

**MECHANISMS BY WHICH HYPERBARIC OXYGEN
THERAPY MAY RESOLVE INFLAMMATION IN CHRONIC
WOUNDS**

by

ANWAR JASIB AL-MZAIEL

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Dedication

To

my husband, Ahmed

my parents

and

my kids, Azal and Rafal

*whose love, interest and encouragement
have steered me to where I am today.*

MECHANISMS BY WHICH HYPERBARIC OXYGEN THERAPY MAY RESOLVE INFLAMMATION IN CHRONIC WOUNDS

ANWAR J AL-MZAIEL

Abstract

Hyperbaric oxygen (HBO) therapy is the intermittent inhalation of 100% oxygen at a pressure greater than one atmosphere absolute. It is an effective treatment for various inflammatory conditions, including chronic wounds which are characterized by an excessive influx of neutrophils and their prolonged persistence at the wound site. Neutrophil apoptosis and clearance have been shown to be required for resolution of inflammation. The mechanisms by which HBO aids wound healing are well documented, but its effects on cellular inflammatory response are not well understood particularly with respect to neutrophils. The hypothesis presented in this thesis is that increased oxygenation via HBO assists chronic wound healing by enhancing non-inflammatory neutrophil defences and cell death through apoptosis.

An investigation was carried out into the effects of HBO on neutrophil antimicrobial function and apoptosis using differentiated HL-60 cells as an *in vitro* neutrophil model. The data clearly showed that a single HBO treatment for 90 min caused an increase in the oxidative burst activity of neutrophil-like cells as shown by increased NBT staining, superoxide (cytochrome *c* reduction) and H₂O₂ production (Kruskal-Wallis, $P < 0.05$), and phagocytosis of *Staphylococcus aureus*. HBO treatment displayed a pro-apoptotic effect, enhancing caspase 3/7 activity both in the presence and absence of a TNF- α stimulus (Kruskal-Wallis, $P < 0.05$) and causing morphological changes (observed using Giemsa

and SYBR[®] Safe staining) associated with apoptosis. Although no consistent pattern was observed, both hyperoxia and pressure alone seemed to contribute to both the increase in antimicrobial activity and the increase in apoptosis induced by HBO in these neutrophil-like cells (Chapters 4 and 5).

HBO-enhanced neutrophil clearance by macrophages was investigated using bovine neutrophils and monocyte-derived macrophages (MDM Φ). A single 90 min HBO exposure significantly increased the clearance of fresh and 22 h-aged neutrophils by MDM Φ (two-way ANOVA, $P < 0.05$), suggesting an increase in phosphatidylserine (PS) exposure in apoptotic neutrophils after HBO treatment (Chapter 6). Importantly, a long-term repetitive exposure to HBO in patients with chronic wounds caused a significant decrease in the antioxidant enzyme defence system (one-way repeated measures ANOVA, $P < 0.05$), plasma TNF- α and IL-1 β after 30 HBO sessions, with down regulation of expression of the anti-apoptotic factors, NF- κ B and Bcl-2 (Chapter 7). These findings may go some way towards explaining the effectiveness of HBO treatment not only for chronic wounds but also for other inflammatory conditions that may be affected by this treatment.

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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.

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Publications

Anwar J. Almzaiel, Gary Smerdon, Richard Billington, A. John Moody. Effects of hyperbaric oxygen treatment on antimicrobial function and apoptosis of differentiated HL-60 cells (neutrophil-like) cells, *Life Sciences*, 93, 125-131

Abbreviations

| | |
|--------------------------------|--|
| aFGF | Acidic fibroblast growth factor |
| ANOVA | Analysis of variance |
| Apaf-1 | Apoptotic protease activating factor-1 |
| ATA | Atmospheres absolute |
| ATP | Adenosine triphosphate |
| bFGF | Basic fibroblast growth factor |
| BSA | Bovine serum albumin |
| C5 α | Complement factor 5 α |
| CAT | Catalase |
| CFDA | Carboxyfluorescein diacetate |
| cGMP | Cyclic guanosine monophosphate |
| COPD | Chronic obstructive pulmonary disease |
| CGD | Chronic granulomatous disease |
| Ct | Threshold cycle |
| DCF | Dichlorofluorescein |
| DCI | Decompression illness |
| DDRC | Diving Diseases Research Centre |
| DISC | Death inducing signalling complex |
| DFU | Diabetic foot ulcers |
| DMF | Dimethylformamide |
| DMEM | Dulbecco's Modified Eagle's medium |
| dH ₂ O ₂ | Distilled water |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxynucleotide triphosphates |
| DTT | Dithiothreitol |
| ECL | Enhanced chemiluminescence |

| | |
|----------------|--|
| ECM | Extracellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| ELISA | Enzyme-linked immunosorbent assay |
| EMSA | Electromobility shift assay |
| eNOS | Endothelial nitric oxide synthase |
| E-selectin | Endothelial selectin |
| FADD | Fas-associated death domain |
| FasL | Fas ligand |
| FGF | Fibroblast growth factor |
| FITC | Fluorescein isothiocyanate |
| FMLP | Formyl-Met-Leu-Phe |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GPCRs | G-protein-coupled receptors |
| GPx | Glutathione peroxidase |
| GR | Glutathione reductase |
| GSH | Reduced glutathione |
| GSSG | Oxidized glutathione |
| HBO | Hyperbaric oxygen |
| HBSS | Hank's balanced salt solution |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HIF-1 | Hypoxia-inducible factor 1 |
| HIF-1 α | Hypoxia-inducible factor 1 alpha |
| HIF-1 β | Hypoxia-inducible factor 1 beta |
| HOCl | Hypochlorous acid |
| HPLC | High-performance liquid chromatography |
| HRP | Horseradish peroxidase |
| ICAM-1 | Intercellular adhesion molecule-1 |
| IFN | Interferon |
| IgG | Immunoglobulin G |
| IKK β | I- κ B kinase β |
| IL | Interleukin |

| | |
|------------------|---|
| iNOS | Inducible nitric oxide synthase |
| IGF-I | Insulin-like growth factor-I |
| I/R | Ischemia reperfusion |
| KGF | Keratinocyte growth factor |
| K _m | Substrate concentration required to give half maximal enzyme activity |
| LPS | Lipopolysaccharide |
| L-selectin | Leukocyte selectin |
| MAPK | Mitogen-activated protein kinase |
| MDMΦ | Monocytes derived macrophages |
| MMP | Matrix metalloproteinase |
| mRNA | Messenger RNA |
| MPO | Myeloperoxidase |
| MTT | 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide |
| NADPH | Reduced nicotinamide adenine dinucleotide phosphate |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| nNOS | Neuronal nitric oxide synthase |
| NOS | Nitric oxide synthase |
| NO _x | nitrite + nitrate |
| NPWT | Negative pressure wound therapy |
| NR | Nitrate reductase |
| ·OH | Hydroxyl radical |
| PAO ₂ | Partial pressure of oxygen in alveoli |
| PaO ₂ | Partial pressure of oxygen in arterial blood |
| PBS | Phosphate-buffered saline |
| PBS-T | PBS-Tween |
| PCR | Polymerase chain reaction |
| PDGF | Platelet-derived growth factor |
| PI3K | Phosphoinositide 3-kinase |
| PGE ₂ | Prostaglandin E2 |
| PGN | Peptidoglycan |
| PGs | Prostaglandins |
| PMA | Phorbol 12-myristate 13-acetate |

| | |
|-------------------|--|
| PMNs | Polymorphonuclear cells |
| PN | Peripheral neuropathy |
| PO ₂ | Partial pressure of oxygen |
| PS | Phosphatidylserine |
| PU | Pressure ulcers |
| RFU | Relative fluorescence unit |
| RNA | Ribonucleic acid |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RPMI 1640 | Roswell Park memorial institute-1640 medium |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SLE | Systemic lupus erythematosus |
| SOD | Superoxide dismutase |
| TCOM | Transcutaneous oxygen monitoring |
| TcPO ₂ | Transcutaneous oxygen tension |
| TF | Tissue factor |
| TGF-β | Transforming growth factor-β |
| TIMP | Tissue inhibitor of matrix metalloproteinases |
| TMB | 3,3',5,5'-tetramethylbenzidine |
| TNF-α | Tumour necrosis factor alpha |
| TNFR | TNF receptor |
| TSP-1 | Thrombospondin-1 |
| UHMS | Undersea and Hyperbaric Medical Society |
| UV | Ultraviolet |
| VCAM-1 | Vascular cell adhesion molecule 1 |
| VEGF | Vascular endothelial growth factor |
| VLU | Venous leg ulcers |

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Chronic wounds such as diabetic foot ulcers and venous or pressure leg ulcers constitute a serious health and economic problem. It has been estimated that 15% of diabetic patients are likely to develop lower extremity ulcers, while 14-24% of diabetic patients with foot ulcers might undergo amputation (James *et al.*, 2008). It is expected that this problem will grow rapidly as the population ages, as 85% of chronic wound patients are over 65 years old (Menke *et al.*, 2007, Diegelmann, 2003).

Hyperbaric oxygen (HBO) therapy is a widely-accepted treatment that is used for chronic wounds. It involves intermittent inhalation of 100% oxygen at a pressure greater than one atmosphere absolute (ATA; Feldmeier, 2003). Hyperbaric oxygen therapy has been demonstrated to assist wound healing through increased oxygen delivery to wound tissue (Gill and Bell, 2004). This has consequences for the functions of various different cell types involved in the wound healing process, including neutrophils, monocytes, fibroblasts and keratinocytes. Of particular interest are neutrophils, which play a critical role in the host's defence against infection in normal wound healing, but which contribute to the prolonged inflammation which is observed in chronic wounds; increased influx of neutrophils to the wound site is associated with increased release of destructive contents (Nwomeh *et al.*, 1998a). The effects of HBO on wound healing may be attributed in part to altered neutrophil recruitment and function and further investigation of these effects could lead to the development of treatment protocols and improved wound healing rates.

1.2 Normal wound healing

Wound healing is normally a well-orchestrated, complex process that serves to repair and restore tissue structure and function that has been disrupted by physical, chemical, bacterial or viral insults (Diegelmann and Evans, 2004, Singer and Clark, 1999). The general sequence of events begins as a response to injury and ends with scar formation. Normal (acute) wounds are those that show obvious signs of healing within a reasonable time frame after injury, and without any complications. Cellular and biochemical activities including phagocytosis, cell proliferation, cell migration, collagen synthesis, angiogenesis and cytokine production are well-coordinated in normal wound healing (Fig. 1.1) Those activities occur in three major overlapping phases: inflammation, proliferation and remodelling (Bonomo *et al.*, 2000). Each phase of wound healing is distinct, although the process as a whole is continuous (Singer and Clark, 1999).

1.2.1 The inflammatory phase

The sequence of wound healing events begins immediately after injury. Injury induces disruption of blood vessels and release of blood constituents into the wound site; this facilitates haemostasis, a first step in wound healing to limit blood loss as well as the restoration of the integrity of the damaged tissue (Diegelmann and Evans, 2004). Haemostasis comprises three main stages: vasoconstriction, platelet plug formation, and formation of a clot. Damage to capillaries promotes vasoconstriction which slows blood flow, and enhances platelet adhesion and activation to produce a platelet plug (Clark, 2001, Lasne *et al.*, 2006). The damaged tissue and activated platelets then produce factors that activate a coagulation signalling cascade with the conversion of fibrinogen to fibrin by thrombin, a major step in the formation of the fibrin clot that serves as a temporary matrix

for cellular migration into the site of injury (Grinnell *et al.*, 1981). Activated platelets trapped in the clot release several growth factors and cytokines that induce the inflammatory phase of wound healing (Schultz *et al.*, 2003, Doughty and Sparks-Defries, 2007). The inflammatory phase is immediate and lasts 2-4 days. The inflammatory growth factors, including platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β), epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1) are vital for wound healing (Tsirogianni *et al.*, 2006). Within 6 h of injury, these growth factors have started to recruit neutrophils to clean the wound site and remove foreign particles; cytokines and adhesion molecules give specificity to this attraction (Blakytyn and Jude, 2006). In response to specific chemotactic factors, such as complement factor 5 α (C5 α), and TGF- β , monocytes are attracted to the wound site within 24 h of injury, and later differentiate into macrophages during the inflammatory phase. Macrophages phagocytose the pathogens and any remaining debris from damaged cells (Lobmann *et al.*, 2005). During phagocytosis, neutrophils and macrophages produce reactive oxygen species (ROS) or proteases, including collagenase, matrix metalloproteinase and elastase that cause extracellular matrix (ECM) degradation. In the latter stages of the inflammatory phase, macrophages also release various inflammatory cytokines including TNF- α , IL-1, IL-6 and IL-8 which provide powerful stimuli to recruit more inflammatory cells to the wound site (Blumenfeld *et al.*, 2000) as well as growth factors which are necessary for initiation of granulation tissue formation. In the normal healing process, the late inflammatory phase is around three to four days after injury, while granulation tissue formation follows four to five days later (Singer and Clark, 1999). After 2-3 days, the number of neutrophils begins to decline as they are cleared from the wound site through phagocytosis (Schultz *et al.*, 2003), while macrophages continue to accumulate at the wound site.

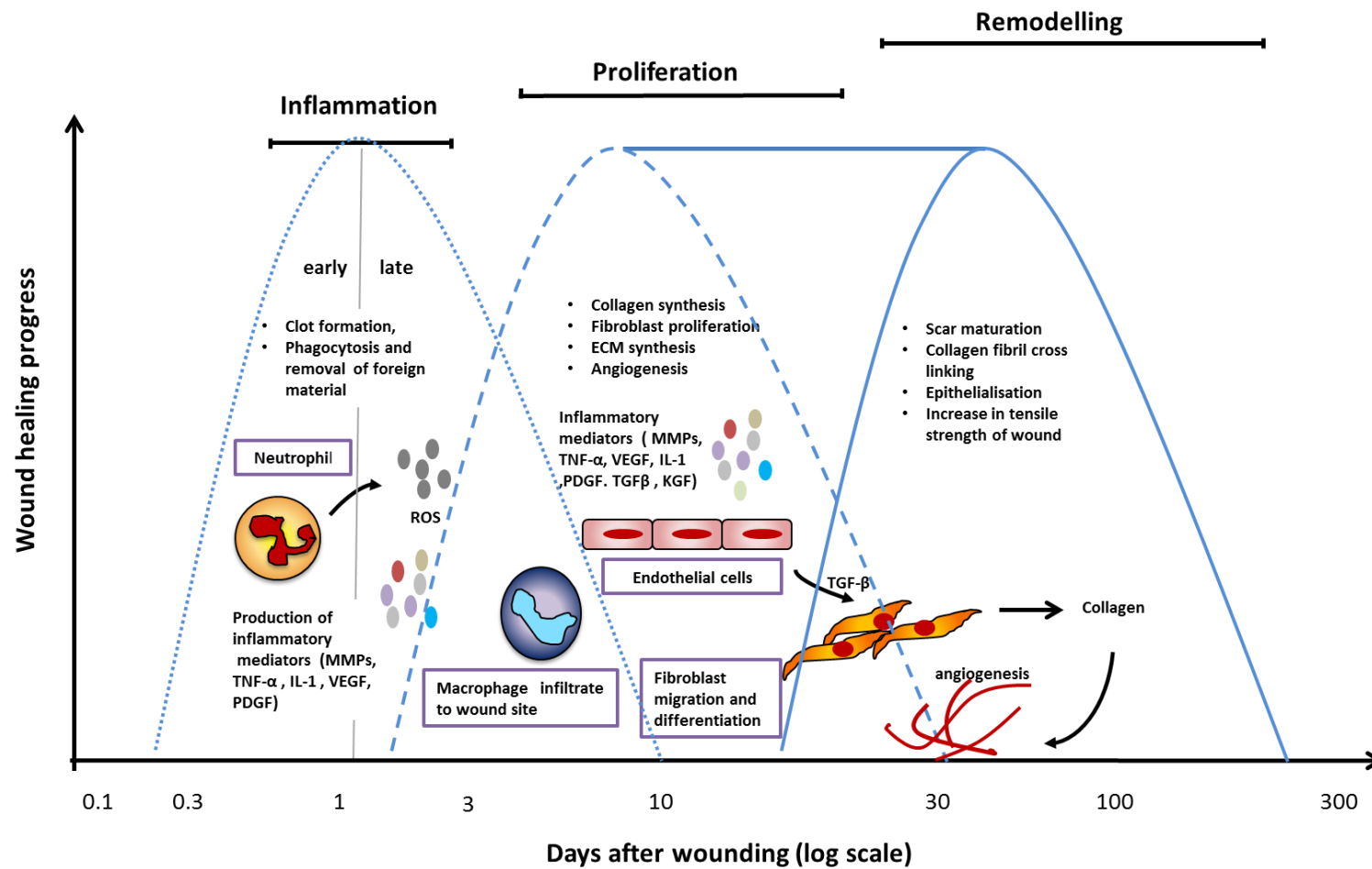


Fig. 1.1 The normal wound healing process. The normal wound healing process consists of three overlapping phases; an inflammatory phase, a proliferative phase and a remodelling phase, all of which involve the participation of different cell types, cytokines and growth factors. Progression through these phases results in normal wound healing (Singer and Clark, 1999).

