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The effect of Hyperbaric Oxygen Therapy on osteoclast and osteoblast function

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By

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Copyright Statement

Dedication

I dedicate this thesis

To those who saw me with their hearts before their eyes, and embraced me with their bodies before their hands… to the two trees that never wither and the shadow that I take shelter once and a while, to the scent of eternity and the most beautiful thing in the existence, to the spring of pure love and supreme tenderness...to the Paradise of the world my homeland… my Mother and Father.

To the flower that fragrant with the best aroma and opened in my home… to whom I will live my life… to all the purity, childhood, and sweetness… to the rain that washed the time hardships… to my moon… my princess Luna.

To the winter nights' breeze and the sea of peace, to the lamp that lightened my way, those who give me without expecting anything in return… the two planets in the giving space… my brother and sister.

To the soul that hugged mine…to the heart that poured his secrets in my heart, to the hand that ignite my passion… from his words and sound I get my strength, from his eyes I get the sun of my life… to whom which draw a smile on my face ….to the hope that never fade… to him who existed when I existed and still my soul… I called him myself… you are me… to my friend and life

Hadil 2013

The effect of Hyperbaric Oxygen Therapy on osteoclast and osteoblast function

Hadil W. Al-Hadi

Abstract

Bone remodelling, the process by which the skeleton adapts to environmental changes, is dependent on the actions of osteoclasts that resorb bone and osteoblasts which make new bone matrix. Aberrant remodelling underpins bone loss in several debilitating skeletal diseases such as osteoporosis, metastatic breast cancer and multiple myeloma. Changes in remodelling activity can also arise as a consequence of therapeutic intervention for instance intravenous bisphosphonate treatment is associated with osteochemonecrosis of the jaw and localised osteoradionecrosis is a common side effect of radiotherapy. Hyperbaric oxygen is often used as an adjunctive therapy in the treatment of these disorders. HBO involves the administration of 100% oxygen at atmospheric pressures greater than one in sealed chambers.

The following studies aimed to evaluate the effect of HBO, hyperoxia, and pressure on RANKL-induced osteoclast differentiation and bone resorption from RAW264.7 and human peripheral blood mononuclear cells (PBMC), and osteoblast differentiation *in vitro.* The study also aimed to further examine the effect of HBO on *ex vivo* osteoclast formation from peripheral blood monocytes obtained from patients undergoing HBO.

Daily exposure to HBO for ninety minutes significantly suppressed osteoclast differentiation and bone resorption in mouse and human monocytes in normoxic and hypoxic conditions *in vitro*. The suppressive action of HBO on osteoclast formation was associated with a significant reduction in *HIF-1α* and *RANK* mRNA expression and HBO also caused a significant reduction in *NFATc1* and *DC-STAMP* expression. This study has for the first time shown that HBO is able to reduce the ability of precursors to form bone resorbing osteoclast.

HBO also suppressed the ability of peripheral blood monocytes to develop into RANKL-induced resorptive osteoclasts. In an *ex vivo* culture system the suppressive effect of HBO was meditated by an action prior to activation of osteoclast differentiation by RANKL and must therefore be an inhibitory effect on the ability of precursors to differentiate along the osteoclastic lineage.

HBO also accelerates the rate of osteoblast differentiation and augments early stages of mineralization and has a more pronounced effect than hyperoxia or pressure alone. HBO enhanced bone nodule formation and ALP activity in human osteoblasts. Furthermore HBO promoted the expression of *type I collagen* and *Runx-2* in both normoxic and hypoxic conditions. HBO had a greater effect on these key markers of osteoblast differentiation than hyperoxia or pressure alone.

This study suggests that HBO suppresses osteoclast activity and promotes osteoblastic bone formation, which may at least in part mediate its beneficial effects on necrotic bone. This provides evidence supporting the use of HBO as an adjunctive therapy to prevent osteoclast formation in a range of skeletal disorders associated with low oxygen partial pressure. The study also provides further support for the use of HBO in the treatment of skeletal disorders associated with excessive resorption such as osteomyelitis, and also provides a potential mechanism through which short term HBO may help fracture healing.

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Abbreviations

$1,25(OH)_2D_3$	Vitamin D3
$Ca_{10}(PO)_4(OH)_2$	Crystal of calcium phosphate salt
ALP	Alkaline phosphatase
AP-1	Activator protein
ATA	Atmosphere absolute
ATM	International reference pressure
ATP	Adenosine triphosphate
β-GP	Beta glycerophosphate
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BP	Bisphosphonate
BSP	Bone sialoprotein
CK	Cathepsin K
CFU-GM	Granulocyte-macrophage colony forming unit
DAP12	DNAX-activating protein of 12 kDa
DC-STAMP	Dendritic specific transmembrane protein
DMSO	Dimethy sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Trypsinethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinases
FCS	Fetal Calf Serum
FGF23	Fibroblast growth factor 23
FcRγ	Fc-gamma receptor

Gla	Carboxyglutamic acid
HBO	Hyperbaric oxygen therapy
HIF	Hypoxia inducible factor
HIF-1α	Hypoxia inducible factor 1 alpha
HIF-1β	Hypoxia inducible factor 1 beta
HRE	Hypoxia response element
IGF	Insulin-like growth factor
IGF-I	Insulin-like growth factor 1
L-AA	L-ascorbic acid
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-7	Interleukin-7
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun NH_2-terminal kinase
MAPK	Mitogen-activated protein kinases
M-CSF	Macrophage-colony stimulating factor
MGP	Matrix Gla protein
MM	Multiple myeloma
MMP	Matrix metalloproteinase
MSCs	Mesenchymal stromal cell
Msx2	Muscle segment homeobox gene
NFAT	Nuclear factor activated T cells
NFATc1	Nuclear factor activated T cells, cytoplasmic 1
NFκB	Nuclear factor-keppa light-chain-enhancer of activated B cells
OCN	Osteocalcin
ON	Osteonecrosis

ONJ	Osteonecrosis of the jaw
OPG	Osteoprotegerin
OPN	Osteopontin
ORN	Osteoradionecrosis
OSCAR	Osteoclast-associated receptor
Osx	Osterix
PBMC	Peripheral blood mononuclear
PHDs	Prolyl hydroxylase domain
PTH	Parathyroid hormone
PTK	Protein tyrosine kinase
PU.1	Purine-rich nucleic acid binding protein 1
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2
Smad	The mothers against decapentaplegic
SNPs	Single nucleotide polymorphisms
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCF	T-cell factor
TGF-β	Transforming growth factor-beta
TNFR	Tumour necrosis factor receptor
TNF-α	Tumour necrosis actor-α
TRAFs	TNFR-associated factors
TRAP	Tartrate resistant acid phosphatase

TREM2	Triggering receptor expressed on myeloid cells 2
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau protein

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee. This study was financed with the aid of Ministry of the Higher Education and Scientific Research/Iraq. Relevant scientific seminars and conferences were attended at which work was presented and papers have been prepared for publication:

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Signed ----------

Date ----------

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Chapter one: Bone formation, resorption and hyperbaric oxygen therapy

1.1 Bone

Bone is a connective tissue which consists of mineralized extracellular matrix and contains three types of cell, which are osteoblasts, osteocytes, osteoclasts and lining cells (Yang and Damron, 2008). The organic phase of the skeleton consists primarily of type I collagen and small amounts of non-collagenous proteins (Hill and Orth, 1998), whereas the inorganic component consists of calcium and phosphorus in the form of hydroxyapatite crystals (Spector, 1994). In bone hydroxyapatite is an imperfect crystal of calcium phosphate salt ($Ca_{10}[PO_4]_6[OH]_2$) having substitutions by magnesium, sodium, strontium carbonate, citrate and fluoride. Crystals are needle shaped, white in colour and measure 100-350 nm (Kandaswamy *et al*., 2000). Hydroxyapatite plays an essential role in the ability of bones to resist mechanical loading.

Bone provides internal support for the body, attachment sites for tendons and muscles, protects body organs and is the main store for calcium and phosphate in the body. It can be sub-divided into two main types, compact or cortical bone (Fig. 1.1), which forms the exterior surface and cancellous or trabecular bone which resides within the cortical cavity (Hill and Orth, 1998). The distribution of type I collagen in trabecular bone can vary; normally in the adult skeleton this is organised into concentric layers with fibres in the same orientation in individual layers; this is termed lamellar bone. The second type, woven bone, typically forms during pathological processes such as fracture repair or diseases associated with high rates of remodelling such as Paget's. Woven bone is rapidly formed and there is little organisation of the collagen fibres. It is structurally less competent than lamellar bone and can provide a 'patch' at fracture sites which is subsequently remodelled to lamellar bone.

Bone tissue is formed through two independent processes, intramembranous ossification and endochondral ossification (Karsenty, 1999). In the case of intramembranous ossification, osteoblasts differentiate directly from mesenchymal cells and form new

bone by a process of appositional growth without the formation of a cartilaginous template. During endochondral ossification bone develops through a sequential process where mesenchymal cells form chondrocytes which proliferate and differentiate to form a cartilaginous template which is subsequently remodeled and replaced by true bone (Chung et al., 1998).

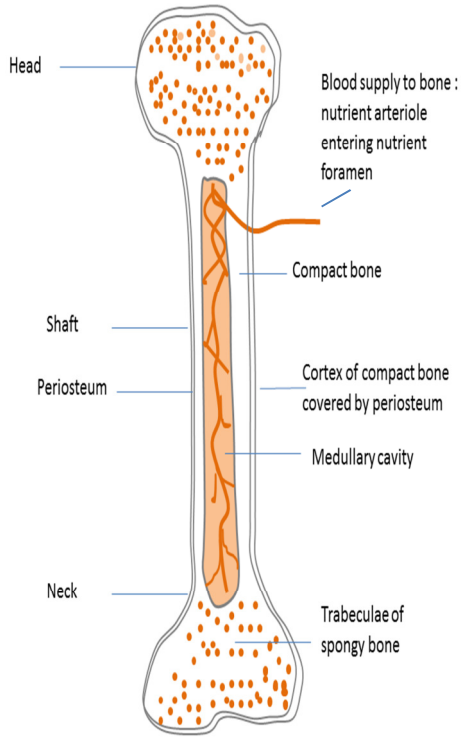


Figure 1.1 Structure of typical long bone. Based on http://medicaldictionary.thefreedictionary.com/bone+matrix

1.2 Bone remodelling

Bone remodelling is the temporally and spatially coupled cellular process that maintains skeletal integrity. It involves the resorption of bone by osteoclasts followed by the synthesis of new bone matrix by osteoblasts. The correct balance between osteoclastic bone resorption and osteoblastic bone formation is important in maintaining physiological bone homeostasis, and disruption of the relationship between these cells can lead to bone disease (Duplomb *et al.*, 2007). There are five phases in bone remodelling: quiescence, activation, resorption, reversal and formation (see Fig. 1.2).

During quiescence the bone surface is inactive, but upon activation osteoclasts are recruited to the bone surface where they resorb bone forming a resorption pit. During reversal, osteoblasts are recruited to the pit and synthesise new bone to replace the resorbed tissue during the formation phase (Hill and Orth, 1998). Under normal conditions in adults there is little net loss of bone during each remodelling cycle ensuring that bone mass remains relatively constant (Eriksen, 1986). Disruption of the remodelling cycle is a common occurrence leading to bone loss in many skeletal diseases such as osteoporosis, metastatic breast carcinoma and multiple myeloma (Fohr *et al*., 2003).

The three cell types that contribute to bone remodelling form from different lineages, osteoblasts, which are derived from mesenchymal osteoprogenitors; osteocytes, which are osteoblasts that become entrapped within lacunae during bone formation; and multinuclear osteoclasts, which are derived from the fusion of mononuclear monocytic precursors present in the marrow and circulation (Ehrlich and Lanyon, 2002). Osteoblast and osteoclast formation are tightly regulated processes dependent on a range of systemic and local signalling factors (Kong *et al*., 1999, Yasuda *et al*., 1998).

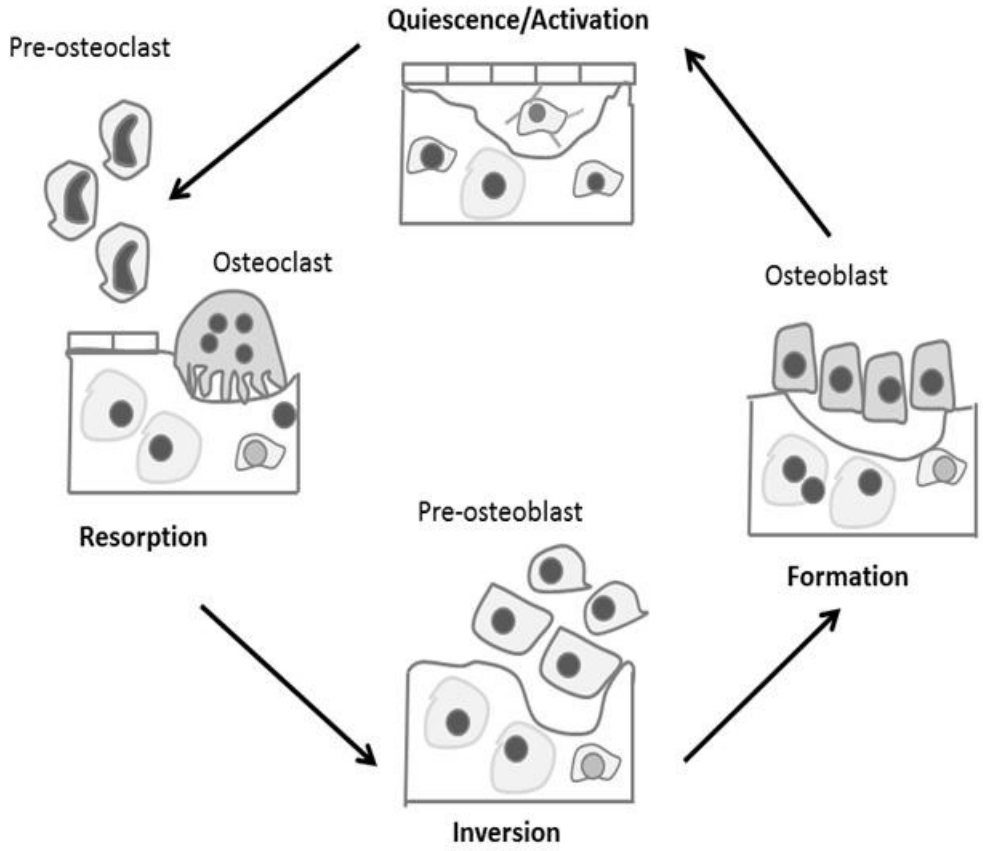


Figure 1. 2 Bone Remodelling process. Based on Brandi (2009)

1.3 Bone cells

There are two bone cell lineages. The first, osteoclasts, resorb bone. The other is the osteoblast family, which consists of osteoblasts that form bone, osteocytes that help maintain bone and lining cells that cover the surface of the bone (Fig. 1.3).

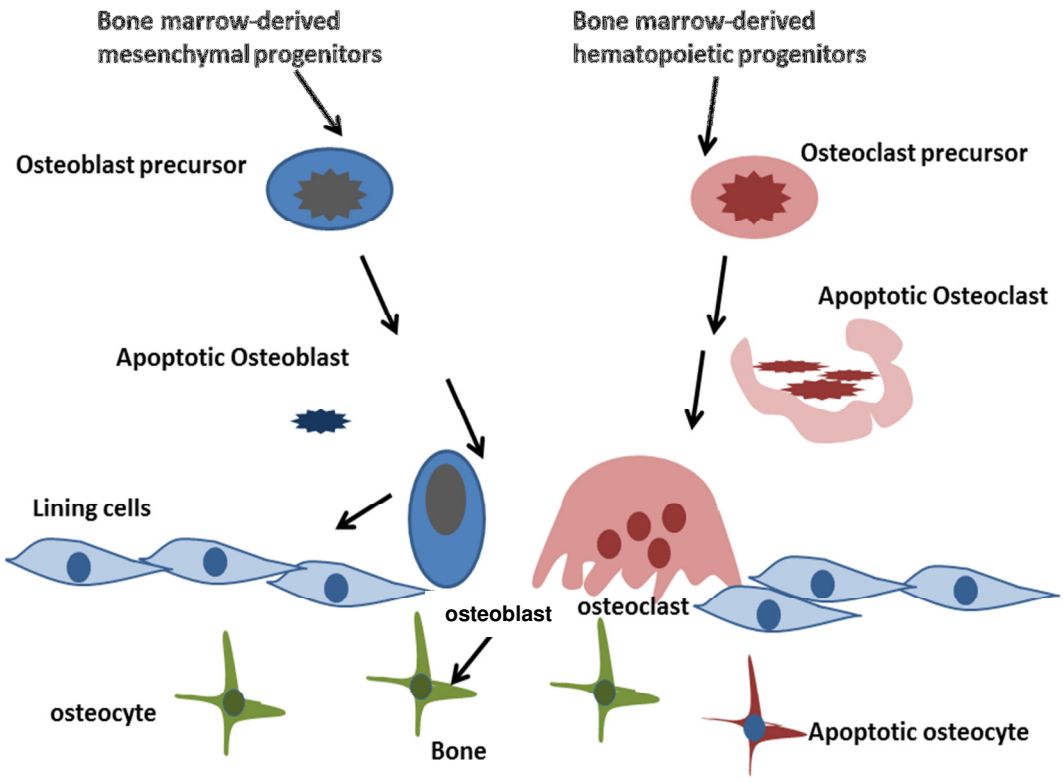


Figure 1.3 The origin and fate of bone cells. Based on washington.edu/bonebio/bonAbout/bonecells.html.

1.4 Osteocytes

Osteocytes, the most common cell found in mature bone, are derived from osteoblasts and are located within the bone matrix (Tate *et al.*, 2004). Manolagas proposed that matrix-producing osteoblasts can become osteocytes, lining cells or undergo programmed cell death (Manolagas, 2000). Osteocytes have long cytoplasmic extensions which cover a vast surface area allowing them to perform a multipurpose role in bone biology, including detection of changes in mechanical loading. These cellular projections are located in thin canaliculi in the mineralised matrix and allow osteocytes to form connections via gap-junctions with neighbouring cells and with the bone surface (Aarden *et al.*, 1999).

1.5 Osteoclasts

Osteoclasts are derived from haematopoietic cells found within the bone marrow and peripheral blood (Fujikawa *et al*., 1996). Osteoclasts are derived from myeloid progenitors that give rise to both the monocyte-macrophage lineage and the osteoclast lineage (Suda *et al*., 1999). During osteoclast differentiation preosteoclasts attach to the bone surface and multinuclear osteoclasts are formed by the fusion of these precursors under the regulation of a complex cell signalling network (Kim *et al*., 2005). The earliest differentiated haematopoietic precursor able to form osteoclasts is the granulocyte-macrophage colony forming unit (CFU-GM) (Knowles and Athanasou, 2009). As detailed below, osteoclast formation is dependent on many locally produced cytokines and systemic regulators. However, central to this are receptor activator of Nuclear Factor kappa B ligand (RANKL) and macrophage colony stimulating factor (M-CSF), which are both essential for the formation and fusion of multinucleated cells. RANKL is a member of the Tumour Necrosis Factor superfamily expressed by osteoblasts and stromal cells in response to resorptive stimuli, which binds to its receptor RANK on osteoclast precursors (Yavropoulou and Yovos, 2008, Jones et al., 2002). RANKL is primarily expressed by osteoblasts but also expressed by activated T cells, fibroblasts and mammary tissue (Anderson *et al*., 1997). It also plays a pivotal role in the regulation of dendritic cell survival, lymphocyte development and lymph node organogenesis (Kong *et al*., 1999). Cell to cell contact between osteoblast/stromal cells and pre-osteoclasts is essential for the activation of RANK, although soluble forms of RANKL have been identified (Khosla, 2001).

RANK is expressed by a number of cells, monocytes/macrophages, fibroblasts, chondrocytes, osteoclasts and osteoblasts as well as B-cells and dendritic cells, and can be induced by IL-1, TNF-α, parathyroid hormone (PTH) and M-CSF (Hofbauer, 1999). The absence of RANK leads to an osteopetrotic phenotype due to the lack of osteoclast

for bone resorption (Kong *et al*., 1999); osteopetrotic is a descriptive term that refers to a group of rare, heritable disorders of the skeleton characterized by increased bone density on radiographs (Stark and Savarirayan, 2009). The binding of RANKL to RANK initiates a complex network of intracellular signal transduction cascades that stimulate the expression of a number of osteoclastic genes (Boyle *et al*., 2003). This involves the induction of TNFR-associated proteins (TRAFs) to RANK which in turn active (Fig. 1.4) Extracellular signal-regular kinase, JNK (c-jun N-terminal kinases), NF-κ B, Nuclear Factor Activated T cells cytoplasmic 1, and Activator Protein-1 (Lee and Kim, 2003). In addition, TNF-α can also directly induce osteoclast formation and can have a synergistic effect with RANKL; it may also increase bone resorption by stimulating the expression of other pro-inflammatory cytokines (Kurokouchi *et al*., 1998).

1.5.1 Osteoclast formation

Osteoclast formation is stimulated in response to falls in circulating Ca^{2+} concentrations, physical inactivity and during fracture repair. Of these stimuli, serum [Ca^{2+}] has the greatest daily influence on osteoclastogenesis; low [Ca^{2+}] leads to the production of parathyroid hormone (PTH) from the parathyroid glands (Bronner and Farach-Carson, 2003). PTH has diverse effects on osteoclasts; it stimulates bone resorption by increasing osteoclastogenesis and enhances the resorptive activity of individual mature osteoclasts. This action is mediated via an effect on osteoblasts; osteoclasts lack PTH receptors and are indirectly regulated via the activation of osteoblastic PTH receptors, which then signal to osteoclast precursors to stimulate their differentiation, fusion and activation (Bronner and Farach-Carson, 2003). There are two main growth factors required for the formation of functional osteoclasts, Macrophage-Colony Stimulating Factor and RANKL, which are expressed by osteoblasts in response to resorptive stimuli such as PTH (Hofstetter *et al*., 2003). M-CSF plays a critical role in the

proliferation, survival and activation of osteoclast precursors (Yavropoulou and Yovos, 2008, Hirayama *et al*., 2005) and is also necessary for the growth, differentiation, activation and survival of cells of the mononuclear system (Felix *et al*., 1994). M-CSF is secreted by a number of cells including osteoblasts, fibroblasts, monocytes and endothelial cells (Leizer *et al*., 1990, Weir *et al*., 1993) in response to IL-1 and TNF-α (Leizer *et al*, 1990). It is essential for the initial stage of osteoclast formation, survival and motility and binds to c-Fms, a dimeric receptor tyrosine kinase (Fuller *et al*., 1993, Lean *et al*., 2000). M-CSF induces the expression of RANK, the receptor for RANKL (Yasuda *et al*., 1998), and activates Mitf and c-Fos which are required for osteoclast differentiation (Kawaguchi and Noda, 2000).

The RANKL/RANK interaction is modified by another factor produced by osteoblasts called osteoprotegerin (OPG), which is a soluble decoy receptor for RANKL (Kong *et al*., 1999b). OPG sequesters RANKL preventing it from binding to RANK, thus OPG suppresses bone resorption and preserves the balance between resorption and formation (Suda *et al*., 1999, Simonet *et al*., 1997). Through the differential production of RANKL/OPG in response to stimuli osteoblasts play a critical role in the differentiation of osteoclasts (Faccio *et al*., 2005). Disruption of this fine balance can lead to inappropriate levels of osteoclast formation and excessive bone loss. The binding of RANKL to RANK activates multiple intercellular signalling cascades that regulate the expression of osteoclastic genes such as cathepsin K, tartrate resistant acid phosphatase (TRAP) and the calcitonin receptor (Matsumoto *et al*., 2004, Kim *et al*., 2008b). The initial signal is propagated by TNF receptor associated factors (TRAFs) which interact with several mitogen activated protein kinase (MAPK) pathways leading to the induction and activation of transcription factors including nuclear factor of activation T cell (NFATc1), NF-κB and AP-1 members. NFATc1 is a transcription factor identified in T-cells. Osteoclastogenesis can be induced in the absence of RANKL with

overexpression of NFATc1 (Takayanagi *et al*., 2002). NFATc1 activation is required for the transcription of osteoclast genes. NFATc1 can activate TRAP, osteoclast associated receptor (OSCAR), Dendritic specific Transmembrane Protein (DC-STAMP), integrin β3, and cathepsin K (Shaulian and Karin, 2001). In addition it promotes expression of its own gene, in a Ca^{2+} calcineurin dependent manner (Kim *et al*., 2005, Matsuo *et al*., 2004) and NFATc1 in many cases also co-operates with other transcription factors such as PU.1, MITF, and AP-1 to augment the transcription of genes (Takayanagi *et al*., 2002). Also osteoclast formation is dependent on the ability of TGF-β to enable receptor activator of NF-kB ligand (RANKL)-induced commitment of haematopoietic precursors to the osteoclastic lineage (Fox *et al*., 2003).

Koga et al. describe a more complex model of osteoclastogenesis in which co-stimulatory mechanisms play an important role in osteoclast development (Koga *et al*., 2004). Co-stimulatory inputs from immunoreceptor tyrosine-based activation motifs (ITAM) such as osteoclast-associated receptor (OSCAR), Triggering receptor expressed on myeloid cells 2(TREM $_2$), Fc-gamma receptor (FcRγ) and DNAX-activating protein of kDa (DAP12) (Kim *et al*., 2008a) regulate osteoclast formation and function (Humphrey *et al*., 2005). OSCAR is a member of the immunoglobulin-like surface receptor family and plays an important role as a co-stimulating receptor for osteoclast differentiation by activating NFATc1 via association with FcRγ (Merck *et al*., 2004). Nemeth et al. demonstrated that OSCAR is involved in the positive feedback circuit of the immunoreceptor-NFATc1 pathway by providing co-stimulatory signals required for RANKL mediated activation of calcium signalling (Nemeth *et al*., 2011). Also RANKL induces osteoclastogenesis by stimulating DC-STAMP internalization, which is necessary for mononuclear osteoclast fusion (Kukita *et al*., 2004). Bone marrow mononuclear cells derived from DC-STAMP deficient mice do not form multinuclear osteoclasts, but have comparable tartrate-resistant acid phosphatase (TRAP) activity *in*

vitro. Moreover, the expression of osteoclast markers and transcription factors required for osteoclast differentiation, including RANK, c-Fos, and NFATc1, was induced by M-CSF and RANKL in DC-STAMP-deficient cells (Matsuo *et al*., 2004), so DC-STAMP efficiently induces the formation of multinuclear osteoclasts indicating that DC-STAMP is indispensable for cell-cell fusion of osteoclasts. Yagi *et al* revealed that DC-STAMP is essential for cell-cell fusion of not only osteoclasts but also of macrophage giant cells (Yagi *et al*., 2005).

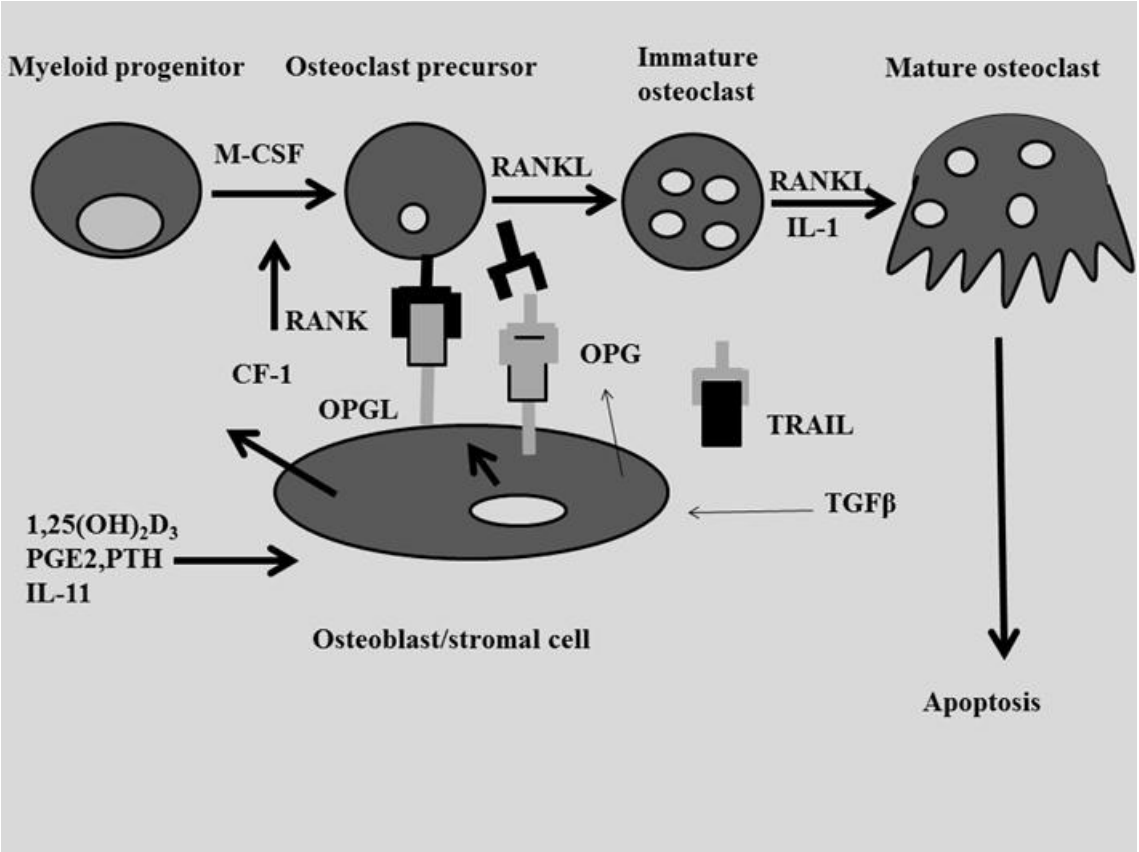


Figure 1.4 The role of RANKL, RANK, and OPG in osteoclastogenesis. RANKL stimulates committed osteoclast precursors and mature osteoclasts via binding to its receptor RANK. RANKL exists in soluble and membrane bound forms. Decoy receptor OPG inhibits RANKL/RANK interactions and OPG appears to be the rate restrictive factor that regulates osteoclast activity and bone mass. Also OPG was found to bind to the apoptosis-inducing TNF-family molecule tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). This figure is based on Kong *et al*. (1999a).

1.5.2 Osteoclastic bone resorption

Bone resorption is a complex process during which osteoclasts break down the organic and inorganic components of bone. This requires the differentiation of osteoclasts from monocytic precursors, their functional polarisation, attachment to the bone surface, formation of a sealing zone, and the release of proteolytic enzymes and protons to form a resorption pit (Dresner-Pollak and Rosenblatt, 2004, Lakkakorpi and Vannanen 1995). Actively resorbing osteoclasts typically possess a highly involute area of membrane opposed to the bone surface termed the ruffled border; this is the resorptive organ of the cell possessing a large surface area for efficient exchange of resorptive factors (Schlesinger *et al*., 1997). The ruffled border is circled by the sealing zone which is rich in F-actin and cell adhesion molecules that attach the cell to the bone matrix.

Osteoclasts express a range of heterodimeric integrins that enable them to interact with specific arginine-glycine-aspartic acid (RGD) containing proteins on the bone surface such as osteopontin (Hughes *et al*., 1993, Nesbitt *et al*., 1993, Helfrich *et al*., 1992, Flores *et al*., 1992). Highly expressed integrins include $\alpha_v\beta_3$, $\alpha_2\beta_v$, $\alpha_v\beta_5$ and $\alpha_2\beta_1$ which have a significant role in the initiation and maintenance of resorption. In particular $\alpha_v\beta_3$ integrin appears to play a particularly important role (Chambers *et al*., 1986), enabling adhesion and migration to areas where resorption is going to occur (Lakkakorpi and Vannanen, 1995). Integrins also maintain the integrity of the sealed zone between the ruffled border and bone surface, and defects in this can lead to poor resorptive capacity (Nakamura *et al*., 1999).

