequence of therapeutic intervention. For instance, disruption of alveolar resorption and formation is often seen in patients receiving intravenous bisphosphonates or radiotherapy.[14]

In the current study Saos-2 cells were used to determine the effects of HBO or its individual constituents, elevated pressure and oxygen partial pressure, on osteoblast function. The results indicate that intermittent HBO exposure similar to that received by patients with osteonecrosis of the jaw accelerates early stages of osteoblast differentiation and increases bone nodule formation. Furthermore, HBO had a greater effect on bone nodule formation than exposure to elevated oxygen or pressure alone. The augmentative action was also noted in conditions that replicate hypoxic oxygen levels observed in necrotic tissue indicating that HBO may be able to rectify aberrant bone formation at these sites. This is similar to the studies of Wang et al. and Kawada et al. where HBO promoted osteoblast activity and improved outcomes when applied during the early stages of tibial healing.[15,16] This beneficial action may in part occur due to increased osteoblast number as HBO significantly increased osteoblast proliferation. Similar rapid changes in osteoblast number were seen in the studies of Wu et al. which demonstrated an initial stimulatory effect of HBO treatment within 3 days.[17]

The response of osteoblasts to low oxygen concentrations has been well documented.[18,19] Osteoblast function is acutely

Table 1 – The effect of HBO, pressure and hyperoxia on Runx-2 and type I collagen expression in normoxic and hypoxic conditions.

Treatment	Runx-2 normoxic conditions RQ	Runx-2 hypoxic conditions RQ	Type I collagen normoxic conditions RQ	Type I collagen hypoxic conditions RQ
Normoxia	1	1	1	1
Hypoxia		0.51 ± 0.33*		0.78 ± 0.54*
Hyperoxia	2.3 ± 0.01	0.91 ± 0.10	1.2 ± 0.03	0.35 ± 0.02*,#
HBO	7.1 ± 0.32*	8.4 ± 0.54*,#	5.2 ± 0.23*	1.5 ± 0.02*
Pressure	1.6 ± 0.05*	0.72 ± 0.34	1.1 ± 0.01	0.71 ± 0.37*

Values are the mean of three separate experiments ± standard error mean.
* Significantly different from normoxia ($p < 0.05$).
\# Significantly different from hypoxia ($p < 0.05$).