1.2.2. The proliferative phase

This phase starts at about day three and lasts for about 3-4 weeks after injury. It comprises of angiogenesis, collagen synthesis, fibroblasia, granulation tissue formation, re-epithelialisation and wound contraction (Midwood *et al.*, 2004). Around two days after injury, the macrophages begin to release growth factors (e.g. TGF- β , TGF- α , IL-1, IGF-1 and PDGF) as well as both acidic and basic fibroblast growth factors (aFGF and bFGF) and vascular endothelial cell growth factor (VEGF, an angiogenic factor), which promote cell migration, proliferation, angiogenesis and synthesis of ECM components (Diegelmann and Evans, 2004). Therefore, macrophages mediate the transition from the inflammatory phase to the phase of granulation tissue formation.

Angiogenesis, the growth of new blood vessels from pre-existing vessels in order to resupply oxygen to ischaemic tissue, is stimulated by macrophage activity (release of TGF and TNF- α) and by hypoxia as a consequence of tissue injury (Lamagna *et al.*, 2006) which, through stabilisation of the transcription factor hypoxia-inducible factor 1- α (HIF- α), leads to the production of VEGF by macrophages and endothelial cells (Lewis *et al.*, 1999). Angiogenesis is modulated by VEGF while cell migration, formation of new extracellular matrix, maturation and keratinization of epidermal cells are modulated by EGF, keratinocyte growth factor (KGF) and PDGF (Gray *et al.*, 1993). During fibroplasia and granulation tissue formation, fibroblasts subsequently differentiate to myofibroblasts which are responsible for wound contraction, collagen production and fibronectin secretion to support cell migration (Schafer and Werner, 2008, Midwood *et al.*, 2004).

Re-epithelialization has been shown to start within hours after injury as clotted blood and damaged stroma are removed from the wound (Singer and Clark, 1999). Epidermal cells migrate across the wound bed and a new basement membrane is formed that

separates the epidermis and dermis. Granulation tissue appears during this phase as early as the 4th day after injury and consists of endothelial cells, fibroblasts and inflammatory cells (Shahzad *et al.*, 2007).

Table 1.1 Cytokines and growth factors that affect wound healing

| Cytokines released | Type(s)of cell(s) | Function in wound healing |
|---------------------------|---|---|
| TNF- α | Neutrophils, macrophages | Inflammation; stimulates angiogenesis and collagen synthesis. |
| IL-1 | Neutrophils, macrophages, keratinocytes | Inflammation; collagen synthesis; enhanced chemotaxis. |
| IL-6 | Neutrophils, macrophages. | Inflammation; re-epithelialization. |
| IL-8 | Neutrophils, macrophages | Inflammation. |
| CSF-1 | Multiple cells | Macrophage activation and granulation tissue formation. |
| TFG- β | Platelets, macrophages, fibroblasts | Granulation tissue formation; angiogenic activity; matrix formation and remodelling. |
| PDGF | Platelets, keratinocytes, macrophages, endothelial cells | Granulation tissue formation; angiogenesis stimulation; matrix formation and remodelling. |
| VEGF | Platelets, neutrophils, macrophages, endothelial cells, smooth muscle cells | Granulation tissue formation; angiogenic activity. |
| IFN- γ | Lymphocytes | Stimulation of macrophages and immune cells. |
| EGF | Platelets, macrophages, fibroblasts, keratinocytes | Re-epithelialization; granulation tissue formation; angiogenic activity. |
| FGF-2 | Macrophages, mast cells, endothelial cells, fibroblasts | Re-epithelialization; granulation tissue formation; matrix formation and remodelling. |

1.2.3 The remodelling phase

The final stage of the wound repair process is the remodelling phase, which is characterized by depletion of metabolic activity, as well as the thickening and strengthening of collagen to produce scar tissue. The remodelling process continues for months after wound closure, and it involves a balance between synthesis of ECM components and their degradation by proteases (Schultz *et al.*, 2003). This phase peaks at the third week after the injury, but the contraction of the wound continues in a process involving the proliferation of myofibroblasts (Guo and Dipietro, 2010), collagen and scar formation (Harding *et al.*, 2002). In the maturation and remodelling phase, Type III collagen as the main component is degraded and stronger Type I collagen is deposited, increasing the tensile strength of the wound (Stadelmann *et al.*, 1998, Singer and Clark, 1999). MMPs such as MMP-9 facilitate collagen and extracellular matrix degradation (Corbel *et al.*, 2000). Contraction of the wound occurs during the second week in a non-infected wound, when new matrix is formed and linked together to achieve maximal tensile strength, usually around the 12th week after wounding (Doughty and Sparks-Defriese, 2007, Werner and Grose, 2003, Gabriel, 2009). The scar from a wound can only recover about 80% of the tensile strength of non-damaged tissue (Bentley, 2004). The decreased activity at the wound site enhances the apoptosis and removal of cells such as endothelial cells, fibroblasts, and other inflammatory cells that are no longer needed for the wound healing process (Desmouliere *et al.*, 1995).

1.3 Chronic wounds

Chronic wounds are wounds that do not heal in an orderly sequence and/or over the expected time frame compared to normal wounds. Such wounds usually fail to heal and persist in an inflammatory state. In general, any wound that fails to heal within three months is termed a chronic wound (Mustoe, 2004, Schultz *et al.*, 2003).

There are several conditions which may cause wounds to become chronic and as a consequence not follow the normal healing progress. Such conditions include diabetes (Marston, 2006), obesity (Wilson, 2003), and age (particularly in patients ≥ 60 years) which has a major impact on healing and physiological processes such as the rate of blood circulation, reduction in collagen formation and basement membrane degeneration (Doughty and Sparks-Defries, 2007). Poor nutrition may prevent wound healing by impacting on synthesis of collagen and other proteins needed for the healing process. Bacteria in high concentrations at the wound site may cause toxin production, and they may also compete with cells in the granulation tissue for available nutrients in the wound site (Evans, 2005).

Chronic wounds include diabetic foot ulcers, pressure ulcers, and venous leg ulcers (Marston, 2006). These wounds have a feature in common in that they are all ischaemic. The neuropathic impairment of musculoskeletal systems as well as leukocyte dysfunction and peripheral vascular disease complications also impairs the healing process (Marston, 2006).

Diabetic foot ulcers (DFU) are the most common complication associated with diabetes, and can lead to either neuropathy or vascular disease as a result of damaged microcirculation and ischaemia (Lobmann *et al.*, 2005), which results in an increased risk of foot ulceration (Jeffcoat and Harding, 2003). Throughout the world, foot lesions accompanied by infection are major causes of hospitalization. It has been estimated that

15% of diabetic patients (110 million worldwide) will develop DFU and about 85% of those patients require lower limb amputations as a result of infection (Jeffcoate *et al.*, 2004). Only 31% of DFU will heal within 20 weeks (Margolis *et al.*, 1999) and the average time for total wound healing of diabetic ulcers is about 89 days (Pecoraro *et al.*, 1991). Clearly, factors like macroangiopathy, neuropathy and increased susceptibility to infection can be responsible for the development of these ulcers. Increased blood glucose impairs neutrophils and macrophages that are critical for immune defences of patients and their ability to repair wounds (Lobmann *et al.*, 2005). Furthermore, reduced blood supply at the wound site may impair wound healing through abnormal expression of growth factors and cytokines involved in the healing process. High levels of MMPs are present in diabetic foot tissue. These enzymes catalyse the breakdown of extracellular matrix proteins and growth factors, impair cell migration (Falanga, 2005) and cause poor neovascularisation (Gallagher *et al.*, 2007, Tepper *et al.*, 2002).

Any ulcer or an area of damaged skin below the knee that takes a long time to heal is known as a venous leg ulcer (VLU; Moffatt *et al.*, 1992, Posnett and Franks, 2008). VLUs can result from the failure of venous valves in the calf, leading to hypertension within veins of the leg, and oedema. Once venous pressure reaches 45 millimetres of mercury (mmHg) VLUs are likely to develop. However, other factors that contribute to VLU include arterial disease, obesity, trauma, immobility and diabetes (Simon *et al.*, 2004). About 80-85% of all leg ulcers are VLU and 54% of patients identified have venous ulcers for more than one year (Kane, 2007). VLUs are associated with age and are therefore less common in people aged < 45 years.

Pressure ulcers (PU) are the result of tissue damage from excessive or constant long term pressure on the tissue. Areas affected by PU are characterised by reduced blood supply of oxygen and vital nutrients which result in damage to tissue and ischaemic necrosis that can cause ulcers to develop. A secondary problem associated with PU is

chronic infection which develops as a result of PU and may lead to death. Despite clinical trials on the prevention of PU, the rate of incidence is as high as 66% in hospitalised patients (Rygiel, 2009) and it is more common in elderly patients (Diegelmann, 2003, Brem *et al.*, 2000). Antibiotics are usually used to treat existing PU to resolve infections. However, the existence of microbial biofilms often results in wound complications as biofilms are inherently more resistant to antibiotic treatments (Wissing, 2009). Biofilms are complex, heterogeneous and integrated community of surface-attached microorganisms of either single or multiple microbial species (Bjarnsholt *et al.*, 2008).

1.3.1 Pathological conditions in chronic wounds

Wound healing is made possible through the restoration of microcirculation and the nutrient supply to the tissue (Thackham *et al.*, 2008). The essential nutrient is oxygen, which is important for the production of granulation tissue and resistance to infection. Hypoxia is the most common feature of chronic wounds, and results from the destruction of blood vessels in the wound area and the high oxygen consumption by leukocytes and fibrocytes (Tandara and Mustoe, 2004, Gottrup, 2002). These hypoxic conditions (5-20 mmHg compared with typical values of 30-50 mmHg in healthy tissue (Sheffield, 1998)) affect various aspects of the wound healing process, including angiogenesis, collagen synthesis and deposition, and epithelialisation.

Hypoxic conditions in a wound make the tissue more susceptible to bacterial infection due to impaired neutrophil bactericidal activity (Gordillo and Sen, 2003, Hopf and Rollins, 2007, Sheffield, 1998). High levels of bacteria can induce degradation of the ECM, which impairs cell migration and/or proliferation of leukocytes and fibroblasts, and delays the immune response. Consequently, infection occurs as the bacteria can easily multiply. Neutrophils at the wound site initiate the inflammatory response to end

the bacterial infection. However, bacteria may form biofilms in chronic wounds, and so protect themselves against the action of the cells and effectors of the immune system as well as antibiotics (Edwards and Harding, 2004). In general, wounds with bacterial loads above 10^5 bacteria/mm³ do not heal well (Fonder *et al.*, 2008, Mustoe, 2004). Various bacterial species have been identified in chronic wounds; the most common species include the Gram-positive species *Staphylococcus aureus* and *Enterococcus faecalis*, and the Gram-negative species *Pseudomonas aeruginosa* (Dowd *et al.*, 2008). Hypoxic conditions and bacterial contamination in chronic wounds act as stimuli for cytokine production from macrophages at the wound site. TNF- α is present in high levels in chronic wounds, and is released from monocytes and endothelial cells in response to endotoxins produced by bacteria (Wallace and Stacey, 1998). TNF- α induces IL-1 β production from macrophages. Both cytokines have effects on the synthesis of collagen by fibroblasts and up-regulate the expression of MMPs while down-regulating tissue inhibitors of MMPs (TIMPs; Mast and Schultz, 1996). Studies on fluids from venous ulcers have shown significant increases in TNF- α and IL-1 β (Tregrove *et al.*, 2000) compared with those from healing wounds. This suggests that pro-inflammatory cytokines are present at higher levels in wound fluids from non-healing ulcers than healing ulcers (Harris *et al.*, 1995).

1.4 Immune cells in wound healing

The wound healing process involves a sequence of cellular and biochemical events that serve to restore tissue integrity after injury. Immune cells are involved in every phase of the wound healing process (Clark, 1996). Inflammatory cells, such as neutrophils and macrophages are common during the initial stages of healing where the priorities are haemostasis and the prevention of infection. Within the inflammatory phase of wound healing, neutrophils migrate into the wound site where their function is to prevent or

limit infection. Prolonged neutrophil infiltration sustains the inflammatory phase, so macrophages enter the area, clearing apoptotic neutrophils and wound debris, and resolving inflammation. Therefore the co-ordinated activity of these two cell types is essential in wound healing. Although T-lymphocytes may become involved at later stages to recognise specific pathogens, they seem to make a less significant contribution to wound healing. However, each type of immune cell also has the potential to attract other immune cells to the wound site, and their mediators also appear to be essential for healing.

1.4.1 Neutrophils

Neutrophils are the most abundant white cells in circulation, constituting 40-65% of the total white blood cell pool (Nisisako *et al.*, 2002). They originate in the bone marrow from myeloid stem cells via a process called myelopoiesis. Neutrophils possess a chromatin-dense, lobulated nucleus; hence they are known as polymorphonuclear cells (PMNs). Also, because of the presence of multiple granules in their cytoplasm they are considered to be a type of granulocyte (Bainton, 1999). The number of neutrophils in circulation is normally in the range $3-5 \times 10^6$ cells ml^{-1} blood, but this can increase during infection. The lifespan of mature neutrophils is quite short, estimated to be between 8 and 20 h before they constitutively undergo programmed cell death or apoptosis (Savill *et al.*, 2002), but this can be prolonged when neutrophils enter the infected or inflamed tissues (Edwards, 1994). Once they are activated by chemical stimuli or surface attachment, they undergo a series of morphological changes and release cytotoxic components that are important for destroying pathogens (Edwards, 1993).

Following transmigration from the circulation into the infected and inflamed site, highly coordinated responses aim to kill the invading pathogens. Neutrophils are able to

recognize microorganisms and begin the process of killing and phagocytosing these particles. Microorganisms may be recognized by neutrophils using surface receptors for microbial sugar molecules, but phagocytosis is more efficient if the pathogen is coated with antibody or complement fragments. Following ingestion, neutrophils initiate a series of components that are able to bind to the Fc receptors (Fc γ RI, Fc γ RII and Fc γ RIIIb) and complement receptors (CR1, and CR3), respectively (Smith, 1994). The microorganisms ingested by neutrophils are internalized into phagosomes and these fuse with granules to form phagolysosomes in which the bacteria are killed.

Following engulfment of microorganisms into phagocytic vacuoles, the bacteria are killed by either oxygen-dependent or oxygen-independent mechanisms (Babior, 1978). In the oxygen-dependent mechanism, phagocytosing neutrophils undergo an oxidative burst during which the NADPH oxidase complex assembles at the phagosomal membrane and produces ROS (Fig. 1.2). These toxic ROS are limited to the intracellular environment of the phagocytic vacuole and are rarely released in the extracellular environment unless the opsonized particles (particles in which antigen is attached to the epitope on the bacteria and consequently can be easily recognized by phagocytes) are too large for engulfment, resulting in disturbed phagocytosis. NADPH acts as an electron donor and phagosomal oxygen acts as the electron acceptor to produce superoxide (Alba-Loureiro *et al.*, 2007), while the oxygen-independent mechanisms involve three neutrophil granule subsets: the azurophilic, specific and gelatinase granules, which each contain characteristic antimicrobial proteins such as defensins, bactericidin, permeability proteins and peptides, and enzymes (Reeves *et al.*, 2002). Antimicrobial proteins such as defensins predominantly function by disrupting anionic bacterial surfaces, whilst proteases, such as neutrophil elastase (NE) and cathepsin G (CG), catalyse the degradation of bacterial proteins.