Breakdown of the inorganic component of bone matrix is dependent on the generation of an acidic environment (pH 4-4.5) within the sealed zone. Acidification of the sealed zone is achieved by the action of vacuolar H^+-ATPases and chloride channels located in the osteoclast ruffled membrane (Edwards *et al*., 2006), which extrude H^+ and Cl^- ions into the sealed zone forming HCl which solubilises hydroxyapatite (Vaananen and

Laitala-Leinonen, 2008). Protons are generated in the osteoclast cytoplasm by carbonic anhydrase II, which catalyses the hydration of CO_2 to HCO_3^- and H^+, and is abundantly expressed on the inner surface of the ruffled border (Fig. 1.5). Cytoplasmic pH is maintained by the action of a chloride bicarbonate exchanger (Cl^-: HCO_3^-) on the apical surface of the osteoclast, which maintains osteoclast functionality by preventing alkalisation (Rousselle and Heymann, 2002). Numerous cell signals influence acid secretion, but one of particular interest is intracellular calcium and the ubiquitous calcium-binding protein, calmodulin. The unique acid-dependent dissolution of calcium salts produces high local extracellular calcium (Silver *et al*., 1988). This is related to a separate osteoclastic calcium regulatory mechanism that includes a calmodulin-dependent calcium ATPase (Bekker and Gay, 1990), and factors effecting osteoclastic intercellular calcium activity like matrix attachment (Miyauchi *et al*., 1991).

Several proteolytic enzymes are responsible for organic matrix turnover; these include the matrix metalloproteinase (MMP) family which are Ca and Zn-dependent endoproteinases (Blavier and Delaisse, 1995, Mannello *et al*., 2006). MMPs play an important role in bone remodelling and osteoclast differentiation and can activate the phospholipase C pathway which regulates expression of the AP-1 transcription factors c-Jun and c-fos (Ricky *et al*., 2005, Engsig *et al*., 2000). MMP-9 in particular is highly expressed in osteoclasts and has a major role in regulating these osteoclastic activities (Blavier and Delaisse, 1995). MMPs are thought to be necessary for the migration of precursor and immature osteoclasts to the bone surface (Inui *et al*., 1999). Bone remodelling and resorption is also dependent on the synthesis of the cysteine protease cathepsin K (CK), which is another osteoclastic enzyme secreted into the sealed zone which degrades type I collagen and other matrix proteins (Troen, 2004). CK expression is up-regulated by RANKL in human osteoclasts via TNF receptor-associated factor 6 (TRAF6) which activates several osteoclastic transcription factors that interact with the

promoter region of CK (Bruce R, 2006, Troen, 2005). MMPs and CK are transported from the endoplasmic reticulum within lysosomes and then released into the resorption lacuna (Delaisse et al., 2003). In the case of CK this is as an active form following intracellular processing of the inactive zymogen at low pH by other proteases or by autocatalysis (Teitelbaum, 2007). CK is critical for the removal of the organic components of bone; humans lacking CK develop pycnodysostosis a rare genetic disorder that is characterised by osteopetrotic bone abnormalities (Motyckova and Fisher, 2002). CK knockout mice also develop osteopetrosis and display features characteristic of pycnodysostosis; osteoclasts isolated from these mice exhibit impaired bone resorption capacity and make few resorption pits (Gowen *et al*., 1999).

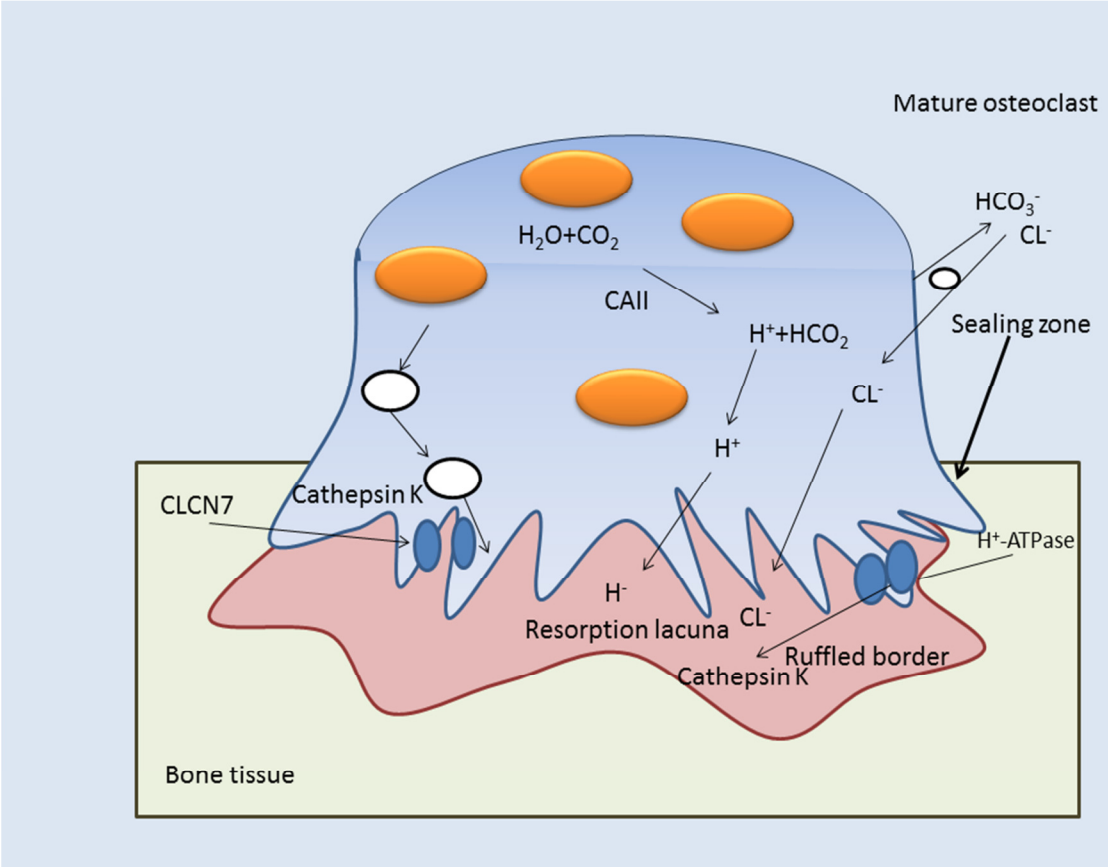


Figure 1.5 Diagram showing an osteoclast attached to bone tissue. Active bone resorption occurs in a sealed compartment between the ruffled border and the bone surface. The bone matrix experiense acidification and demineralisation initially. Later carbonic anhydrase II (CAII) produces protons in the cytoplasm. They are transported over the ruffled border into the resorption lacuna by an osteoclast-specific V-ATPase. Chloride Channel 7 (CLCN7) transports chloride ions from the cytoplasm into the resorption lacuna to equilibrate the charge of ions across the membrane. Posteriorly, the degradation of the organic matrix is done by several proteolytic enzymes including the cysteine proteinases, for example, cathepsin K (CTSK), and the matrix metalloproteinases. This diagram is based on Balemans *et al*. (2005).

1.6 Osteoblasts

Osteoblasts are derived from mesenchymal stem cells and are responsible for the deposition and mineralization of bone matrix. The events during osteoblast differentiation are outlined in Fig. 1.6. Osteoblasts arise from common progenitors which can also form chondrocytes, muscle and adipocytes under the regulation of various hormones and local factors (Yamaguchi and Sugimoto, 2000). Osteoblast formation is a consecutive process that involves the development of immature osteoblast before mature cell formation.

Osteoblasts can be characterised by several markers with important roles in bone matrix synthesis and mineralization. These include alkaline phosphatase (ALP) and type I collagen (Murshed *et al*., 2005). Osteoblasts also secrete further extracellular matrix proteins including osteocalcein (OCN), matrix Gla protein (MGP), bone sialoprotein (BSPs), osteonectin and osteopontin (OPN) (Young *et al*., 1992, Harada and Rodan, 2003).

Several transcription factors are crucial for skeletogenesis and osteoblast differentiation including RUNX2 and osterix (Nakashima *et al*., 2002). They target osteoblast-related genes such as OCN, BSP, OPN and collagen type I (Ducy, 2000). Osteoblasts express receptors for various hormones including PTH (Dempster *et al*., 1993), 1α,25-dihydroxyvitamin D_3[1α,25$(OH)_2D_3$] (Lain *et al*., 1999), oestrogen (Boyce *et al*., 1999) and corticosteroids. Osteoblast derived osteocalcin has also recently been shown to function as an endocrine regulator of metabolism. Fulzele *et al* indicated that insulin signalling in osteoblasts regulates glucose metabolism via OCN (Fulzele *et al*., 2010). OCN stimulates insulin release from pancreatic beta cells and at the same time directs fat cells to release the hormone adiponectin, which increases sensitivity to insulin (Lee *et al*., 2007). In addition osteoblasts play an important role in osteoclast differentiation via the release of RANKL (Katagiri and and Takahash, 2002).

1.6.1 Osteoblast differentiation

Osteoblasts differentiate from pluripotent mesenchymal progenitor cells under the control of various transcription and growth factors (Katagiri and and Takahash, 2002). Mesenchymal stem cells differentiate into immature osteoblasts, which express bone matrix protein genes. Through the actions of Runx2, and osterix (osx), the immature osteoblasts, which express high levels of OPN, differentiate into mature osteoblasts, which express high levels of OCN (Karsenty and Wagner, 2002, Phan and Zheng, 2004). Finally mature osteoblasts are embedded in the bone matrix to become osteocytes (Liu *et al*., 2001). The expression of Runx and osx are themselves under the control of BMP, TGF beta and other systemic factors as shown in Fig. 1.6.

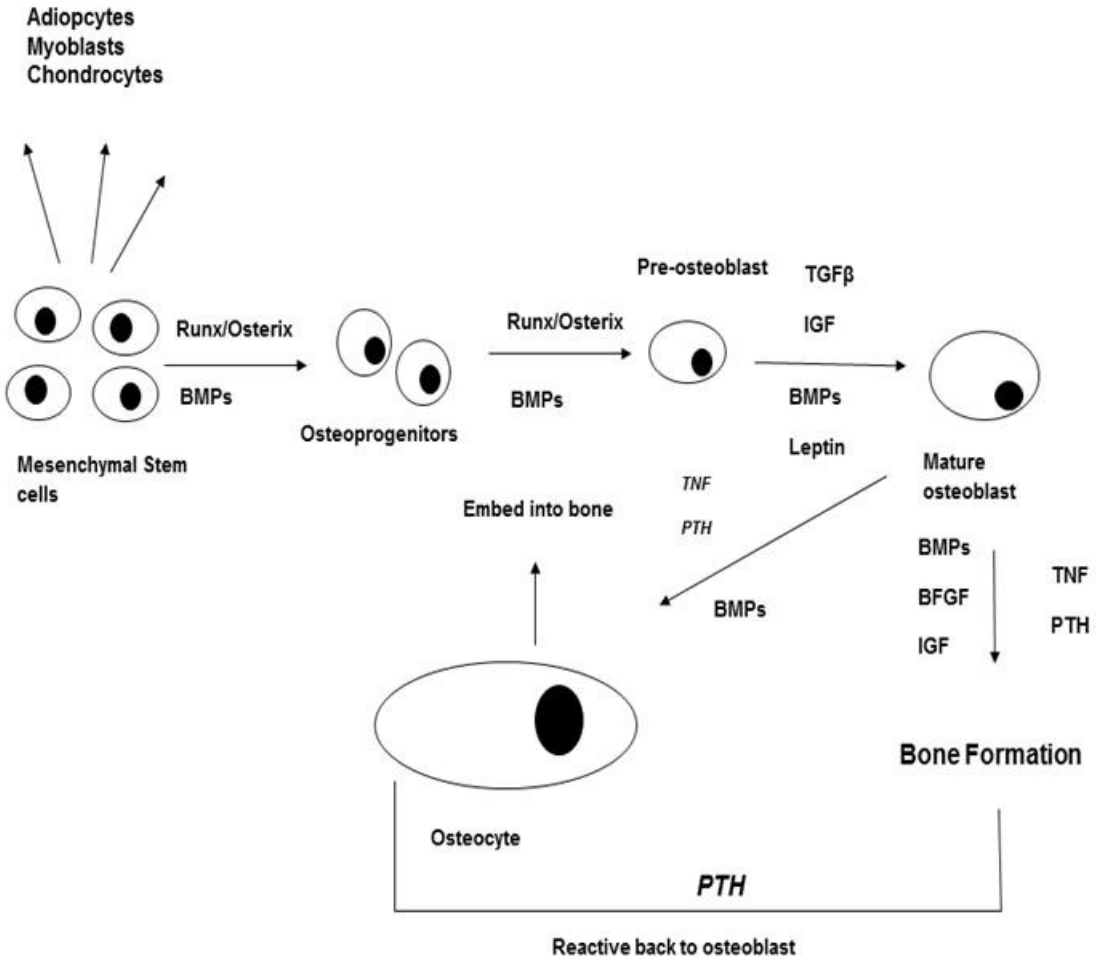


Figure 1.6 Mesenchymal stem cells differentiate into osteoblasts under Runx2 and osterix, Mesenchymal cells can also form adipocytes, myocytes, and chondrocytes under the control of transcription factors. Runx2 is a key regulator of osteoblast differentiation pathway, while *osterix* is downstream of Runx2 and is important for osteoblast maturation. This figure is based on Phan and Zheng (2004).

Osteoblast formation is regulated by several cytokines including bone morphogenetic proteins (BMPs), transforming growth factors (TGF), fibroblast growth factor (FGFs), Wnt, and hedgehog proteins (Hu *et al*., 2004) with members of the TGF family arguably being the most important of these (Cohen, 2002). The TGF superfamily is comprised of over forty members such as TGF-β, activin and BMPs (Guo and Wang, 2009). BMPs are osteoinductive factors that stimulate ectopic cartilage and bone formation subcutaneously or intramuscularly in experimental animals (Rosen and Thies, 1992). BMPs regulate multiple processes such as proliferation, apoptosis, differentiation, cell-fate determination and morphogenesis. BMPs are expressed in a wide range of tissues, for instance BMP3, BMP4 and BMP7 are expressed in the kidney; BMP2, BMP4, BMP5 and BMP7 in limb buds; and BMP2, BMP4 and BMP7 during tooth development (Chang *et al*., 1994). Chen et al demonstrated that BMP2 exerts this osteogenic action by activating Smad (The mothers against decapentaplegic) signalling which regulates transcription of osteogenic genes (Chen *et al*., 1997). BMPs bind type II and type I serine/threonine kinase receptors, which activate specific Smad proteins (Itoh *et al*., 2000). BMPs induce canonical Smad1/5/8 phosphorylation in osteoblasts (Zhou *et al*., 2010, Fuentealba et al., 2007) and osteoblast-specific Smad1 knockout mice develop an osteopenic phenotype, whereas combined loss of Smads 1/5/8 results in severe chondrodysplasia (Wang *et al*., 2011, Retting *et al*., 2009). BMP action is modified by a range of antagonists such as noggin and chordin which regulate BMP receptor binding. In the same way several lines of evidence indicate that Indian and sonic hedgehog modulate BMP function during pattern formation including skeletal formation in vertebrates (YamaguchiI *et al*., 2000). Moreover FGF signalling was reported to modify BMP signalling and bone formation. FGF-2 and FGF-9 increase BMP-2 and TGF-β1 expression and endogenous FGF/FGFR signalling is a positive upstream regulator of the BMP-2 gene in calvarial osteoblasts (Fakhry *et al*., 2005),

potentially through the upregulation of Runx2 (Choi *et al*., 2005). Furthermore, FGF-2 and BMP-2 have a synergistic effect on fracture healing: FGF-2 has a critical function at early stages while BMP-2 promotes mineralization at later stages (Hughes-Fulford and Li, 2011). FGF-2 null mice have impaired nuclear accumulation of Runx2 and hindered BMP-2 induced bone formation and ALP activity (Naganawa *et al*., 2008).

Wnt signalling also influences osteoblast proliferation, function and survival. Wnts are secreted, lipid-modified glycoproteins that activate cell surface receptor-mediated signal transduction pathways to regulate cell fate, proliferation, migration, polarity, and gene expression (Moon *et al*., 2002). Wnts activate at least three distinct intracellular signalling cascades: the Wnt-β-catenin pathway, the Wnt-Ca^{2+} pathways or the Wnt-planar polarity pathway. The Wnt-β-catenin pathway is commonly referred to as the canonical pathway. It promotes cell fate determination, proliferation and survival by increasing β-catenin levels and altering gene expression through lymphoid enhancer factor-T cell enhancer factor (Lef-Tcf) transcription factors (Behrens *et al*., 1996). In addition β-catenin, a factor that mediates the canonical WNT signalling pathway, also regulates low-density lipoprotein receptor-related protein 5 (LRP5), a WNT co-receptor that regulates bone formation (Mundy, 2002). In addition to promoting osteoblast maturation Wnts play a role in lineage determination of mesenchymal precursor cells (Westendorf *et al*., 2004).

1.6.2 Osteoblastic transcription factors

Runx2 has been identified as a master transcription factor for skeletal formation and mineralisation by promoting early stages of osteoblast differentiation (Kern *et al*., 2001). *Runx* expression is first detected in preosteoblasts, upregulated in immature osteoblasts but downregulated in mature osteoblasts. It triggers the expression of major bone matrix genes such as OCN, type I collagen and OPN (Ducy *et al*., 1997) during the

early stages of osteoblast differentiation but is not essential for the maintenance of these genes in mature osteoblasts (Komori, 2010). Runx2 is expressed exclusively in mineralized tissues and its expression precedes osteoblast differentiation and OCN expression by several days (Ducy *et al*., 1997). It promotes osteoblast maturation by supporting exit from the cell cycle and activating genes that facilitate osteoblast differentiation, indicating that Runx2 has a dual biological role in the osteogenic lineage attenuating proliferation and promoting maturation (Pratap *et al*., 2003). However, *Runx2* expression in distinct proliferating mesenchymal cell types does not necessarily result in activation of mature bone phenotypic markers raising the question of whether *Runx2* has a regulatory function in proliferating osteoblasts before osteoblast maturation (Stricker *et al*., 2002).

Osteoblasts must synthesise collagenous extracellular matrix (ECM) before they will differentiate and express osteoblast-related genes such as OCN, BSP and ALP and ultimately mineralize (Franceschi, 1999). This requires Runx2 to cooperate with several other transcription factors to enable its action on osteoblast differentiation. Some of these factors provide co-stimulatory signals while others repress Runx2 activity by affecting DNA binding activity or transactivation potential. One of these is osx which is a zinc finger-containing transcription factor required for the differentiation of preosteoblasts into mature osteoblasts (Cao and Chen, 2005, Nakashima *et al*., 2002). Osx is expressed in osteoblasts of all endochondral and membranous bones and is necessary but not sufficient for hMSC osteoblast differentiation. In the absence of osx, no cortical or trabecular bone forms through intramembranous or endochondral ossification. Osx acts downstream of Runx2 because osx null preosteoblasts express chondrocyte marker genes, suggesting that Runx2-expressing preosteoblasts are still bi-potential (Nakashima *et al*., 2002). Osx regulates the expression of a set of ECM proteins, which are involved in terminal osteoblast differentiation (Zhou, 2011). Osx

was found to form a complex with NFAT, promoting osteoblastic bone formation through the activation of COLIA1 promoter activity (Koga *et al*., 2005). Osx expression is induced by a range of factors know to promote bone formation including BMP2 and BMP6 (Friedman *et al*., 2006). Matsubara et al found that Smad signaling is required for induction of osx and that expression is regulated by Runx2-dependent and independent mechanisms via BMP2 signaling (Matsubara *et al*., 2008).

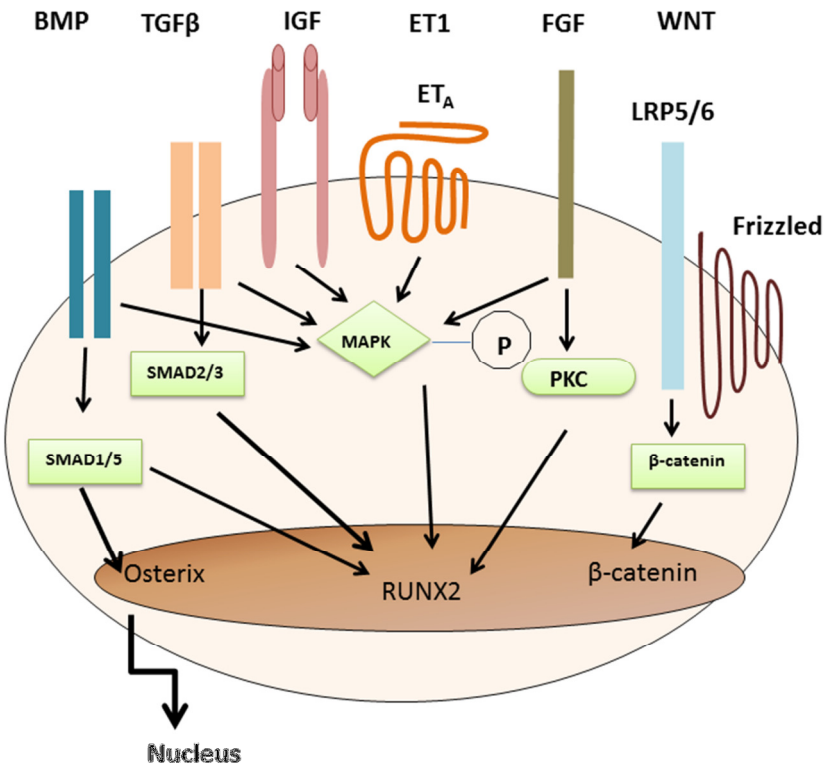


Figure 1.7 This diagram shows the signal transduction pathways that regulate osteoblast function. Bone morphogenetic protein (BMP) binds to its receptor and induces the formation of a complex in which the type II BMP receptor phosphorylates and activates the type I BMP receptor. RUNX2 and osterix (two transcription factors that control osteogenesis) are upregulated by phosphorylation of the SMAD proteins. BMP2 was also shown to activate p38 mitogen-activated protein kinase (MAPK), leading to an increase in RUNX2 transcription. In the same way, TGFβ regulates RUNX2 transcription by phosphorylating SMAD2 and SMAD3 also by activating p38 MAPK. On the other hand, the WNT proteins interact with WNT receptor frizzled and co-receptor LRP5 or LRP6 to activate a signalling pathway that stabilizes cytoplasmic β-catenin. Diagram is based on Logothetis and Lin (2005).

1.7 Bone formation

There are two types of bone formation, the first, intramembranous, occurs in parts of the flat bone of the skull and clavicle. Intramembranous formation beings with the condensation of mesenchymal cells which differentiate directly into osteoblasts that produce a mineralised extracellular matrix. The structure of the bony element formed is determined genetically and does not require mechanical inputs. The second type of formation, endochondral ossification, occurs in long bones such as the femur. During endochondral bone formation a cartilaginous template is generated by chondrocytes, this is subsequently replaced by true bone through the action of chondroclasts and osteoblasts (Mundy, 1999).

The formation of bone by either endochondral or intramembranous ossification involves two principle steps, firstly, production of extracellular organic matrix (osteoid), then mineralization of the matrix to form bone. Osteoid is primarily composed of type I collagen fibres which give bone tensile strength and also provides a backbone for the deposition of bone mineral (Hollinger *et al*., 2004). The production of type I collagen in itself may provide a stimulus for later events during bone formation. Previous studies demonstrated that osteosarcoma cells, calvarial osteoblasts and preosteoblasts show increased expression of ALP and other markers of the osteoblastic phenotype when plated on type I collagen (Aronow *et al*., 1990, Shi *et al*., 1996) and other studies have shown that ALP expression depends on type I collagen (Masi *et al*., 1992, Lynch *et al*., 1995, Celic *et al*., 1998). Furthermore, Lynch et al suggested that type I collagen was needed for the formation of mature osteoblast phenotype and mineralization of the extracellular matrix (Lynch *et al*., 1995) and showed abnormal expression and synthesis of type I collagen in subchondral osteoblasts coupled with low mineralization, which mimics the *in vivo* situation.

Non-collagenous proteins also play a key role in regulating the nucleation and maturation of mineral as well as the activity, localisation and differentiation of bone cells. These proteins are primarily secreted by osteoblasts during formation and include OPN, ON and OCN as well as cytokines such as TGF-β (Nefussi *et al*., 1997). OPN is the primary protein induced by cellular transformation and it is produced at late stages of osteoblastic maturation during matrix formation. In osteoclasts it has been shown to induce intracellular signalling pathways (Sodek *et al*., 2000). ON, an acidic glycoprotein, plays an essential role as a regulator of bone remodelling. It is essential for the maintenance of bone mass and for balancing bone formation and resorption. ON also promotes osteoblast differentiation and cell survival (Kapinas *et al*., 2009). OCN is an extracellular matrix protein produced by osteoblasts; it has a role in the early stages of bone healing and has been shown to stimulate adhesion of osteoblast-like cells (Ingram *et al*., 1993). It was first discovered as a calcium binding protein which can undergo vitamin K dependent γ-carboxylation. The γ-carboxylated form binds hydroxyapatite and is abundant in bone extracellular matrix. OCN is expressed by osteoblasts, odontoblasts, and hypertrophic chondrocytes at the onset of tissue mineralization and accumulates in the bone extracellular matrix (Hauschka and Wians, 2005). OCN avidly binds hydroxyapatite via 3-carboxylated glutamic acid residues and was originally thought to play a role as a regulator of mineral nucleation (Shanahan *et al*., 2000). However, Lain et al implicated OCN as a potential inhibitor of bone formation and a role for OCN in bone resorption is strongly supported by a series of *in vivo* and *in vitro* studies relating OCN in bone to osteoclast recruitment and activity (Lain *et al*., 1984). Therefore, OCN can no longer be considered a passive marker of mineralization. OCN deficiency has long been associated with increased bone formation (Price, 1989). Young et al suggested that osteocalcin expression is restricted to growing bone implicating its role in the modulation of mineralization (Young *et al*., 1992). Ducy

et al indicated that OCN deficient transgenic mice have increased bone formation without impairing bone resorption or affecting mineral content, suggesting OCN prevents excessive bone formation (Ducy *et al*., 1996).

Bone formation involves the proliferation of primitive mesenchymal cells, their differentiation into osteoblast precursors (osteoprogenitor, pre-osteoblast), formation of mature osteoblasts, formation of matrix and finally mineralization. Osteoblasts converge at the bottom of the resorption cavity and form osteoid which begins to mineralize until the cavity is filled (Hill and Orth, 1998). During mineralisation matrix vesicles are exocytosed from osteoblasts into sites where calcification will occur. Mineral crystals then begin to form within the vesicles which are augmented by ALP and pyrophosphatase and calcium binding proteins such as annexin I. The next phase of the process is when the crystals are released from the vesicles exposing them to the extracellular fluid. This contains high concentrations of Ca^{2+} and PO_4 allowing the crystals to act as nuclei around which further crystals can develop by homologous nucleation. Mineralisation is further controlled by interactions with the inorganic components of the matrix which act as initiators or inhibitors of nucleation thereby assuring that crystal growth is spatially and temporally appropriate (Balcerzak, 2003). Mineralisation is assisted by the release of osteoblastic ALP. In both bone and calcifying cartilage ALP is expressed early in development and is soon observed on the cell surface and in matrix vesicles. The principal regulatory pathways controlling ALP expression is the BMP/RUNX2/ osterix system and the WNT signalling cascade, which also interact with each other (Gaur et al., 2005). Gong et al demonstrated that a number of Wnt proteins are capable of inducing ALP in mesenchymal cells (Gong *et al*., 2001). Rawadi et al shows that Wnt/LRP5 controls the expression of ALP through the canonical β-catenin cascade and that the capacity of BMP-2 and Shh to induce the expression of

ALP in mesenchymal cells is dependent on the integrity of this pathway (Rawadi *et al*., 2003).

1.8 Hyperbaric Oxygen Therapy

Hyperbaric oxygen therapy (HBO) is the breathing of pure oxygen in a sealed chamber that has been pressurised at 1.5 to 3 times normal atmospheric pressure (London *et al*., 1993). This differs from normobaric oxygen therapy (NBO), where raised oxygen levels are administered at one atmosphere (Singhal *et al*., 2005). The use of NBO as a simple first aid tool for decompression sickness has been advocated for a long time (Balestra *et al*., 2004) but is less effective than HBO (Beynon *et al*., 2007). The use of HBO as a medical therapy has a long history; in 1662 Henshaw, a British clergyman, first suggested that a rise in the ambient pressure around a patient may have therapeutic benefits, since this time, reports of beneficial effects from increased pressure have increased (Kindwall, 2002). Subsequently in 1877 a French surgeon called Fontaine developed the first mobile hyperbaric operating room, and at this time hyperbaric chambers were available in all large European cities. In the early 1900s Dr Orville Cunningham, a professor of anaesthesia at the University of Kansas, constructed the largest hyperbaric chamber in the world and used hyperbaric oxygen to treat various altered health states (Jain 2004). Modern clinical use of hyperbaric oxygen began in 1955 with Churchhill-Davis who helped to attenuate the effects of radiation treatment in cancer patients using high oxygen environments.

There are two types of hyperbaric chamber design. The first type is multiplace chambers that allow two or more people to be treated simultaneously. Multiplace chambers are usually metallic and are always pressurized with air, 100% oxygen being delivered to patients via an oronasal mask, head tent or endotracheal tube. The second type is monoplace chambers, which are designed to treat a single patient and are pressurized with oxygen (Grim *et al*., 1990).

HBO can be employed as an intervention in a number of different disorders where body tissues have suffered from a decrease in oxygen levels leading to hypoxia due to a range

of causative factors (Kindwall, 2002, Albert 2008). These include decompression illness, severe carbon monoxide poisoning, smoke inhalation, treatment of chronic wounds and some infections, wound healing after reconstructive surgery, radiation necrosis, acute blood loss where a blood transfusion is not possible, relief of symptoms in multiple sclerosis, sports injuries, diabetic foot ulcer and refractory osteomyelitis. HBO has also been used as an adjuvant therapy for the treatment of skeletal disorders such as osteonecrosis of the jaw, osteomyelitis and complicated fractures (Bartlett, 2000, Bennett *et al*., 2005). While positive effects of HBO on patient outcomes have been noted the cellular and molecular mechanism through which HBO may affect remodelling is poorly understood.

1.8.1 Mechanisms of action

HBO increases oxygen transfer to tissues by two mechanisms, first increasing the saturation of haemoglobin and secondly and potentially more importantly increasing the concentration of oxygen dissolved in plasma. Hyperbaric oxygen therapy increases the haemoglobin saturation with oxygen from 97% to 100%. HBO also increases plasma oxygen saturation (London *et al*., 1993). Arterial oxygen tension is raised six fold when breathing 100% oxygen at 1 ATA, 14 fold under 2 ATA and 22 fold when under 3 ATA. Tissue oxygen tension rises to a maximum of 500 mmHg at 3 ATA, and increases the oxygen delivery up to 60 ml per litre of blood. This is enough to meet the basic metabolic tissue needs of reparative tissues in the human body without the contribution of haemoglobin (Tibbles and Edelsberg 1996, Evans, 2002). Normoxia, the concentration necessary to maintain aerobic metabolism and homeostasis in the body, is around 15%–21% O_2. Oxygen tensions outside this normal range are defined as follows: anoxic (less than 0.01% O_2), hypoxia (12% O_2 or less), hyperoxia (45%–100% O_2), and hyperbaric oxygen (any O_2 tension greater than 1 atmosphere absolute pressure or 760 mm Hg) (Widiyanti, 2011).

1.8.2 Evidence supporting the use of HBO in osteonecrosis and osteomyelitis

HBO has been used to improve healing and restoration from infection in the patients (Zamboni *et al*., 2003). HBO increases oxygen transfer to tissues by two mechanisms, first increasing the saturation of haemoglobin and secondly and potentially more importantly increasing the oxygen concentration dissolved in plasma (London *et al*., 1993). The efficacy of HBO in altered health states is attributed to a range of actions including the reversal of hypoxia, changes in vascular reactivity, reduced oedema, modulation of nitric oxide and reactive oxygen species production, modification of growth factor and cytokine production and modulation of the immune response (Wang *et al*., 1995). Refractory osteomyelitis is considered a form of chronic osteomyelitis that has reoccurred after appropriate intervention; HBO has been proved effective in the treatment of osteomyelitis promoting the repair and removal of necrotic bone (Davis *et al*., 1986). In animal studies, HBO has been shown to improve both bone generation (Inoue *et al*., 2000) and the removal of dead or abnormal bone (Jones *et al*., 1991). HBO is often utilised in the treatment of chronic necrotizing infections of the skin usually as a consequence of a traumatic or surgical wound. The increase in tissue oxygen levels after HBO enhances leukocyte mediated killing of bacteria and inhibits the growth of anaerobic organisms reviewed by (Tibbles and Edelsberg 1996).