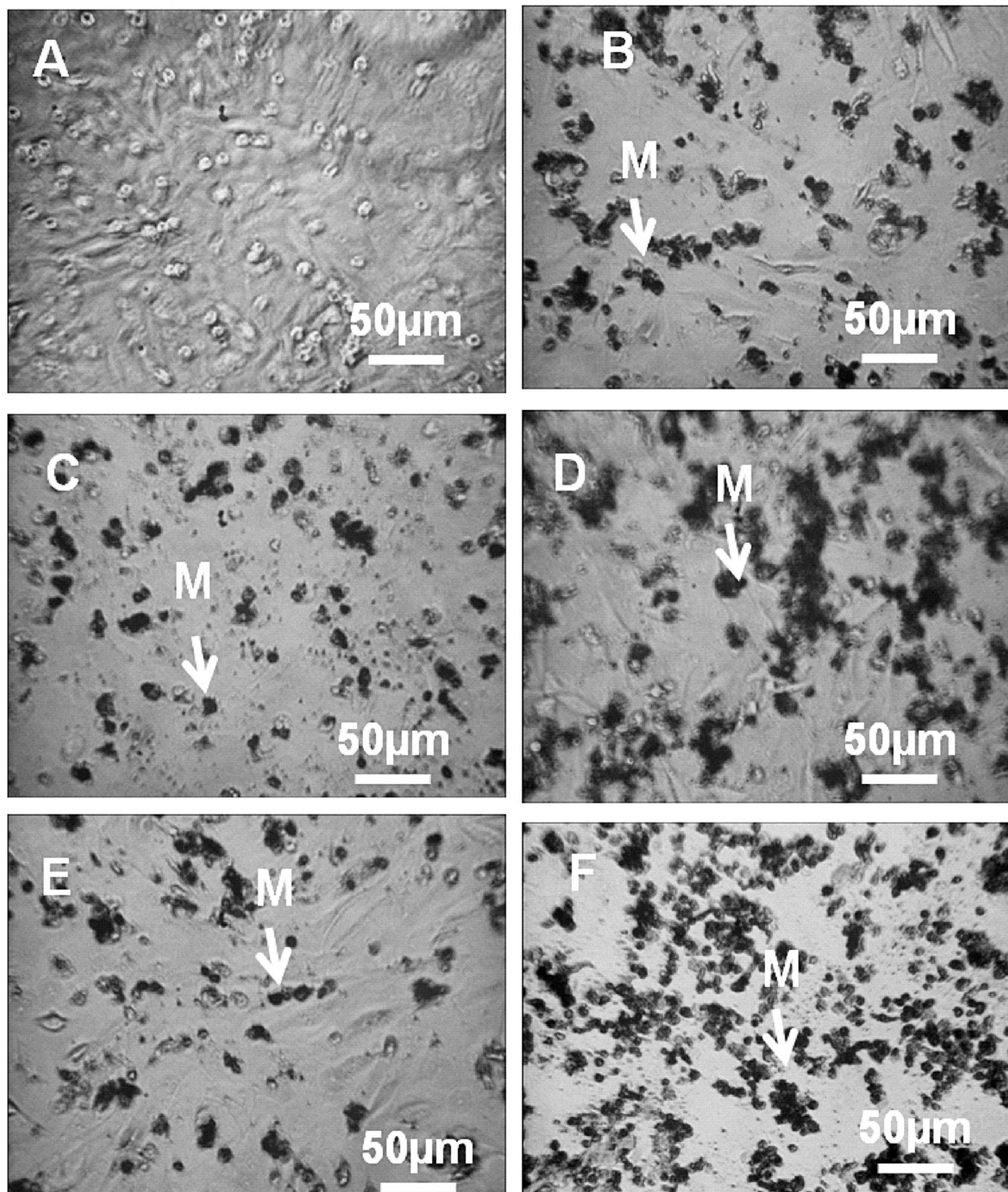

Fig. 4 – Saos-2 cells cultured for 14 days (A) control no differentiation reagents (β-GP and L-AA), (B) differentiation reagents in normoxic control conditions, (C) differentiation reagents in hypoxic conditions, (D) differentiation reagents with exposure to elevated oxygen conditions, (E) differentiation reagents with exposure to elevated pressure, and (F) differentiation reagents in HBO. Arrows highlight mineralised nodules. Images taken at 40× magnification.

sensitive to changes in oxygen partial pressure; bone nodule formation and osteoblast proliferation are strongly inhibited when oxygen concentration is decreased to 5% and almost completely abolished at 1%.[20] Salim et al. indicated that brief exposure to hypoxia down-regulated Runx-2 expression, thus inhibiting critical steps in the osteogenic differentiation of pluripotent mesenchymal precursors[21] and was also shown to decrease alkaline phosphatase activity in primary foetal rat calvarial osteoblast cultures.[19] In the current studies hypoxia reduced nodule formation at 7 and 14 days which was associated with a suppression of osteoblast number and ALP activity. This was consistently reversed by daily application of HBO.

To assess the molecular mechanisms through which HBO affected osteoblast function we examined the effect of HBO on key markers of osteoblast differentiation Runx-2 and type I collagen. Type I collagen is the major organic component of bone matrix and is a marker of mature osteoblasts. HBO enhanced Runx-2 expression at 7 days and this coincided with increased bone nodule formation. Ontiveros et al. demonstrated that hypoxia decreased Runx-2 in osteoblast and this was also noted in this study.[22] They also suggested that modulation of oxygen concentration could differentially regulate bone cell phenotypes and thereby stimulate skeletal homeostasis. HBO also promoted the expression of type I collagen a marker of early mature osteoblast and had a greater effect than treatment with elevated oxygen or pressure alone, which is in keeping with Ishii's studies suggesting that intermittent HBO enhances collagen synthesis and is beneficial for producing extracellular

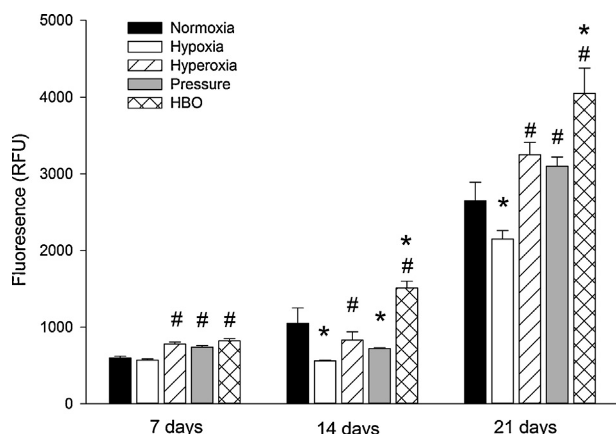

Fig. 5 – HBO enhances early stages of mineralisation and prevents the inhibitory effects of hypoxia. Saos-2 cells were treated with β-GP (10 mM) and L-ascorbic acid (L-AA 50 mg/l) for 7, 14 and 21 days and exposed to elevated pressure, elevated oxygen levels or HBO daily for 90 min. Mineralisation was then assessed using a calcein incorporation assay. *Significantly different from normoxic control ($p < 0.05$), #significantly different from hypoxic control ($p < 0.05$). Values are expressed as the mean ± S.E.M. of three replicate experiments.

matrices in tissue engineering.[23] However, while it is clear that HBO has a beneficial effect on bone cell function *in vitro* this may be limited by compromised blood flow to the necrotic area that may restrict the delivery of oxygen to the affected tissue.

5. Conclusion

These findings suggest that HBO accelerates the rate of osteoblast differentiation, augments early stages of mineralisation and has a more pronounced effect than treatment with elevated oxygen levels or pressure alone. This supports the use of HBO as an adjunctive therapy to prevent bone loss in a range of skeletal disorders associated with low oxygen partial pressure and also provides a potential mechanism through which short term HBO therapy may help in fracture healing.

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