Plasma membrane

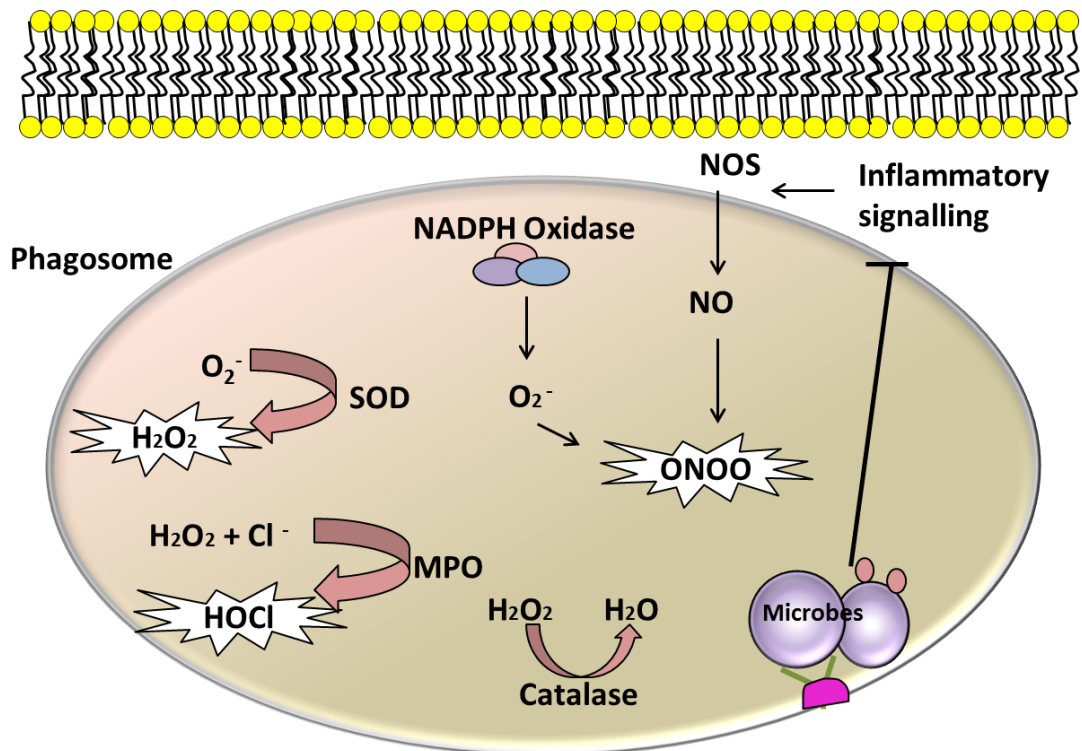


Fig. 1.2 Phagocytosis and bacterial killing in neutrophils. Neutrophil phagocytosis is initiated by microorganisms. NADPH oxidase activation leads to superoxide ($O_2^{\cdot-}$) production which is dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 acts as an intermediate that interacts with myeloperoxidase (MPO) found in neutrophilic granules to form hypochlorous acid (HOCl) or is converted to H_2O by catalase activity. Additionally, NO is produced by nitric oxide synthase (NOS) activity in response to inflammatory stimuli and reacts with $O_2^{\cdot-}$ to form ONOO $^-$ (Hampton *et al.*, 1998).

1.4.2 Neutrophils in wound healing

The first cells that infiltrate the wound site after tissue damage are neutrophils; these cells mediate the first line of defence and initiate the inflammatory response during wound healing. The main function of neutrophils at the wound site is to remove pathogens (Dovi *et al.*, 2004). Foreign materials like bacteria and non-functional host cells initiate chemical signals, attracting neutrophils, which ingest bacteria by phagocytosis, followed by release of ROS into the phagosome to kill them (Hart, 2002, Sylvia, 2003). A rapid and effective response by neutrophils decreases the chance of complications caused by infection, while macrophages, which are also essential for

wound healing (Leibovich and Ross, 1975), arrive at the wound site after neutrophils (up to 48 hours later), whereas other cells of the adaptive immune response do not arrive until 7 days post injury. Neutrophils provide a rapid but non-specific immune response to injury (Dovi *et al.*, 2004). Neutrophils clean the wound site and are then cleared by macrophages (Tsirogianni *et al.*, 2006).

An additional beneficial role of neutrophils in wound healing is to produce growth factors such as GM-CSF (Canturk *et al.*, 2001), VEGF (Mccourt *et al.*, 1999) and IL-8 (Rennekampff *et al.*, 2000), which can promote revascularization and tissue repair. However, most studies suggest there is also a negative role of neutrophils in normal wound healing due to the production of bioactive substances that can cause tissue damage, in addition to the secreted proteases that themselves cause substantial tissue damage (Ashcroft *et al.*, 1999, Ashcroft and Roberts, 2000).

In chronic wounds the inflammatory response is characterized by a continued influx of inflammatory cells, especially neutrophils, into the wound site. Neutrophils produce pro-inflammatory cytokines that attract more inflammatory cells, and release further toxic mediators causing extensive tissue damage (Nwomeh *et al.*, 1998b). Chronic wound conditions such as hypoxia act as stimuli for over-recruitment of neutrophils to the wound site via endothelial cells through multi-step adhesion (Sumpio *et al.*, 2002). The respiratory burst is impaired as very little oxygen is present and hence NADPH oxidase activity is limited. The partial pressure of oxygen (PO_2) in chronic wounds is often 5-20 mmHg, whereas the K_m of NADPH oxidase is 40-80 mmHg (Gordillo and Sen, 2003, Sheffield, 1998). It is generally accepted that neutrophils are affected not only by hypoxia, but also by the bacteria community and cytokine profile of the wound (Yager and Nwomeh, 1999). There are many mechanisms by which neutrophils may impair the healing process; activated neutrophils release an abundance of antimicrobial substances such as ROS, cationic peptides, eicosanoids, and proteases that harm other

cells and directly damage any newly-formed structure in the healing wound, and as result delay the healing process. For example, neutrophil elastase can catalyse the degradation of the components of the extracellular matrix as well as complement, immunoglobulin and cytokines (Weiss, 1989). Moreover, neutrophils are responsible for exacerbating the hypoxic environment in chronic wounds due to their consumption of any available oxygen for respiratory burst activity (Gordillo and Sen, 2003). However, some studies suggest a positive effect of neutrophils in wound healing while others suggest a negative effect. Studies on PU.1 null mice, which are genetically incapable of raising the standard inflammatory response because they lack macrophages and functional neutrophils, have reported an ability to heal skin wounds without implementation of an inflammatory response. In addition, it has been shown that neither neutrophils nor macrophages are needed in these mice for wound healing when there is a low risk of infection (Martin *et al.*, 2003). Dovi *et al.* (2003) reported that neutrophil depletion in the mice resulted in increased re-epithelialization and had no effect on collagen content or on the wound strength, while a decrease in re-epithelialization and vascularisation has been shown in CXCR2 knockout mice with defective neutrophil recruitment (Devalaraja *et al.*, 2000). However, neutrophils are a major source of inflammatory cytokines, such as IL-1, IL-6 and TNF- α which can stimulate the attracted monocytes to differentiate into M1 macrophages (Hübner *et al.*, 1996, Werner and Grose, 2003).

1.4.3 Macrophages in wound healing

At the wound site, macrophages consist of two populations, both of which originate from bone marrow. The first is the resident tissue macrophages that are present in tissues at all times. Normal tissue contains resident macrophages at a low density of 1-2 per mm² (Dipietro *et al.*, 1995), whilst the second is derived from the differentiation of

circulating monocytes after recruitment to the injury site. Once the monocytes migrate through the vessel wall, they release enzymes that degrade ECM proteins to facilitate their migration into the wound site. In response to stimuli such as LPS or IFN- γ present in the micro-environment, monocytes differentiate into macrophages (Martin and Leibovich, 2005). Macrophages can display different functional phenotypes and physiological properties in the wound site (Mantovani *et al.*, 2002). These cells can be divided into two groups: the M1 phenotype (classically activated) and the M2 phenotype (alternatively activated; Schwacha, 2003). In response to mediators such as LPS or IFN- γ , macrophages are differentiated into M1 macrophages which have been attributed with cytotoxic functions in acute and chronic inflammation (Mosser and Edwards, 2008, Gordon, 2003). Macrophages are alternatively activated by IL-4 and IL-13 into M2 macrophages (Gordon, 2003) that have been attributed with roles in proliferation of connective, endothelial and epithelial tissue in wound healing (Martinez *et al.*, 2008). The influence of macrophages on each stage of repair varies with the specific phenotype. During the inflammatory phase, macrophages exhibit pro-inflammatory functions such as antigen presentation, phagocytosis, production of inflammatory cytokines and growth factors that enhance the wound healing process. Fibroblasts, keratinocytes and endothelial cells are stimulated by macrophages to induce ECM formation, re-epithelialization, and angiogenesis, while in the remodelling phase, macrophages can change the ECM composition by the release of degrading enzymes. As such, macrophages play an important role in the transition from the inflammatory phase to the proliferative phase in which they are able to switch and sustain wound healing events (Singer and Clark, 1999). It seems that the balance between the two phenotypes is important in the phases of wound healing and any disturbance in this balance results in a poor healing process. The macrophage phenotype in wound healing is not fully characterized and it appears to change during the healing process (Daley *et*

al., 2010, Lucas *et al.*, 2010). Furthermore, different macrophage phenotypes produce different cytokines and growth factors that stimulate capillary growth, collagen synthesis and fibrosis (Mirza *et al.*, 2009).

Studies on wound healing in which macrophages have been depleted (Table 1.2) show a delayed infiltration of fibroblasts and defective fibrosis in guinea pigs (Leibovich and Ross, 1975). Furthermore, recent results on wounds in macrophage-depleted mice indicate decreased re-epithelization, granulation tissue formation, angiogenesis, wound cytokine production, myofibroblast-associated wound contraction, vascularization and scar formation in mice depleted before injury or 3 days after injury (Goren *et al.*, 2009, Mirza *et al.*, 2009, Lucas *et al.*, 2010).

Table 1.2 The effects of macrophage depletion on wound healing

| Depletion stage | Wound closure | Granulation tissue formation, angiogenesis | Collagen/fibrosis | study |
|------------------------|----------------------|---|--------------------------|------------------------------|
| early | decrease | ND | decrease | (Leibovich and Ross, 1975) |
| early | decrease | decrease | decrease | (Mirza <i>et al.</i> , 2009) |
| early | decrease | decrease | decrease | (Goren <i>et al.</i> , 2009) |
| early | decrease | decrease | decrease | (Lucas <i>et al.</i> , 2010) |
| middle | decrease | decrease | ND | (Lucas <i>et al.</i> , 2010) |
| late | ND | ND | ND | (Lucas <i>et al.</i> , 2010) |

ND: No difference

1.4.4 Interactions between neutrophils and macrophages

Neutrophils have a short life span; 8-20 h in circulation and 1-4 days in tissue (Akgul *et al.*, 2001). Macrophages play an important role in the control of cellular events of wound healing through their ability to induce apoptosis and phagocytosis of a variety of wound cells. Macrophages target neutrophils during the inflammatory phase; the same is true of fibroblasts and endothelial cells during the proliferative and remodelling phases. Tissue injury is characterised by infiltration of neutrophils and later macrophages, with the number of neutrophils decreasing as the macrophages become the predominant inflammatory cell. Neutrophils undergo apoptosis in the wound, and are then recognised and ingested by macrophages (Meszaros *et al.*, 1999; Fig. 1.3). The macrophages recognize apoptotic signals including phosphatidyl serine (PS), and thrombospondin 1 (TSP1), by several surface receptors such as integrin $\alpha_v\beta_3$, and CD36, a class B scavenger receptor with a very short C-terminal cytoplasmic domain, has been proposed to cooperate with the vitronectin receptor for internalisation of TSP-1-opsonised apoptotic neutrophils by MDM Φ (Meszaros *et al.*, 2000).

Phagocytosis of human apoptotic neutrophils has been shown to suppress the release of the pro-inflammatory mediators GM-CSF, IL-8, MCP-1, eicosanoids and chemoattractants from phagocytic cells (Meagher *et al.*, 1992, Hughes *et al.*, 1997). For instance, ingestion of apoptotic cells triggers an anti-inflammatory response from monocytes/macrophages such as decreased production of TNF- α , IL-1 and IL-12, and upregulation of IL-10 (Voll *et al.*, 1997, Fadok *et al.*, 1998). It has been suggested that apoptotic neutrophils at wound sites release factors, e.g. low-molecular weight lipid-soluble factors, that suppress the production of pro-inflammatory mediators from macrophages (Daley *et al.*, 2005). Recent studies in CD18 knockout mice as models for leukocyte adhesion deficiency syndrome 1, a disorder characterised by infections and impaired wound healing in humans, have provided evidence regarding interactions

between neutrophils and macrophages in wounds (Peters *et al.*, 2005). In these animals, skin wounds failed to recruit neutrophils, assembled a normal complement of macrophages, and exhibited retardation of closure by contraction. These changes occurred in the context of increased pro-inflammatory cytokine production and decreased TGF- β 1 and its type II receptor. It was suggested from these findings that the lack of apoptotic neutrophils in the wounds of the knockout animals deprived macrophages, resulting in a decrease in TGF- β 1 production, a main regulator of phagocytosis. After resolution of the inflammatory phase of wound healing, macrophage numbers decrease in wounds. Some die at the site of the wound, as evidenced by the presence of intracellular macrophage molecules such as arginase I in the wound extracellular fluids (Albina *et al.*, 1990); others are likely to migrate to draining lymph nodes (Brancato and Albina, 2011).

Cell death can occur via two distinct processes, necrosis and apoptosis. Necrosis describes a pathological form of cell death resulting from severe trauma or acute cellular injury followed by loss of membrane integrity (Zhivotovsky, 2004), while apoptosis is a more controlled form of cell death in which dying cells exhibit distinct morphological features that mark them for phagocytosis by neighbouring cells with no loss of membrane integrity (Fig. 1.3). Apoptotic cells are characterised by several common features that were first described by Kerr and colleagues (1972), such as cell shrinkage, condensed chromatin, nuclear fragmentation (pyknosis) and finally cytoplasmic budding to form membrane-bound apoptotic bodies. More importantly, during apoptosis membrane integrity is maintained and PS, a molecular marker of apoptotic cells, appears on the outer leaflet of the plasma membrane, and can be measured via the binding of annexin V, a specific phospholipid-binding protein (Hongbo and Fabien, 2008). A balance between anti- and pro-apoptotic signals in the cell determines whether or not apoptosis will take place.

In contrast to necrosis, the cell remains intact (as may be indicated by the exclusion of vital dyes), and maintains its contents (Hongbo and Fabien, 2008).