Osteonecrosis (ON) is the death of tissue due to hypoxia which has occurred as a consequence of trauma, surgery or chemo or radio therapy. Osteoradionecrosis (ORN) is relatively common in individuals who have undergone radiotherapy of the head and neck to remove a tumour and osteochemonecrosis (OCN) is increasingly seen in individuals who have received anti-resorptive bisphosphonates to treat bony metastases (Vanderpuye and Goldson 2000). This primarily affects alveolar bone of the jaw due to its higher remodelling rate and as a consequence oxygen requirement in comparison to other systemic bone sites. The incidence of this disorder varies considerably depending

on many factors such as treatment regime, dental health and follow-up time (Hansen *et al*., 2006, Wahl, 2006).

The pathogenesis of ORN and in particular bisphosphonate-induced osteonecrosis (BRONJ) is not completely understood, but in the case of ORN is thought to occur when exposure to radiation damages blood vessels and osteocytes within the bone matrix leading to reduced vascularity and bone marrow fibrosis (Khoo, 2003). Hypovascularity generates hypoxia within these regions, which in turn reduces the total number of bone cells at these sites and thereby disrupts the remodelling process (Maier *et al*., 2000). Marx described in 2003 the positive relationship between osteonecrosis and the use of bisphosphonates (Marx, 2003, Marx *et al*., 2005). Bisphosphonates are used to prevent resorption and skeletal disease in patients with osteoporosis, Paget's disease and skeletal metastases.

HBO stimulates monocyte and fibroblast function and collagen synthesis and increases vascular density (Annane *et al*., 2004). HBO is effective for the treatment of BRONJ, it increases local concentrations of reactive nitrogen species (RNS) and reactive oxygen species (ROS), which influence osteoclast differentiation and activity and regulate other critical aspects of bone metabolism. ROS enhance the expression of RANKL (Bai *et al*., 2005), changing the RANKL/osteoprotegrin ratio and favouring osteoclast differentiation (Khosla, 2001). HBO generated ROS and RNS also induce stem cell mobilization and vasculogenesis; these effects help reduce the area of poorly vascularised bone and promote remodelling of necrotic areas.

Osteomyelitis is a chronic or acute infection of bone which may remain dormant for many years (Gill and Bell, 2004). It is caused by bacterial or fungal infections and is associated with a pronounced osteolytic response. HBO has been used to treat chronic refractory osteomyelitis since 1965 (Cierny *et al*., 1985) and can play an important role in the management of osteomyelitis (Mader *et al*., 1999). HBO therapy increases tissue

oxygen tension and promotes bone and soft tissue healing in ischemic tissue (Esterhai *et al*., 1987). HBO increases the oxygen concentration in infected tissue which inhibits the growth of anaerobic organisms and enhances the influx of immune cells (Mader *et al*., 1989). The increase in oxygen tension also increases oxygen-dependent killing mechanisms of polymorphonuclear leukocytes. Hyperbaric oxygen therapy is effective and safe for chronic refractory osteomyelitis provided that patients have received appropriate medical and surgical management; this treatment modality remains encouraging for chronic osteomyelitis because of the high rate of wound complications and recurrence in this disease (Chen *et al*., 2003).

1.8.3 Effect of HBO, oxygen and pressure on bone cells

Oxygen availability plays a significant role in the regulation of bone cells. Hypoxia occurs when the blood supply to tissues is reduced or disrupted. Oxygen tension (pO_2) in arterial blood is about 95mmHg (12%), and in venous and capillary blood it is about 40mm Hg (5%), approximately a quarter of that in atmospheric air (Lewis *et al*., 1999). When the blood supply is reduced or disrupted oxygen levels fall and hypoxia can occur. The normal oxygen tension within bone tissue remains unknown. Lack of oxygen can result in a failure to generate sufficient ATP to maintain essential cellular functions, whereas excess oxygen (hyperoxia) results in the generation of damaging reactive oxygen intermediates.

Changes in oxygen tension have a rapid and opposing effect on osteoblast and osteoclast activity. Hypoxia stimulates osteoclast formation, resulting in resorption of significant amounts of bone (Arnett *et al*., 2003). Hypoxia enhances osteoclast differentiation through a direct effect on monocytic precursors (Zhao *et al*., 2011). Hypoxia has also been shown to reduce levels of IL-10, an inhibitor of osteoclast differentiation (Naldini *et al*., 1997). Exposure to hypoxia for longer than 24 hours or continuous HIF-1α expression is associated with a loss of membrane integrity and

reduced osteoclast number (Knowles and Athanasou, 2009). Utting *et al* (2006) demonstrated that hypoxia inhibits the proliferation of mature osteoblast precursors and leads to the failure of cell differentiation and formation *in vitro* (Utting *et al*., 2006). Also the exposure of mesenchymal stromal cell transplanted *in vivo* to hypoxia may affect their bone forming potential (Potier *et al*., 2007). Bone nodule formation was inhibited by hypoxia at both early and late stages (Utting *et al*., 2006).

In contrast previous studies indicate that HBO has a positive effect on osteoblast differentiation, promoting ALP expression, bone nodule formation and mineralisation *in vitro* (Wu *et al*., 2007). HBO enhances bone accruement during experimental tooth movement and mandible distraction (Muhonen *et al*., 2004, Gokce *et al*., 2008) and promotes osteoblast proliferation and reduces apoptosis *in-vivo* (Wong *et al*., 2008). Ishii *et al* (1999) reported that intermittent HBO enhances collagen synthesis and is beneficial for producing extracellular matrices in tissue engineering (Ishii *et al*., 1999). Previous studies demonstrated that HBO decreased tissue damage and induced osteodentine formation in the rat mandible after vertical osteotomy (Nilson *et al*., 1987). Freiberger 2009 demonstrated that adjunctive HBO may benefit patients with bisphosphonate associated osteonecrosis of the jaw. This may be due to changes in the distribution of mononuclear cells (Bitterman *et al*., 1993, Freiberger, 2009).

Recent studies have shown that oxygen tension regulates the formation and differentiation of various bone cells, including chondrocytes, osteoblasts and osteocytes (Hirao et al., 2006). Furthermore, while the effect of HBO on osteoclastogenesis has until now been poorly documented the importance of oxygen in osteoclast differentiation is more widely appreciated. Yamasaki *et al* (2009) demonstrated that a high oxygen tension promotes the survival of human osteoclast precursors via up-regulation of M-CSF (Yamasaki *et al*., 2009).

Another force acting on cells residing in the bone tissue is pressure. Cells in bone and connective tissue are also sensitive to variations in pressure. Rubin *et al* (1997) demonstrated that pressure is known to generate responses from bone cells and that continuous hydrostatic pressure at physiological levels decreased osteoclast formation in marrow cultures (Rubin *et al*., 1997). The effect of continuous static pressure on the differentiation of osteoblast was examined and it found that differentiation was inhibited as were collagen synthesis and ALP activity (Ozawa *et al*., 1990). Further, subtle changes in pressure accelerate the mineralization front in fetal bone rudiments (Burger *et al*., 1991).

1.8.4 Cellular mechanisms for sensing changes in oxygen

The level of hypoxia inducible factor (HIF-1α), a transcription factor responsible for the activation of hypoxia responsive genes, is rapidly modified by changes in oxygen concentration. Under normoxic conditions HIF-1α and the closely related HIF-2α, are the targets of a family of prolyl-hydroxylases, which utilize molecular oxygen and 2-oxoglutarate to selectively hydroxylate two conserved proline residues on the HIF-1α molecule (Epstein *et al*., 2001). These hydroxylated residues are then targeted by the ubiquitin ligase von Hippel-Lindau protein (pVHL), which polyubiquinates HIF targeting it to the proteasome for proteolytic destruction. During hypoxia hydroxylation of HIF is inhibited allowing it to accumulate when prolyl hydroxylase domain (PHDs) are inhibited due to the lack of O_2. This allows the accumulation of HIF heterodimers that drive the expression of oxygen sensitive genes (Maxwell *et al*., 1999). HIF-1α then diffuses into the nucleus, where it forms a complex with the HIF-1β subunit and recruits transcriptional co-activators such as p300/CBP for full transcriptional activity (Fig. 1.8). This binds to HIF responsive elements in the promoter region of a wide range of target genes required for the cellular adaptation to oxidative stress and induces their transcription. These target genes include genes involved in angiogenesis such as

vascular endothelial growth factor (VEGF), erythropoiesis, glucose metabolism, apoptosis, cell proliferation and survival, pH regulation and proteolysis (Carroll and Ashcroft, 2005).

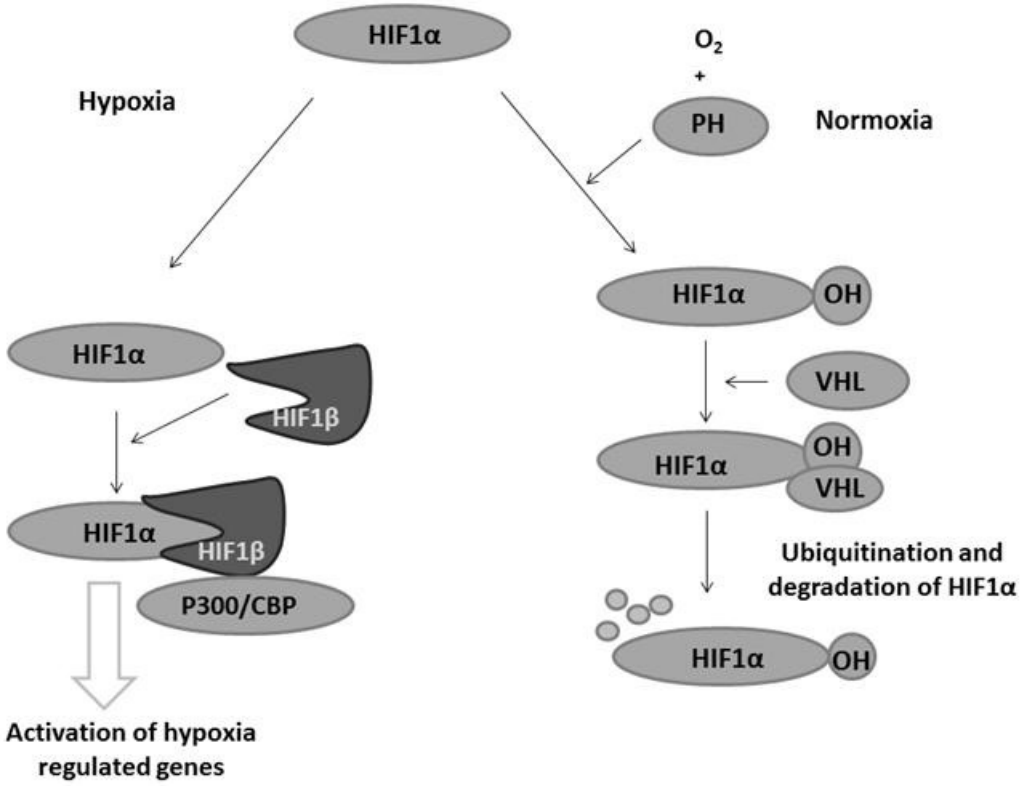


Figure 1.8 HIF-1α regulations by proline hydroxylation. In normoxic conditions HIF-1α is hydroxylated, polyubiquitinated, recognised by the proteasome and subsequently degraded. In hypoxic conditions proline hydroxylation is inhibited and thus HIF-1α is stabilised and accumulates. It translocate to the nucleus where it forms a complex and initiates transcription of oxygen responsive genes. This figure is based on Ke and Costa (2006) and Hoeben *et al*. (2004).

1.9 Aims and objectives

While it is clear that HBO has skeletal effects, the cellular and molecular mechanisms mediating this action are not well understood. Importantly the effect of HBO on osteoclastogenesis in human cells has not been studied. Similarly the mechanisms by which human osteoclasts sense changes in O_2 have not been ascertained. Thus, to increase our knowledge relating to HBO and bone remodelling the current study aimed to try to understand the effect of HBO on osteoclast formation and bone resorption, and osteoblast formation to assess if one mechanism through which HBO helps skeletal healing is by an inhibition of osteoclastogenesis and stimulation of osteoblast differentiation.

Objectives:

1- to determine the molecular action of HBO on osteoclast differentiation, to evaluate the effects of HBO, hyperoxia and elevated pressure on RANKL-induced osteoclast differentiation and bone resorption using RAW264.7 and human peripheral blood mononuclear cells (PBMC);

2- to examine the effect of HBO on *ex vivo* osteoclast formation from peripheral blood monocytes obtained from patients undergoing HBO; and

3- to evaluate the direct effect of HBO on osteoblast differentiation and bone nodule formation, and also to examine the effect of HBO, pressure and hyperoxia on proliferation, expression of key regulators and markers of osteoblast differentiation, and mineralised nodule formation such as Runx-2 and type I collagen; compared to normoxic and hypoxic conditions.

Hypotheses:

HBO will suppress osteoclast formation from circulating monocyte, and increase osteoblast differentiation.

HBO will suppress the expression of key genes required for osteoclast formation and increase genes required for osteoblast formation.

Chapter two: General materials and methods

2. Materials and methods

2.1 Media and reagents

Recombinant mouse RANKL, human RANKL and human M-CSF were purchased from Insight Biotechnology (Insight Biotechnology, Wembley, and London, UK). Ampicillin, streptomycin, DMEM, α-MEM culture medium and trypsin were obtained from Sigma Aldrich (Poole, Dorset, UK). All plastics, flasks and tubes were purchased from Fisher Scientific (Nunc, UK). The Cell Titer 96 AQueous non-reactive cell proliferation assay kit was purchased from Promega (Promega, Madison, USA). Antibodies were obtained from Abcam (Cambridge, UK). Hyperbaric oxygen chambers were obtained from the Diving Diseases Research Centre (DDRC), Plymouth, UK. Cylinders containing custom mixtures of O_2, CO_2 and N_2 were purchased from DDRC.

2.2 Cell culture

Monocytic RAW264.7 cells were purchased from LGC standards (Teddington, London, UK) and human osteogenic sarcoma cells (Saos-2) were purchased from the European Collection of Cell Culture (ECCAC, UK). All experiments were performed in culture medium supplemented with 10% charcoal stripped fetal calf serum (Autogen Bioclear, UK), 2 mmol/l glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin all from Sigma Aldrich (Poole, Dorset, UK). Unless otherwise stated, incubations were performed at 37 °C in a humidified 5% CO_2 incubator. Cultures were fed every 2-3 days by replacing half of the medium with fresh medium and cytokines.

2.3 Cryopreservation of cells

Cells were cryopreserved and then used for experiments as required. Subconfluent cells were removed from culture vessels using trypsin-EDTA; 0.5% trypsin containing 0.5 mM EDTA, at a dilution of 1:5, was added for 5 min at 37 °C with gentle agitation to help the cells detach. Following this, 5 ml of medium was added, and the mixture was centrifuged at 130 ×*g* for 5 min and resuspended in a solution of (v/v) 90% serum and 10% DMSO. Cells were then stored at -80 °C in cryovials.

2.4 Isolation and culture of peripheral blood monocytes

Peripheral blood was obtained from healthy volunteers by venipuncture, using heparin to prevent coagulation in accordance with approved ethical guidelines. The blood was collected by a research nurse at the DDRC. Blood was diluted 1:1 in un-supplemented media (α-MEM). Mononuclear cells were isolated by centrifuging 15 ml of αMEM blood suspension over 25 ml of Histopaque-1077 (Sigma-Aldrich, UK), at 700 ×*g* for 30 min at 4 °C. The buffy layer containing monocytes was removed and washed in 10 ml of non-supplemented α-MEM then centrifuged at 400 ×*g* for 10 min at 4 °C (Fig. 2.1). The cell pellet was resuspended in culture medium containing 10% FCS and red cells lysed using a 10% acetic acid solution. Peripheral Blood Monocyte cells (PBMC) were counted in the resulting suspension using a haemocytometer.

For the induction of osteoclast formation, 1×10^4 PBMCs were cultured in 96 well plates containing α-MEM supplemented with 50 ng/ml M-CSF and 30 ng/ml RANKL. For the assessment of bone resorption slices of devitalised cortical bovine bone were placed into the wells prior to the addition of cells. Normoxic experiments were performed by incubating plates at 37 °C in a humidified atmosphere of 5% CO_2 when cultures were not receiving experimental treatments. During hypoxic experiments plates were incubated in humidified air-tight chambers prepared at the DDRC at a temperature of 37 °C, which were flushed on a daily basis after experimental treatments with a gas

mixture consisting of 2% O_2, 93% N_2 and 5% CO_2. All cultures were fed every two-three days by replacing half the medium with fresh medium and cytokines. Experiments were stopped after twelve days to assess osteoclast differentiation by staining for tartrate-resistant acid phosphatase (TRAP) and twenty days for bone resorption.

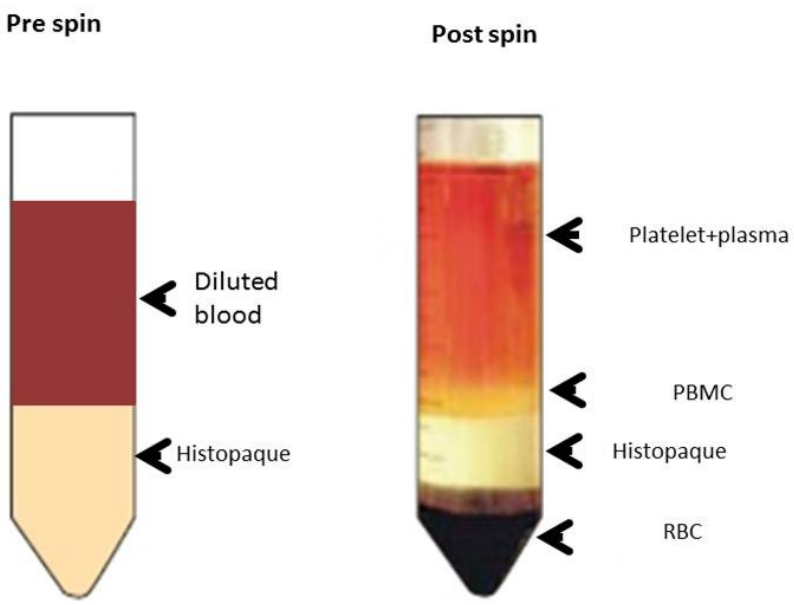


Figure 2.1 Schematic view of monocyte separation. Samples layering before and after Histopaque-1077 density gradient centrifugation. This figure is based from www.biocompare.com.

2.5 Assessment of osteoclast differentiation

Osteoclast formation was assessed by staining for the specific osteoclastic marker tartrate resistant acid phosphatase (TRAP), which was performed using an acid leukocyte (TRAP) kit (Sigma, Poole, Dorset, UK). Prior to staining cells were fixed with 10% formaldehyde/PBS for 10 min and then washed with distilled water. Cultures were then TRAP stained for one hour in a humidified chamber at 37 °C using naphthol AS-BI phosphate as a substrate and fast garnet GBS base as a chromogen. After washing in distilled water the number of TRAP-positive multinucleated cells (>3 nuclei per cell) and TRAP-positive mononucleated cells were scored at five predetermined sites of each well using an inverted light microscope (Olympus, Japan) fitted with an eyepiece graticule at with a ×40 objective. The number of TRAP positive cells per cm^2 was then calculated and a mean ± SEM recorded for each group.

2.6 Assessment of bone resorption

For the assessment of bone resorption cells were cultured on slices of devitalised cortical bovine bone. Bone slices were prepared from frozen sections of bovine femur obtained from an abattoir (Ashburton, Devon) using a low speed diamond saw (Buehler, Coventry, UK).

Slices were then cleaned by ultrasonication in distilled water for 15 min and washed with acetone for 10 min. Slices were then sterilized in ethanol at room temperature and stored dry at -20 °C until used. After appropriate incubation times cells were removed from the surface of the bone slice by immersion in sodium hypochlorite for five minutes, washed in distilled water for five minutes and stained with 1% toluidine blue solution for ten minutes. Slices were then air-dried and mounted on glass slides using double sided tape. Resorption pits appeared as darkly stained, clearly marginated areas using reflected light microscopy and resorption area was quantified across the entire

surface of the slice using an eye-piece graticule with a ×40 objective. Resorption was then calculated as the percentage of bone surface displaying pits.

2.7 Measurement of proliferation

Cell proliferation was measured using the Cell Titer 96 AQeous non-reactive cell proliferation kit. This uses mitochondrial NADH/NADPH-dependent dehydrogenase activity as a marker of cell proliferation, which converts a substrate (3-(4,5-dimethylthiazol-2-gl)-5-(3-carboxymethoxylphenyl)-2-(4-sulfhophenyl-2H) tetrazolium compound (MTS) into a soluble formazan. Measurement of the absorbance of the formazan in experimental wells and wells with known cell numbers enabled the quantification of the number of viable cells.

The assay was performed by discarding 20 µl of culture medium from each well and adding 20 µl of MTS solution. Plates were then incubated at 37 °C for one hour and then absorbance was measured at 490 nm using a 96 well plate reader (Molecular Devices, USA). The viable cell number in each well was calculated by using A_{490} of the known standards and the equation of the line $y = mx + c$.

2.8 Measurement of mineralization and alkaline phosphatase activity

Mineralization was assessed using a modification of Hale's methodology (Hale et al., 2000) by measuring calcein incorporation. Saos2 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). Culture medium was removed, and cells were washed with PBS and incubated in culture medium containing 1 mg/ml calcein for four hours at 37 ºC. Fluorescence was measured with a Cytofluor II fluorescence multi-well plate reader (Preseptive Biosystems, USA) at 485 nm excitation and 530 nm emission.

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 were then normalized to total cell number.

2.9 Molecular biology

2.9.1 RNA Extraction

Total RNA was isolated from cultures using a Sigma Genelute on-column RNA isolation kit. Cells were washed with PBS and RNA was extracted according to the manufacturer's protocol. Genomic DNA was removed with an On-column DNase-I treatment step. The purity and concentration of RNA was measured using a Nanodrop spectrophotometer (ND-1000) (Labtech, UK). To assess the resolution of samples, absorbance was measured at 260 nm and 280 nm, and anA_{260}/A_{280} ratio of 2.0-2.23was considered satisfactory). All reagents and the consumables were RNase and DNase free.

2.9.2 Reverse transcription

RNA (1 µg) was reversed transcribed to cDNA using the ImPromII Reverse Transcription System (Promega, Southampton, UK). The initial reaction consisted of 1 µg of RNA and 0.5 µM random nonamers, in a final volume of 5 µl. This was incubated at 70 °C for 5 min and then quickly chilled on ice for 5 min. The reverse transcription reaction mix was then prepared by combining the following in a thin wall PCR tube on ice: nuclease-free water (to a final volume of 15 µl); ImProm-II™ 5× Reaction Buffer, 4.0 µl; $MgCl_2$ (final concentration 1.5-8.0 mM), 1.5 µl; dNTP mix (final concentration 0.5 mM for each dNTP); recombinant RNasin inhibitor, 1.0 µl; and ImProm-II™ Reverse Transcriptase, 1.0 µl. Tubes were then placed in a GeneAmp PCR System 9700 machine and cycled under the following conditions; 10 min at room temperature followed by 37 °C for 50 min and 94 °C for 5 min. cDNAs were then stored at 4 °C until used.

2.9.3 PCR

The primers were designed using the NCBI website's primer Blast software, and purchased from Eurofins MWG Operons (Germany).

Each PCR reaction contained 2 µl of cDNA; 2.5 µl of 10× buffer; 0.5 µl of forward and reverse primer (10 µM); dNTPs (0.5 mM); and 0.2 µl Taq DNA polymerase made to a final volume of 25 µl in RNAse free water. Reaction conditions were 94 ^{o}C for 2 min followed by 40 cycles at 94 °C for 30 s, 60 °C for 30 sand 72 °C for 30 s.

Product size was confirmed using agarose gel electrophoresis. PCR samples had 2.5 µl of loading buffer (orange G and 10% glycerol) added before being loaded onto a 2% TAE agarose gel. Gels were made by dissolving agarose in an adequate volume of TAE buffer (Invitrogen, UK) which was heated in a microwave for 2-3 min, then cooled to 50 °C before being set in a gel tray with appropriate sized well combs. A 1 kb DNA ladder was used (0.5 µg/lane). For the visualization of DNA under UV light, 1 µl of ethidium bromide (10 mg/ml) was added before gels were poured. Gels were run at 70-100 V for 30 min and band size and presence checked using a UV gel documentation system (UViTech, Japan) linked to a PC (Toshiba, Japan).

2.9.4 Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was used to detect the expression levels of key regulators of osteoclast and osteoblast differentiation using the $\Delta\Delta C_T$ methodology. Cells were plated in six well plates at an appropriate density and incubated for an appropriate time with a complete change of medium every three to four days. Total RNA was then isolated using a Sigma Genelute RNA isolation kit and used in a revere transcription reaction to produce cDNA as detailed in Section 2.9.2. Real time PCR was performed on a StepOne PCR system linked to Step One v.2 software (Applied Biosystems,UK) using the DNA-binding dye SYBR green for detection of PCR products. β-actin was

used as a house keeping gene to normalise mRNA levels. 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, PCR buffer (300 nM ROX reference dye) and specific primers (0.2 µM). Reaction conditions were 94 °C for two min, followed by forty 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.

2.10 *In vitro* exposure of cells to HBO, altered pressure and oxygen conditions

For *in vitro* treatment of cells with HBO (97.9% O_2, 2.4 ATA), elevated pressure (2.4 ATA, 8.8% O_2, 2.1% CO_2, and 89.1% N_2) and hyperoxic conditions (95% O_2), cells were incubated in air-tight stainless steel chambers prepared at the DDRC (Fig. 2.2). Chambers were flushed for 4 min with relevant gas mixes and then pressurised to 2.4 atmosphere absolute (ATA) over 2 min. Cultures were exposed to HBO or pressure for ninety minutes to replicate the duration of treatment received by hyperbaric therapy patients; pressure chambers were decompressed over 8-10 min.

Figure 2.2 Miniature hyperbaric oxygen chambers. These enable high pressure 2.4 ATA and controlled oxygen exposure for cell research.

2.11 Exposure of cells to normoxia (21% O_2), hypoxia (2% O_2), and hyperoxia (95% O_2)

For hypoxia (2% O_2) normoxia (21% O_2) and hyperoxia (95% O_2) treatments cells were incubated in special air tight chambers prepared in Plymouth Hyperbaric Medical Centre in a total volume of five litres. Cells were also flushed with a gas mixture containing a 95% O_2, 5% CO_2 or 5% O_2, 5% CO_2 (balance N_2) for 2-4 min at a rate of 4 l min^{-1} in gas-tight plastic boxes (21.5 cm × 21.5 cm × 11 cm). This achieved the correct concentration inside the chamber box, based on direct measurement with an air flow meter (PCE-007, Dwyer Instruments Ltd High Wycombe, UK). Chambers were re-gassed daily as above with appropriate O_2 concentrations (Fig. 2.3).

Figure 2.3 Oxygen cylinders and boxes used in the experiments to provide normoxia, hypoxia and hyperoxia.

2.12 Statistical Analysis

Statistical analysis was performed using one way ANOVA for comparison between groups. All results are expressed as mean ± S.E.M. and significance was considered at $P < 0.05$. The analysis was performed using Statview statistical software (Abacus Concepts, California, USA).

Chapter three: The effect of hyperbaric oxygen therapy on osteoclast formation and bone resorption *in vitro*

3.1 Introduction

HBO is the breathing of pure oxygen in a sealed chamber pressurised to greater than normal atmospheric pressure (Grim *et al*., 1990). HBO is widely used as a treatment for decompression sickness and severe carbon monoxide poisoning, and is also used as an adjunctive treatment in non-healing diabetic wounds and necrotic skeletal disorders such as ORN and BRONJ. HBO induces a tenfold increase in tissue oxygen tension primarily by elevating plasma oxygen (Tibbles and Edelsberg, 1996) and this enables a rapid delivery of oxygen to areas of ischaemic tissue damage as this is erythrocyte independent. The subsequent transient increase in oxygen tension is thought to promote tissue regeneration through multiple mechanisms including changes in vascular reactivity and angiogenesis, reduced oedema, modulation of free radical production, bactericidal effects and modification of cytokine production and the immune response (Gill and Bell, 2004).

Bone cells are acutely sensitive to changes in oxygen level and hypoxia is a hallmark of many skeletal disorders associated with aberrant levels of osteoclast activity (Hiraga *et al*., 2007, Kurowska-Stolarska et al., 2009). In contrast, elevated oxygen levels promote M-CSF expression and osteoclast precursor survival (Yamasaki *et al*., 2009). In healthy bone oxygen levels range from 6-9% but this falls to 1-3% at fracture sites and within necrotic bone (Maurer *et al*., 2006). Unsurprisingly in light of the association between low oxygen level and pathological bone loss, hypoxia has been shown to augment osteoclast differentiation and bone resorption *in vitro*. Arnetts *et al.'s* (2003) studies in mouse models indicate that hypoxia enhances osteoclast formation (Arnett *et al*., 2003) with similar results reported by other investigators (Muzylak *et al*., 2006, Srinivasan and Avadhani, 2007, Knowles and Athanasou, 2008, Utting *et al*., 2010). Cells, including osteoclasts, sense changes in oxygen tension via the oxygen-dependent degradation of hypoxia-inducible transcription factors (HIFs) (Knowles and Athanasou,

2008, Majmundar *et al*., 2010, Utting *et al*., 2010). In normoxic conditions HIF-1α is rapidly hydroxylated by HIF prolyl hydroxylases (PHD). Hydroxylation allows the ubiquitin kinase von Hippel-Lindau protein (pVHL) to bind HIF-1α and target it for proteasomal degradation. Low oxygen levels limit PHD activity which enables an accumulation of HIF that drives expression of genes associated with the cellular response to low oxygen tension (Trebec-Reynolds *et al*., 2010).

The augmentative effect of hypoxia on osteoclast formation is blunted by transient exposure to elevated oxygen *in vitro* (Knowles and Athanasou, 2009). However at present there is little information regarding the effect of HBO on osteoclast differentiation and activity and it is unclear if HBO is more effective than elevated oxygen or pressure alone (Gray and Hamblen, 1976). Similarly the molecular action of HBO on osteoclast differentiation has also to be determined. To address these questions the current study evaluated the effects of HBO, hyperoxia and elevated pressure on RANKL-induced osteoclast differentiation and bone resorption from RAW264.7 and human peripheral blood mononuclear cells (PBMC).

3.2 Experimental design

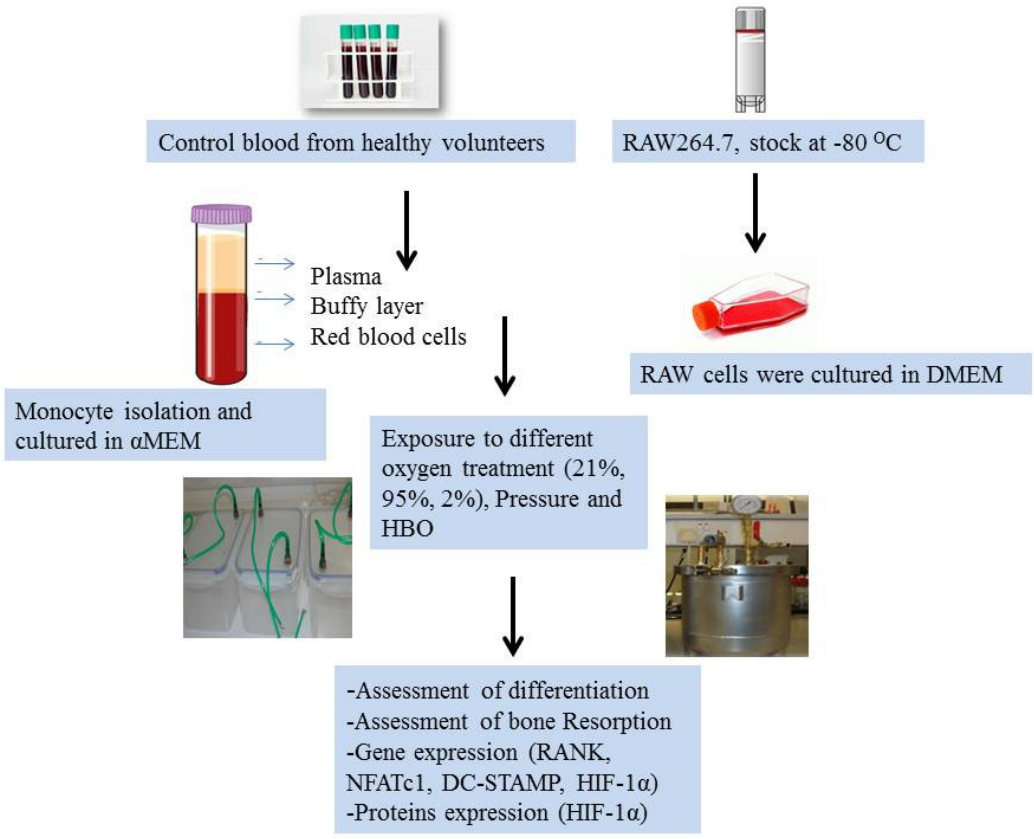


Figure 3.1 Experimental design. Monocytes, obtained from healthy volunteers, and RAW264.7 cells were treated with RANKL and M-CSF, then incubated in normoxic conditions (21% O_2, 1 ATA) or normoxic conditions, with ninety minutes per day exposure to HBO (97.9% O_2 at 2.4 ATA), hyperoxia (95% O_2, 1ATA) or elevated pressure (8.8% O_2, 2.4 ATA). For RAW 264.7 cells, experiments were stopped after four days to assess osteoclast differentiation, after eight days for resorption. For hPBMC experiments were stopped after twelve days to assess osteoclast differentiation and after twenty days for resorption.