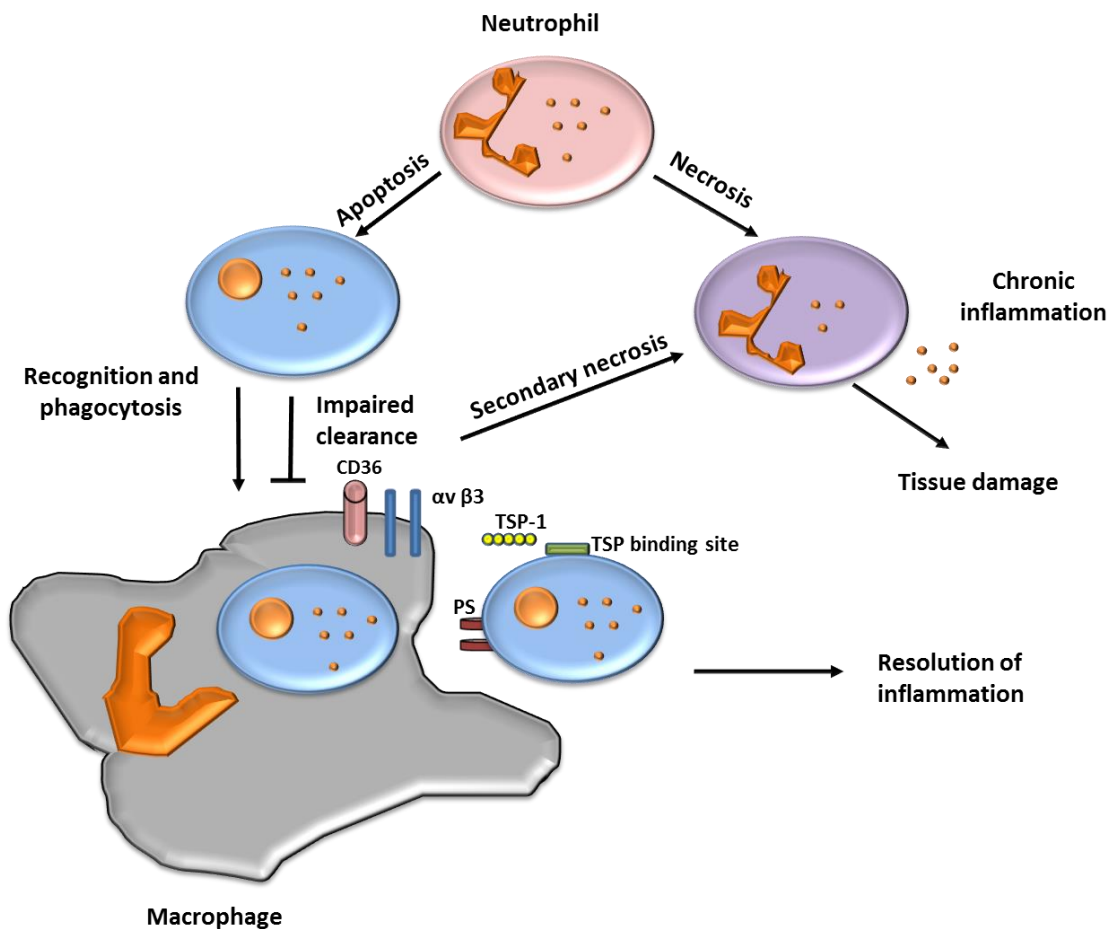


Fig. 1.3 The fate of neutrophils. Neutrophils are removed from tissue in a non-inflammatory process to ensure that they do not cause tissue damage through release of their intracellular contents. Neutrophils undergo apoptosis followed by recognition and phagocytosis by macrophages, a process associated with release of mediators such as TGFβ by macrophages. The apoptotic cells display various signals such as phosphatidylserine (PS) and surface-bound thrombospondin (TSP) that are recognized by phagocyte receptors CD36 in conjugation with integrin α_vβ₃. Necrotic cell death results in loss of membrane integrity and subsequent release of contents that cause tissue injury and induce further recruitment of inflammatory cells (Gregory and Devitt, 2004).

Neutrophils are terminally differentiated cells which spontaneously undergo apoptosis in the absence of cytokines, pro-inflammatory agents, or any other extracellular stimuli (Savill *et al.*, 1989). This process is followed by the removal of apoptotic neutrophils by macrophages, which prevents them from releasing their cytotoxic contents extracellularly, as would occur if the cells died by necrosis (Haslett, 1992). Neutrophils undergoing apoptosis lose their abilities to undergo chemotaxis and phagocytosis, and to generate a respiratory burst or to degranulate (Wyllie *et al.*, 1980).

Cells have two major pathways that lead to apoptosis: the internal and the external pathway. The latter is mediated by death receptors while the internal is mitochondria-dependent; both pathways involve cleavage of and thereby activation of pro-caspase3 into caspase-3 which in turn affects other components in the apoptotic pathway (Riedl and Shi, 2004). Caspases (*Cysteine Aspartic-Specific Proteases*) are a family of cysteine proteases which are central to the neutrophilic apoptotic machinery. The active enzymes have a heterotetrameric structure comprised of two large and two small subunits with two active sites per molecule (Thornberry and Lazebnik, 1998). Caspases catalyse the cleavage of peptide bonds after aspartate residues in their substrate (Nicholson, 1996). Depending on the functional and pro-domain lengths which can modulate caspase activation, caspases can be divided into initiator or effector caspases; upstream initiator caspases have large N-terminal pro-domains, and include caspases -2, -8, -9 and -10, while downstream effector caspases possess short apparently non-functional terminal pro-domains and include caspases -3, -6 and -7 (Thornberry *et al.*, 1997).

The extrinsic initiation of apoptosis is originated by death receptors, the tumour necrosis factor receptor (TNFR) family and FasR (Degterev *et al.*, 2003). The ligand for TNFR is soluble and is secreted during initiation of inflammation, while the ligand for FasR is FasL expressed as a trimer during lymphocyte development and homeostasis (Krammer, 2000); binding of this ligand to its receptor leads to the formation of a cytoplasmic death domain that triggers an intracellular signalling cascade and facilitates activation of caspases -8 and -3 (Nagata, 1997). The intrinsic pathway of apoptosis is mediated via various mechanisms resulting in mitochondrial release of cytochrome *c* into the cytosol in response to cellular damage and stress. Cytochrome *c* is a key component of the apoptosome, a complex that activates caspase-9, which in turn cleaves and activates pro-caspase-3 (Riedl and Shi, 2004). Caspase-3 is involved in the inactivation of the DNA repair system (Casciola-Rosen *et al.*, 1996). A study by Murphy *et al.* (2008) has

confirmed that although human neutrophils have only small amounts of cytochrome *c*, the apoptotic protease activating factor 1 (Apaf-1) which interacts with cytochrome *c*, is essential in their apoptosis. Release of cytochrome *c* from mitochondria is regulated by the Bcl-2 family of proteins (Akgul *et al.*, 2001; Fig. 1.4).

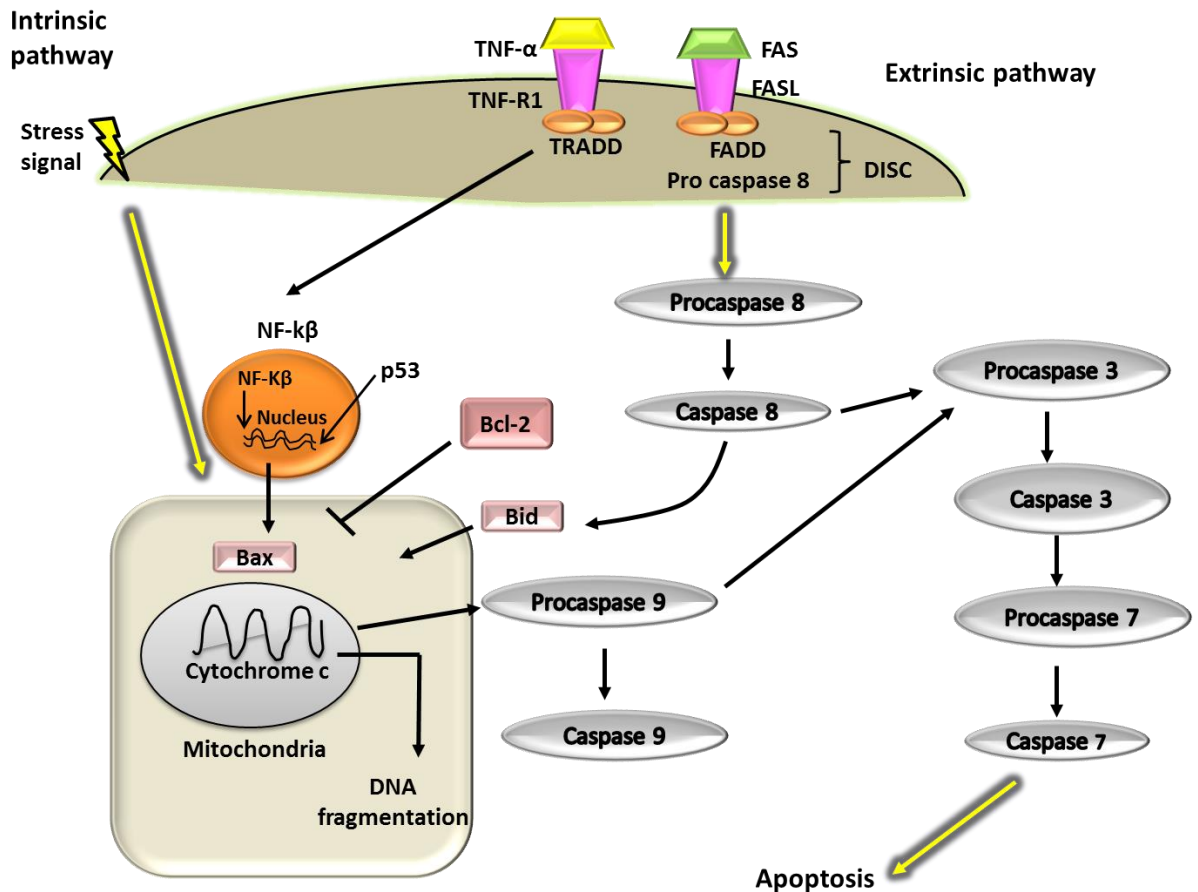


Fig. 1.4 The extrinsic and intrinsic pathways of apoptosis. The extrinsic pathway engages the cell surface death receptors such as TNFR1 and Fas with their specific ligands, which leads to procaspase-8 activation, which in turn cleaves and activates the effector caspase-3 that degrades essential proteins and confers the apoptotic morphological changes. The intrinsic pathway involves mitochondria; it is activated following a wide variety of intercellular signals such as DNA damage and oxidative stress. Transduction of these signals requires the permeabilization of the mitochondrial outer membrane by proteins of the Bcl-2 family and results in the release of mitochondrial apoptogenic factors including cytochrome *c* (Riedl and Shi, 2004).

1.5 Role of oxygen in wound healing

In wound healing, oxygen is a basic requirement for the production of adenosine triphosphate (ATP). ATP is synthesized by oxidative phosphorylation in mitochondria and is essential for intracellular processes such as biosynthesis, movement and transport. Therefore, oxygen is required to provide adequate energy for proper cellular function (Gordillo and Sen, 2003, Tandara and Mustoe, 2004). However, not all cells are oxygen-dependent, for example neutrophils.

Collagen synthesis and deposition are critical for wound healing in providing the matrix for angiogenesis and tissue remodelling. This process is oxygen-dependent. Conversion of proline into hydroxyproline is an important step in the formation and stabilisation of the pro-collagen triple helix and is catalysed by prolyl hydroxylase, which requires oxygen as a substrate as well as α -ketoglutarate. The concentration of oxygen at which prolyl hydroxylase displays 50% of its activity has been determined to be in the PO_2 range of 20-25 mmHg (Hopf *et al.*, 2001). Thus depletion of tissue oxygen results in production of an immature polypeptide in the rough endoplasmic reticulum which is excreted as non-functional gelatinous protein (Gordillo and Sen, 2003). *In vivo* studies have shown that collagen deposition in wound healing is directly related to tissue oxygenation, so increasing wound oxygenation results in increased collagen deposition and tensile strength (Jonsson *et al.*, 1991).

At the cellular level, NADPH oxidase is responsible for the respiratory burst that occurs in neutrophils during the inflammatory phase of the healing process. This NADPH-linked enzyme produces oxidants by consuming high amounts of oxygen, which contributes to successful wound healing, as these oxidants are required for killing bacteria and limiting wound infection (Soneja *et al.*, 2005). Neutrophils and other phagocytes use oxygen to produce ROS by respiratory burst activity, including

oxidizing species such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). These ROS also serve as cellular messengers to promote essential processes in wound healing (Hopf and Rollins, 2007), such as cytokine production, angiogenesis, cell motility and extracellular matrix formation (Sen, 2003).

Tissue hypoxia is known to initiate angiogenesis, while increased oxygen levels are needed to create the oxygen gradient that promotes and maintains it (Neil, 2007), because angiogenesis is an oxygen and growth factor-dependent process (Knighton *et al.*, 1981). It has been established that VEGF is the major long term angiogenic stimulus at a wound site. It promotes endothelial cell proliferation and migration, inhibits apoptosis, and increases vascular permeability (Neufeld *et al.*, 1999). *In vitro*, VEGF has been reported to up regulate endothelial ICAM-1 expression and to promote neutrophil adhesion and then migration into the wound site as part of the inflammatory phase of wound healing (Lu *et al.*, 1999, Radisavljevic *et al.*, 2000, Kim *et al.*, 2001). These processes all stimulate angiogenesis (Dvorak, 2000). Hypoxia stimulates VEGF production and angiogenesis through HIF-1 α as well as other pathways involving MEK/ERK molecules and the gene for early growth response-1 (EGR-1), a transcription factor produced at the wound site (Semenza, 2000, Michiels *et al.*, 2000, Lo *et al.*, 2001). Treatment with oxygen induces VEGF expression in endothelial cells and macrophages (Maniscalco *et al.*, 1995, Deaton *et al.*, 1994, Darrington *et al.*, 1997), and increases VEGF protein expression in wounds *in vivo* (Sheikh *et al.*, 2000). Exposure to oxygen triggers ROS production which in turn stimulates cells such as fibroblasts, keratinocytes and macrophages to release VEGF and enhance angiogenesis. For example, H_2O_2 , produced by the respiratory burst activity of neutrophils, up regulates VEGF expression in keratinocytes (Kuroki *et al.*, 1996).

1.6 Hyperbaric oxygen (HBO) therapy

Hyperbaric oxygen therapy is defined by the Undersea and Hyperbaric Medical Society (UHMS) as a mode of medical treatment that involves intermittent inhalation of 100% oxygen whilst inside a treatment chamber compressed to a pressure greater than one 1 ATA (Jain, 2004b). In order for this treatment to be delivered to the patient, monoplace and multiplace chambers are used. Monoplace chambers, as the name indicates, are designed to treat a single patient at a time. Multiplace chambers, on other hand, are larger and can accommodate more than one person (up to 12 patients). Oxygen can be delivered through face masks, nasal hoods or endotracheal tubes (Rowe, 2001, Broussard, 2004).

The development of hyperbaric medicine is linked to the history of diving medicine. The first hyperbaric chamber was designed in 1662 by the British physician Henshaw who used compressed air for medical purposes, as oxygen was not discovered until 1775, by Joseph Priestley. In 1834, Junod used a hyperbaric chamber to treat pulmonary diseases with two to four atmospheres of pressure. The Junod trial report in 1800 triggered an expansion of the use of hyperbaric chambers worldwide. For treating diving accident victims, a system using HBO was proposed by Dräger in 1917 (Yarbrough and Behnke, 1939, Jain, 2004b), but it was not until 1937 that the first medical application of HBO was reported for the treatment of decompression sickness (Severinghaus, 2003).

Since the 1930s, HBO therapy has been widely used in diving medicine and for numerous other medical conditions. The UHMS evaluates and regulates the current and potential indications for hyperbaric oxygen therapy and they update their guidelines periodically. Using HBO for these indications is evidence-based with literature showing significant results in the treatment outcomes (Broussard, 2003, Feldmeier *et al.*, 1997).

The UHMS recommends HBO as treatment of choice for several conditions:

- a) Air or gas embolism, carbon monoxide poisoning, and decompression illness.
- b) As an adjunctive therapy for clostridial myonecrosis, crush injury, compartment syndrome and other acute ischaemia, enhanced healing in selected problem wounds, exception anaemia, interaction abscess, necrotizing soft tissue infections, refractory osteomyelitis, radiation tissue damage, compromised skin grafts and flaps and thermal burns.

Patients with chronic wounds which have not responded to standard treatments, including antibiotics, topical dressings, debridement of non-vital tissue and correction of vascular problems within at least 30 days may be considered for HBO (Broussard, 2004). To identify patients that should respond well to HBO, transcutaneous oxygen monitoring (TCOM) is used. This is a quantitative measure of oxygen availability to tissue, demonstrated as the wound transcutaneous oxygen tension (TcPO₂) at atmospheric pressure. Patients with wound TcPO₂ < 40 mmHg that improves to >100 mmHg whilst breathing 100% oxygen at 1 ATA are considered suitable for adjunctive HBO (Fife *et al.*, 2009, Sheffield, 1998). The wound TcPO₂ is then tested under HBO, with values of 200 mmHg in non-diabetic patients and 400 mmHg in diabetic patients being considered as indicators of a patient exhibiting a good response to HBO.

Patients with hypoxic wounds are subjected to HBO treatment usually delivered at 1.9-2.5 ATA for sessions of 90-120 min each. During treatment the patient breathes 100% oxygen while the chamber is compressed over 5 min. To minimise the risk of oxygen toxicity, patients have 5 min “air-breaks” after 30 min and 65 min of treatment, during which they breathe air instead of oxygen. The treatments are usually given once a day, and up to five times per week in conjunction with other appropriate treatment(s), including antibiotics, wound debridement and dressing.

1.6.1 Side effects and counter indications

Although HBO is a relatively safe treatment, there are several potential side effects caused by the increased pressure and hyperoxia, which occur in less than 1% of all patients. The commonest side effect is middle ear barotrauma, of which there is an estimated incident rate of 52 cases per every 10,000 patients (Thackham *et al.*, 2008), and occurs in any closed air space where the patient's ears are not properly cleared during compression and decompression. Boyle's law states that as pressure increases volume decreases at a constant temperature, so in spaces where air may be trapped, such as the ear or sinuses, the air is compressed and then, during decompression, increases in volume, leading to the barotrauma (Thackham *et al.*, 2008, Broussard, 2000). Another side effect of HBO is myopia, a lens deformation resulting from oxidative damage to the lens protein (Foster, 1992, Tibbles and Edelsberg, 1996, Gill and Bell, 2004) which can develop after extensive HBO treatment (more than 20 treatments), but usually resolves within eight weeks post-treatment (Kindwall, 1995, Jain, 2004b). Oxygen toxicity is a rare side-effect of HBO. It involves either the pulmonary or central nervous system with an incident rate estimated at between 1 and 3 cases per 10,000 treatments (Al-Waili and Butler, 2006, Kulikovskiy *et al.*, 2009).

Recent studies on HBO have reported that toxic effects correlate with high pressures (4-5 ATA) and/or long exposure durations of over 2 h (Harabin *et al.*, 1990, Kleen and Messmer, 1999, Wada *et al.*, 2001). The cellular damage through oxidation of lipids, protein and DNA is the most characteristic feature of oxidative stress induced by HBO (Speit and Bonzheim, 2003) in both human and animal models, and has been found to be related to pressure and time of exposure (Table 1.3). The "air breaks" mentioned above are designed to minimise the risk of oxygen toxicity. Overall, the side effects of HBO treatment are rare and most can be avoided or treated without any long-term effect on patients.

An absolute contraindication to HBO is an untreated pneumothorax, which must be excluded before treatment (Hampson, 1999). Other absolute contraindications include chemotherapy treatment with antibiotics (e.g. doxorubicin and bleomycin or adriamycin), the cardiac and pulmonary toxicity of which is exacerbated by HBO. Relative contraindications include respiratory infections, severe asthma/chronic obstructive pulmonary disease and steroid treatments, seizure disorders and pregnancy.

Table. 1.3 Oxidative effects of prolonged exposure to HBO

| HBO treatment | Model | Treatment effects | Reference |
|---|---|--|----------------------------------|
| 100% oxygen at 2.5 ATA for total 30 min (1 session/day) | Patient with pathological conditions related to hypoxia. | Repeated exposure to HBO (15th session) led to a significant increase in malondialdehyde, a significant decrease in SOD and catalase expression by erythrocytes compared to 1 st HBO session. | (Benedetti <i>et al.</i> , 2004) |
| 2.5 ATA for 60 min | Patient with pathological conditions related to hypoxia. | Repeated HBO sessions did cause significant changes on erythrocyte antioxidant capacity and lipid peroxidation. | (Eken <i>et al.</i> , 2005) |
| 100% oxygen at 1, 1.5, 2, 2.5 and 3 ATA, respectively for 3 h | Sprague-Dawley rats. | Elevated levels of oxidative markers TBARS and SOD were found in lung, brain and erythrocytes after 2 h of HBO exposure. | (Oter <i>et al.</i> , 2005b) |
| 3 ATA for 30, 60, 90 and 120 min | Rat brain tissue. | TBARS and SOD were found to increase in a time-dependent manner. | (Korkmaz <i>et al.</i> , 2008) |
| 2.8 ATA For 2 h (1 session/ day) over seven days | Sprague-Dawley rats with streptozotocin (STZ)-induced diabetes. | The levels of blood glucose and TBARS increased significantly and the activity of SOD decreased significantly in the erythrocytes and all organs of rats with diabetes subjected to HBO exposure. | (Matsunami <i>et al.</i> , 2010) |

1.6.2 Physical mechanisms of HBO

Ambient air contains 21% oxygen, 79% nitrogen, and 0.04% carbon dioxide at sea level. The total pressure of this mixture at sea level is defined as 1 atmosphere absolute (ATA). This is equivalent to 760 mmHg. This value is calculated using “Dalton’s law” which states that “the total pressure exerted by a gases mixture equals the sum of the partial pressures of different gases in the mixture”. According to this law, the partial pressure of oxygen (PO₂) in the room air we breathe equates to 160 mmHg.

$$PO_2 = 760 \text{ mm Hg} \times 21\%/100\% = 160 \text{ mm Hg}$$

However, the partial pressures exerted by gases dissolved in water or body fluids differs from those in ambient air (Bookspan, 2003). Henry’s law describes the solution of gases in liquids, and states that “at constant temperature, the amount of a gas dissolved in a liquid is proportional to the partial pressure of the gas above the liquid” (Gill and Bell, 2004). Henry’s law is represented by the following formula:

$$\textit{Concentration of dissolved gas} = \textit{gas partial pressure} \times \textit{solubility coefficient}$$

Oxygen is the example of the gas in this case, whilst the plasma represents the liquid. The solubility coefficient decreases with increasing temperature.

Boyle’s law states that “at constant temperature for a fixed mass of gas, the absolute pressure and the volume of a gas are inversely proportional”. The law can be stated in another way, that is, that the product of volume and absolute pressure will remain constant.

$$\textit{Pressure} \times \textit{Volume} = K (\textit{constant})$$

Together, Boyle’s and Henry’s laws constitute the basis for hyperbaric therapy. Increasing the PO₂ of oxygen has little effect on the oxygen bound to haemoglobin (Hb), as Hb is 97-98% saturated at sea level in normal air, giving an O₂ content of

approximately 200 ml O₂ l⁻¹ arterial blood. Additionally, 3.2 ml O₂ l⁻¹ blood is dissolved in plasma and is carried in solution (Hunt *et al.*, 2004). Oxygen passes from the ambient air to the alveolar air and continues through the pulmonary alveoli, diffusion from the pulmonary capillary and venous blood to the systemic arterial and capillary blood. Finally, oxygen moves into the interstitial and intracellular fluids where it is consumed in the endoplasmic reticulum, and mitochondria. The concentration gradient controls oxygen diffusion from the alveoli to the blood. The partial pressure of oxygen differs in the arterial and venous circulations. The partial pressure of oxygen in the alveoli (PAO₂) equals 104 mmHg, while in the arterial blood (PaO₂) equals 90 mmHg and in venous blood (PvO₂), 40 mmHg. The difference between PAO₂ and PvO₂ (64 mmHg) causes oxygen to diffuse from the alveolar side to the arterial side and then to the tissue capillaries where it is released from Hb for use by cells (Tibbles and Edelsberg, 1996, Jan *et al.*, 2006). Normally 97% of oxygen carried in the arterial blood is chemically bound to haemoglobin while the remaining 3% is dissolved in plasma. One gram of Hb can combine with a maximum of 1.34 ml of oxygen. The normal concentration of Hb is 15 g per 100 ml of blood; when Hb is fully saturated (100%) with oxygen, 100 ml of blood can transport 20 ml of oxygen. After leaving the lung, Hb carries about 19.5 ml of oxygen per 100 ml of blood. In passing through tissue capillaries, this amount is reduced to 5 ml of oxygen per 100 ml blood. Hyperbaric oxygen increases the amount of oxygen dissolved in plasma. By applying the previously-described laws to the air that we breathe (containing 21% O₂), at 1 ATA the amount of dissolved oxygen in 100 ml of plasma equals 0.499 ml, when inhaling 100% oxygen at 1 ATA, the O₂ concentration increases to 1.5 ml/100 ml of plasma, and when the pressure is increased to 3 ATA, the amount of dissolved oxygen in 100 ml of plasma increases to 6.422 ml (Tibbles and Edelsberg, 1996, Jan *et al.*, 2006, Marx *et al.*, 1990) which is enough to meet the basic

metabolic needs of healing processes in tissues without need for the contribution of Hb (Gabb and Robin, 1987, Leach *et al.*, 1998). This is the principle mechanism of HBO.

1.6.3 Evidence for the effectiveness of HBO in the treatment of chronic wounds

Several studies have been conducted into the healing potential of HBO, the majority investigating chronic non-healing wounds (Table 1.4). Wattle *et al.* (1991) reported in a non-controlled study that HBO accelerated the healing of diabetic foot lesions. Another prospective controlled study with chronic diabetic foot lesions carried out by Doctor *et al.* (1992) found better control of infection and less need for amputation in the group treated with conventional management and four sessions of HBO compared with the group treated only with conventional management. In a randomised study, Faglia *et al.* (1996) compared HBO with comprehensive protocols for decreasing the risk of amputation in 70 patients with diabetic foot ulcers who received either HBO (35 patients) or conventional treatment (35 patients). The study demonstrated a significant difference in amputation rates between the two groups, with less amputation in the HBO group, and concluded that HBO in addition to conventional therapy was effective in decreasing the need for amputation. A study by Zamboni *et al.* (1997) showed that HBO treatment caused significant decreases in wound size area in comparison to wounds in control subjects. The wound PO₂ in the HBO-treatment group was raised from 12 mm Hg in room air to 560 mm Hg under HBO. Baroni *et al.* (1987) conducted a non-randomized study in patients with diabetic gangrene. The study reported significant wound healing in patients who received HBO compared with subjects who had not received HBO. In a small randomised study by Kessler *et al.* (2003), a doubling of the healing rate of non-ischaemic chronic foot ulcers was found in selected diabetic patients with HBO treatment. The study consisted of 28 patients with chronic foot ulcers subjected to conventional treatment, with 14 of them additionally receiving HBO. The

wounds responded well to HBO with an increase in TcPO₂ near ulcers from a mean of 21.9 mmHg to 454.2 mmHg during the treatment. Another small randomized study, by Abidia *et al.* (2003), demonstrated an improved healing rate with HBO treatment in diabetic ulcers compared to the placebo group receiving hyperbaric air.

The effects of HBO on non-diabetic problem wounds, including venous stasis ulcers, were examined in a study by Hammarlund and Sundberg (1994) in which 16 patients were subjected to HBO. A significant decrease in wound size area was reported in the HBO group compared to the control group.

Table 1.4 *In vivo* clinical trials using HBO as adjunctive treatment for chronic wounds

| No. of patients | Description of wounds | HBO treatment | Control treatment | outcomes | Studies |
|-----------------|---|---|---|---|--------------------------------|
| 59 | Diabetic foot lesions, ischaemic or neuropathic wounds | Multi-disciplinary wound care plus HBO 29 times, 45 min, 2.5 ATA | Patients with diabetic foot lesion treated with multi-disciplinary wound care only. | Complete healing: 52 (88%) of 59, major amputation 7 (11.8) of 59. | (Wattel <i>et al.</i> , 1991) |
| 30 | Diabetic patients with chronic foot lesions (over 2 year). | Multi-disciplinary wound care plus HBO 4 times, 45 min, 3 ATA over 2 weeks. | Surgery, antibiotics, local wound care. | Above ankle amputations: HBO, 2 (13%) of 15; control, 7 (47%) of 15; minor (other) amputations: HBO, 4 (27%) of 15; control, 2 (13%) of 15. | (Doctor <i>et al.</i> , 1992) |
| 70 | 3 month foot ulcers, Wagner grade 2-4, signs of neuropathy similar in both groups | Multi-disciplinary wound care plus HBO 30 times, 90 min, 2.2-2.5 ATA. | Diabetic patients with chronic wounds treated with multi-disciplinary wound care only. | Major amputation: HBO, 3 of 35 (8.6%); control 11 of 35 (33.3%). | (Faglia <i>et al.</i> , 1996) |
| 10 | Diabetic chronic non-healing lower extremity wounds.. | Multi-disciplinary wound care plus HBO 30 times, 90 min, 2.0 ATA. | Diabetic patients treated with multi-disciplinary wound care only. | Complete healing: HBO, 4 (80%) of 5; control, 1 (20%) of 5; no amputation in either group. | (Zamboni <i>et al.</i> , 1997) |
| 29 | Duration not specified, chronic Wagner grade 0-2 foot ulcers | Control treatment not specified plus HBO 30 times, 120 min, 2.5 ATA. | Control treatment not specified plus air at 2.5 ATA. | The ulcers resolved completely following a course of HBO. | (Lin <i>et al</i> 1996) |
| 18 | Ischaemic ulcers > 6 weeks, 1-10 cm in diameter. | Multi-disciplinary wound clinic plus HBO therapy 30 times, 90 min, 2.4 ATA. | Diabetic patients with chronic wounds treated with air at 2.5 ATA plus multi-disciplinary wound clinic. | Proportion of ulcers healed, minor/major amputation. | (Abidia <i>et al.</i> , 2003) |

Table continued

| No. of patients | Description of wounds | HBO treatment | Control treatment | outcomes | Studies |
|------------------------|--|---|--|--|---------------------------------|
| 28 | >3 month, chronic Wagner grade 1-3 foot ulcers, signs of neuropathy in all patients. | Multi-disciplinary wound care plus HBO 30 times, 90 min, 2.4 ATA. | Patients with chronic wound treated with multi-disciplinary wound management. | Wound size reduction, proportion of ulcers healed. | (Kessler <i>et al</i> 2003) |
| 16 | 1 year duration, normal distal blood pressure at the ankle and the first digit. | Usual care plus HBO 30 times, 90 min, 2.5 ATA. | Patients with chronic wounds treated with air at 2.5 ATA plus multi-disciplinary wound clinic. | Proportion of ulcers healed, wound size reduction. | (Hammarlund and Sundberg, 1994) |

1.6.4 Effects of HBO on wound healing

The primary effect of hyperbaric oxygenation is to produce a favourable gradient for oxygen diffusion from capillaries to ischaemic tissue sites, because under HBO (2-2.5 ATA), haemoglobin becomes fully oxygenated and oxygen dissolves in plasma in direct proportion to the PO_2 thereby becoming sufficient to meet the requirements for tissue oxygen without needing the support of oxygen bound to haemoglobin (Fife *et al.*, 2002). Secondary effects include reduction of oedema formation, neovascularisation, stimulation of neutrophil-oxidative killing mechanisms, phagocytosis of bacteria (Dellinger, 2005) and improved collagen synthesis and cross-linking (Sheikh *et al.*, 2000).

Oxygen is an important component of the healing process; HBO improves wound healing in patients with chronic leg ulcers as tissue oxygen tension increases (Bishop, 2008, Jain, 2004a). Increased oxygen pressure, above 2 ATA, but below 3 ATA, increases oxygen diffusion into the tissue. Hypoxic areas benefit substantially from this increase (Gill and Bell, 2004).

Studies have shown that HBO has a direct effect on anaerobic bacteria, which lack antioxidant defences such as superoxide dismutase (SOD) and catalase, so any oxidative stress caused by HBO is toxic to these bacteria (Barriere *et al.*, 2001, Thom, 1990). For example, HBO has a bactericidal effect in, and is used as treatment for, necrotizing fasciitis and osteomyelitis, due to the high oxygenation of wound tissue and production of ROS. Not only that, HBO has been shown to enhance the activity of various antibiotics, for example aminoglycosides against *Pseudomonas aeruginosa* (Muhvich *et al.*, 1989, Park *et al.*, 1991).

Hyperbaric oxygen has been shown to have a positive effect on collagen synthesis; HBO increases arterial PO_2 resulting in an increase in wound oxygen tension in hypoxic

wounds (Broussard, 2004). Consequently collagen production is at an optimum with an PO₂ of 150 mmHg (Hopf *et al.*, 2001). Hyperbaric oxygen also promotes granulation tissue formation, epithelialisation (Lavan and Hunt, 1990) and fibroblastic activity (Hehenberger and Hansson, 1997).