3.2.1 RAW264.7 osteoclast formation assay

RAW 264.7 cells were cultured under standard conditions prior to the start of experiments (5% CO_2 in a humidified environment at 37 °C) 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). For the induction of osteoclast formation cells were cultured at 5×10^4 cells/well in 96-well plates and treated with RANKL (30 ng/ml). For the assessment of bone resorption cells were cultured on slices of devitalised bovine cortical bone. Normoxic experiments were performed by incubating plates at 37 °C in a humidified atmosphere of 5% CO_2 when cultures were not receiving experimental treatments. During hypoxic experiments plates were incubated in humidified airtight chambers prepared by the DDRC at a temperature of 37 °C, which were flushed on a daily basis after experimental treatments with a gas mixture consisting of 2% O_2, 93% N_2 and 5% CO_2. All cultures were fed every two-three days by replacing half the medium with fresh medium and cytokines. Experiments were stopped after four days to assess osteoclast differentiation and eight days for resorption (Fig. 3.1).

3.2.2 Isolation and culture of peripheral blood monocytes

Peripheral blood was obtained from healthy volunteers by venipuncture using heparin to prevent coagulation in accordance with approved ethical guidelines, as described in Chapter 2, Section 2.4 (Fig. 3.1).

3.2.3 Exposure of cells to normoxia (21% O_2), hypoxia (2% O_2) and hyperoxia (95% O_2)

For hypoxia (2% O_2), normoxia (21% O_2) and hyperoxia (95% O_2) treatments cells were incubated in airtight chambers, as described in Chapter 2, Section 2.11.

3.2.4 Exposure of cells to HBO and elevated pressure

For HBO (97.9% O_2, 2.1% CO_2, 2.4 ATA) and pressure control (2.4 ATA, 8.8% O_2, 2.1% CO_2, and 89.1% N_2) cells were incubated in stainless steel hyperbaric chambers. Chambers were flushed for four minutes with relevant gas mixes and then pressurised to 2.4 atmosphere absolute (ATA) over two minutes. To investigate the effect of pressure alone cells were incubated at 37 °C in 8.8% O_2, 2.1% CO_2, and 89.1% N_2 at 2.4 ATA. 8.8% O_2 is equivalent to 21% at 1 ATA. Cultures were exposed to HBO or pressure for ninety minutes to replicate the duration of treatment received by hyperbaric therapy patients as described in Chapter 2, section 2.10.

3.2.5 Assessment of osteoclast differentiation

Osteoclast formation was determined using the specific osteoclastic marker tartrate resistant acid phosphatase (TRAP) (Burstone, 1958), as described in Chapter 2, Section 2.5.

3.2.6 Assessment of bone resorption

Slices of bovine cortical bone used as substrates for osteoclastic resorption were prepared as previously described (McSheehy and Chambers, 1986) (see Chapter 2, Section 2.6). Resorption pits appeared as darkly stained, clearly marginated areas using reflected light microscopy and resorption area was quantified across the entire surface of the slice using an eye-piece graticule at a magnification of ×40.

3.2.7 Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was used to detect gene expression for key regulators of osteoclast differentiation using the $\Delta\Delta C_T$ methodology. Details are described in Chapter 2, Section 2.9.4. Primer sequences are shown in Table 3.1.

Table 3.1 Primer sequences

Name	Product size (base pairs)	Sequences
β-actin human	153	F:GCGCGGCTACAGCTTCA R:TGGCCGTCAGGCAGCTCGTA
RANK human	235	F:GGTGGTGTCTGTCAGGGCACG R:TCTCCCCCACCTCCAGGGGT
Dc-STAMP human	166	F:GTTGGCTGCCCTGCACCGAT R:TCCCTCATCCTGGGGCTGCC
NFATc1human	161	F:GGTCTCGAACACTCGCTCTGCC R:GCAGTCGGAGACTCGTCCCTGC
HIF1-α human	295	F:CAGAAATGGCCTTGT R:CAGGCTGTGTCGACTGAG

3.3 Western blotting

3.3.1 Preparation of cell lysate

Total cell lysates were obtained by lysing cells in lysis buffer (0.1% Triton X-100, 20 mM Tris-chloride, pH 7.9, 1% NP-40, 150 mM $NaCl_2$, 2.5 mM $MgCl_2$ and 5% glycerol), with 10× protease inhibitor cocktail (100µl/ml). Cells were grown in six well plates, washed with ice cold PBS on ice, scraped from wells and transferred to 1.5 ml Eppendorfs. Lysis buffer was added to the cells in the Eppendorfs and then centrifuged for five minutes at 8000 ×*g*. The supernatant was removed and analysed for protein content using the BSA assay. This solution was stored at -80 °C until used in western blot experiments.

The Bradford assay and a bovine serum albumin (BSA) standard curve (up to 2 mg/ml) were used to quantify the amount of protein in each lysate. 50 µl of each standard or cell lysate was mixed with 250 µl of Bradford reagent and the absorbance measured at 595 nm against a blank composed of 50 µl of water and 250 µl of Bradford reagent. Two identical measurements were made for each sample. Absorbance was then plotted against BSA concentration and the equation of the line ($y = mx + c$) used to calculate the amount of protein in each sample (Fig. 3.2). The protein concentration of each sample was then normalised to controls. Samples were stored at -80 °C until used.

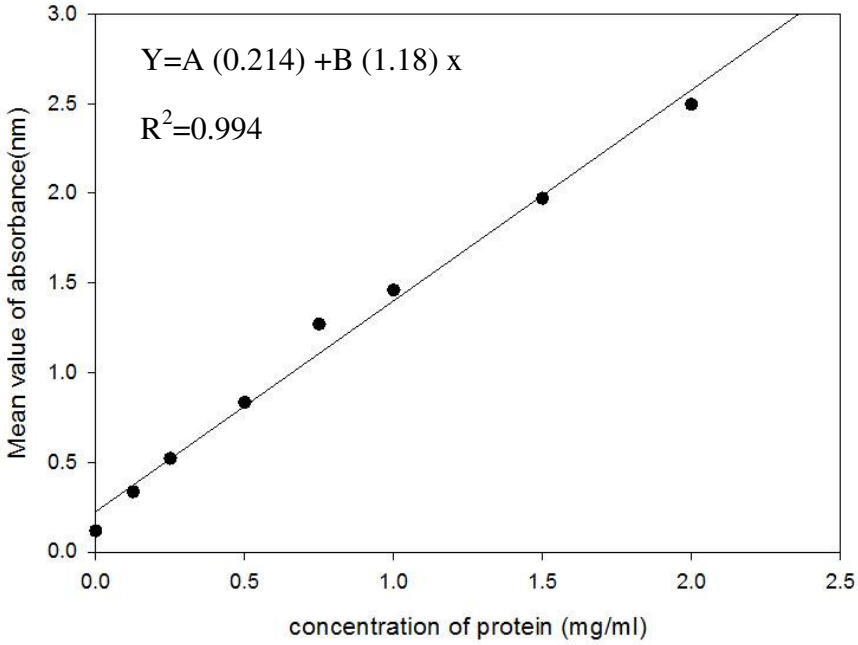


Figure 3.2 Calibration graph for protein determination

3.3.2 SDS-PAGE stand for gel electrophoresis

10% SDS PAGE gels were prepared for separation of proteins. These were prepared with 3.3 ml of 30% acrylamide/bis-acrylamide, 2.5 ml of 1.5 M Tris-chloride, 0.4% SDS, pH 8, 4.2 ml of ddH_2O and 10% ammonium persulphate (30µl) (APS). TEMED (5 µl) was then added to start the crosslinking reaction. Thegel mixture was immediately transferred into the cast with a plastic pipette and overlaid with isopropanol; to stop oxygen interfering with the polymerisation reaction, then overlaid with 150 µl H_2O.

Stacking gels were prepared by mixing 0.67 ml of 30% acrylamide/bis-acrylamide, 1.0 ml of 0.5 M Tris-chloride, 0.4% SDS, pH 6.8, 10% (30µl) APS and 5 µl of TEMED. Liquid was removed from the main gel and the stacking gel poured on top. A plastic comb consisting of 15 teeth, matching the thickness of the gel, was then inserted into the stacking gel, with care taken not to create any bubbles. The comb was left in the stacking gel as it set for 30-40 min. The gel was taken out of the casting equipment and put into the gel tank, containing 1× electrophoresis running buffer (12 g Tris base (1.3%), 57 g glycine (5.7%), 4 g (3%) SDS in 800 ml, pH 8.3). Cell lysates were removed from -80 °C and non-reducing solubilising buffer added to them (0.12 M Tris base, 4% (w/v) SDS, 20 %(w/v) glycerol, 0.004% bromophenol blue, pH 6.8). Lysates were then heated to 95 °C for 5 min, loaded into wells with 10 µl of biotinylated protein ladder and then run for 45-50 min at 200 V. Protein was transferred from SDS-PAGE gels onto a polyvinylidene fluoride PVDF membrane prepared for transfer by soaking in methanol for 30 s, dH_2O for two min and transfer buffer (0.025 M Tris base, 0.192 M glycine, 20 % methanol (v/v)) for at least five minutes. Sponges and blotting paper were also soaked in cold transfer buffer. Gels were removed from the electrophoresis tank, sandwiched in the transfer apparatus and electrophoresis performed at 100 V for 30-40 min. The membrane was then blocked with blocking buffer (500 ml PBS, 250 µl of

0.05% Tween 20, 5% (w/v) milk protein) for 30-60 min with gentle rocking followed by three washes of five minutes with (PBS, 0.05% Tween 20). Following this primary antibody was diluted according to supplier's instructions (1:1000) and incubated with the membrane overnight at 4 °C. Three washes of five minutes were then made with 5 ml of (PBS, 0.05% Tween 20) before addition of HRP conjugated secondary antibody for 45 min diluted according to supplier's instructions (1:1000). Finally three washes of five minutes were then made with 5 ml of (PBS, 0.05% Tween 20). The blot was then developed by incubating with 3 ml of Luminata Crescendo Western HRP substrate (Millipore, UK) for five minutes. Membranes were then placed in cling film and bands digitally photographed using UVP Bioimaging System. The optical density of bands was then measured using Image J software (NCBI, USA).

Blots were then stripped in buffer (1.5% glycine, 0.1% w/w SDS, and 1% v/v Tween 20, pH 2.2) for one hour and reprobed for GAPDH as described above to check for protein levels in lanes. The optical density of the GAPDH band was then measured using Image J and these values used to normalise the target band level by calculating relative densities.

3.4 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, California, USA).

3.5 Results

3.5.1 HBO, hyperoxia and pressure suppress osteoclast differentiation in RAW 264.7 cells

RANKL directly stimulated the formation of TRAP positive osteoclasts within four days in normoxic conditions, which similar to previous findings readily formed resorption pits on bone slices (Figs 3.3 and 3.4). Exposure to HBO, pressure or hyperoxia for ninety minutes daily significantly reduced the number of TRAP positive osteoclasts compare to normoxic conditions, with HBO and pressure having a significantly greater effect compared to hyperoxia (Fig. 3.3 A). Osteoclast formation in HBO, hyperoxia and pressure treated cultures was 0.51, 0.75 and 0.50 of normoxic control. Osteoclast activity was also significantly reduced to a similar extent by all treatments. The percentage of bone surface displaying resorption pits was 0.36, 0.38 and 0.44 of control in HBO, hyperoxia and pressure treated cultures, respectively (Fig. 3.3 B).

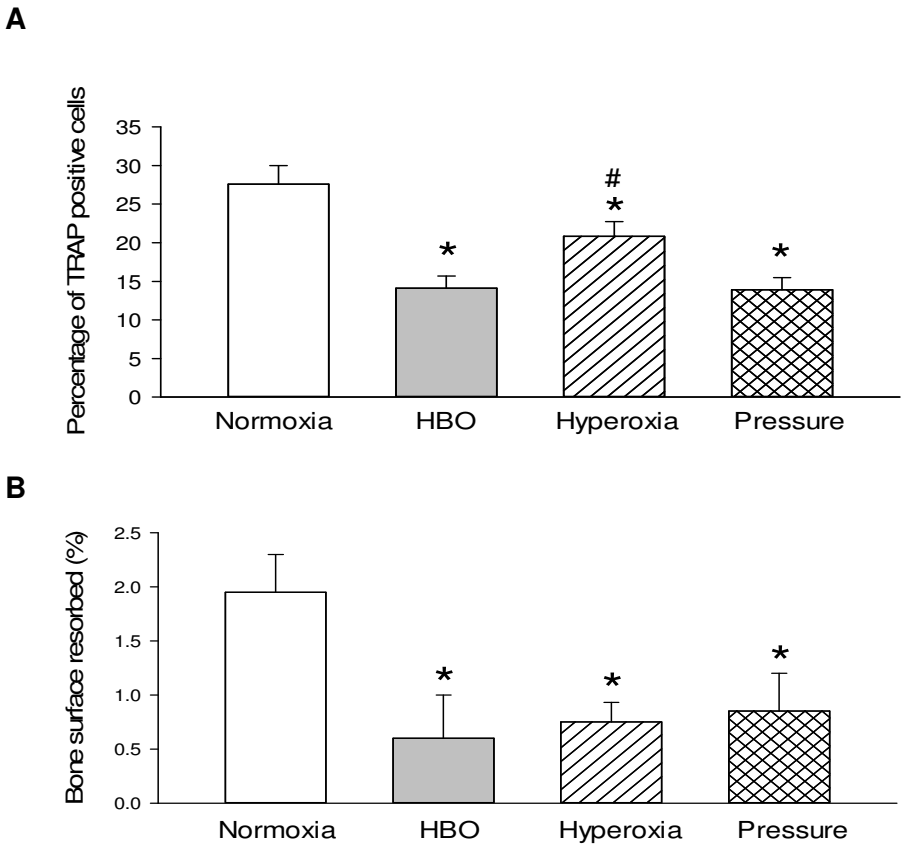


Figure 3.3 Daily exposures to HBO, hyperoxia or pressure inhibited RANKL-induced osteoclast formation and bone resorption. RAW264.7 cells were treated with RANKL (30 ng/ml) and incubated in normoxic conditions (21% O_2 1 ATA) or normoxic conditions with ninety minutes per day (4 days for TRAP, 8 days for resorption) exposure to HBO (97.9% O_2 at 2.4 ATA), hyperoxia (95% O_2, 1ATA) or elevated pressure (8.8% O_2, 2.4 ATA). Results are expressed as the mean ± SEM of three experiments. (A) Percentage of cultures expressing TRAP positivity, (B) percentage of bone surface displaying resorption pits. * $P < 0.05$ versus normoxia, # $P < 0.05$ versus HBO and pressure.

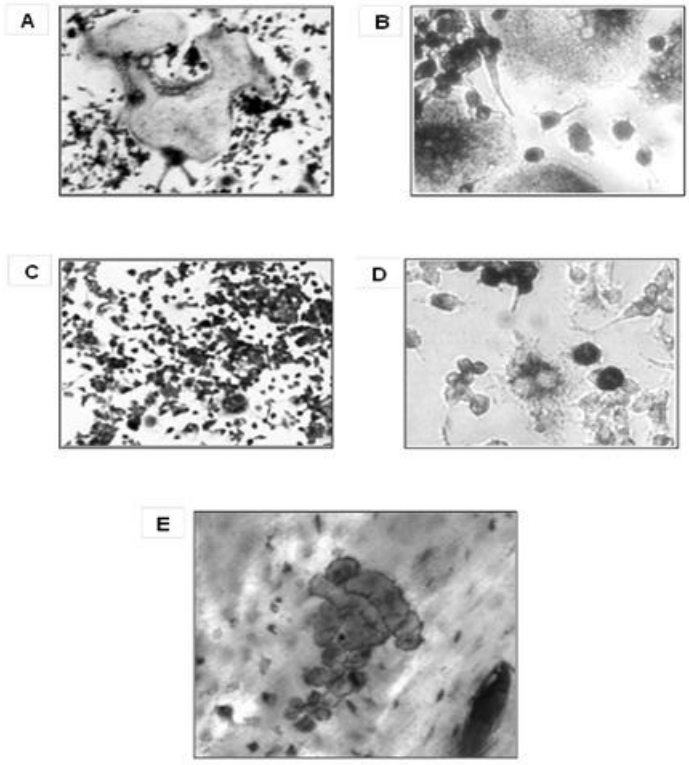
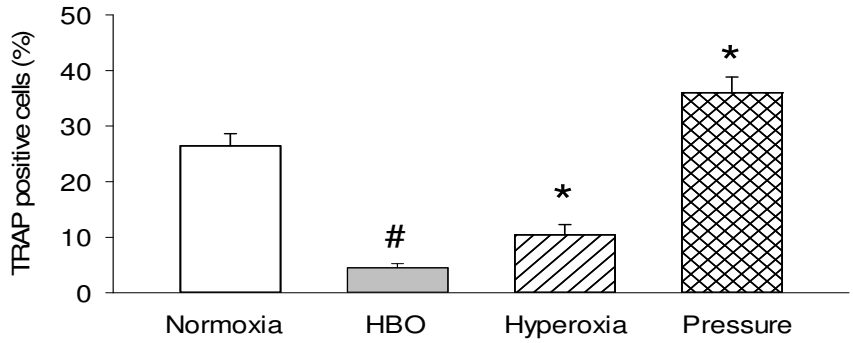


Figure 3.4.Images of TRAP stained cultures from normoxia (A-B) and HBO (C-D) treated cultures at magnifications of ×100 and ×400. Image of representative resorption pits taken by reflected light microscopy at a magnification of ×200 from a normoxic culture. Resorption pits appear as darkly stained, clearly marginated areas.

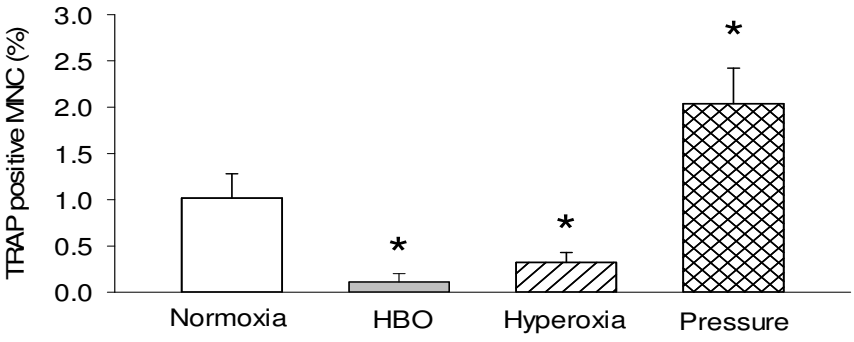
3.5.2 HBO and hyperoxia suppress osteoclast differentiation in human PBMC cultures

To determine the response of human cells PBMC were exposed to daily HBO, hyperoxia and pressure. HBO and hyperoxia significantly reduced the number of RANKL-induced TRAP positive mononuclear and multinuclear osteoclasts formed under normoxic conditions (Fig. 3.5). HBO had a significantly greater inhibitory action on osteoclast differentiation than hyperoxia or pressure alone, with pressure increasing osteoclast formation compared to the normoxic control. Mononuclear and multinuclear osteoclast formation was 0.16/0.11, 0.39/0.31 and 1.36/2.0 fold changes compared to the control in HBO, hyperoxia and pressure treated cultures, respectively. RANKL-induced bone resorption was significantly decreased by HBO and hyperoxia, with HBO having a more pronounced effect than pressure or hyperoxia alone, whereas pressure had no significant effect on osteoclast activity. The extent of bone surface resorbed in HBO, hyperoxia and pressure treated cultures was 0.4, 0.5 and 0.65 of the control.

A



B



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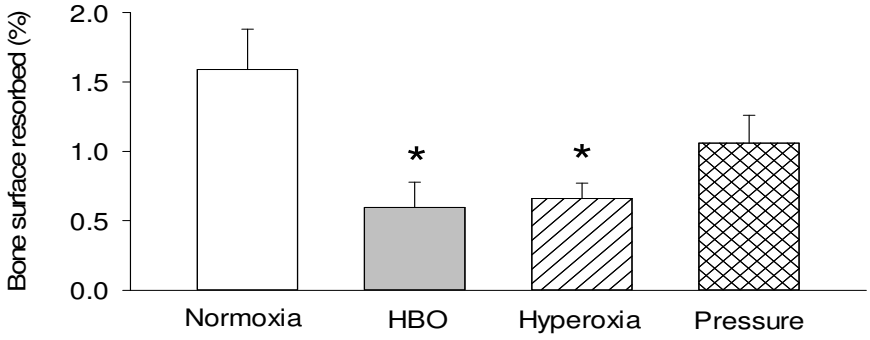


Figure 3.5 Daily exposures to HBO and hyperoxic conditions inhibit RANKL-induced osteoclast formation and bone resorption. hPBMC were treated with RANKL (30 ng/ml) and M-CSF (30 ng/ml) incubated continuously in normoxic conditions (21% O_2, 1 ATA) or normoxic conditions with a ninety minute daily exposure 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 expressed as the mean ± S.E.M. of three experiments. (A) Percentage of TRAP positive mononuclear cells within culture (B) percentage of TRAP positive multinuclear cells within culture (C) percentage of bone surface displaying resorption pits. # $P < 0.05$ versus all groups, * $P < 0.05$ versus normoxia.

3.5.3 HBO has a greater effect on osteoclast differentiation than hyperoxia or pressure alone in hypoxic conditions

Osteonecrotic bone tissue is poorly vascularised and as a consequence hypoxic. Hypoxia augments osteoclast formation which in turn leads to further osteolysis. HBO is often used as an adjunct to debridement of osteonecrotic bone; with patients receiving HBO for ninety minutes per day for 30 days before and ten days after surgery. However the effect of HBO on osteoclast formation in hypoxic conditions has not been studied, hPBMC were therefor cultured in hypoxic conditions (2% O_2) and exposed daily (8 days for TRAP and 21 days for resorption) to HBO, hyperoxia or elevated pressure for ninety minutes.

Hypoxia significantly augmented RANKL-induced osteoclast formation in PBMC cultures (Fig. 3.6). Exposure to HBO, hyperoxia or pressure for ninety minutes 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. 3.6), with osteoclast number still significantly elevated above normoxic conditions in pressure treated cultures.

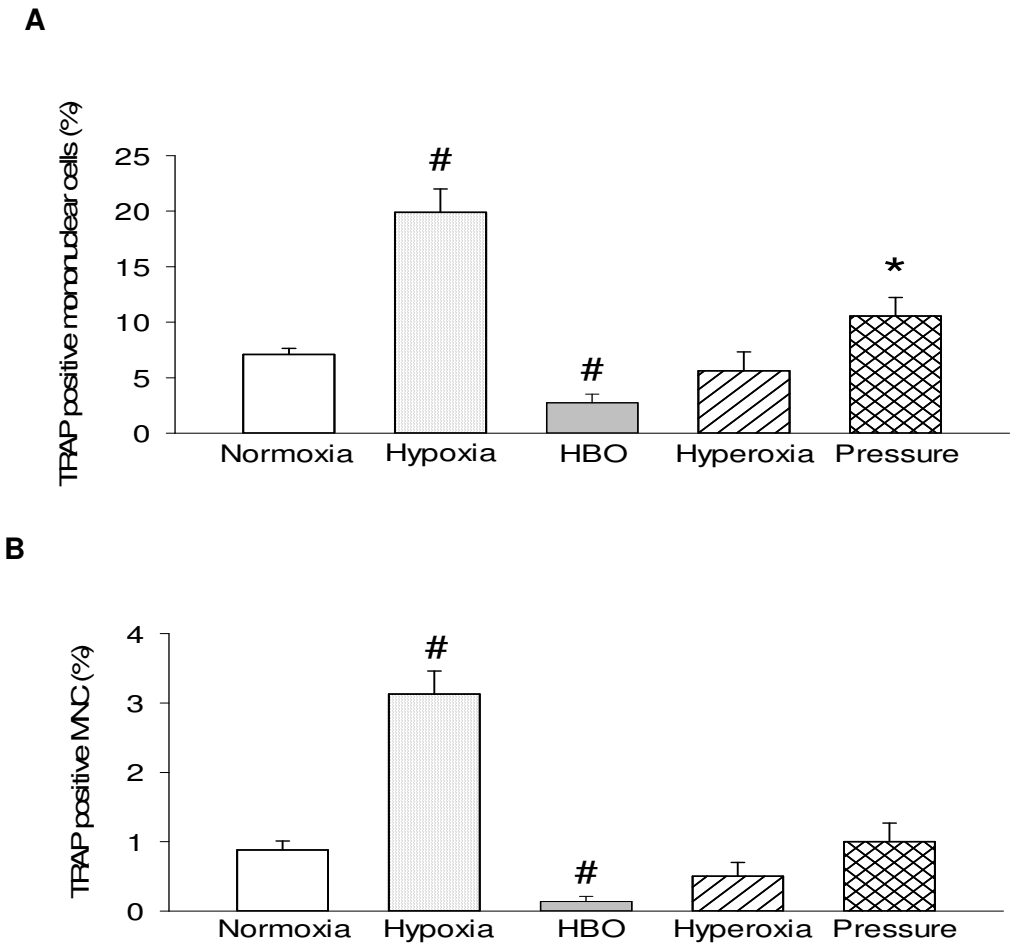


Figure 3.6 HBO has a greater suppressive effect on hypoxia-induced osteoclast formation than hyperoxia or pressure alone. PBMC were treated with RANKL (30 ng/ml) and M-CSF (30 ng/ml) and incubated continuously in hypoxic conditions (2% O_2, 1 ATA) or hypoxic conditions with a ninety minute daily exposure to HBO (97.9% oxygen at 2.4 ATA), hyperoxia (95% oxygen at 1 ATA) or elevated pressure (8.8% oxygen at 2.4 ATA). # $P < 0.05$ versus all other groups, * $P < 0.05$ versus normoxia.

3.5.4 HBO suppresses expression of key regulators of osteoclast differentiation in hypoxic conditions

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 signalling 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 through which HBO suppresses osteoclast differentiation, RANK, DC-STAMP and NFATc1 mRNA expression were examined using real time quantitative PCR. Hypoxia stimulated a significant 5.57 fold increase in RANK expression in developing osteoclasts, which was prevented by daily HBO (Table 3.1). Hypoxia also significantly augmented RANKL-induced DC-STAMP and NFATc1 mRNA expression compared to normoxic conditions (Table 3.2) 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 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-1 protein expression (Table 3.3). 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. HBO and hyperoxia, but not pressure, significantly decreased HIF mRNA expression, and HBO, but not hyperoxia or pressure significantly decreased HIF protein levels in comparison to normoxic controls (Table 3.3).

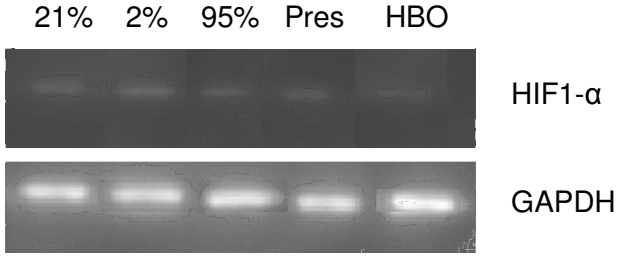
Table 3.2 HBO suppresses the stimulatory effect of hypoxia on RANKL-induced NFATc1, DC-STAMP and RANK mRNA expression.

	NFATc1	DC-STAMP	RANK
Normoxia	1	1	1
Hypoxia	197.7 ± 63.7 #	19.3 ± 9.7 #	5.57 ± 1.7 #
HBO	0.77 ± 0.15 #	0.53 ± 0.16 #	1.10 ± 0.4

Values are expressed as RQ relative to normoxic conditions using ΔΔCT, all group's expression are normalised to β-actin. Values are the means ± S.E.M. of three experiments. # $P < 0.05$ versus all other groups.

Table 3.3 Hypoxia elevates whereas HBO suppresses HIF-1α mRNA and protein expression.

	HIF-1α mRNA	HIF-1α protein
Normoxia	1	1
Hypoxia	1.33 ± 0.01 #	1.50 ± 0.33 #
HBO	0.14 ± 0.04 # *	0.59 ± 0.10 # *
Hyperoxia	0.35 ± 0.08 # *	0.75 ± 0.12 *
Pressure	0.76 ± 0.28 *	0.88 ± 0.13 *



Values are expressed as RQ relative to normoxic conditions using ΔΔCT, all group's expression are normalised to β-actin. Protein expression values are expressed relative to the optical density of normoxia bands, all group's values normalised to GAPDH expression. Values are the means ± S.E.M. of three experiments. # $P < 0.05$ versus normoxia. * $P<0.05$ versus hypoxia.

3.6 Discussion

The work in this chapter investigated the effect of HBO, pressure and hyperoxia on RANKL-induced osteoclastogenesis. The major outcomes suggest that daily exposure to HBO for ninety minutes significantly suppresses osteoclast differentiation and bone resorption in mouse and human monocytes compared to normoxic and hypoxic conditions *in vitro*. Furthermore, HBO had a greater effect on human osteoclast formation than either hyperoxia or elevated pressure alone. The exposure regime used in these experiments replicated that typically administered to patients undergoing hyperbaric therapy who receive a course of daily exposures at 2-3 ATM for ninety minutes per session. HBO is employed as an adjuvant therapy in several conditions including refractory osteomyelitis and ORN or BRONJ (Ahmed *et al*., 2009, Vescovi and Nammour, 2010, Peleg and Lopez, 2006). HBO has been shown to improve bone graft and dental implant incorporation (Sirin *et al*., 2011) and is also used to promote healing of complicated fractures with mixed outcome (Bennett *et al*., 2005). Previous studies examining the effect of HBO have primarily focused on changes in osteoblast activity. Data from these investigations indicate that HBO has a positive effect on osteoblast differentiation; promoting ALP expression, bone nodule formation and mineralisation *in vitro*. HBO has also been shown to enhance bone accruement during experimental tooth movement (Gokce *et al*., 2008) and accelerates the healing rate in rabbit and rat models of calvarial and mandibular bone regeneration *in vivo* (Jan *et al*., 2006, Muhonen *et al*., 2004). However, only Gray and Hamblen studies have examined the effect of HBO on resorption noting a decrease in ^{45}Ca release from calvarial organ cultures (Gray and Hamblen, 1976). Due to the heterogeneous nature of organ culture it is unclear if this represents a direct effect of HBO on monocyte differentiation or an indirect action mediated through osteoblast or stromal derived regulators of osteoclast formation. Data from the current studies using RAW264.7 cells, which lack stroma or

osteoblasts, suggest that monocytes themselves are directly sensitive to HBO, although this does not preclude additional osteoblast-mediated actions in mixed cell populations.