1.6.4.1 Effect of HBO on the immune system

Hyperbaric oxygen is used as adjunctive therapy for various inflammatory conditions (as described in section 1.6), including gas gangrene, refractory osteomyelitis, necrotizing soft tissue infection, burns and chronic wounds (Camporesi, 1996). Effects of HBO on the immune response have been previously reported (Benedetti *et al.*, 2004, Benson *et al.*, 2003, Bitterman *et al.*, 1994, Brenner *et al.*, 1999). Some changes in cellular and humoral components of the immune system were indicated. Hyperbaric oxygen has been shown to affect some aspects of host defence such as enhancing the respiratory burst activity of neutrophils and subsequently bacteria killing, although the results of studies on this are conflicting (Table 1.5). However, overall HBO effects seem to be immunosuppressive and lead to an anti-inflammatory response (Brenner *et al.*, 1999). Several mechanisms have been proposed, including inhibition of the production of inflammatory mediators such as cytokines, a decrease in lymphocyte proliferation (Gadd *et al.*, 1990), and impairment of leukocyte and macrophage function (Murphy *et al.*, 1975).

Table 1.5 Effects of HBO on respiratory burst activity of neutrophils

| stimuli | HBO treatment | outcome | study |
|---|---|--|----------------------------------|
| None | HBO: 2.4ATA for 90 min. | Demonstrated a marked increase in the magnitude of a signal with properties consistent with free radicals by low temperature electron spin resonance spectroscopy in human blood following HBO exposure. | (Narkowicz <i>et al.</i> , 1993) |
| Phorbol ester | HBO: 2.8 ATA or 3ATA for 45 min. | HBO had no effect on respiratory burst (using DCF-diacetate by human neutrophils). | (Thom <i>et al.</i> , 1997) |
| 10 ⁻⁷ M FMLP | HBO: 1.8ATA for 45 min. | HBO induced phagocytosis by neutrophils and H ₂ O ₂ in 10 human volunteers. | (Labrouche <i>et al.</i> , 1999) |
| Zymosan, PMA and FMLP | HBO: 2.4ATA for 90 min. | Zymosan-induced respiratory burst was reduced by HBO, while FMLP- or PMA-induced respiratory was not affected. | (Kaln <i>et al.</i> , 2002) |
| <i>Escherichia coli</i> , or priming with 1 µg ml ⁻¹ TNF-α followed by 10 ⁻⁷ M FMLP | HBO treatment (2.8 ATA for short and long-term (five days) 90 min exposure. | No significant changes in respiratory burst (using dihydrorhodamine) and phagocytic activity (determined by intake of FITC-labelled opsonized <i>E. coli</i>) in human neutrophils after HBO treatment. | (Jüttner <i>et al.</i> , 2003) |
| FMLP | HBO treatment (2.8 ATA for 45 min) | HBO increases intracellular ROS production, in both FMLP-stimulated and unstimulated neutrophils. | (Thom <i>et al.</i> , 2008) |

Hyperbaric oxygen treatment may also enhance wound healing by reducing pathological inflammation (immunosuppressive effects; Al-Waili and Butler, 2006, Zhang *et al.*, 2008). Hyperbaric oxygen treatment significantly decreased TNF-α and IL-1β production in LPS-stimulated monocytes and macrophages from rats or human peripheral blood (Lahat *et al.*, 1995, Benson *et al.*, 2003; Table 1.5). In animal models of ischaemia, HBO has been shown to reduce immunocompetent cell sequestration and synthesis of TNF-α (Yang *et al.*, 2001), due to a decrease in ICAM-1 expression levels

(Buras *et al.*, 2000). The immunosuppressive effect of HBO is also demonstrated by the inhibited development of Th cells, suppression of autoimmune systems, decreased responses to antigens, a decrease in circulating lymphocytes and leukocytes, and increased allograft survival (Satio *et al.*, 1991, Xu *et al.*, 1997, Hansbrough *et al.*, 1980, Erdmann *et al.*, 1995). Additionally, HBO reduces the expression of the cyclooxygenase-2 (COX-2) mRNA, an enzyme involved in inflammation, and the expression of hypoxia induced factor-1 α (HIF-1 α), a transcription protein associated with hypoxia (Yin *et al.*, 2002, Li *et al.*, 2005). HBO treatment every day at 2 ATA causes significant increase in Bcl-2, an inhibitor of apoptosis (Wada *et al.*, 2000).

Moreover, HBO has been shown to reduce the number of inflammatory cells and protein levels in the aqueous humour in rabbits (Ersanli *et al.*, 2005). HBO inhibits inflammation in various clinical conditions including cerebral ischaemia (Helms *et al.*, 2005), acute colitis (Akin *et al.*, 2002), severe acute pancreatitis (Balkan *et al.*, 2006) and compartment syndrome (Abdullah *et al.*, 2006). This suggests that the anti-inflammatory effects of HBO contribute to decreasing the inflammatory mediators such as NO that play important roles in immunological responses and wound repair (Bredt and Snyder, 1992, Garthwaite, 1991). Nitric oxide production and bioactivity has been established as a major signalling messenger in physiological processes such as angiogenesis, vasodilation, granulation tissue formation, and collagen deposition that are critical to normal wound healing (Schwentker and Billiar, 2003, Fukumura *et al.*, 2001). NO is produced continuously by nitric oxide synthase activity (NOS); this enzyme catalyses NO formation from the amino acid L-arginine, with consumption of oxygen. To date, tissue oxygen is known to significantly affect endogenous NOS production in articular cartilage where the tissue PO₂ is comparable to that in ischemic wounds (Fermor *et al.*, 2007). Studies in rodents investigating the effect of HBO on ROS and NO formation have provided evidence of increased NO concentrations as a

result of NOS stimulation (Thom, 1993). Another group of inflammatory mediators is the prostaglandins (PGs) that are generated in response to injury and inflammation. HBO treatment causes a significant decrease in prostaglandin E₂ (PGE₂) production, a potent immunosuppressive in alveolar bone, splenic macrophages and brain (Chen *et al.*, 2002, Mialon and Barthelemy, 1993, Inamoto *et al.*, 1991). These studies confirm that HBO might possess anti-inflammatory properties.

1.6.4.2 HBO, growth factors and cytokines in wound healing

Hypoxia at the site of a wound due to injury to the blood vessels develops and triggers production of inflammatory cytokines which play an important role in the wound healing process. Hyperbaric oxygen affects the release of several types of cytokines and growth factors that are vital in wound healing (Table. 1.6). While hypoxia is required to initiate angiogenesis, oxygen supplied via HBO therapy has additional angiogenic effects through continued release of VEGF (Sheikh *et al.*, 2000). VEGF promotes endothelial cell proliferation and migration, inhibits apoptosis, and increases vascular permeability (Neufeld *et al.*, 1999). It has been observed that VEGF upregulates endothelial ICAM-1 expression *in vitro*, and promotes neutrophil apoptosis (Lu *et al.*, 1999, Radisavljevic *et al.*, 2000, Kim *et al.*, 2001). A previous study by Fok *et al* (2008) has shown that HBO increased the levels of VEGF in rabbits with bone lesions after 6 weeks of treatment. VEGF production is related to oxygen tension; as oxygen tension increases the VEGF production increases which in turn enhances angiogenesis and the progress of wound healing (Ozaki *et al.*, 1997). Studies on the ischaemic rabbit ear ulcer model suggest that HBO stimulates PDGF production thereby enhancing granulation tissue formation (Kang *et al.*, 2004). These effects seems to be mediated by an increase in ROS (Sen *et al.*, 2002).

Studies have shown various effects of HBO on cytokines. HBO exposure transiently suppressed stimulus-induced pro-inflammatory cytokines in monocytes (Benson *et al.*, 2003). In another study, exposure of healthy volunteers to hyperoxia, high pressure alone, or HBO for 90 min, stimulated IFN- γ secretion from lymphocytes after HBO (Granowitz *et al.*, 2002). In ischaemia-reperfusion injury, treatment with HBO increased TNF- α and TGF β 1 in tissue (Grunenfelder *et al.*, 2002). HBO has been shown to have an immunosuppressive effect; this is clear from the marked decrease in IL-1 and IL-2 production and a significant decrease in PGE₂ production by monocytes (Inamoto *et al.*, 1991). This suggests that the reduction of PGE₂ production may play an important role in the anti-inflammatory effects of HBO.

Table 1.6 HBO effects on cytokines and growth factors

| cytokine | source | HBO treatment | Comment/Outcome | Author, year |
|---------------------------------|------------------------------------|--------------------------------|--|--|
| IL-1 | Monocytes-macrophages | HBO, 2.4 ATA, 90 min | Inhibits LPS-induced IL-1 β synthesis | (Benson <i>et al.</i> , 2003) |
| TNF- α , IL-1 β | Monocytes-macrophages | HBO, 2.0 ATA, 90 min | Inhibits stimulus-induced TNF- α and IL-1 β synthesis. | (Inamoto <i>et al.</i> , 1991, Lahat <i>et al.</i> , 1995) |
| IL-2 | Monocytes | HBO, 2.5ATA, 90 min | Decrease in IL-2 production and IL-2 receptor expression. | (Ginaldi <i>et al.</i> , 1991) |
| TNF- α | Monocytes-macrophages | HBO, 2.4ATA, 90 min | Inhibits TNF- α production. | (Benson <i>et al.</i> , 2003) |
| TGF β | Chronic ischaemic wounds in rabbit | HBO, 2.4 ATA, 90 min | Increases levels of TGF β and produces a synergistic effect on a chronic wound model in young rabbits, but not in old rabbits. | (Zhao <i>et al.</i> , 1998, Bonomo <i>et al.</i> , 2000) |
| PDGF- β | Acutely ischaemic wounds in rabbit | HBO, 2.0 ATA, 90 min | Increases expression of PDGF- β receptor. | (Bonomo <i>et al.</i> , 2000) |
| VEGF | HUVECs | HBO, 2.5 ATA, 2-8 h | Up regulates VEGF production and release. | (Lee <i>et al.</i> , 2006, Eliss <i>et al.</i> , 2005) |
| IFN- γ | Lymphocytes | HBO, 2 ATA, 90 min | Reduces PMA/PHA-stimulated release of IFN- γ by lymphocytes. | (Granowitz <i>et al.</i> , 2002) |
| FGF | Muscle cells | HBO, 3 ATA, 1 h/day for 14 day | Up regulates FGF expression. | (Asano <i>et al.</i> , 2007) |

1.7 Hypotheses and aims

1.7.1 Hypotheses

In chronic wounds, the healing sequence appears to halt at the inflammation stage. Failure to resolve the inflammatory phase is critical to the prevention of wound healing. This study focused on two cell types central to the resolution of inflammation and progression of healing in chronic wounds, neutrophils, whose primary function is to prevent or limit infection, which tends to sustain the inflammatory phase, and macrophages, which serve to clear apoptotic neutrophils and initiate the proliferative phase. Therefore, the interplay between these two cells types is of key importance. HBO enhances chronic wound healing by increasing the amount of oxygen available to the wound tissue, while some of the therapeutic effects of HBO involve revascularisation and improved bacterial killing.

The main hypothesis is that increased oxygenation also assists chronic wound healing by reducing tissue damage resulting from over-recruitment of neutrophils. Specifically, the hypothesis is that HBO mediates two effects: (a) it improves the non-inflammatory antimicrobial function of neutrophils, and (b) it enhances neutrophil apoptosis and hence phagocytosis by macrophages, leading to resolution of the inflammatory response (Fig 1.5).

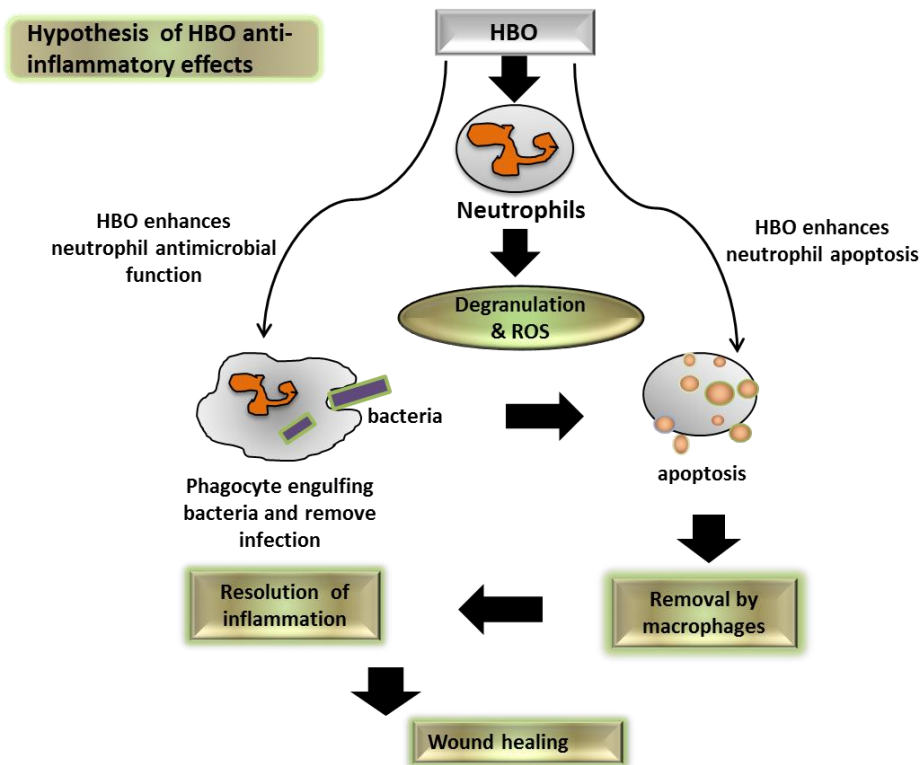


Fig. 1.5 Schematic diagram showing hypothesised HBO-induced anti-inflammatory pathways and their effects on wound healing. HBO enhances the respiratory burst activity of neutrophils and ROS. The antimicrobial effects of HBO are associated with enhanced neutrophils apoptosis as a consequence of an increase in ROS production by the cells themselves. Apoptotic neutrophils are safely removed by macrophages, leading to resolution of inflammation and subsequent wound healing.

1.7.2 Aims and objectives

The aims of this study were therefore to investigate the effects of HBO on neutrophils, to elucidate those elements that are affected by oxygen tension and also the interplay between these cells and macrophages. Differentiated HL-60 cells and bovine neutrophils were used as *in vitro* models for experiments on neutrophils and in co-culture experiments. An *in vivo* study on blood from patients with chronic wounds was also used to investigate the long-term effects of HBO treatments on some biochemical parameters.

The specific objectives were:

1. To develop a model for neutrophil function and the inflammation cascade that could be investigated after treatment with HBO.
2. To examine the effects of HBO on the function of human neutrophils and longevity (apoptosis).
3. To establish the effects of HBO on the inflammatory mediator molecules such as pro- and anti-inflammatory cytokines, in order to understand the molecular mechanism of inflammation.
4. To examine the effects of HBO on macrophage phagocytosis of apoptotic neutrophils.
5. To conduct an *in vivo* study tracking changes in biochemical parameters from patients with chronic wounds over a course of HBO treatment.

CHAPER TWO

GENERAL METHODS

2.1 Material and methods

2.1.1 Hyperbaric oxygen and pressure control treatments

Hyperbaric chambers were obtained from Process Projects Limited (Hampshire, UK) and connecting hoses from Pressure Lines Ltd (Plymouth, UK). Different gas mixes were kindly donated by the DDRC (Plymouth, UK). To obtain hyperbaric oxygen conditions, the gas in the chambers was flushed for 4 min at 3 l min⁻¹ with 97.9% O₂, 2.1% CO₂, and then pressurized to 2.4 atmosphere absolute (ATA) over 2 min; 2.1% CO₂ at 2.4 ATA was used to achieve a CO₂ concentration equivalent to 5% CO₂ at 1 ATA in a conventional incubator (Fig. 2.1). To investigate the effect of pressure alone, cells were treated with 8.8% O₂, 2.1% CO₂ and 89.1% N₂ at 2.4 ATA; 8.8% O₂ was used such that the O₂ concentration would be equivalent to 21% at 1 ATA. For normobaric hyperoxic or hypoxic treatments, cells were also flushed with a gas mixture containing a 95% O₂, 5% CO₂ or 5% O₂, 5% CO₂ balanced with N₂ (Table 2.1) for 2-4 min at a rate of 4 l min⁻¹ in gas-tight plastic boxes (21.5 cm × 21.5cm × 11cm). 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). Since it has been estimated that physiological hypoxia is 1-5 % O₂ (Ivanovic, 2009), 5% O₂ was chosen as the hypoxic environment for neutrophils, although this is slightly higher than observed in chronic wounds (2% O₂). Control cell cultures for each experiment were placed in similar boxes under 21% O₂, 5% CO₂, 74% N₂, at 1 ATA. All gas mixes were prepared by the DDRC. Treatments were continued for 90 min, after which the cells were removed (pressure chambers were decompressed over 8-10 min) and centrifuged for 5 min at 400 g followed by washing and re-suspension to either 1 × 10⁶ or 2 × 10⁶ cells ml⁻¹ in PBS, supplemented RPMI-1640 medium or Hank's Balanced Salt Solution

(HBSS) as appropriate. Cells were used immediately in the assays described below. All treatments were carried out at room temperature.

Table 2.1 *In vitro* conditions. Two pressure treatments were used in this study, an HBO treatment, representing patient treatment conditions, and a pressure control treatment, used to examine the effect of treating cells with increased pressure, but with no increase in the amount of available oxygen (Table 2.7). CO₂ (5 %) was included in both conditions to represent the presence of CO₂ at the cellular level. Hyperoxic conditions were used to examine the effect of increased oxygen.

| Treatment | Gas composition | Pressure |
|--------------------|---|----------|
| HBO | 97.9% O ₂ , 2.1% CO ₂ | 2.4 ATA |
| Pressure control | 8.8% O ₂ , 2.1% CO ₂ , balance N ₂ | 2.4ATA |
| Hyperoxia | 95% O ₂ , 5% CO ₂ | 1 ATA |
| Hypoxia | 5% O ₂ , 5% CO ₂ , balance N ₂ | 1 ATA |
| Normoxia (control) | 21% O ₂ , 5% CO ₂ , balance N ₂ | 1 ATA |

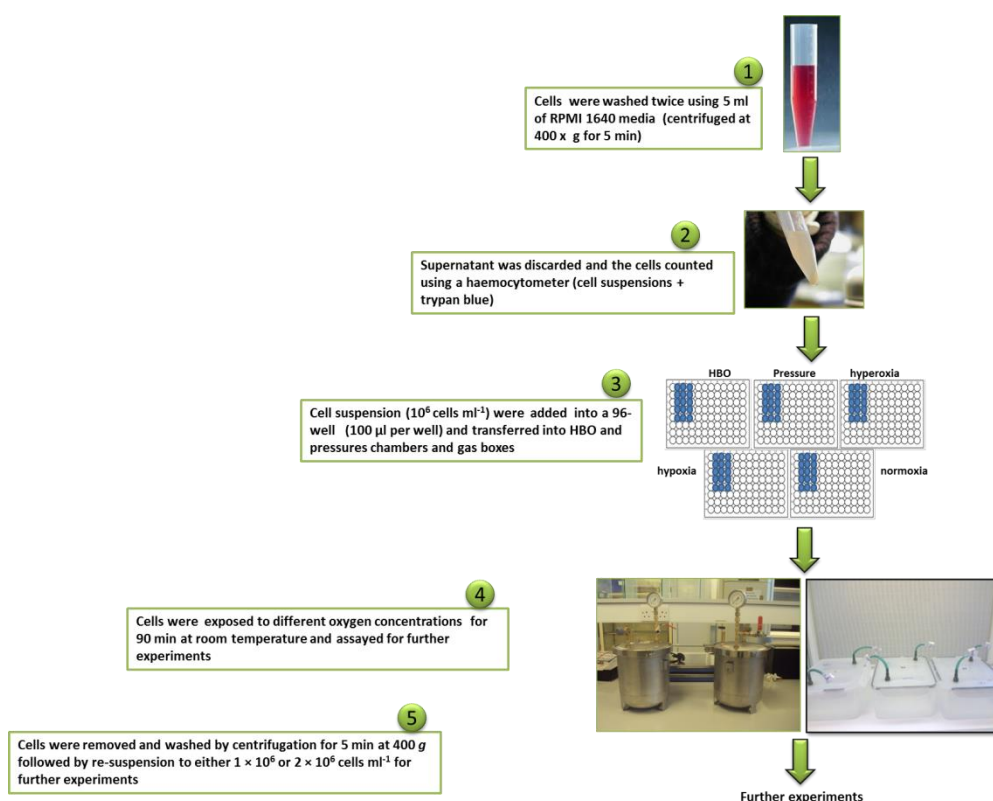


Fig. 2.1 Experimental design Cells were placed inside the hyperbaric chamber, which was then, sealed using a clamp seal. The chamber was compressed with a gas mix (depending upon the experiment) to 2.4 ATA, and then the outlet valve was sealed. For normobaric hyperoxic or hypoxic treatments, cells were flushed with a gas mixture containing a 95% O₂, 5% CO₂ or 5% O₂, 5% CO₂ balance N₂ (Table 2.1) for 2-4 min at a rate of 4 l min⁻¹ in gas-tight plastic boxes. After 90 min of treatment, the chamber was decompressed by slowly releasing the outlet valve over 8-10 min until the gauge pressure fell to 0 ATA.

2.1.2 Measurement of NBT reduction

Nitroblue tetrazolium (NBT) is a yellow low water-soluble nitro-substituted aromatic tetrazolium compound that reacts with superoxide ions produced by the respiratory burst activity of neutrophils to form blue-black formazan deposits. The ability of NBT to be reduced by cells was determined by light microscopy.

Respiratory burst activity measurements using NBT were performed as described by Verstuyf *et al.* (1995). Neutrophil-like cells (100 μl of 10^6 cells ml^{-1} in RPMI-1640) (section 3.2.2) were mixed with 100 μl of 100 ng ml^{-1} PMA and 100 μl of 5 mg ml^{-1} NBT (Sigma, Poole, UK) and incubated for 30 min at 37 °C. After incubation, the percentage of cells containing intracellular reduced blue-black formazan deposits was then determined by microscopy following preparation of cytopsin slides of the incubated cells (400 g for 5 min using a Shandon Cytospin centrifuge). At least 200 cells were assessed per slide ($\times 100$ magnification). Conversion of NBT to the blue-black formazan (NBT positive) after stimulation indicates the oxidative burst characteristic of mature granulocytes.

2.1.3 Assay of extracellular hydrogen peroxide

The horseradish peroxidase (HRP)-catalysed oxidation of the fluorogenic probe homovanillic acid by H_2O_2 can be used to assess the H_2O_2 release from neutrophils during respiratory burst activity. With an excess of HRP, the change in fluorescence of homovanillic acid (excitation 320 nm / emission 420 nm) is proportional to the amount of H_2O_2 , and a standard curve can be constructed and used to calculate the H_2O_2 in samples (Marco *et al.*, 1986).

An HRP -linked assay was used to measure production of H_2O_2 by neutrophils or neutrophil like cells (section 3.2.2), using homovanillic acid. Briefly, 100 μl of cell

suspension in HBSS, containing 10^6 cells ml^{-1} (cells counted as described in section 2.1.5) were added to each well of a 96-well plate. Then 100 μl of HBSS containing 200 μM homovanillic acid, 10 U ml^{-1} HRP, sigma (Pool, UK) and 100 ng ml^{-1} PMA were added per well, and the fluorescence measured after 120 min at 37 °C using a CytoFluor II fluorescence microplate reader (PerSeptive Biosystems, Framingham, USA). A standard curve (0-100 $\mu\text{M H}_2\text{O}_2$) was used to determine the H_2O_2 concentration in each well (Fig. 2.2).

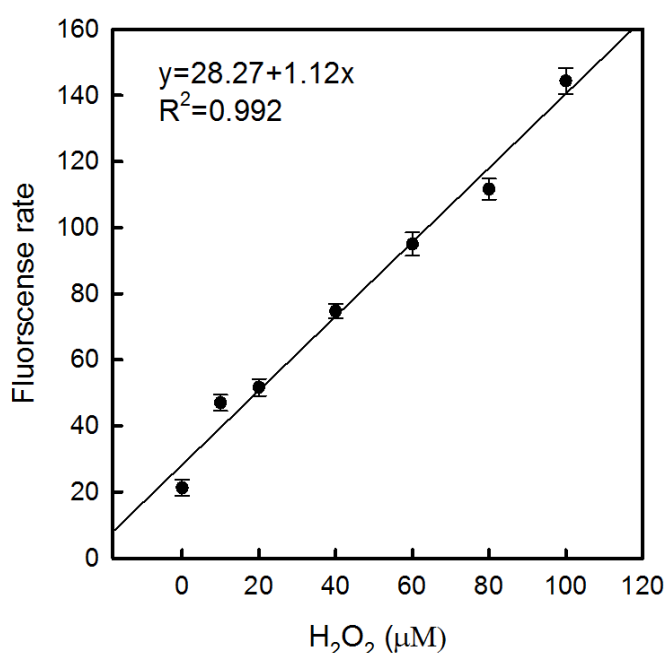


Fig. 2.2 Standard curve of H_2O_2 assay. H_2O_2 concentrations (0 to 100 μM) were prepared in dH_2O and the data points are the mean of triplicates \pm SEM.

2.1.4 Assay of phagocytic activity

This assay is based on the phagocytosis of particles or bacterial cells (in this case *Staphylococcus aureus* NCIMB 6571) that are incubated with the neutrophils. The neutrophil-like cells that had ingested bacteria were stained with modified Wright-Giemsa stain and were counted using light microscopy.

The stock bacteria cultures were freeze-dried and kept at -20 °C. Each week, sample cultures were thawed and plated onto sheep blood agar (Sigma, Poole, UK), incubated overnight at 37 °C, and then stored at 4 °C. Before use, a single colony was transferred from a plate into 15 ml of tryptone soya broth (TSB, Sigma, Poole, UK) and incubated overnight at 37 °C. The bacteria were separated by centrifuging the broth at 400g for 5 min and washing in PBS. A standard curve of bacterial growth (A_{600}) versus the number of colony forming units (CFU) was used to calculate bacterial numbers required for each experiment (Fig. 2.3). Neutrophil-like cells (10^6 cells ml⁻¹ in PBS; section 3.2.2) were incubated with *Staphylococcus aureus* NCIMB 6571 (re-suspended in PBS) at a ratio of 1:10 for 30 minutes at 37 °C in a shaking water bath. Phagocytosis was stopped by adding of ice-cold PBS into the cell-bacteria mixture followed by centrifugation at 400 g for 5 min. After another wash with ice-cold PBS, cytopsin slides of the cells that had ingested bacteria were prepared (400 g for 5 min using a Shandon Cytospin centrifuge), prior to being fixed in methanol for 5 min. Slides were stained using 20% modified Wright-Giemsa stain (pH 7.0, Sigma, Poole, UK) for 10 min, washed with three changes of distilled water and air dried thoroughly before evaluation. A minimum of 500 cells were examined by light microscopy ($\times 100$ magnification), and the phagocytic index was calculated as described by Campbell *et al.* (2001), according to the following formula: phagocytic index = (percentage of cells containing at least one bacterium) \times (mean number of bacteria per positive cell). This takes into account both the number of phagocytes containing bacteria and the mean number of phagocytised bacteria in these cells.

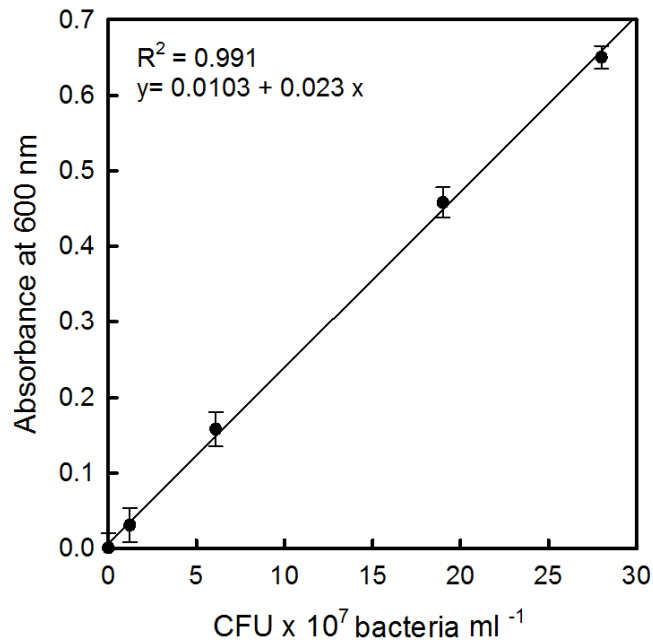


Fig. 2.3. An example standard curve for bacteria grown in TSB. *Staphylococcus aureus* NCIMB 6571 dilutions (1×10^7 to 28×10^7 bacteria ml^{-1}) were prepared in TSB and data points are the mean of triplicates \pm SEM.

2.1.5 Assay for cell viability

The trypan blue assay is based on the principle that viable cells possess intact cell membranes that exclude the dye, whilst non-viable cells, with damaged membranes stain blue. The assay gives an indication of the overall viability of the cell population, but does not distinguish between apoptotic and necrotic cells. Samples of each suspension of cells ($50 \mu\text{l}$ of 10^6 cells ml^{-1} in supplemented RPMI-1640) were mixed with $50 \mu\text{l}$ of 0.4% trypan blue solution and incubated at room temperature for 5 min. Total and non-viable (i.e. trypan blue stained) cells were counted using a haemocytometer.

2.1.6 MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is an electron acceptor that is easily reduced by cellular dehydrogenases in metabolically-active cells,

causing cleavage of the tetrazolium ring in the soluble yellow MTT to form an insoluble purple crystal (MTT formazan; Vistica *et al.*, 1991). DMSO is added to dissolve the insoluble purple MTT formazan product. The absorbance of MTT formazan can be measured at 540 nm to indicate the level of MTT reduction, which is proportional to the number of viable cells. Cells were resuspended in RPMI-1640 at 10^6 cells ml^{-1} . Samples of each cell suspension (100 μl) were placed in the wells of 96-well plates and then 10 μl of MTT (5 mg ml^{-1} in PBS; Sigma, Poole, UK) were added to each well and incubated for 3 h at 37 °C. After incubation, 100 μl DMSO was added to each well and incubated for 30 min at room temperature to solubilize any formazan product. The plates were read on a VersaMax plate reader (Molecular Devices, Sunnyvale, CA, USA) at 540 nm.

2.1.7 Gene expression

Real-time polymerase chain reaction (RT-PCR) enabled the analysis of mRNA expression of multiple genes. For the present study, 4 genes (NF- κ B, Bcl-2, TNF- α and IL-10) involved in both apoptosis and inflammation, and known to be expressed by neutrophils, were quantified, along with an endogenous control (β -actin; Table 2.2).

2.1.7.1 RNA extraction

Total RNA of neutrophils was extracted using the GenElute™ Mammalian Total RNA Miniprep Kit from (Sigma Aldrich, Poole, UK) according to the manufacturer's instructions. Briefly, 1×10^6 cells were washed with PBS (1 ml). Cells were lysed and homogenised by mixing with lysis buffer (250 μl containing 2.5 μl 1% β -mercaptoethanol) in Eppendorf tubes (1.5 ml) for 10 s to ensure complete lysis of the cells in order to improve the yield of extracted RNA. The cell lysates were centrifuged for 2 min at $8,000 \times g$ (MSE Micro Centaur). The supernatant containing the RNA was

gently pipetted into a filtration tube and centrifuged for 2 min at $8,000 \times g$. 350 μ l of molecular grade 70% ethanol was added to the eluate which was then transferred to the binding spin column to assist the selective binding of RNA. The column was then centrifuged for 15 s at $13000 \times g$ at room temperature; the eluate was discarded and the sample washed with solution 1 and solution 2.

2.1.7.2 DNA digestion

In order to remove any contaminating genomic DNA from total RNA, DNA digestion was used to digest any DNA contamination using the on-column DNaseI Digestion set (Sigma Aldrich, Poole, UK). DNase (80 μ l), was added to RNase-free tubes containing RNA samples, before incubation at room temperature for 15 min for DNA digestion and then centrifuged at $13000 \times g$ in a microcentrifuge (Micro Centaur, MES, UK). The impurities were removed by washing three times with different wash buffers before being eluted with 40 μ l elution buffer into a new collection tube for 1 minute at $13000 \times g$ at room temperature.