While the effect of HBO on osteoclastogenesis has until now been poorly documented the importance of oxygen in osteoclast differentiation is more widely appreciated. The partial pressure of oxygen in healthy bone is similar to that measured in other tissues (6-9%), however in diseased or necrotic bone much lower partial pressures of 0.5 to 4% have been recorded (Maurer *et al*., 2006). Osteoclast differentiation and activity is acutely sensitive to changes in tissue oxygen levels. Hypoxia enhances osteoclast differentiation through a direct effect on monocytic precursors (Zhao *et al*., 2011, Srinivasan and Avadhani, 2007) and indirectly by promoting the release of cytokines such as VEGF, IGF and IL-6 that support osteoclast formation (Arnett *et al*., 2003, Fukuoka *et al*., 2005, Knowles and Athanasou, 2008, Utting *et al*., 2010). Hypoxia has also been shown to reduce levels of IL-10, an inhibitor of osteoclast differentiation (Naldini *et al*., 1997). Prolonged hypoxia also augmented osteoclast formation in the current studies and this was prevented by daily HBO exposure. This is in keeping with the studies of Muzylak and Arnett (Arnett *et al*., 2003, Muzylak *et al*., 2006) but contrasts somewhat with Knowles and Leger where exposure to hypoxic conditions for longer than twenty four hours or continuous HIF-1α expression was associated with a loss of membrane integrity and reduced osteoclast number (Knowles and Athanasou, 2009, Leger *et al*., 2010). The reason for this discrepancy is unclear but may relate to differences in culture methods employed. The current experimental cultures and those of Arnett and Muzylak *et al* (2003) required daily re-gassing, during which short periods of higher O_2 partial pressures may have occurred, whereas Knowles and Leger's cultures were maintained at a constant 2% O_2. Knowles and Leger showed that exposing cells to normoxic conditions for one hour per day prevented the anti-osteoclastic effect of hypoxia. 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 (Bejar *et al*., 2005, Kim *et al*., 2006) and bone resorption is a characteristic of metastatic breast tumours in spite of the low partial pressure within and adjacent to tumours (Hiraga *et al*., 2007). Furthermore, oxygen levels 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 represent a more realistic model. Whatever the answer, it is clear from the current studies that HBO suppressed RANKL-induced osteoclast formation and bone resorption in cells that were otherwise maintained in normoxic and hypoxic conditions.

While HBO consistently suppressed osteoclast formation, human and murine precursors displayed a differential response to pressure under normoxic conditions. Similar to Rubin's studies using mouse marrow cultures (Rubin *et al*., 1997) elevated pressure had an anti-osteoclastic effect on RAW264.7 cells but augmented 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 a small numbers of 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 (Chen *et al*., 2007, Benson *et al*., 2003), 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.

The anti-osteoclastic effect of HBO was more pronounced than either hyperoxia or elevated pressure alone indicating that appropriate combinations of pressure and hyperoxia are the most effective therapeutic strategy. To assess the molecular

mechanism mediating the anti-osteoclastic effect of HBO this study examined expression of key mediators of RANKL-induced osteoclast differentiation. RANKL binds to its membrane bound receptor RANK on monocytic precursors, which, via TRAF 2, 3 and 6, activates downstream transcription factors that control expression of genes typical of osteoclasts such as TRAP and DC-STAMP. Pivotal to this is the c-fos and Ca^{2+} dependent activation of NFATc1 (Matsuo *et al*., 2004). RANK or NFATc1 deficient mice lack osteoclasts and are severely osteopetrotic as a consequence and it is suggested that NFATc1 alone may be sufficient to induce osteoclast formation under appropriate conditions (Matsuo *et al*., 2004). Culturing PBMC in hypoxic conditions significantly elevated RANK expression in the presence of RANKL and this may in part mediate the augmentative effect of hypoxia on osteoclast differentiation. This is in keeping with the recent studies of Tang which suggest that hypoxia-induced RANK expression in MDA-MB-231 breast cancer cells contributes to the osteolysis associated with breast metastases (Tang *et al*., 2011). Furthermore, SNPs in RANK are associated with an increased risk of developing BRONJ (Katz *et al*., 2011), suggesting a central role for aberrant RANK signalling in ischaemic bone loss. HBO may at least in part reduce osteoclast formation in compared to hypoxic conditions by preventing the elevation in RANK expression. However, HBO also suppressed osteoclast formation in compared to normoxic conditions when RANK was not elevated, suggesting that this is not the only mechanism by which HBO acts. 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 fusion protein DC-STAMP mRNA expression in HBO treated cultures. The mechanism through which HBO suppresses NFATc1 expression may relate to an effect on upstream control elements such as NF-κB or c-fos. However, it is unlikely that c-fos represents the primary target for HBO as the c-fos promoter lacks oxygen responsive elements and

HBO has been shown to have contrasting effects on c-fos expression, decreasing LPS-induced expression in the brain stems of rats (Lin and Wan, 2008) and increasing expression in endothelial cells (Godman *et al*., 2010). In contrast the NF-κB promoter contains oxygen responsive elements and its expression and activity are acutely sensitive to changes in oxygen, with hypoxia elevating and HBO suppressing NF-κB levels and activity (Walmsley *et al*., 2005, Yu *et al*., 2009).

The ability of osteoclasts to sense changes in oxygen level is dependent on the HIF pathway which regulates the cellular response to hypoxia in many tissues. Under normoxic conditions HIF proteins are rapidly degraded following hydroxylation of conserved proline residues by PHD. These hydroxylated residues are targeted by the ubiquitin ligase VHL. In ischaemic tissues PHD activity is suppressed allowing the accumulation of HIF heterodimers 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 (Knowles and Athanasou, 2008); osteoclasts lacking HIF-1α do not respond to changes in oxygen partial pressure (Knowles and Athanasou, 2008, Zhao *et al*., 2011) whereas inducers of HIF-1α stimulate resorption (Knowles et al., 2010). 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 in normoxic controls. Hyperoxia also reversed the effect of hypoxia; however it did not reduce HIF protein levels below that of normoxic conditions. In contrast, HIF mRNA and protein levels were not drastically affected by pressure which corresponds to pressure's modest effect on osteoclast differentiation. This suggests that at least in part the anti-osteoclastic action of HBO may be mediated through a reduction in HIF-1α, which would be expected to decrease expression of hypoxic responsive genes such as NF-κB and thereby limit NFATc1 expression and osteoclast formation.

HBO has a direct suppressive effect on osteoclast differentiation and activity in normoxic and hypoxic conditions. 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 low oxygen partial pressure.

Chapter four: Hyperbaric oxygen therapy suppresses the osteoclast forming ability of peripheral blood monocytes: an *ex-vivo* study

4.1 Introduction

HBO increases the partial pressure of oxygen dissolved in plasma and tissues, which reduces hypoxia and stimulates angiogenesis in hypovascular tissue (Grim *et al*., 1990). This can help in a number of pathologies where tissues have experienced decreased oxygen levels (Cohn, 1986). For instance in oral and maxillofacial surgery HBO is often used to treat osteoradionecrosis (ORN;Vudiniabola *et al*., 1999). Osteonecrosis is a complication of radiotherapy or high dose bisphosphonate therapy employed in the treatment of a range of secondary bone cancers. Irradiation decreases hard and soft tissue vascularity leading to hypoxia which then in turn promotes bone necrosis, and dysfunctional macrophage and fibroblast activity during wound healing (Marx, 1983). HBO is often used in these patients prior to reconstructive surgery to repair damaged skeletal elements. A single HBO session for ORN commonly consists of the patient breathing 100% oxygen at 2-2.4 ATA for 90-120 min; usually treatment occurs on a daily basis, five to six days per week until the required number of sessions is completed (Vudiniabola *et al*., 1999). HBO is considered a valuable adjunct for the healing of bone lesions in different anatomical regions with ischemic perfusion and also has uses in the treatment of osteomyelitis and the potential to augment bone turnover (Broussard, 2004, Freiberger, 2009)

Following on from the previous chapter in which *in vitro* application of HBO inhibited RANKL induced osteoclast formation and bone resorption in cell lines and human PBMC, the effect of HBO on osteoclast formation was further examined by establishing the effect of *in vivo* HBO on osteoclast formation and bone resorption *ex vivo*. Blood was obtained from consenting individuals undergoing HBO at the DDRC and the ability of their monocytic precursors to form bone resorptive osteoclasts prior to and following HBO therapy was assessed. The effect of HBO on the intracellular mechanisms that regulate osteoclast formation was also examined.

4.2 Expermintal setup

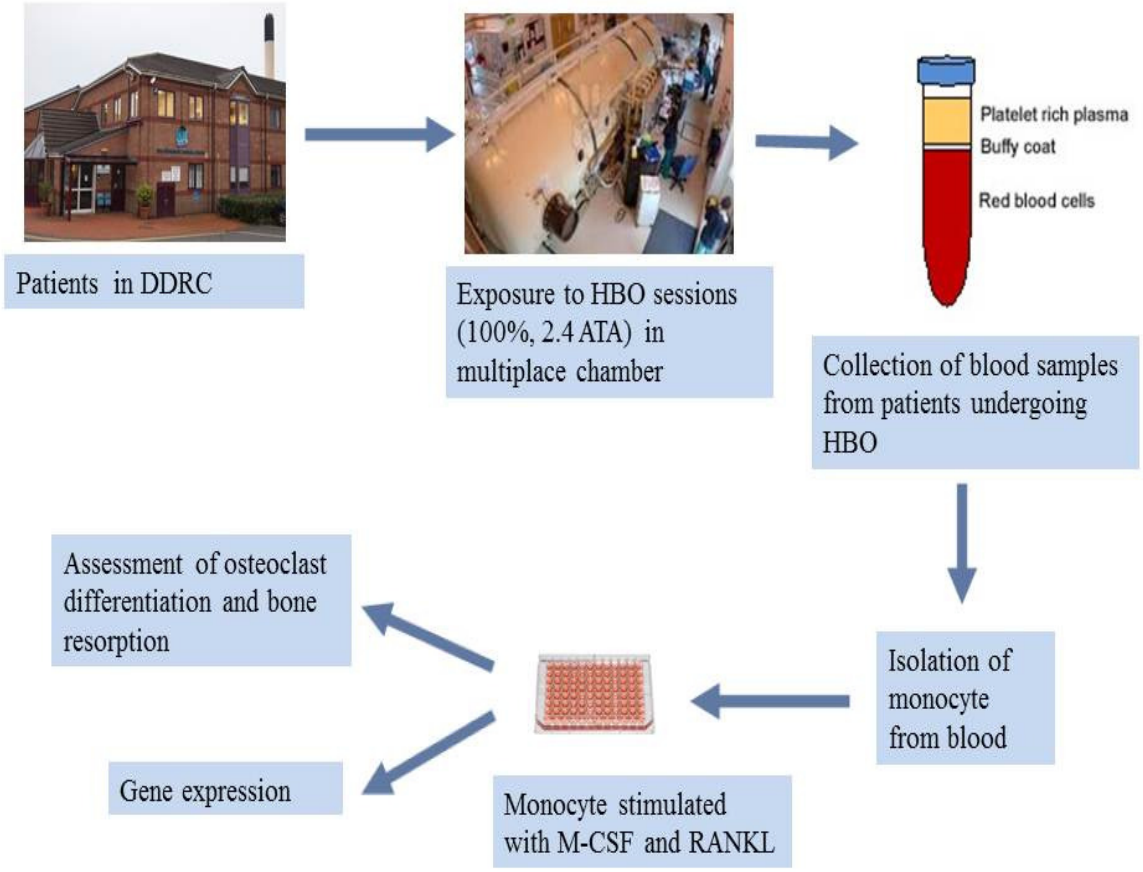


Figure 4.1 Experimental design. Six patients from DDRC were enrolled in this study. Patients were exposed to HBO treatment (1 session per day) according to a routine therapy protocol in the multiplace chamber. Blood samples were taken from each after repeated exposure to HBO (pre HBO, 10 and 25 HBO treatments).

4.2.1 Ethical Considerations and sample preparation

All studies reported in this chapter were approved by Plymouth and Cornwall NHS Ethics Committee and all subjects provided written informed consent. Blood samples were taken from consenting patients referred for HBO by their consultants to the Plymouth Hyperbaric Medical Centre (Diving Disease Research Centre, DDRC). Patients, who were over 18 and had English as first language, were considered for inclusion. Patients were given a minimum of 24 h to consider participation before being consented by the DDRC research nurse. Subjects who were considered by the clinical team to be too frail or unwell to have blood taken were not invited to participate in the study.

The clinical history of consenting individuals was ascertained by the research nurse at the DDRC. Six male patients were recruited with an age range of (49-83 years); three were receiving HBO for chronic wound healing and the other three for radiation tissue damage (Table 4.1). Patients were exposed to HBO inside a multiplace chamber. Patients were provided with a sealed breathing mask from which they received 100 % O_2 at 2.4 ATA for 90 min daily, including air breaks for 5 min to minimise the risk of oxygen toxicity. The first blood sample was taken on the same day as the first HBO session one hour before treatment. Subsequent samples were taken before the patient had their eleventh (10 treatment group) and twenty sixth treatment (25 treatment group), about 21 h after HBO the previous day. Patients were asked to donate a total of 60 ml blood collected in Vacutainers via venepuncture by the research nurse/doctor at the DDRC. Blood was collected into potassium EDTA Vacutainer tubes to prevent clotting and kept at room temperature until cell cultures were set up after 2 h.

Table 4.1 Patient information

Patient Number	Age at Time of Treatment	Sex	Reason for HBO
1	63	Male	Problem Wound (diabetic – type 1)
2	70	Male	Radiation Tissue Damage (not diabetic)
3	66	Male	Radiation Tissue Damage (not diabetic)
4	49	Male	Problem Wound (diabetic – type 2)
5	49	Male	Radiation Tissue Damage (not diabetic)
6	83	Male	Problem Wound (diabetic – type 2)

Figure 4.2 Representation of a multiplace chamber with patients undergoing HBO at DDRC

4.2.2 Isolation and culture of peripheral blood monocytes

Peripheral blood was obtained from healthy volunteers by venipuncture, using heparin to prevent coagulation in accordance with approved ethical guidelines. The blood was collected by a research nurse at the DDRC. Blood was diluted 1:1 in un-supplemented media (α-MEM). Mononuclear cells were isolated by centrifuging 15 ml of α-MEM blood suspension over 25 ml of Histopaque-1077 (Sigma-Aldrich, UK), as described in Chapter 2, Section 2.4 (Fig 4.1).

4.2.3 Assessment of osteoclast differentiation

Osteoclast formation was determined using the specific osteoclastic marker tartrate resistant acid phosphatase (TRAP), as described in Chapter 2, Section 2.5. The number of TRAP-positive and negative cells was recorded at five predetermined sites in each well using a light microscope fitted with an eye-piece graticule (Olympus, Japan) at 40× magnification. The percentage of TRAP positive cells was recorded for each well and a mean ± S.E.M. was calculated for the group.

4.2.4 Assessment of bone resorption

Slices of bovine cortical bone used as substrates for osteoclastic resorption were prepared as previously described Chapter 2, Section 2.6. After appropriate incubation times cells were removed from the surface of the bone slice by immersion in sodium hypochlorite for five minutes, washed in distilled water for five minutes and staining with 1% toluidine blue solution for ten minutes. Slices were then air-dried and mounted on glass slides. Resorption pits appeared as darkly stained, clearly marginated areas using reflected light microscopy and resorption area was quantified across the entire surface of the slice using an eye-piece graticule at a magnification of ×40.

4.2.5 Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was used to detect gene expression for key regulators of osteoclast differentiation using the $\Delta\Delta C_T$ methodology, as described in Chapter 2. The forward and reverse primer sets used for PCR are shown in Table 4.2.

Table 4.2 Primer sequences

Name	Product size	Sequences
Human β-actin	153	F:GCGCGGCTACAGCTTCA R:TGGCCGTCAGGCAGCTCGTA
Human RANK	235	F:GGTGGTGTCTGTCAGGGCACG R:TCTCCCCCACCTCCAGGGGT
Human Dc-STAMP	166	F:GTTGGCTGCCCTGCACCGAT R:TCCCTCATCCTGGGGCTGCC
Human NFATc1	161	F:GGTCTCGAACACTCGCTCTGCC R:GCAGTCGGAGACTCGTCCCTGC

4.3 Statistical Analysis

Statistical analysis was performed using one way ANOVA for comparison between groups. All results are expressed as mean ± S.E.M. and significance was considered at $P < 0.05$. The analysis was performed using Stat view statistical software (Abacus Concepts, California, USA).

4.4 Results

4.4.1 The effect of HBO on osteoclast formation and bone resorption

HBO suppressed the ability of PBMC to form RANKL-induced osteoclasts. Daily HBO treatment significantly reduced RANKL-induced TRAP positive mononuclear cell formation in a treatment number-dependent manner (Figs 4.3 and 4.4). RANKL induced a significant five-fold increase in the number of mononuclear osteoclast compared to M-CSF controls. Ten HBO sessions for ninety minutes per day significantly reduced the number of RANKL-induced TRAP positive osteoclasts that formed, causing a 50% decrease in mononuclear osteoclast number compared with pre HBO, although osteoclast number did not return to control levels. TRAP positive mononuclear osteoclast formation was, however, suppressed to control levels after 25 HBO sessions which caused an 80% decrease compared with pre HBO number (Figs 4.3 and 4.4).

Similar suppressive effects of HBO were noted on multinuclear osteoclast formation. Once more, RANKL induced a significant increase in osteoclast number compared with the pre HBO treatment group. However, in contrast to the effect on mononuclear cells 10 HBO sessions had no significant suppressive effect on RANKL-induced multinuclear osteoclast formation whereas 25 sessions significantly suppressed multinuclear osteoclast formation again returning numbers to control levels (Figs 4.4 and 4.5).

When the effects of HBO on mononuclear and multinuclear cells were assessed together, RANKL induced a significant 5 fold increase in TRAP positive osteoclast number. Ten HBO treatments significantly suppressed the number of TRAP positive cells (60% of pre-HBO), but did not decrease numbers to control levels. Osteoclast formation

following 25 HBO treatments was not significantly different from control and was significantly less than pre-HBO (20% of pre-HBO) and 10 treatments (Figs 4.4 and 4.6).

Next, this study investigated the ability of hPBMC to form resorption pits on slices of bovine cortical bone. As expected in the RANKL treated pre HBO group osteoclast produced characteristic resorption pits after 21 days, which was decreased in a treatment-dependent manner by HBO (Fig. 4.8 B-D). Similar to its effect on mononuclear osteoclast 10 HBO treatments significantly reduced bone resorption area to 20% of the area of pre-HBO cultures. Resorption was further reduced after 25 treatments being 10% of pre HBO values and not significantly different from M-CSF controls (Figs 4.7 and 4.8).

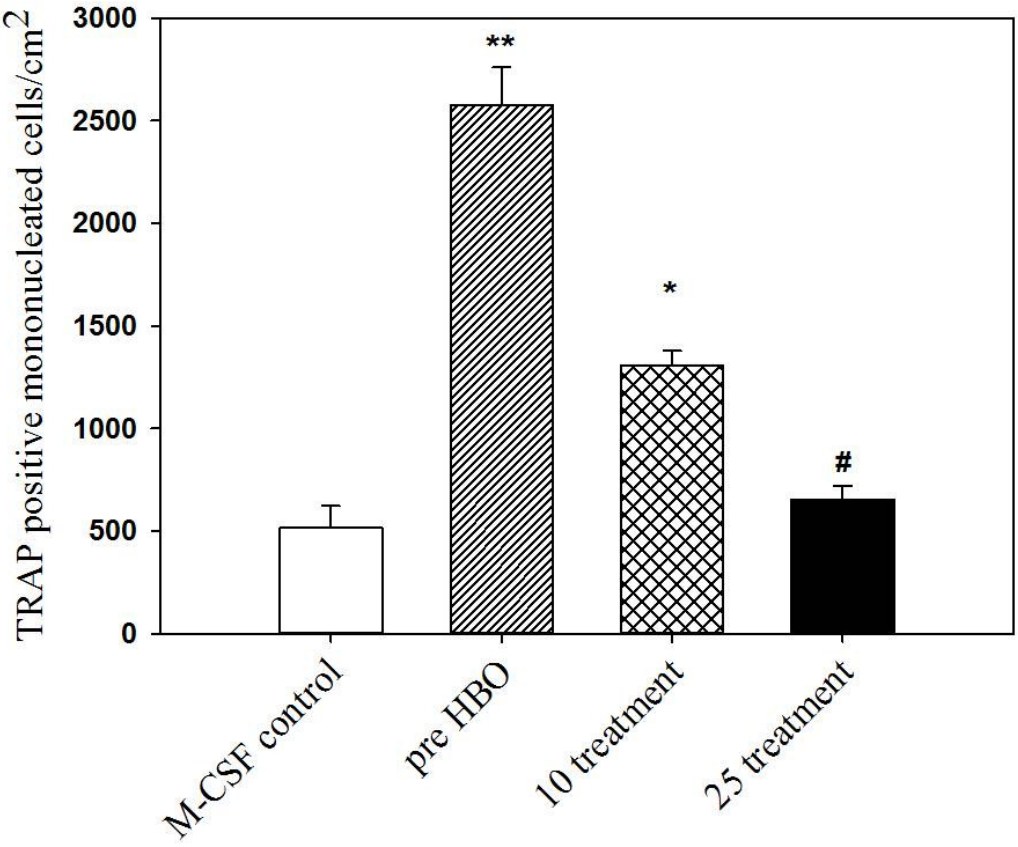


Figure 4.3 HBO (100% O_2, 2.4 ATA) supressed RANKL-induced mononucleated osteoclast formation, hPBMC were obtained from patients undergoing HBO therapy and treated with RANKL (30 ng/ml) and M-CSF (30 ng/ml) for 12 days. Data are expressed as mean ± S.E.M., n = 6. ** $P < 0.05$ compared to control, * $P < 0.05$ compared to pre HBO, $^{\#}$ $P < 0.05$ compared to 10 treatments.

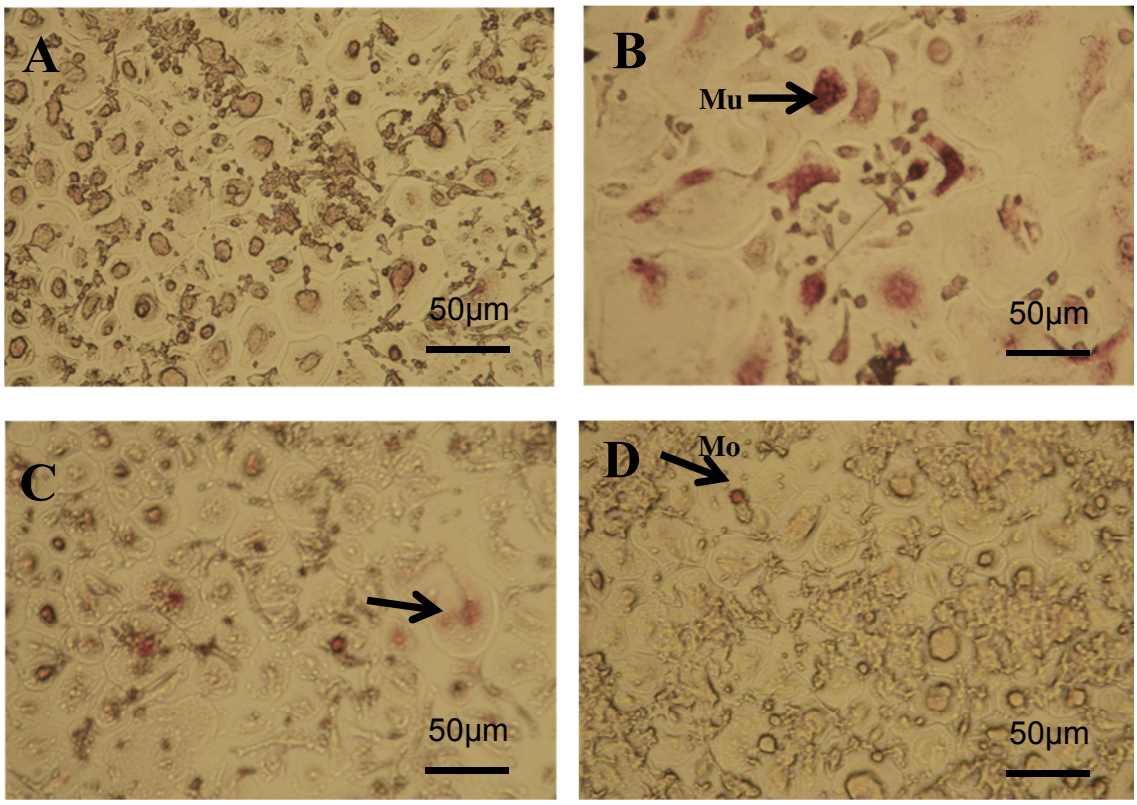


Figure 4.4 The effect of HBO (100% O_2, 2.4 ATA) treatment on hPBMC treated with RANKL (30 ng/ml) and M-CSF (30 ng/ml) on TRAP staining on day 12 of culture (magnification ×40). Samples were obtained from patients undergoing HBO at the DDRC. Digital images taken from inverted microscope (A) M-CSF control (B) osteoclast formation pre HBO (C) osteoclast formation after 10 HBO treatments (D) osteoclast formation after 25 HBO treatments. TRAP positive mononuclear (Mo) and multinuclear (Mu) cells by arrowheads.

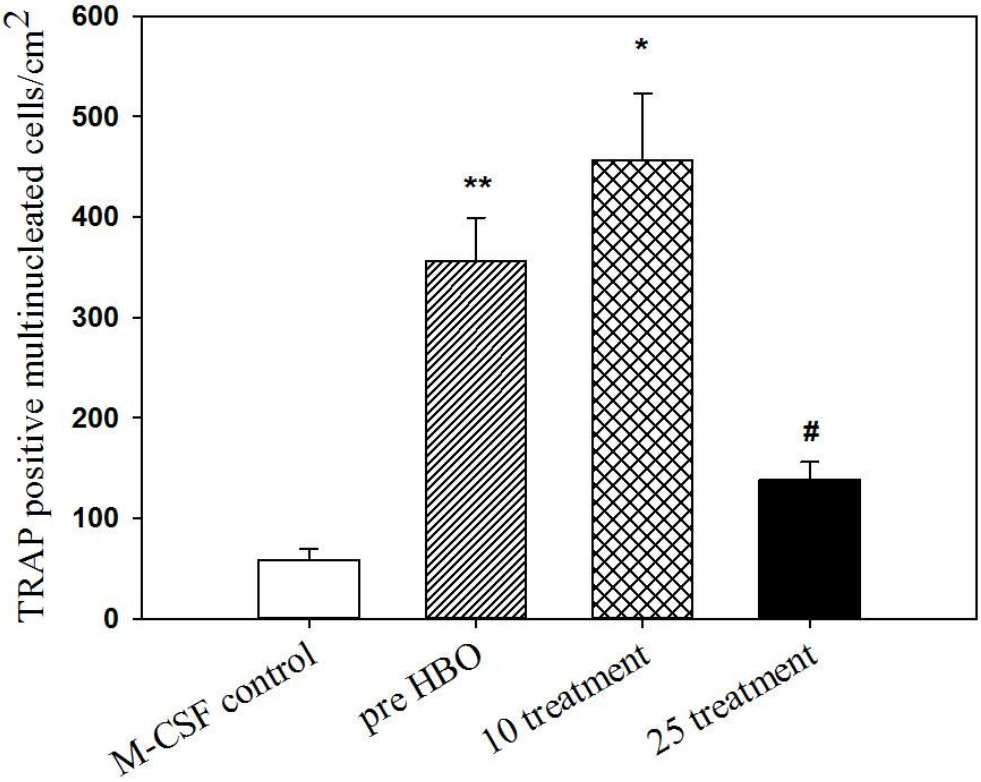


Figure 4.5 HBO (100% O_2, 2.4 ATA) supressed RANKL-induced multinucleated osteoclast formation. hPBMC were obtained from patients undergoing HBO at the DDRC and treated with RANKL (30 ng/ml) and M-CSF (30 ng/ml) for 12 days before staining for TRAP. Data are expressed as mean ± S.E.M., n = 6. ** $P < 0.05$ compared to control, * $P < 0.05$ compared to pre HBO, $^{\#}$ $P < 0.05$ compared to 10 treatments.

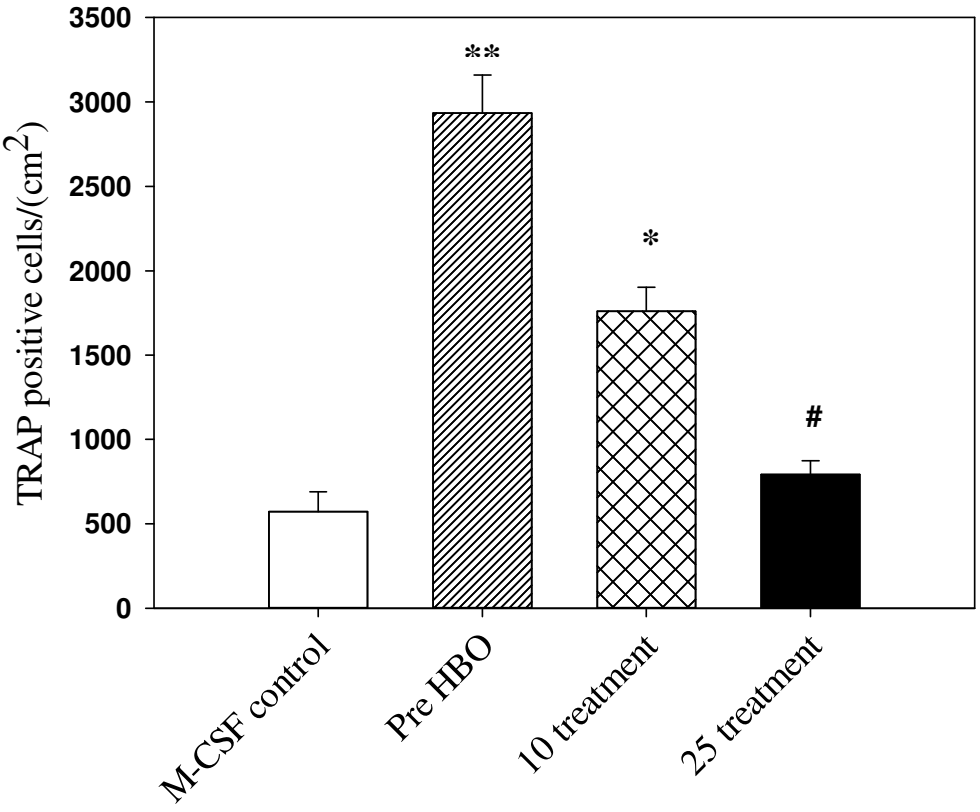


Figure 4.6 HBO (100% O_2, 2.4 ATA) supressed RANKL-induced osteoclast formation in hPBMC obtained from patients undergoing HBO at the DDRC. hPBMC were treated with RANKL (30 ng/ml) and M-CSF (30 ng/ml) for 12 days, and then stained for TRAP. Data are expressed as mean ± S.E.M., n = 6. ** $P < 0.05$ compared to control, * $P < 0.05$ compared to pre HBO, $^{\#}$ $P < 0.05$ compared to 10 times treatment.

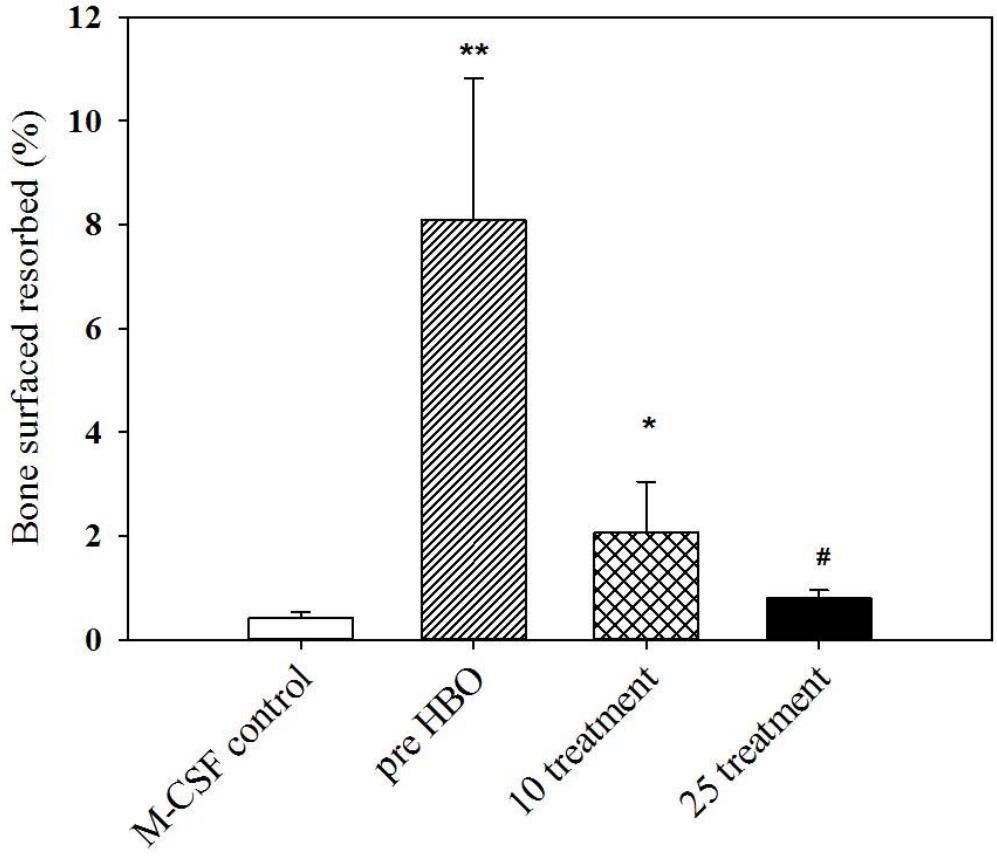


Figure 4.7 Daily exposure to HBO inhibits RANKL-induced bone resorption. hPBMC obtained from patients undergoing HBO therapy at the DDRC were treated with RANKL (30 ng/ml) and M-CSF (30 ng/ml) for 21 days before resorption was assessed. Results expressed as the mean ± S.E.M., n = 6. $^{\#}$ $p < 0.05$ versus pre HBO treatment, ** $p < 0.05$ versus control, * $p < 0.05$ versus 10 treatment.

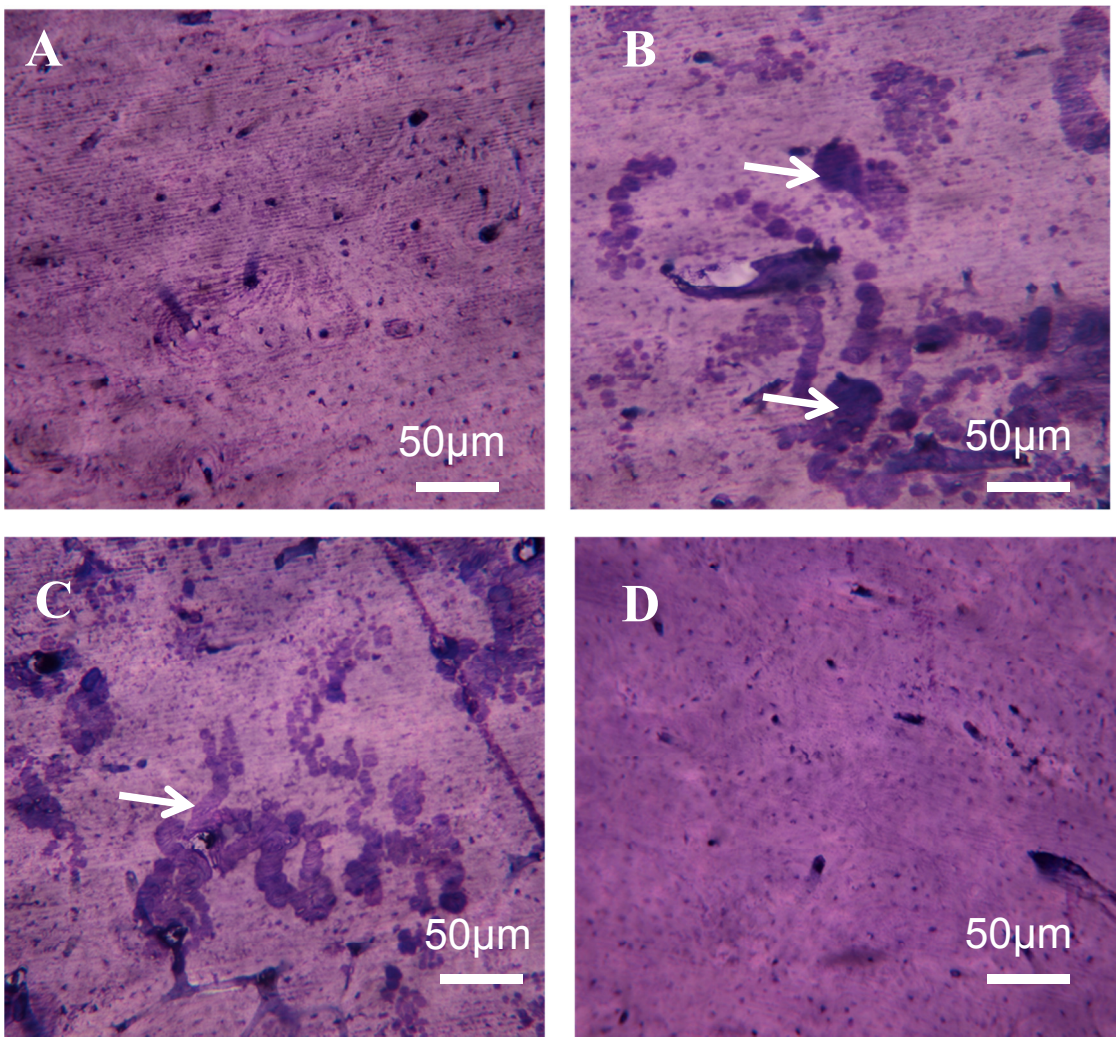


Figure 4.8 Images of resorption pits taken by reflected light microscopy. Resorption pits appear as darkly stained, clearly marginated areas. Resorption was quantified across the entire surface of the slice using an eyepiece graticule at a magnification of ×40. (A) M-SCF control (B) pre HBO (C) 10 treatments (D) 25 treatments. Arrows point to pits.

4.4.2 Effect of HBO on osteoclastic gene expression

The effect of HBO on RANK, DC-STAMP and NFATc1 mRNA expression was examined. RANK mRNA was significantly increased by 10 HBO treatments with a 3.5 fold increase compared with pre HBO treatment group. However there was a significant decrease in RANK expression after 25 treatments, which was 50% of pre-HBO levels (Fig. 4.9). The expression profile of DC-STAMP also displayed a similar change in expression. DC-STAMP expression significantly increased after 10 HBO treatments with a 2.3 fold increase compare with pre HBO treatment group. This again was followed by a significant decrease in expression after 25 HBO treatments (Fig. 4.10). In contrast HBO had a less pronounced effect on NFATc1 expression 10 treatments significantly reduced NFATc1 expression by 30% compared with pre HBO, but no significant effect was noted after 25 treatments (Fig. 4.11).

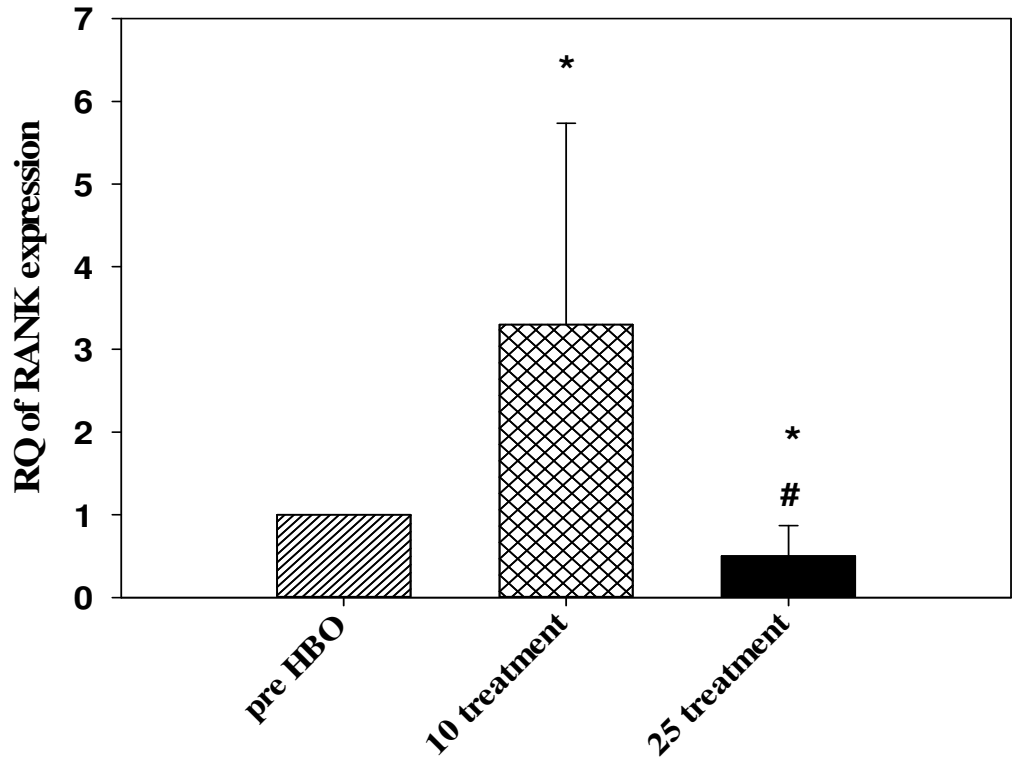


Figure 4.9 Effect of HBO on RANK mRNA expression. Monocytes were isolated from patients before and after HBO, cultured with M-CSF (30 ng/ml) and RANKL (30 ng/ml) and mRNA extracted after 14 days. cDNA was synthesised from extracted RNA and qPCR used to determine RANK expression. Gene expression was normalised against an endogenous control (β-actin). Data expressed as the mean ± S.E.M., n = 6. $^{\#}P < 0.05$ versus 10 treatments, $*p < 0.05$ versus pre HBO.

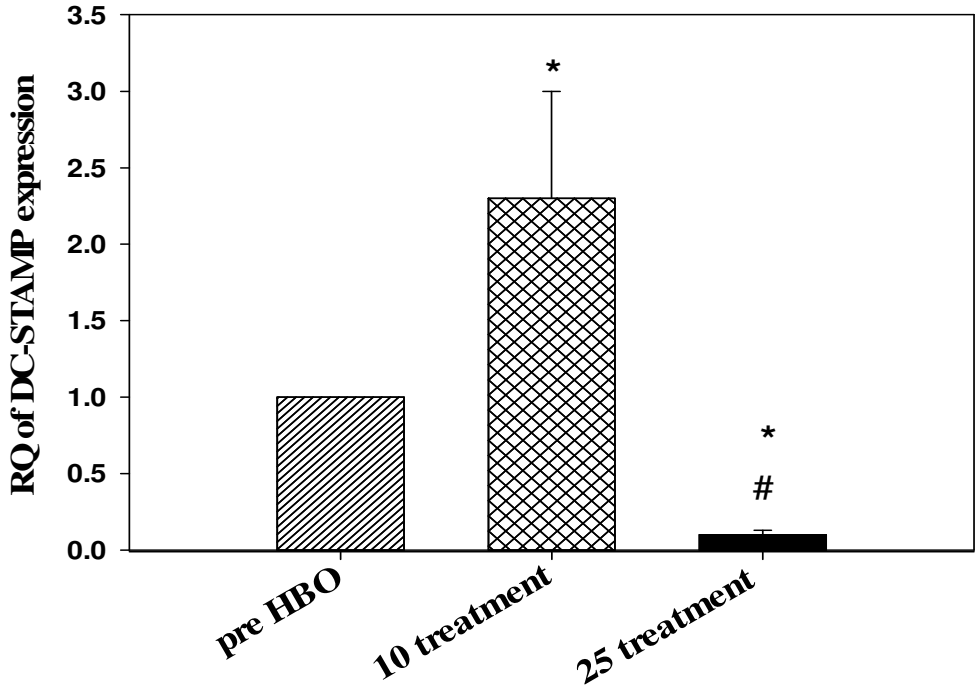


Figure 4.10 Effect of HBO on DC-STAMP mRNA expression. Monocytes were isolated from patients before and after HBO, cultured with M-CSF (30 ng/ml) and RANKL (30 ng/ml) and mRNA extracted after 14 days culture. cDNA was synthesised from extracted RNA and qPCR used to determine DC-STAMP expression. Gene expression was normalised against an endogenous control (β-actin). Data expressed as the mean ± S.E.M., n=6. $^{\#}P < 0.05$ versus 10 treatment, * $p < 0.05$ versus pre HBO.

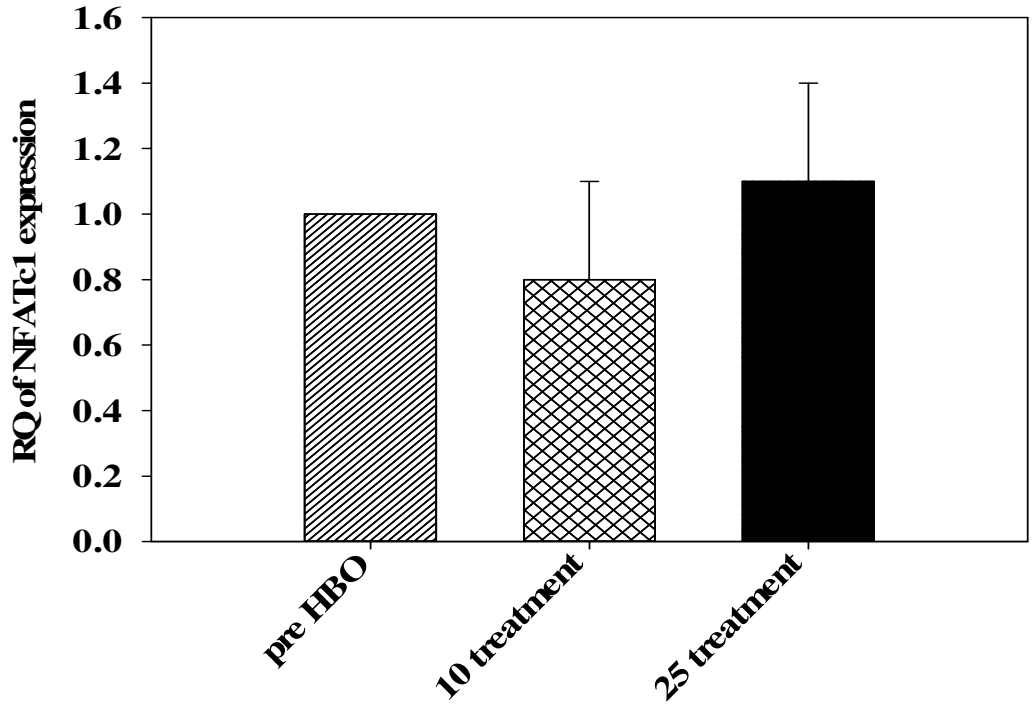


Figure 4.11 Effect of HBO on NFATc1 expression. Monocytes were isolated from patients before and after HBO cultured with M-CSF (30 ng/ml) and RANKL (30 ng/ml) and mRNA extracted after 14 days. cDNA was synthesised from extracted RNA and qPCR used to determine NFATc1 expression. Gene expression was normalised against an endogenous control (β-actin). Data expressed as the mean ± S.E.M., n = 6.

4.5 Discussion

The previous chapter suggests that HBO has a direct inhibitory effect on osteoclast differentiation and bone resorption. In the current study hPBMC of patients undergoing HBO were used to study the effect of HBO on the ability of precursors to respond to RANKL *ex vivo*. The outcomes from this study confirm the results from the previous chapter as HBO suppressed the ability of peripheral blood monocytes to develop into RANKL-induced resorptive osteoclast. As this is an *ex-vivo* culture system this suggests that the suppressive effect was meditated by an action of HBO prior to RANKL activation *in vitro* and must therefore be mediated via an inhibitory effect on the ability of precursors to differentiate along the osteoclastic lineage. This may reflect a direct action on the precursors themselves or alternatively an indirect action mediated through stromal cells that support precursor development. The effect of HBO would also appear to be accumulative with a greater effect noted with further treatments. There was also an initial differential response of multinuclear and mononuclear cells to HBO. While HBO suppressed mononuclear osteoclast formation within ten treatments, multinuclear osteoclast formation was not suppressed by this exposure number. The apparent resistance of multinuclear osteoclasts may reflect an accumulative effect of HBO on mononuclear cell formation. Multinuclear cells form by the fusion of mononuclear cells and it is likely that the number of mononuclear cells able to differentiate into osteoclasts falls as the number of exposures increases. Therefore during the earlier stages of treatment there will be precursors present that would be able to differentiate into mononuclear osteoclasts and fuse to form multinuclear osteoclasts. This assertion is supported by the lack of a significant reduction in DC-STAMP expression, which facilitates membrane fusion, at ten treatments. DC-STAMP would support fusion and therefore maintain multinuclear osteoclast number. The subsequent

fall in DC-STAMP expression correlates with a decrease in multinuclear number providing further weight to this.

In addition, the inhibitory effect of HBO could be due to changes in the distribution of mononuclear cells (Bitterman *et al*., 1993). The monocyte-macrophage is an important source of IL-1 and IL-6, HBO has generally been shown to suppress inflammation by reducing inflammatory and increasing anti-inflammatory cytokine production (Brenner *et al*., 1999). For instance in patients with Crohn's disease treated with 100% O_2 for 90 min at 2.4 ATA, LPS-stimulated monocyte-macrophages secreted less IL-1, IL-6 and TNF-α than cells obtained prior to treatment (Weisz *et al*., 1997). Benson et al found that HBO attenuates inflammatory cytokine production *in vivo* in animal models of systemic inflammation and they found that HBO exposure transiently supresses stimulus-induced proinflammatory cytokine production (Benson *et al*., 2003). Jimi et al indicated that IL-1 induces the multinucleation and bone-resorbing activity of osteoclasts even in the absence of osteoblast/stromal cells (Jimi *et al*., 1999) and IL-1 is a potent stimulator of bone resorption *in vivo* (Boyce *et al*., 1989). Also TNF-α stimulates osteoclastogenesis *in vitro* and *in vivo* directly stimulating osteoclast formation from human or mouse monocytes (Kudo *et al*., 2002) and augmenting RANKL-induced osteoclastogenesis (Fox *et al*., 2008). Other studies have shown that HBO increases IL-10 production by stimulated splenic T cells suggesting that HBO treatment has a broad effect on the adaptive immune response (Kudchadkar *et al*., 2008). IL-10 is an important suppressor of bone resorption *in vivo*, and it appears that the anti-osteoclastic mechanisms induced by IL-10 are more effective than those mediated by IL-4 (Sasaki *et al.*, 2000). Hong *et al* found that the inhibitory effect of IL-10 on OC formation is mediated on OC precursor cells and this inhibitory effect may be useful in a wide variety of osteoporotic disease (Hong *et al*., 2000). Fox and Evans also demonstrated that IL-10 acts directly on mononuclear precursors and indicated that IL-

10 directly inhibits osteoclastogenesis by suppressing NFATc1 activity (Evans and Fox, 2007).

Whatever the mechanism it is clear that one way HBO induces resistance to RANKL-induced osteoclast formation is by modifying RANK expression. In Chapter 3 it was shown that HBO significantly reduces RANK expression. Similarly in the current studies 25 HBO sessions caused a significant decrease in RANK expression. HBO may therefore at least in part reduce osteoclast formation by preventing the elevation in RANK expression. However the *in-vivo* response to HBO is not as straight forward as that noted *in vitro* as 10 exposures increased RANK expression. This suggests that *in vivo* the response of monocytic cells may depend on their differentiation stage, with newly formed or immature precursors displaying a decrease in RANK whereas more mature precursors respond with an increased expression. This concept is again supported by changes in multinuclear osteoclast formation and DC-STAMP expression, with 10 exposures increasing whereas 25 exposures suppressing expression and multinuclear osteoclast number. As DC-STAMP is a marker of late stage osteoclast formation, and enables fusion of mononuclear cells, this suggests that *in vivo* HBO in the short term may potentially accelerate the development of osteoclasts by facilitating the response to RANKL.

This data shows that NFATc1 expression was little effected by repeated HBO treatment in contrast to the previous chapter. This may reflect the nature of the treatment sessions. The implications of this data for patient treatment regimens also needs further clarification as it is unclear if the suppressive effect would be present if only 10 sessions were administered or if the further 15 sessions are needed for this to occur. This is of clinical importance as shorter but equally effective treatment regime would greatly reduce the financial cost and issues with patient compliance.

Previous studies demonstrated that osteonecrosis is a common complication of radio or high dose bisphosphonate therapy for secondary bone cancer. HBO is often used in these patients prior to reconstructive surgery to repair damaged skeletal elements and it also used in the management of other skeletal disorders. Evidence for its effectiveness comes from a range of sources. Chen et al concluded that HBO is effective and safe for chronic refractory osteomyelitis provided that patients had received appropriate medical and surgical management (Chen *et al*., 2003). HBO has been used in the treatment of stage I avascular necrosis of the head of the femur (Ries *et al*., 2002). Granstrom recommended the uses of HBO as an adjunctive treatment in the rehabilitation of oral cancer patients (Granstrom, 2003). Freiberger et al demonstrated that adjunctive HBO may benefit patients with BRONJ (Freiberger, 2009). In 1983 Marx demonstrated that HBO and aggressive surgery in a progressively staged manner was able to achieve complete resolution of ORN (Marx, 1983). However, HBO is widely employed the cellular and molecular mechanism of its action is poorly understood. Previous work addressing this issue has shown that both resorptive osteoclasts and formative osteoblasts respond to changes in oxygen concentration.

These findings suggest that HBO suppresses osteoclast formation and bone resorption from circulating monocytes. This would appear to be due to an action on early stages of precursor development and is associated with appropriate changes in the expression of osteoclastic genes. This study advances our knowledge of the effect of HBO on osteoclast formation, and hence adds to knowledge and understanding of the mechanistic action of this therapy in the treatment of osteonecrotic bone loss.

Chapter five: The effect of hyperbaric oxygen therapy on osteoblast differentiation and bone formation *in vitro*

5.1 Introduction

Bone homeostasis depends on appropriate osteoblast and osteoclast activity. Aberrant remodelling underpins bone loss in several debilitating skeletal diseases such as osteoporosis, metastatic breast cancer and multiple myeloma. Hyperbaric oxygen is used as an adjunctive therapy in the treatment of osteoradionecrosis and chronic osteomyelitis of the jaw after radiotherapy or in cases of BRONJ. HBO has been used to promote healing of irradiated mandibular bone since 1973 (Mainous and Hart, 1975) and is also used to promote bone healing (Bennett *et al*., 2005). For instance HBO has been used in patients with bone grafts (Kerwin *et al*., 2000) dental implants (Granstrom *et al*., 1999) and destruction osteogenesis (Clark *et al*., 2006).

Several studies have shown a positive effect of HBO on bone healing and bone formation (Penttinen *et al*., 1972, Nilsson *et al*., 1988, Nilsson, 1989, Jan *et al*., 2010). Jan *et al* (2009) demonstrated that the hypoxic state that commonly follows bone and blood vessel injury inhibits fibroblast proliferation, collagen synthesis and granulation tissue formation (Jan *et al*., 2009). Penttinen *et al* (1972) found that HBO produced a state of intermittent hyperoxia which alternated with periods of hypoxia maintaining the stimulus for healing at least in part through an ability to promote angiogenesis. Further evidence for this is provided by studies using rabbit irradiated mandible bone harvest chambers in which HBO stimulates ALP activity and angiogenesis (Muhonen *et al*., 2004). Therefore through an effect on capillary proliferation HBO may help restore osteoblast formation at formally hypoxic sites. It is clear also that oxygen partial pressure directly impacts on osteoblast function as hypoxic conditions similar to those associated with anaemia, diabetes and arthritis decrease formation and nodule mineralization in cultured osteoblast *in vitro* (Utting *et al*., 2006). In addition it is possible that HBO may also have direct actions on osteoblasts that further enhance its regenerative capacity; however this has not been extensively examined. Therefore to

determine the direct effect of HBO on osteoblast differentiation and bone nodule formation, this study examined the effect of HBO, pressure and hyperoxia on proliferation, expression of key regulators and markers of osteoblast differentiation, and mineralised nodule formation compared to normoxic and hypoxic conditions.

5.2 Experimental design

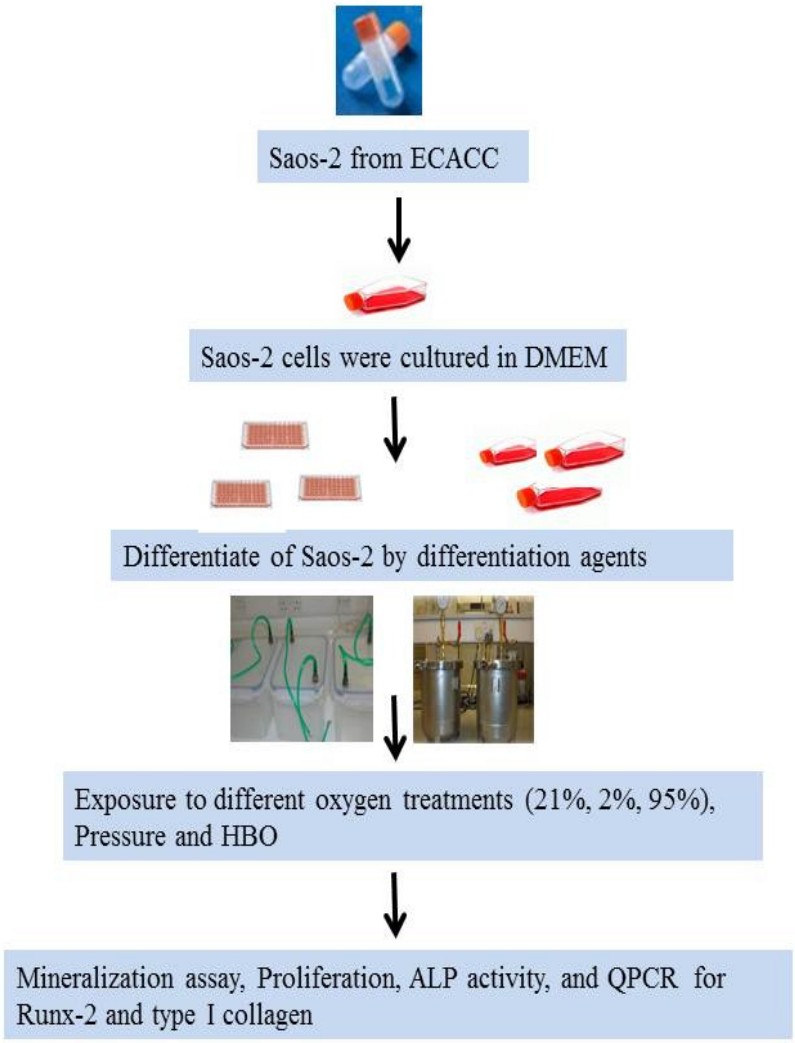


Figure 5.1 Experimental design. Saos-2 cells were differentiated using differentiation agents and were exposed to different oxygen treatments.

5.2.1 Cell culture

Saos-2 human osteoblast-like cells were obtained from ECACC (Porton Down, UK), (ECACC cat. no.8905205) and cultured in Dulbecco's minimum essential medium supplemented with 10% charcoal stripped fetal 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, and cultures were fed every 2-3 days by replacing half the medium with fresh medium.

For *in vitro* treatment of cells with HBO (97.9% O_2, 2.4 ATA), elevated pressure (2.4 ATA, 8.8% O_2, 2.1% CO_2 and 89.1% N_2) and hyperoxic conditions (95% O_2) cells were incubated in air-tight stainless steel chambers prepared at the DDRC. Chambers were flushed for four minutes with relevant gas mixes and then pressurised to 2.4 atmosphere absolute (ATA) over two minutes as described in Chapter 2, Sections 2.10 and 2.11. Cultures were exposed to HBO or pressure for ninety minutes to replicate the duration of treatment received by hyperbaric therapy patients.

For hypoxia (2% O_2), normoxia (21% O_2) and hyperoxia (95% O_2) treatments cells were incubated in special air tight chambers prepared at the DDRC in a total volume of five litres. Chambers were flushed with appropriate gas mixtures for ninety minutes and then sealed. Chambers were re-gassed daily as above with appropriate O_2 concentrations.

5.2.2 Measurement of proliferation

This assay was measured by using using the Cell Titer 96 AQeous non-radioactive cell proliferation kit, as described in Chapter 2, Section 2.7.

5.2.3 Measurement of mineralization

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), and then mineralization was assessed using a modification of Hale's methodology (Hale et al., 2000) by measuring calcein incorporation, as described in Chapter 2, Section 2.8.

5.2.4 Alkaline phosphatase activity (ALP)

Saos-2 human osteoblast-like cells were cultured and treated as described above in Section 5.2.3. 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 normalized to total cell number.

5.2.5 Real time quantitative PCR analysis

Saos-2 cells (1×10^6 cells per well) were incubated in six well plates for four or seven days while receiving relevant experimental treatments. Total RNA was then isolated and quantified on a Nanodrop spectrophotometer, cDNA was synthesised from RNA isolates using the ImPromII Reverse Transcription System (Promega, Southampton, UK). 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 as described in Chapter 2, Section 2.9.4. The forward and reverse primer sets used for PCR were as follows (Table 5.1).

Table 5.1 Primer sequences of osteoblastic transcriptional factors

Target sequence	Product size (base pairs)	Sequences
β-actin	153	F:GCGCGGCTACAGCTTCA R:TGGCCGTCAGGCAGCTCGTA
Runx-2	157	F:AGACCCCAGGCAGGCACAGT R:GCGCCTAGGCACATCGGTGA
type I collagen	153	F:CCTGGCAGCCCTGGTCCTGA R:CTTGCCGGGCTCTCCAGCAG

5.3 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.

5.4 Results

5.4.1 HBO augments early stages of mineralization

The effect of HBO on mineralization was assessed in cultures of Saos-2 cells incubated in the presence of differentiation agents (L-AA and β-GP). All treatment groups were significantly different from the control group (no β-GP 10 mM or L-AA) at all time points examined. Compared to normoxic conditions, HBO significantly enhanced mineralization at seven days inducing a 1.55 fold increase in comparison with the normoxia group. At 14 days HBO induced a significant 1.4 fold increase in mineralization in comparison to normoxia. In contrast, at 21 days HBO had no significant effect when compared to time matched normoxic groups (Figs 5.1 and 5.2). At 7 days hyperoxia induced a significant 1.3 fold decrease in mineralization compared with normoxia and at 14 and 21 days had no significant effect on calcein incorporation. Pressure alone had no significant effect on mineralization at 7, 14 or 21 days compared with normoxia (Figs 5.2 5.3).

To replicate the hypoxic environment associated with necrotic bone, experiments were then performed in hypoxic conditions. Hypoxia inhibits growth and differentiation of osteoblasts as shown from the literature (Utting *et al*., 2006) and at 14 and 21 days hypoxia caused a significant 1.9 and 1.2 fold reduction in mineralization. Similar to the effect of HBO on calcein incorporation in normoxic conditions, HBO had a pronounced augmentative effect on mineralization reversing the suppressive hypoxic action at all time points. At 7 days HBO induced a significant 1.3 fold increase in mineralization compared with normoxia and 1.5 fold compared with hypoxia. At 14 days HBO had the greatest effect compared to the other treatments and significantly increased mineralization 1.4 fold versus normoxia and 2.7 fold versus hypoxia. At 21 days HBO also significantly enhanced mineralization inducing a 1.5 fold increase compared with normoxia and 1.8 fold increase compared with hypoxia (Figs 5.2 and 5.4).

At seven days hyperoxia significantly enhanced osteoblastic mineralization with a 1.2 fold increase compared to cultures continuously grown in normoxic conditions and a 1.4 fold increase compared to cell cultures exposed to intermittent hypoxic conditions. At 14 days hyperoxia reversed the suppressive effect of hypoxia, but did not increase mineralisation beyond that of normoxic control. Hyperoxia at 21 days induced a significant 1.2 and 1.5 fold increase in comparison with normoxia and hypoxia groups (Figs 5.2 and 5.4).

At 7 days pressure stimulated a significant 1.2 and 1.3 fold increase in calcein incorporation compared with normoxia and hypoxia, respectively. At 14 days pressure reversed the suppressive effect of hypoxia but did not enhance mineralization beyond that of normoxic control with a 1.2 fold increase in comparison with hypoxia treatment group. In contrast at 21 days pressure significantly enhanced mineralisation with 1.2 and 1.5 fold increases compared to normoxic and hypoxic cultures (Figs 5.2 and 5.4).

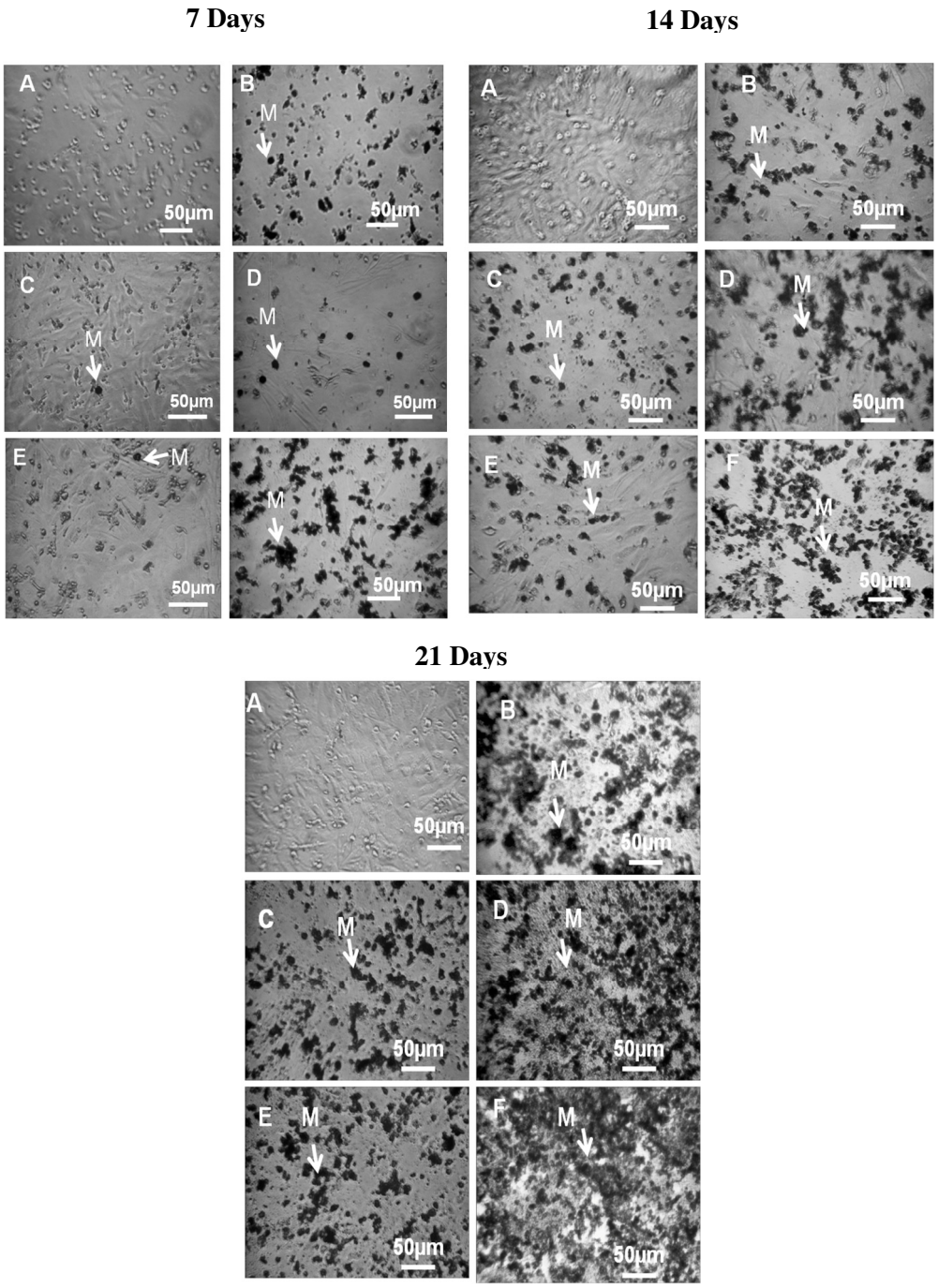


Figure 5.2 Bone nodule formation Saos-2 cells cultured for 7, 14 and 21 days in the presence of differentiation agents (β-GP and L-AA), pictures taken from inverted microscope (A) control (no β-GP or L-AA) (B) normoxia (C) hypoxia (D) hyperoxia (E) pressure (F) HBO. Arrows highlight mineralised nodules.

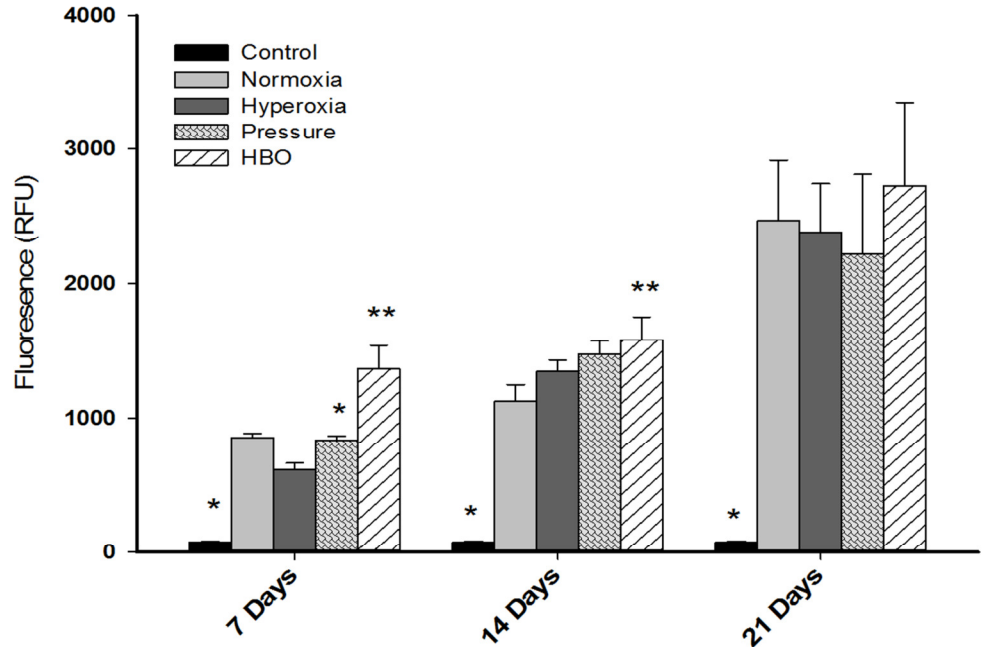


Figure 5.3 HBO enhances early stages of mineralization in normoxic conditions. Saos-2 cells were treated with β-GP (10 mM) and L-ascorbic acid (L-AA, 50 mg/l) for 7, 14 and 21 days and exposed to various treatments daily for 90 minutes. Mineralization was then assessed using a calcein incorporation assay. 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 all groups ($P < 0.05$). ** Significantly different from normoxic group ($P < 0.05$).

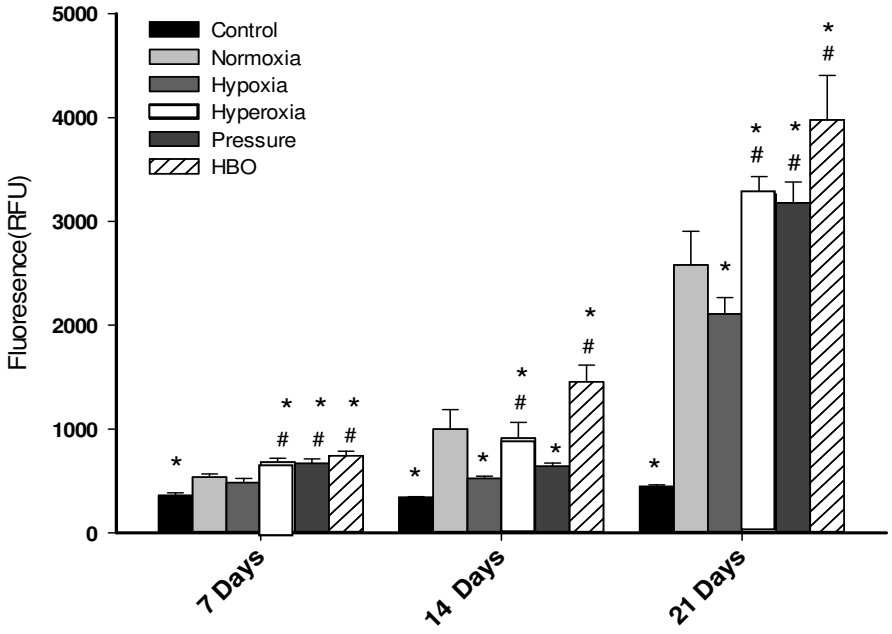


Figure 5.4 The effect of HBO, pressure and hyperoxia on osteoblast activity in hypoxic conditions. Saos-2 cells were treated with β-GP (10 mM) and L-AA, (50 mg/l) for 7, 14 or 21 days. Values are expressed as the mean of three replicates with standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. * Significantly different from normoxia group ($P < 0.05$). #Significantly different from hypoxic group ($P < 0.05$).

5.4.2 HBO promotes osteoblast proliferation in normoxic and hypoxic conditions

The effect of treatment on cell number was assessed at days 4, 7 and 14. Under normoxic conditions HBO increased cell number with an 8.7 fold increase at four days, 1.1 fold increases at seven days and a 9.6 fold increase at 14 days (Fig. 5.5).

In contrast, hyperoxia only had a positive effect on cell number at four days inducing a 7.7 fold increase in number compared to normoxia. At seven days hyperoxia reduced cell number causing a 1.4 fold decrease compared with normoxia. At 14 days hyperoxia had no significant effect on cell number compared to normoxia (Fig. 5.5).

Pressure alone induced a 2.7 fold increase in cell number at four days, had no significant effect at seven days and significantly enhanced osteoblast proliferation at fourteen days with a 2.3 fold increase compared to normoxia (Fig. 5.5).

Hypoxia caused a significant 1.6 and 7.7 fold decrease in osteoblastic proliferation at four and seven days. But no significant effect was noted at 14 days. HBO had no significant effect on cell number at four days compared to normoxia and hypoxia groups; at seven days HBO reversed the suppressive effect of hypoxia but did not augment cell number beyond that of normoxic control. At 14 days HBO had the greatest effect on osteoblast proliferation in hypoxic conditions inducing a 9.6 fold increase compared with normoxia and 1.9 fold increase compared with hypoxia.

At 4 days hyperoxia reversed the suppressive effect of hypoxia but did not augment cell number beyond that of normoxic control with 1.6 fold increase compared to hypoxic group. At 7 days hyperoxia was unable to prevent the suppressive effect of hypoxia. Similarly at 14 days hyperoxia had no significant difference compared to normoxia or hypoxia groups.

At 4 days pressure had no effect on cell number. However, it reversed the suppressive action of hypoxia at seven days, augmenting cell number beyond that of normoxic

control with a 2.8 fold increase compared to hypoxia group. Pressure at 14 days had no effect compared with normoxia and hypoxia treatment groups (Fig. 5.6).

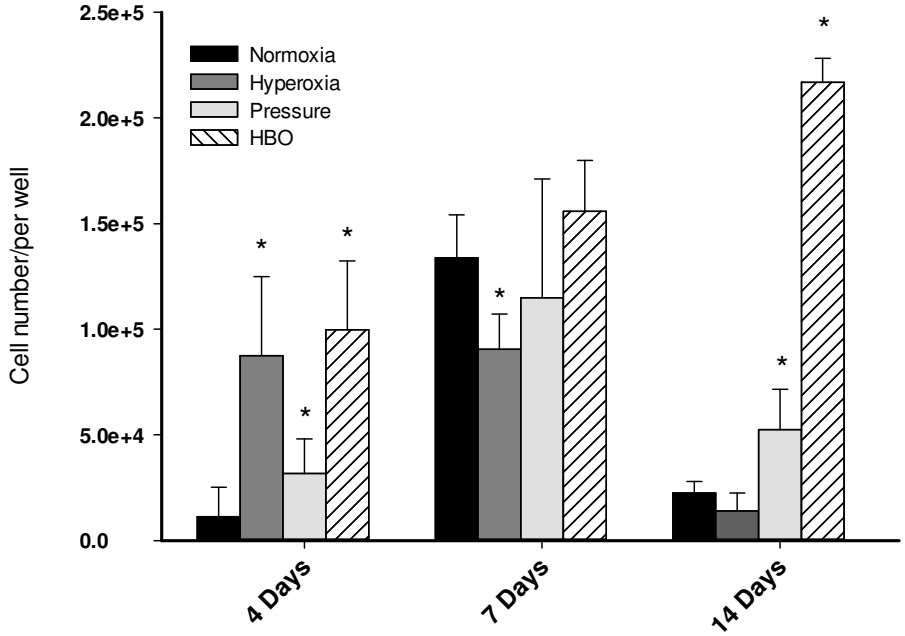


Figure 5.5 The effect of HBO, pressure and hyperoxia on osteoblast number in normoxic conditions. Saos-2 cells were treated with β-GP (10 mM) and L-ascorbic acid (L-AA, 50 mg/l) and cell number assessed using an MTS assay at 4, 7, and 14 days Values are the mean of three replicates with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. *Mean values of group were significantly different from normoxic control ($P < 0.05$).

Osteoblast number in hypoxic conditions

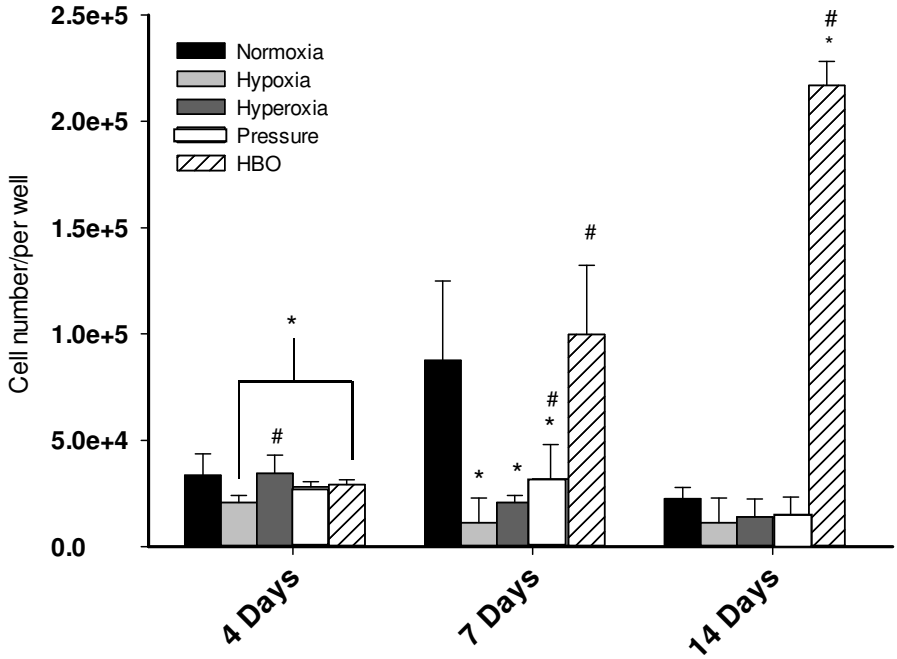


Figure 5.6 The effect of HBO, pressure, and hyperoxia on osteoblast number in hypoxic conditions. Saos-2 cells were treated with β-GP (10 mM) and L-ascorbic acid (L-AA, 50 mg/l) and cell number assessed using an MTS assay at 4, 7 and 14 days. Values are mean of three replicates with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. * Significantly different from the normoxia control ($p< 0.05$). # Significantly different from hypoxia treatment groups ($P < 0.05$).

5.4.3 HBO treatment stimulates ALP activity

Unsurprisingly ALP activity, a marker for osteoblast differentiation, increased in the presence of the known promoters of osteoblast differentiation β-GP and L-AA as the cultures progressed. Under normoxic conditions ALP activity was significantly enhanced in HBO-treated cultures at four and seven days which induced a 1.8 and 1.2 fold increase, respectively, whereas HBO had no significant effect on ALP activity at 14 days. At 4 days hyperoxia had no significant effect on ALP activity compared with normoxia; at seven days hyperoxia reduced ALP activity significantly with 1.2 fold decrease compared to normoxia. This suppression was not seen at fourteen days and ALP activity was not different from normoxia. Pressure significantly increased ALP activity at four days compared with normoxic group inducing a 1.8 fold increase. Unexpectedly pressure decreased ALP activity at 7 days and again this suppressive action was not seen at fourteen days (Fig. 5.7).

At 4 days hypoxia induced a modest increase in ALP activity compared to normoxia. At seven days hypoxia significantly decreased ALP activity with a 1.8 fold reduction compared to normoxia. While ALP activity was suppressed at day 14 compared with normoxia this did not reach statistical significance (Fig. 5.8).

At 4 days HBO significantly elevated ALP activity in comparison with other groups with a 1.7 fold increase compare with normoxia. At 7 days HBO reversed the suppressive effect of hypoxia but did not augment ALP activity beyond that of normoxic control, inducing a 1.5 fold increase compared to hypoxia group. At 14 days HBO significantly stimulated ALP activity compared to normoxia (1.2 fold) and hypoxia (1.3 fold) treated cultures (Fig. 5.8). At four days hyperoxia had no significant effect on ALP activity. At 7 days hyperoxia was unable to prevent the suppressive effect of hypoxia. However at fourteen days hyperoxia significantly enhanced ALP

activity with 1.2 and 1.3 fold increases compared to normoxia and hypoxia respectively (Fig. 5.8).

Pressure enhanced ALP activity in comparison to normoxia at four days but was not as strongly as hypoxia or HBO. At 7 days pressure partly reversed the suppressive effect of hypoxia but did not restore ALP to normoxic levels (Fig. 5.7). At 14 days daily exposure for ninety minutes to pressure significantly stimulated ALP activity inducing a 1.3 and 1.4 fold increase compared to normoxia and hypoxia treated cultures respectively (Fig. 5.8).

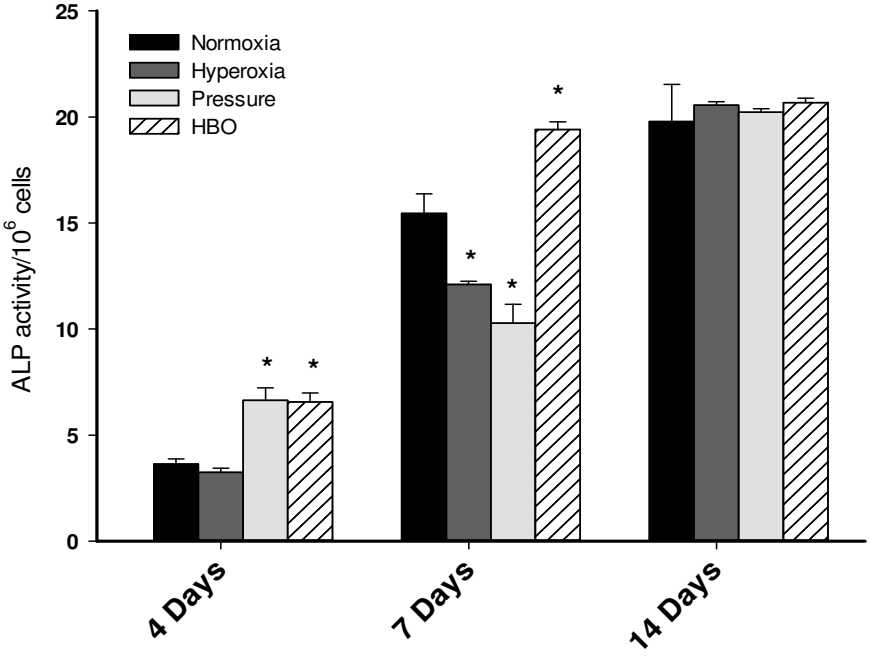


Figure 5.7 The effect of HBO, pressure, and hyperoxia on ALP activity in normoxic conditions. Saos-2 cells were treated with β-GP (10 mM) and L-ascorbic acid (L-AA, 50 mg/l) and ALP activity assayed after 4, 7 and 14 days. Values are mean of three replicates with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. * Significantly different from normoxia ($P < 0.05$).

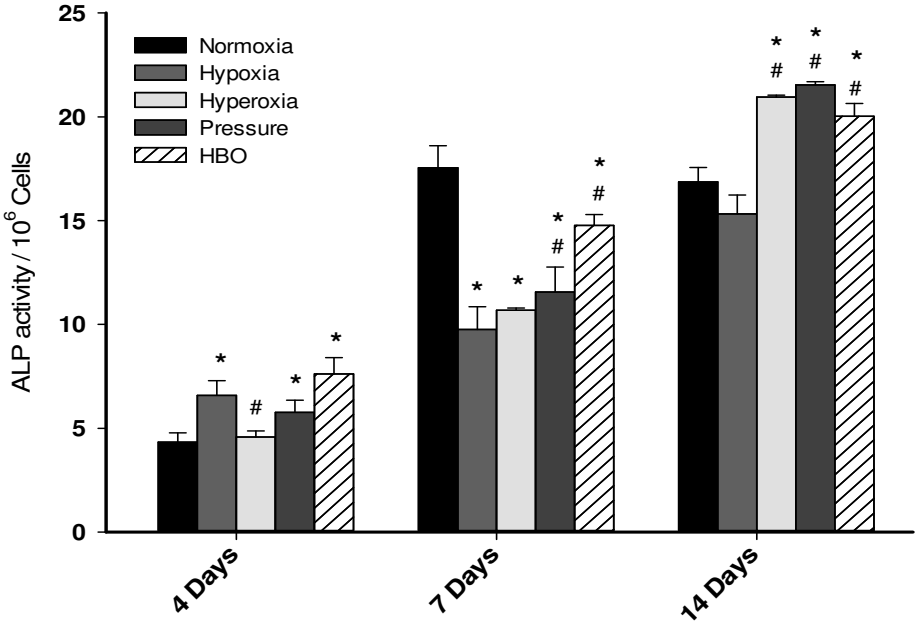


Figure 5.8 The effect of HBO, pressure, and hyperoxia on osteoblast activity in hypoxic conditions, ALP activity assay for 4, 7, and 14 days. Saos-2 cells were treated with β-GP (10 mM) and L-ascorbic acid (L-AA, 50 mg/l) and ALP activity assayed after 4, 7 and 14 days. Values are mean of three replicates with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. * Significantly different from normoxia group ($P < 0.05$). # Significantly different from hypoxia ($P < 0.05$).

5.4.4 HBO enhances Runx-2 and type I collagen expression

Osteoblast differentiation is regulated by transcription factors that control gene expression. Therefore to determine the potential molecular mechanism by which HBO enhances early stages of osteoblast differentiation Runx-2 expression was examined using real time quantitative PCR. HBO significantly increased Runx-2 expression stimulating a 7.1 fold increase compared to normoxia. Hyperoxia also significantly enhanced Runx2 expression stimulating a 2.7 fold increase compared with normoxia. In contrast pressure had no significant effect on Runx-2 expression (Fig. 5.9). Hypoxia significantly reduced mRNA expression of Runx-2 with a 2.8 fold decrease compared to normoxia. Daily exposure to HBO for ninety minutes significantly increased Runx-2 expression inducing an 8.3 fold increase compared with normoxia and 22.6 fold increase compared with hypoxia. Hyperoxia and pressure had no significant effect on Runx-2 expression compared to normoxia and hypoxia groups (Fig. 5.10).

Type I collagen is highly expressed in osteoblast and is the most abundant protein of bone extracellular matrix. Type I collagen is expressed in osteoblast at all stages during development (Kern *et al*., 2001). In normoxic conditions HBO had a great effect on *type I collagen* expression compared to other treatment groups, stimulating a 4.5 fold increase compared to normoxia. In contrast hyperoxia and pressure had no effect on type I collagen expression (Fig. 5.11). Interestingly hypoxia significantly decreased type I collagen expression; hypoxia reduced a 1.4 fold decrease compared with normoxia, whereas HBO significantly enhanced type I collagen mRNA expression; HBO induced a 1.6 fold increase compared with normoxia and 2.4 fold increase compared with hypoxia. In contrast hyperoxia significantly reduced type I collagen mRNA expression compared with normoxia and hypoxia, and pressure was unable to reverse the suppressive effect of hypoxia (Fig. 5.12).

Runx-2 expression in normoxic conditions

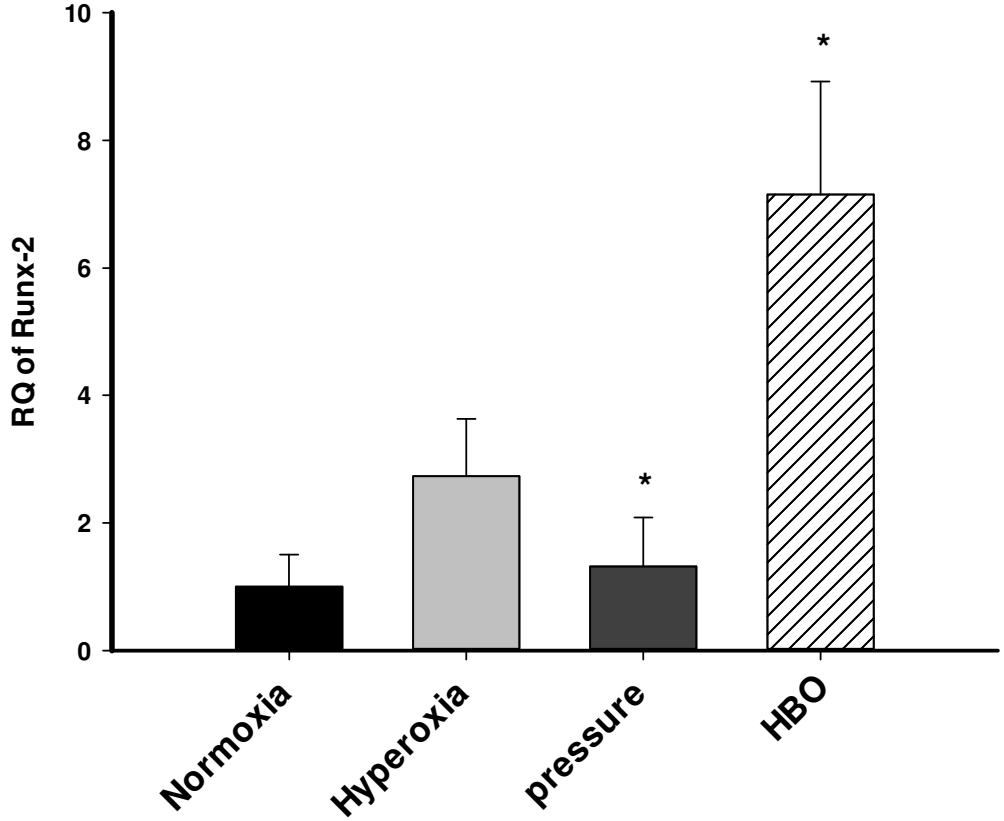


Figure 5.9 The effect of HBO, pressure and hyperoxia on Runx-2 expression, in normoxic conditions for 7 days. Saos-2 cells were treated with β-GP (10 mM) and L-ascorbic acid (L-AA, 50 mg/l). Quantification of this mRNA expression normalized against β-actin mRNA level. Values are mean of three replicates with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. * Significantly different from normoxia ($P < 0.05$).

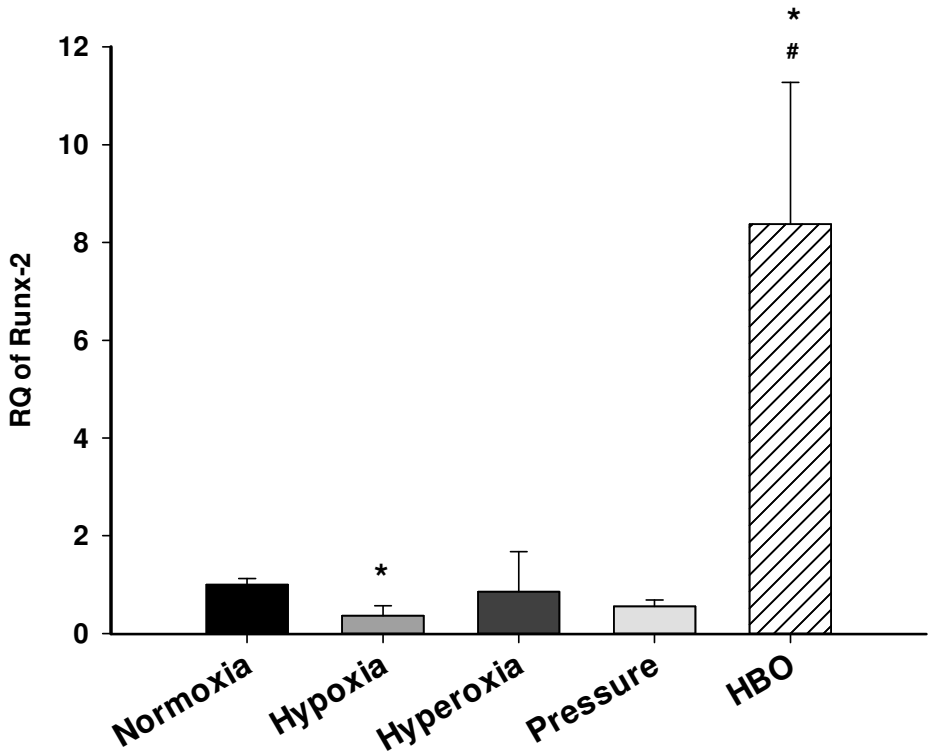


Figure 5.10 The effect of HBO, pressure and hyperoxia on Runx-2 expression in hypoxic conditions for 7 days. Saos-2 cells were treated with β-GP (10 mM) and L-ascorbic acid (L-AA, 50 mg/l). Quantification of this mRNA expression normalized against β-actin mRNA level. Values are mean of three replicates with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. * Significantly different from normoxia group ($P < 0.05$). # Significantly different from hypoxia group ($P < 0.05$).

Type I collagen expression in normoxic conditions

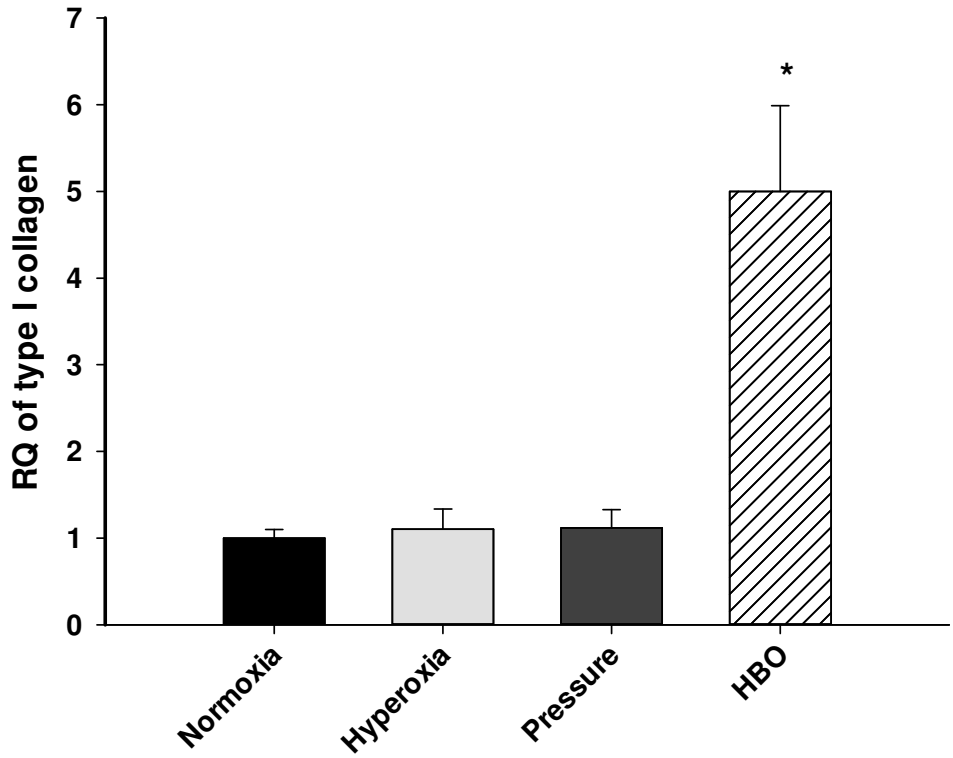


Figure 5.11 The effect of HBO, pressure and hyperoxia on type I collagen expression in normoxic conditions for 7 days. Saos-2 cells were treated with β-GP (10 mM) and L-ascorbic acid (L-AA, 50 mg/l). Quantification of this mRNA expression normalized against β-actin mRNA level. Values are means of three replicates with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. * Significantly different from normoxia ($P < 0.05$).

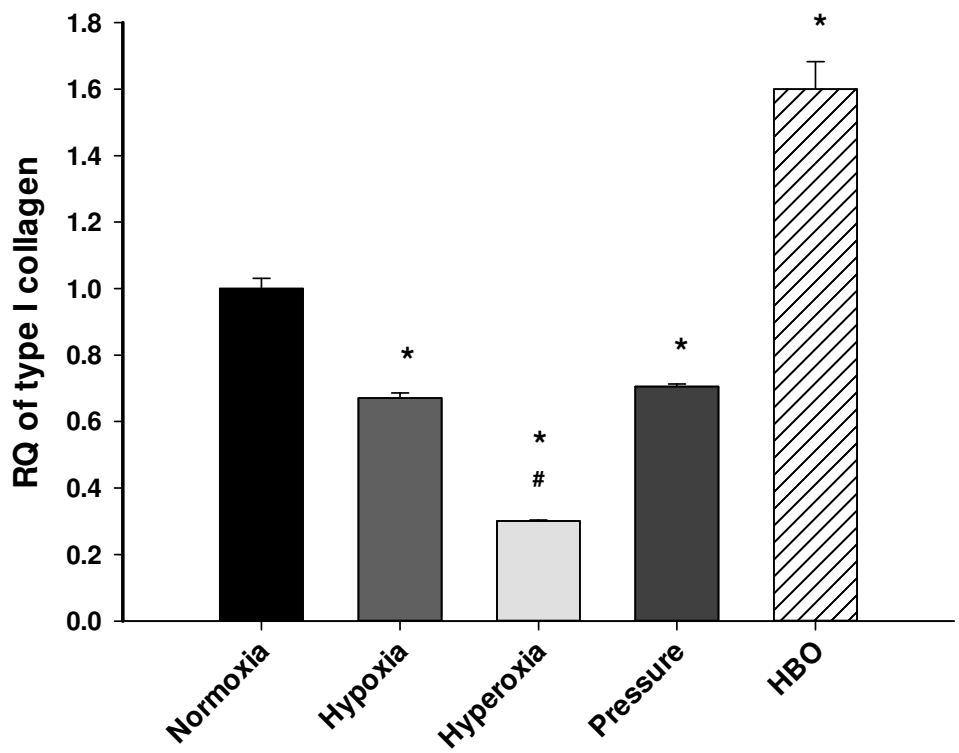


Figure 5.12 The effect of HBO, pressure and hyperoxia on type I collagen expression, in hypoxic conditions for 7 days. Saos-2 cells were treated with β-GP (10 mM) and L-ascorbic acid (L-AA, 50 mg/l). Quantification of this mRNA expression normalized against β-actin mRNA level. Values are mean of three replicates with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. * Significantly different from normoxia groups ($P < 0.05$). # Significantly different from hypoxia groups ($P < 0.05$).

5.5 Discussion

Appropriate bone remodelling is dependent on a delicate balance between osteoblastic bone formation and osteoclastic resorption. Disruption of the balance between these cell types is often seen in bone diseases such as osteoporosis, metastatic breast cancer, multiple myeloma and osteomyelitis (Duplomb *et al*., 2007). Changes in remodelling activity can also arise as a consequence of therapeutic intervention for instance intravenous bisphosphonate treatment is associated with BRONJ and localised ORN is a common side effect of radiotherapy (Migliorati *et al*., 2006).

In the current study Saos-2 cells were used to study the effects of HBO, pressure and hyperoxia in normoxic and hypoxic conditions. The results indicate that intermittent HBO exposure, similar to that received by patients with osteonecrosis of the jaw or complicated fractures, augments early stages of bone nodule formation in normoxic conditions. Furthermore, HBO had a greater effect on bone nodule formation than either hyperoxia or pressure alone. This beneficial action was also noted in hypoxia and the effect appeared to be extended beyond that seen in normoxic conditions, indicating that HBO is able to reverse the suppressive action of hypoxia at later stages of osteoblast differentiation and bone formation. This is similar to the studies of Wang in which HBO promoted osteoblast activity and improved outcomes when applied during the early stages of the tibial healing process (Wang *et al*., 2005). This augmentative action may at least in part be due to an increase in the number of osteoblasts as HBO significantly increased osteoblast number in both normoxic and hypoxic conditions and HBO had the largest effect in comparison with hyperoxia and pressure alone; this is similar to the study of Mainous (1977) which demonstrated that treatment of mandibular osteoradionecrosis with HBO increased collagen formation, fibroblast proliferation, capillary budding, osteoblastic and osteoclastic activity, callus formation and mineralization. This action, however, was not consistent with any effect on

osteoblast number noted in hypoxic conditions at day four of culture. Similar rapid changes in osteoblast number were seen in the studies of Wu et al (2007) which demonstrated an initial significant stimulatory effect of HBO treatment within three days (Wu *et al*., 2007). Ozawa *et al* (1990) examined the effect of a continuous static pressure on the differentiation of osteoblast and found that differentiation was inhibited, as were collagen synthesis and alkaline phosphatase activity. Rubin *et al*. (1997) demonstrated that pressure is also known to generate responses from bone cells and continuous hydrostatic pressure at physiological levels decreased osteoclast formation in marrow cultures.

The response of osteoblasts to hypoxia has been well documented (Tuncay *et al*., 1994, Steinbrech *et al*., 2000). Osteoblast function and bone formation are strongly oxygen dependent; bone nodule formation was strongly inhibited when oxygen concentration was decreased to 5% and almost completely prevented when oxygen concentration was 1% (Utting *et al*., 2006). Hypoxia inhibits the proliferation of mature osteoblast precursors and leads to the failure of cell differentiation and formation *in vitro* (Utting *et al*., 2006). Potier *et al* (2007) suggested that exposure of mesenchymal stromal cell (MSCs) transplanted *in vivo* to hypoxia may affect their bone forming potential (Potier *et al*., 2007). Salim *et al* (2004) indicated that brief exposure to hypoxia down-regulated *Runx2* expression, thus inhibiting critical steps in the osteogenic differentiation of pluripotent mesenchymal precursors and committed osteoblasts (Salim *et al*., 2004). In the current studies hypoxia reduced nodule formation at seven and fourteen days which was associated with a suppression of osteoblast number, ALP activity and Runx2 expression. This was consistently reversed by the application of daily HBO, which elevated mineralisation, cell number and Runx-2 expression beyond that of normoxic controls. In keeping with Utting *et al* (2006) bone nodule formation was inhibited by hypoxia at both early and late stages (Utting *et al*., 2006). In addition as this cultures

lack any other cell types this suggests that HBO has direct actions on osteoblasts to promote differentiation and activity in addition to any further angiogeneic effects that may occur *in vivo*.

There have been *in vivo* studies on HBO; Gokce *et al* (2008) found that HBO enhanced bone formation during experimental tooth movement and demonstrated that osteoblastic activity might be modulated by changes in oxygen tension (Gokce *et al*., 2008). Muhonen *et al* (2004) concluded that radiotherapy disturbs distraction bone formation and neovascularization, and HBO increased osteoblast activity and angiogenic response in rabbit mandibular distraction (Muhonen *et al*., 2004). Some authors suggested that the proliferative effects of hyperbaric oxygen therapy on osteoblasts may contribute to the recruitment of osteoblasts at fracture sites and could promote the proliferation of growth-arrested osteoblasts (Hsieh *et al*., 2010). Elevated oxygen conditions induced mesenchymal tissue differentiation toward osteoblasts (Warren *et al*., 2001). Also this data shows that HBO elevated ALP activity at all time points in normoxic conditions and a similar action was noted in hypoxic conditions at 4 and 14 days similar to the studies of Okubo *et al* (2011) who found that alkaline phosphatase activity and calcium content were significantly greater with HBO at day 7 and 21 (Okubo *et al*., 2001). Further studies demonstrated the role of oxygen in bone remodelling by the direct effect on collagen synthesis, ALP activity, and the production of transcriptional regulators of osteoblast differentiation and bone formation (Warren *et al*., 2001).

To assess the molecular mechanisms, this study 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 (Lynch *et al*., 1995). This study showed that HBO enhances Runx-2 expression at seven days which coincides with a significant increase in nodule formation in normoxic and hypoxic conditions. Ontiveros *et al* (2004) demonstrated that hypoxia decreased Runx-2

in osteoblast grown in standard tissue culture plates and it also noted this in my studies (Ontiveros *et al*., 2004). Hypoxia also has been shown to decrease alkaline phosphatase activity in primary fetal rat calvarial osteoblast cultures exposed to hypoxia as was noted at day 7 in these experiments (Tuncay *et al*., 1994). They also suggested that modulation of oxygen concentration could differentially regulate bone cell phenotypes and thereby stimulate skeletal homeostasis. This data also shows that HBO appears to promote the expression of the major component of bone matrix type I collagen a marker of early mature osteoblast in both normoxic and hypoxic conditions. HBO had a greater effect on these key markers of osteoblast differentiation than hyperoxia or pressure alone, which is in keeping with Ishii's studies that suggest that intermittent HBO enhances collagen synthesis and is beneficial for producing extracellular matrices in tissue engineering (Ishii *et al*., 1999).

HBO has been used as an adjunctive therapy in many of these disorders with varying outcomes. Several studies demonstrate that oxygen concentration influences bone remodelling, and HBO has a positive effect on osteoblast activity (Warren *et al*., 2001, Wu *et al*., 2007). Previous studies (Sawai *et al*., 1996) undertaken to evaluate the effect of hyperbaric oxygen therapy on autogenous free bone grafts transplanted from iliac crest to the mandibles of rabbits indicate that HBO accelerates the rate of union. Similarly the effect of HBO treatment on the healing of normally regenerate bone injuries has been extensively investigated using different experimental settings. In earlier studies adjunctive HBO was found to amend calcium binding and opposition of femur fracture (Coulson *et al*., 1966). In animal studies HBO has been shown to improve both bone generation and the removal of dead or abnormal bone (Tkachenko *et al*., 1988). Ueng *et al* suggested that the bone healing of tibia is enhanced by intermittent HBO (Ueng *et al*., 1998). Previous studies demonstrated that HBO decreased tissue damage and induced osteodentine formation in the rat mandible after

vertical osteotomy (Nilson *et al*., 1987). Mainous (1977) treated mandibular osteoradionecrosis with HBO and concluded that when the partial pressure of oxygen increased, collagen formation and fibroblastic proliferation, capillary budding, osteoblastic and osteoclastic activity, callus formation and mineralization were all increased (Mainous, 1977). Wray and Rogers 1968 supported the healing of standard tibial fracture in rats treated with hyperbaric oxygen for six hours per day at 2 ATA for 20 to 26 days post-fracture revealed that although callus formation was increased the breaking strength was reduced as compared to atmospheric controls (Wray and Rogers, 1968). Sirin et al assessed the potential effect of HBO on artificial bone grafts in rat tibiae and they suggested that HBO had beneficial effects on the healing of unfilled bone defects and those filled with β-tricalcium phosphate (Sirin *et al*., 2011). Thus, the general consensus from the limited *in vivo* studies is that HBO typically has a beneficial effect on osteoblast activity.

Although the potential mechanisms underlying the changes in gene expression with HBO remain unclear, previous studies report that HBO applied during the early stage of tibial healing was more effective than at later stages in terms of increasing bone mineral density and mechanical properties (Wang *et al*., 2005). This is in keeping with the data obtained here, which showed that in normoxic conditions HBO enhanced the early stages of osteoblast differentiation. These findings suggest that HBO accelerates the rate of osteoblast differentiation and augments early stages of mineralization, and has a more pronounced effect than hyperoxia 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.

Chapter six: General discussion

6.1 Discussion

Bone remodelling requires the coordinated regulation of osteoblast and osteoclast activity (Nair *et al*., 1996). Remodelling is disrupted in many skeletal diseases including osteoradionecrosis and osteomyelitis. Hyperbaric oxygen therapy may support remodelling at these sites (Freiberger, 2009). This current study however shows that HBO can regulate bone cell activity by several mechanisms; it can directly suppress osteoclast differentiation and activity in normoxic and hypoxic conditions and accelerate the rate of osteoblast differentiation and mineralization. In Chapter 3 the results show that HBO for ninety minutes suppressed osteoclast differentiation and bone resorption from mouse and human monocytes in normoxic and hypoxic conditions *in vitro*. Furthermore, HBO had a greater effect on osteoclast formation than either hyperoxia or elevated pressure alone in both culture systems, indicating that appropriate combinations of pressure and hyperoxia are the most effective therapeutic strategy. HBO suppressed RANKL-induced osteoclast differentiation at least in part by decreasing RANK, NFATc1 and DC-STAMP expression. The suppressive effect of HBO on NFATc1 expression may relate to an effect on upstream control elements such as NF-κB or c-fos. HBO also inhibited HIF-1α gene and protein expression in the presence of RANKL. This suggests that the anti-osteoclastic action of HBO may be mediated through a reduction in HIF-1α, which would be expected to decrease expression of hypoxic responsive genes such as NF-κB and thereby limit NFATc1 expression and osteoclast formation. HIF-1α is stabilized by NADPH oxidase during hypoxia (Goyal *et al*., 2004). Therefore, suppression of NADPH oxidase by HBO (Ostrowski *et al*., 2006) may mediate redox-dependent HIF-1α degradation; also the accumulation of HIF-1α in ischaemic or hypoxic tissues might promote adaptive mechanisms for cell survival (Goyal *et al*., 2004). The mechanisms by which HIF moderate osteoclast functional longevity remain poorly understood and are an area for future study.

HBO also inhibits the ability of precursors to form osteoclasts, HBO is often used in patients prior to reconstructive surgery to repair damaged skeletal elements and it is also used effectively in the management of other skeletal disorders. This study therefore examined the effect of HBO on the ability of precursors to subsequently respond to RANKL *ex vivo*. HBO suppressed the ability of peripheral blood monocytes to develop into RANKL-induced resorptive osteoclasts. HBO only caused a significant decrease in RANK and DC-STAMP expression after 10 treatment sessions so the response of monocytes may depend on their differentiation stage, with newly formed or immature precursors displaying an increase in RANK whereas more mature precursors responding with decreased expression. This concept is again supported by changes in multinuclear osteoclast formation and DC-STAMP expression. This indicates that as the suppressive effect was meditated by HBO prior to activation of osteoclast differentiation this may be mediated via an inhibitory effect on precursor lineage switching.

In addition to the direct effect of HBO on osteoclast formation HBO in my studies also had direct effects on osteoblasts. The results in Chapter 5 indicate that HBO accelerates the rate of osteoblast differentiation and augments early stages of mineralization, and has a more pronounced effect than hyperoxia or pressure alone. HBO enhances Runx-2 mRNA expression and also causes a significant increase in bone nodule formation in normoxic and hypoxic conditions. It also promotes expression of the major component of bone matrix type I collagen a marker of early mature osteoblast in both normoxic and hypoxic conditions. HBO had a greater effect on these markers of osteoblast differentiation than hyperoxia or pressure alone. This also provides a potential mechanism through which short term HBO therapy may help in fracture healing. In keeping with this, several studies have proved the efficacy of HBO therapy in improving bone healing or bone formation. Penttinen *et al* (1972) found in their study that intermittent exposure of rats to HBO resulted in hypertrophy of the cartilage and an

increase in the rate of bone formation, also in animal studies showed that HBO can be used to treat delayed fracture healing (Ueng *et al*., 1998, Bennett *et al*., 2005).

Previous work evaluating the effect of HBO on injured or irradiated sites showed increased bone apposition or decreased osteoclastic activity (Gokce *et al*., 2008). For instance Jan et al concluded that HBO stimulated bone formation in defects grafted with demineralized bone matrix and resulted in a large reduction in fibrous tissue and an increase in its replacement with marrow (Jan *et al*., 2010). In animal studies HBO improved bone generation (Inoue *et al*., 2000, Tkachenko *et al*., 1988) and assisted the removal of necrotic bone (Jones *et al*., 1991, Strauss *et al*., 1982). The benefits of HBO were less clear in a more recent study where cats with experimentally induced non-union bone showed increased bone formation but not improved vascularisation (Kerwin *et al*., 2000). There have been reports of clinical improvement following the application of HBO to individuals with established non-union (Atesalp *et al*., 2002). Enrico *et al* (2010) demonstrated an effective treatment modality that involves the use of HBO to treat patients with femoral head necrosis (Enrico *et al*., 2010). An increase in oxygen tension within regenerating tissue could promote collagen and adenosine-triphosphate (ATP) synthesis (Ishii *et al*., 1999) leading to changes in osteoblastic and osteoclastic activity (Nilsson *et al*., 1988). The potential mechanisms by which HBO effects bone remodelling have not been fully determined.

In conclusion this study provides evidence that HBO has direct beneficial effects on osteoblast and osteoclast activity in addition to further potential angiogenic effects *in vivo*. Suppression of osteoclastic resorption and promotion of bone formation would be expected to increase bone tissue mass and help provide a more suitable site for reconstructive surgery or potentially enhance fracture repair rate. The studies add to the knowledge and understanding of the potential mechanistic action of this therapy in the

treatment of osteonecrotic bone loss and complicated fracture and also indicate that HBO may promote the formation of new tissue for dental implants.

6.2 Future work

While this work adds to the field further investigations are still needed to examine the effect of HBO on serum biochemical markers of remodelling such as n-mid OCN. Serum OCN is widely used as a marker of bone formation and studies have shown that OCN levels correlate well with direct invasive measures of bone formation (Takahashi *et al*., 2000). Similarly the effect of HBO on resorption markers, such as c-terminal telopeptides of type-I collagen (CTx) would also be useful (Bonde *et al*., 1995). This would enable the assessment of HBO on remodelling activity *in vivo*, which was not possible in the present study due to the requirement of overnight fasting and the frailty of the study cohort.

The impact of HBO on the levels of TNF-R, IL-1R, c-fos, NFkB, p38, TREM2, DAP12, and OSCAR has not been studied in human subjects and would further enhance understanding of the mechanism of action. In addition the effect of HBO on the oxygen sensing mechanism in monocytes and differentiating osteoclasts has also not been established, so it is better to measure the level of oxygen sensing proteins pVHL, HIF-2α, and PHD1-3. The effect of HBO on HIF-1α may be better investigated by other technique, for instance electromobility shift assay (EMSA). This would reveal the extent of HIF-1-binding to DNA indicating not just the amount of HIF-1α formed following HBO treatment but also the degree of activity (Kim *et al*., 2006). To further elucidate the role of HIF-1α in osteoclast and osteoblast function HIF-1α levels could be knocked down with small interfering RNA (siRNA) and the response of osteoclast and osteoblast in hypoxic conditions examined (Knowles & Athanasou, 2009).

The minimum number of treatments to see beneficial effects on bone cell function is also unclear. The results of the current study suggest that this lies somewhere between 10 and 30 treatments. However, whether 10 treatments are sufficient or more are required cannot be ascertained from the current time points. Therefore in order to

establish the effect of accumulative treatments a more in depth study of HBO treatments with samples taken after each session between 10 and 30 treatments needs to be conducted. Bone cell activity at these times would then be compared to time matched controls that had only received 10 sessions.

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Appendices

Hyperbaric Oxygen Therapy Augments Early Stages of Osteoblast Differentiation and Mineralisation *in vitro*

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Introduction

❖Hyperbaric oxygen therapy (HBO), the breathing of 100% oxygen in a sealed chamber at greater than normal atmospheric pressure is used to promote tissue repair(1).

❖In the skeleton HBO has been employed as an adjuvant therapy in cases of osteomyelitis, bisphosphonate-induced osteonecrosis of the jaw and to augment fracture repair. The beneficial effect of HBO may occur through an action on osteoblast differentiation or activity(2).

❖Osteoblast play a pivotal role in the regulation of bone formation and are derived from mesenchymal stem cells. Osteoblast formation is a complex process regulated by multiple factors. Key to this is the expression of transcription factors such as Runx2 that promote the expression of type I collagen and other osteoblastic genes (3).

Aims

The aim of this study was to determine the effects of HBO, pressure and hyperoxia on osteoblast differentiation and bone formation.

Methodology

❖ Mineralisation assay

Saos2 human osteoblast-like cells were cultured in 96 well plates (5 x 10^4 per well) in the presence or absence of b-glycerophosphate (β-GP; 10 mM) and L-ascorbic acid (L-AA; 50 mg/l). Cultures were exposed daily for 90 min to:
1.HBO(2.4 ATA, 97%O2).
2.Pressure (2.4 ATA, 8.8%O2).
3.Hyperoxia(95% O2).
Outside of treatment cultures were maintained in air with (5% CO_2).

Figure 1: Hyperbaric oxygen chambers, these chambers enable high pressure 2.4 ATM and controlled oxygen exposure for cell research.

Mineralization was measured by incorporation of the fluorescent calcium binding marker calcein into mineralised nodules. Culture medium was removed and cells washed with PBS and incubated in culture medium containing 1 mg/ml calcein for 4 h at 37°C. Fluorescence was measured with a cytofluor II fluorescence multi-well plate reader at 485 nm excitation and 530 nm emission.

❖ QPCR analysis of gene expression

Saos-2 cells (1x10^6 cells per well) were incubated in six-well plates for 4, 7, and 14 days for quantitative PCR analysis. This was performed on a StepOne PCR system using the DNA-binding dye SYBR green for investigation of the key regulators of osteoblast differentiation Runx-2, and type one collagen.

Results

HBO stimulates mineralisation

The results from our current study indicate that HBO significantly enhances mineralization, at early stages of nodule formation (seven days), whereas no significant effect was noted at later culture times.

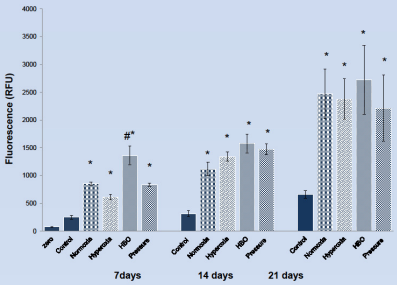

Figure 2: Saos-2 cultures exposed to HBO, hyperoxia, and pressure alone, cells were cultured for seven, 14 and 21 days in the presence of 50mg/L L-AA and 10 mM βGP.
HBO significantly enhanced mineralization, at early stage of nodule formation (seven days), whereas no significant effect was noted at later culture times. Values are means of three separate experiments, with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA.* All values were significantly different from zero day and control group (P<0.001).#Mean value of HBO was significantly different from hyperoxia and pressure groups. RFU, relative fluorescence ratio.

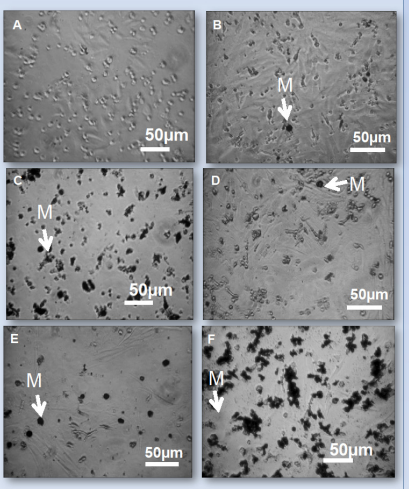

Figure 3: Mineralized nodules in Saos-2 osteoblastic cells (M arrows) (A) un-treated control (B) 21% oxygen at 7days (C) HBO (2.4 ATA, 97%O_2) at 7 days (D) Pressure (2.4 ATA, 8.8%O_2) at 7 days, (E) 95% oxygen at 7days (F) HBO (2.4 ATA, 97%O_2) at 14 days. Cells were cultured for 7,14 days in the presence of 50mg/L L-AA and 10 mM βGP.

Methodology

HBO enhances expression of osteoblast differentiation markers

HBO, hyperoxia and pressure significantly enhanced mRNA expression of the major organic component of bone matrix type one collagen and the osteoblastic transcription factor Runx2 at day four. HBO, pressure and hyperoxia also induced Runx2 and type I collagen mRNA expression in the absence of L-AA and BGP at day four. HBO but not pressure or hyperoxia also enhanced expression at day seven of culture.

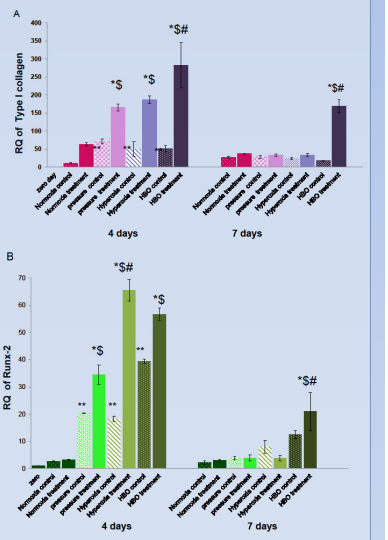

Figure 4: HBO stimulates mRNA expression of (A) type I collagen (B) Runx-2 four and seven days. Hyperoxia and pressure alone stimulate expression at day 4 only. mRNA levels were normalized relative to the expression of type I collagen, and Runx-2 at day 0, and results are shown as mean RQ (±SEM) of triplicate experiments. *p<0.001 versus normoxia,$^{\$}$p<0.001 versus their control, #p<0.001 versus hyperoxia and pressure treatment .**p<0.001 versus to the control. Cells were treated for 4,7 days in the presence of 50mg/L L-AA and 10 mM βGP.

Conclusion

HBO therefore appears to accelerate the rate of osteoblast differentiation and augments early stages of mineralized nodule formation as a consequence. 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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The Effect of Hyperbaric Oxygen Therapy on Osteoclast Formation and Bone Resorption

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Introduction

- Hyperbaric oxygen therapy (HBO) is the breathing of pure oxygen in a sealed chamber at greater than normal atmospheric pressure (1).
- Bone remodelling is the temporally and spatially coupled process that maintains skeletal integrity. It involves the resorption of bone by osteoclast followed by the synthesis of new bone matrix by osteoblast. The correct balance between osteoclastic bone resorption and osteoblastic bone formation is important in maintaining physiological bone homeostasis and disruption of this relationship which can lead to bone disease (2).
- HBO is employed as an adjunctive therapy in a number of skeletal disorders such radio and biosphosphonate induced osteonecrosis of the jaw and chronic osteomylitis (3).
- The formation of osteoclasts from non-committed monocytic precursors is a complex and tightly regulated process that is dependent on regulatory factors of resorption, such as parathyroid hormone, which stimulate osteoclastogenesis by increasing osteoblastic RANKL expression, which binds to its receptor RANK on monocytic precursors (4).
- RANK activation stimulates a cascade of intercellular signals that induce the expression of TRAP, a marker of osteoclast differentiation and DC-STAMP, which regulates mononuclear osteoclast formation (4).

Study objective

The purpose of the present study was to evaluate the effect of HBO, pressure and hyperoxia on RANKL-induced osteoclast formation in RAW 264.7 cells and human peripheral blood monocytes (PBMC).

Methodology

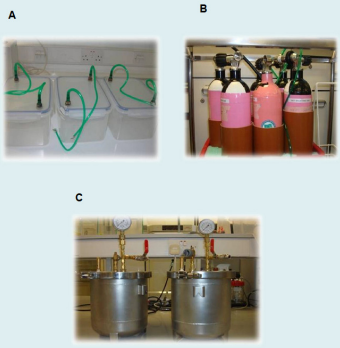

Figure 1: (A) Boxes used in the experiments to provide normoxia, hypoxia and hyperoxia., (B) Oxygen cylinders used in the experiments to provide normoxia, hypoxia and hyperoxia, (C) Hyperbaric oxygen chamber, these chambers controlled at high pressure 2.4 ATM and with (97.9 $\%O_2$) and (8.8 $\%O_2$), specially designed for cells research.

Methodology

Human peripheral blood monocytes

RAW264.7 cells

Cell culture

Cells were seeded into 96 well plates containing bovine bone slices (4-mm diameter) at a density $5x10^5$cells per well with fresh αMEM or DMEM medium and cytokines (RANKL 30ng/ml, and M-CSF 30ng/ml). Cells were fed every 2-3 days by replacing half the medium with fresh medium, culture were maintained at 37°C .

Normoxic culture

Cells were maintained in Normoxic conditions(21% O_2) then exposed daily for 90 min to:
1.HBO(2.4 ATA, 97%O_2).
2.Pressure (2.4 ATA, 8.8%O_2).
3.Hyperoxia(95% O_2).

Hypoxic culture

Cells were maintained in hypoxic conditions then exposed daily for 90 min to:
1.HBO(2.4 ATA, 97%O_2).
2.Pressure (2.4 ATA, 8.8%O_2).
3.Hyperoxia(95% O_2).
4.Normoxia (21%O_2).

Assessment of osteoclast formation and activity

Osteoclast formation was evaluated by osteoclastic marker tartarate resistant acid phosphatase (TRAP) and quantifying resorption area on bovine bone slice.

QPCR

Quantitative PCR was performed on a StepOne PCR system using the DNA-binding dye SYBR green for investigation of the key regulators of osteoclast differentiation RANK, and DC-STAMP(multinuclearity).

Statistical analysis was performed using one way analysis of variance (ANOVA) for comparison between groups. All results were expressed as mean ± SEM and significance at p<0.05

Results of RAW cells

Normoxic conditions

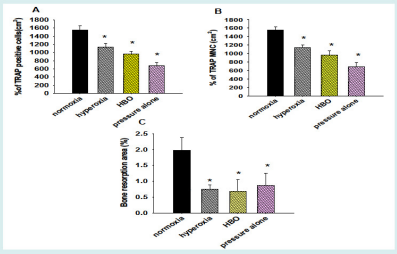

Figure 4: HBO, hyperoxia, and pressure alone exposure inhibits RANKL-induced osteoclast formation, and bone resorption, RAW264.7,treated with RANKL 30 ng/ml incubated in 97.9% oxygen at 2.4 ATA (HBO), 8.8% oxygen at 2.4 ATA(HBO), 95% oxygen alone, and 21 % oxygen alone for 90 min. The mean ± S.E. of three experiments. (A) TRAP positive mononuclear cells, (B) TRAP positive multinuclear cells (C) Bone resorption. *(P < 0.05) significant versus normoxic control.

Results of Human cells

Normoxic Exposure

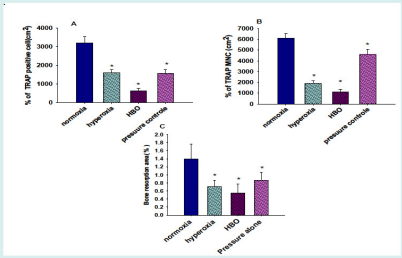

Figure 5: HBO, hyperoxia, and pressure alone exposure inhibits RANKL-induced osteoclast formation, and bone resorption, HPBMC, treated with RANKL 30 ng/ml and M-CSF 30 ng/ml incubated in 97.9% oxygen at 2.4 ATA (HBO), 8.8% oxygen at 2.4 ATA(pressure alone), 95% oxygen alone, and 21 % oxygen alone for 90 min, The mean ± S.E. of three experiments. *P < 0·05 for (A) HBO suppressed RANKL-induced TRAP positive (B) multinucleated cells (C) Bone resorption , *(P < 0.05) significant versus normoxic control.

Results of Human TRAP and QPCR

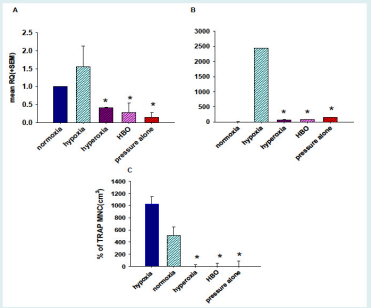

Figure 6: HBO, Hyperoxia, and pressure alone reduced TRAP multinuclear cells and mRNA gene expression of RANK, and DC-STAMP respectively in hypoxic conditions, (A) DC-STAMP (B) RANK (C) multinuclear cell. mRNA was measured using real-time PCR. mRNA levels were normalized relative to the expression of RANK, and DC-STAMP, and results are shown as mean RQ(±SEM) of triplicate determinants.*p<0.05 versus hypoxia.

Results

- Under normoxic conditions, daily, HBO (2.4 ATA, 97% O_2, 90 minutes per day) or hyperoxia (1 ATM, 95% O_2, 90 minutes per day) significantly decreased the number of RANKL-induced TRAP positive mononuclear and multinuclear osteclasts forming in RAW and HPBMC cultures (p <0.05).
- Similarly, HBO and hyperoxia significantly reduced RANKL-induced bone resorption as assessed by the bone slice assay in RAW and HPMC.
- Pressure alone (2.4 ATA, 8.8% O_2, 90 minutes per day) also significantly suppressed osteoclast differentiation and bone resorption to a similar extent as HBO and hyperoxia.
- Furthermore, under hypoxic conditions, typically seen in osteonecrosis, HBO, hyperoxia and pressure (daily, 90 minutes per day) also significantly reduced RANKL-induced osteoclast formation in hypoxic conditions (2% O_2, 22.5 hours per day).
- Quantitative PCR analysis of key regulators of osteoclast differentiation (RANK) and multinuclearity (DC-STAMP) indicated that HBO, hyperoxia and pressure all significantly suppressed RANK and DC-STAMP mRNA expression.

Conclusion

This data suggests that HBO, elevated O_2 and pressure suppress osteoclast differentiation and bone resorption in mouse and human monocytes. Furthermore the data indicates that elevated HBO, pressure and O_2 also directly inhibit osteoclast formation in hypoxic conditions; a hallmark of many skeletal disorders. This provides evidence supporting the use of HBO as an adjunctive therapy to prevent bone loss in a range of skeletal disorders associated with low oxygen partial pressure.

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Hyperbaric 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% 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.

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 1×10^6 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,

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