2.1.7.3 Quantitation of RNA

The concentration and purity of the RNA was quantified using a NanoDrop UV spectrophotometer (ND-1000) (Labtech International Ltd., Luton, UK). The absorbance was measured at 260 nm for determination of RNA concentration, and samples with acceptable purity (i.e. A_{260}/A_{280} ratio 2.0-2.23) were used for reverse transcription. An A_{260}/A_{280} ratio greater than 1.8 is usually considered an acceptable indicator of good RNA (Sambrook 1989). RNA samples were stored at $-80 \text{ }^{\circ}\text{C}$ until use.

2.1.7.4 Reverse Transcription for synthesis of complementary DNA (cDNA)

DNA is more stable than RNA and so is a useful starting point for RT-PCR. The complementary DNA (cDNA) is made from RNA by reverse transcription. The reaction is primed by random nonamer primers that anneal to the mRNA. The resulting cDNA is then used as a template for the polymerase chain reaction (PCR) or quantitative PCR where the gene of interest is amplified until there are millions of copies.

Reverse transcription was employed to convert mRNA into cDNA by using ImProm-II™ Reverse Transcriptase (Promega, Southampton, UK). Briefly 1 µg of total RNA was incubated with random nonamers (1 µM; Sigma, R7647) and nuclease-free water to a final volume of 10 µl in thin-walled PCR tubes. The mixture was placed in a Gen Amp PCR system 9700 Thermal cycle. RNA was denatured at 70 °C for 10 min. The reactions were quickly cooled on ice for 5 min and subsequently 10 µl of master mix (Table 2.2) containing 1 unit of ImProm-II™ Reverse Transcriptase were added to each reaction. Reactions were then incubated at room temperature for 10 min to ensure the elongation of random primers. The cycling conditions were: 25 °C for 5 min, 42 °C for 60 min and 70 °C for 15 min for heat-inactivation of the ImProm-II™ Reverse Transcriptase. Samples were then stored at 4 °C for up to a month or at -20 °C until used. Negative controls consisting of reactions lacking RT were run in all cDNA experiments.

Table 2.2 Preparation of Reverse Transcription Mix.

| Reagent | Volume |
|--|-------------|
| RNA template (1 µg) | up to 1 µg |
| Random nonamers (Polymerase containing nine repeating units) | 1 µl |
| Molecular biology quality water to final volume | 10 µl |
| 10 min at 70 °C then cooled on ice for 5 min | |
| ImProm-II™ Reverse Transcriptase | 1 µl |
| 5× ImProm-II™ reaction buffer | 4 µl |
| 10 mM dNTP | 1 µl |
| 25 mM MgCl ₂ | 3 µl |
| Molecular biology quality water | up to 15 µl |

To check the purity of DNA and the molecular weight of the PCR product, the cDNA agarose gel electrophoresis was run after amplification. The primers were designed using the mRNA sequence of genes which obtained from a Primer Blast in the NCBI (National Centre for Biotechnology Information) (<http://www.ncbi.nlm.nih.gov/BLAST/>). The use of a housekeeping gene in the same well reduces potential error caused by differences in the contents of each well, i.e. template or primer concentrations. The criteria for designing primers for qPCR included a GC content of 50-60%, an annealing temperature of 55-60°C and that primers be in the range of 18 to 28 nucleotides in length (Table. 2.3). β-actin was used as a house keeping gene control in this case to normalize RNA levels. A total of 23 µl mix was made up as described in Table 2.4 and added for each sample, in which specific primers were used. Then, amplifications were performed using a Gen Amp PCR system 9700 instrument according to the conditions in Table 2.4.

Table 2.3. List of primer sequences for NF- κ B, Bcl-2, TNF- α , IL-10 and β -actin RT-PCR analysis and their selection criteria.

| Primer | Primer Sequence (5-3 | Size bp | GC content% | Annealing temperature (C ^o) |
|--------------------------|--|------------|----------------|---|
| NF- κ B | F: GGGACCGGCAGCTGGGAGAT R: CGGGTTCCAGGCGCCTTGTG | 150 | 65.5 | 60 |
| Bcl-2 | F:TGGATGACTTGAGTACCTGAAC R: ACAGCCAGGAGAAATCAAAC | 121 | 57.6 | 55.3 |
| TNF- α | F: GGTGGCCCCTCCATCAACAGC R: GGCCACGAGGGCATTGGCATA | 150 | 60.87 | 63.5 |
| IL-10 | F: CCTGGAAGAGGTGATGCCACAGG R: CCTTCTCCACCGCCTTGCTCT | 155 | 61.91 | 60 |
| Human β - Actin | F: GCGCGGCTACAGCTTCACCA R: TGGCCGTCAGGCAGCTCGTA | 120 | 60 | 60 |
| Bovine β -actin | F:CGCCATGGATGATGATATTGC R: AAGCCGGCCTTGACAT | 120 | 60 | 60 |

Table 2.4 Preparation of PCR mix for one reaction.

| Reaction mix | Volume | Amplification conditions |
|---|--------------|--------------------------|
| Sample | 2 μ l | |
| Taq polymerase (Sigma) | 0.2 μ l | 94 °C 2 min |
| PCR reaction buffer | 2.5 μ l | 94 °C 30 s |
| 25 mM MgCl ₂ | 1.5 μ l | 60 °C 30 s (40 cycles) |
| 10 mM dNTP | 1.0 μ l | 72 °C 30 min |
| Forward primer (10 pmol μ l ⁻¹) | 0.5 μ l | 4 °C hold |
| Reverse primer 10 pmol μ l ⁻¹) | 0.5 μ l | |
| Molecular biology-grade water | 16.8 μ l | |

PCR mix (10 μ l) was resolved on 1% agarose gels in order to confirm the size and the quality of the PCR product for specific primers. Electrophoresis gels (1%) were made with 1 \times Tris-Acetate-EDTA buffer (TAE, Fisher Scientific, Loughborough, UK). For example, 0.7 g of agarose (Fisher, Loughborough, UK) were dissolved in 70 ml TAE. SYBR[®] safe (DNA gel stain, 3 μ l) was added to enable UV visualisation of the amplified target. Then, 2.5 μ l of Orange G dye were added to each sample (1/10 sample volume) as a colour marker to monitor the progress of agarose gel electrophoresis. Ladder (25 μ l of a 1 kb) was prepared as follows; 16 μ l molecular water, 2.5 μ l dye and 2.5 μ l of ladder (100 bp, New England Bio Labs). Samples (25 μ l) were loaded into the wells and the gel was run in 1 x TAE buffer at 90 V for 1 h. DNA band images were taken using a visualizer (UVItec Limited, England). The amplified product was visualized as a single compact band of the expected size under UV light dependent on primer set used.

2.1.7.5 Real-time quantitative polymerase chain reaction (Q-PCR)

In the typical PCR, only large changes in starting mRNA can be measured. To remove this limit, quantitative PCR (qPCR) was developed, which enables accurate and absolute quantitation, high-throughput processing as well as multiplexing of samples. The newly-synthesised DNA is fluorescently probed, the increase in fluorescent signal being proportional to the amount of DNA synthesised at each cycle. An internal control is used to normalise the sample and run on the same plate, which gives the qPCR relative quantitation. The cycles at threshold fluorescence (C_t) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceed background level). C_t levels are inversely proportional to the amount of starting template. The earlier the fluorescence passes above the threshold, the more abundant the initial target. This allows quantification of the initial amount of target relative to a reference gene (normally a housekeeping gene such as β -actin or GAPDH).

Quantitative RT-PCR using the DNA-binding dye SYBR[®] green system was used for detection of PCR products. RNA was extracted and cDNA synthesised as described above. All qPCR reactions were performed in a 48-well plate using a 48 well Stepone PCR system linked to Stepone v.2 analytical software (Applied Biosystems Warrington, Cheshire, UK). The amplification was carried out in a final reaction volume of 25 μ l containing PCR SYBR Green master mix prepared for one reaction (Table 2.5). The PCR protocol was designed for 40 cycles. The fluorescence signal was measured once in each cycle. For each gene of interest a negative control was used in which samples of cDNA were not added. The difference between the C_t of the reference and target gene was calculated and is known as ΔC_t . The Relative Quantity (RQ) of the treated sample can be calculated by comparing the ΔC_t of the treated sample to a control sample (known as $\Delta\Delta C_t$):

$$\Delta\Delta C_t = \Delta C_{t \text{ sample}} - \Delta C_{t \text{ reference control}}$$

$$RQ = 2^{-\Delta\Delta C_t}$$

Table 2.5 Preparation of Sybr[®] Green PCR master mix for one reaction

| Reaction mix | Volume (μl) | Amplification conditions |
|---|-------------|---|
| SYBR [®] Green JumpStart <i>Taq</i> ready mix | 12.5 | 94 °C 2 min 94 °C 30 s 60 °C 30 s (for 40 cycles) 72 °C 30 s |
| Reference dye | 0.25 | |
| Forward primer (10 pmol μl ⁻¹) | 0.5 | |
| Reverse Primer (10 pmol μl ⁻¹) | 0.5 | |
| Template cDNA | 2 | |
| Water molecular biology reagent (RNase and DNase free) | 9.5 | |
| Total volume | 25.25 | |

2.1.7.6 Stability of β-actin following different treatments

Previous studies on HBO treatment have used β-actin as a reference control gene (Gu *et al.*, 2008, Matsunami *et al.*, 2009, Speit and Bonzheim, 2003, Gaydar *et al.*, 2011). However, β-actin, a cytoskeletal protein has been reported to be influenced by factors such as mechanical stress (Janmey, 1998). To check the selected reference gene, the expression of β-actin was determined by qPCR in both stimulated and unstimulated ATRA-differentiated cells following different treatments. The Ct values demonstrated that β-actin expression did not alter after exposure to HBO, pressure, hyperoxia and hypoxia compared to the normoxia condition (Fig. 2.4A). The PCR product for β-actin was confirmed by using 1% agarose gel electrophoresis (as described in section 2.2.7.4). No difference in bands was seen after different treatments (two-way ANOVA, $P > 0.05$; Fig. 2.4A and B).

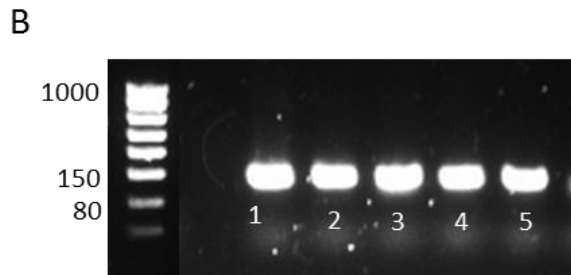
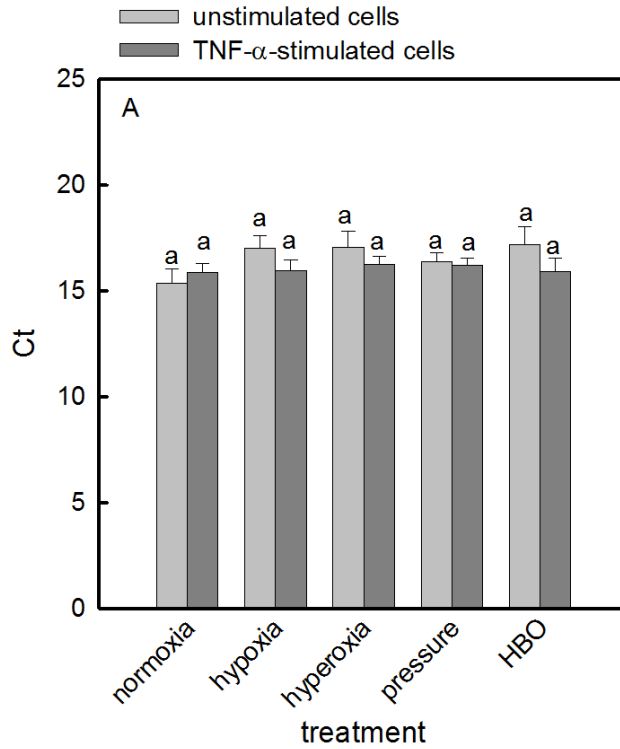


Fig. 2.4 The expression of β -actin in ATRA-differentiated cells. The expression of β -actin was measured by qPCR in ATRA-differentiated cells stimulated with TNF- α (10 ng ml^{-1}) or control (unstimulated). Cells were subjected to different treatments for 90 min and stimulated with TNF- α (10 ng ml^{-1}) for 3 h. The expression for β -actin is expressed as Ct value. Data are means \pm SEM for three separate experiments (passages numbers 20, 24 and 28, $n = 3$) with triplicate measurements. Bars with the different lower case letters are significantly different (two-way ANOVA, $P > 0.05$; A) RT-PCR product for β -actin in ATRA-differentiated cells stimulated with TNF- α was loaded in agarose gel 1% to confirm the product quality, bands description: (1) normoxia (2) hypoxia, (3) hyperoxia, (4) pressure, (5) HBO (B).

2.1.8 Measurements of pro-and anti-inflammatory cytokines using multiplex

Luminex bead assays

The Luminex[®] system by Millipore (Billerica MA, USA) uses internally colour-coded beads (microspheres) with two fluorescent dyes. By using different intensities of two dyes, 100 distinctly coloured bead sets can be created, each coated with a specific capture antibody (Fig. 2.5). After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with streptavidin-PE conjugate, to complete the reaction on the surface of each microsphere.

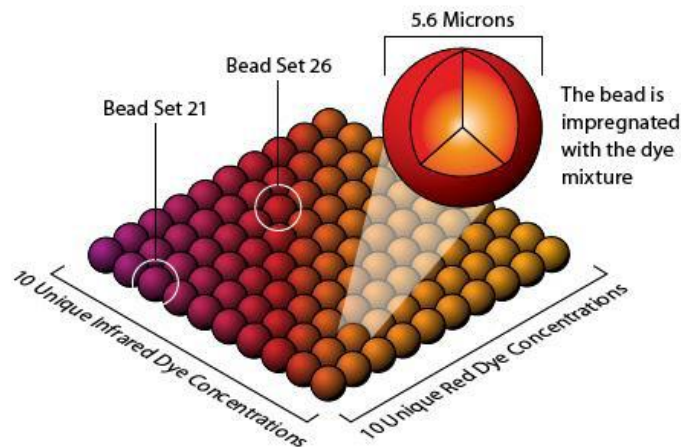


Fig.2.5: The Luminex[®] system: The beads on which the antibodies are fixed, are impregnated with different amounts of red and infrared fluorophores (from http://www.panomics.com/images/96_2_Bead_v1.jpg).

Specific antibodies for specific cytokines are attached to each bead type such that one bead type will bind to one cytokine in a sample. After adding the biotinylated detection bodies and the PE-conjugated streptavidin, PE will be indirectly attached to the cytokines. The microspheres are then allowed to pass through a laser which excites the internal dyes marking the microsphere set, and finally allows identification and quantification of each individual microsphere based on the fluorescent reporter signals.

Supernatant or plasma levels of TNF- α , IL- β 1, IL-6 and IL-10 were quantified using the Luminex[®] system (Millipore, UK), according to the manufacturer's instructions. Antibody-immobilized beads were prepared by mixing 60 μ l from each of the four antibody bead vials to the mixing bottle and bringing the volume to 3 ml with bead diluent. Mixtures were then vortexed for 30 sec and then for 1 min immediately before use. Standards of the cytokines of interest were prepared at concentrations of 3.2-10000 pg ml^{-1} in the multiplex assays with standard buffer (1x). Samples or standard (25 μ l per well) were incubated with the microspheres in a 96-well Luminex plate for 1 h at room temperature. After washing twice with washing buffer, 25 μ l of biotinylated detection antibodies were added to each well and incubated for 1 h. After incubation, 25 μ l of streptavidin-phycoerythrin mixture (0.5 mg ml^{-1}) were added to each well containing detection antibodies and incubated for 30 min. After washing twice, the fluorescence of the PE bound to the microspheres was analysed using the Luminex 100[™] system with the mean fluorescence intensity (MFI) being directly proportional to the concentration of cytokines present. Concentrations (pg ml^{-1}) of different analytes were determined by using the standard curves (Fig.2.6). Two quality controls were prepared with 250 μ l of deionized water and used in the assay to take into account any quenching and autofluorescence.

$$y = a + \frac{(b-a)}{1 + ((x/c)^d)^f}$$

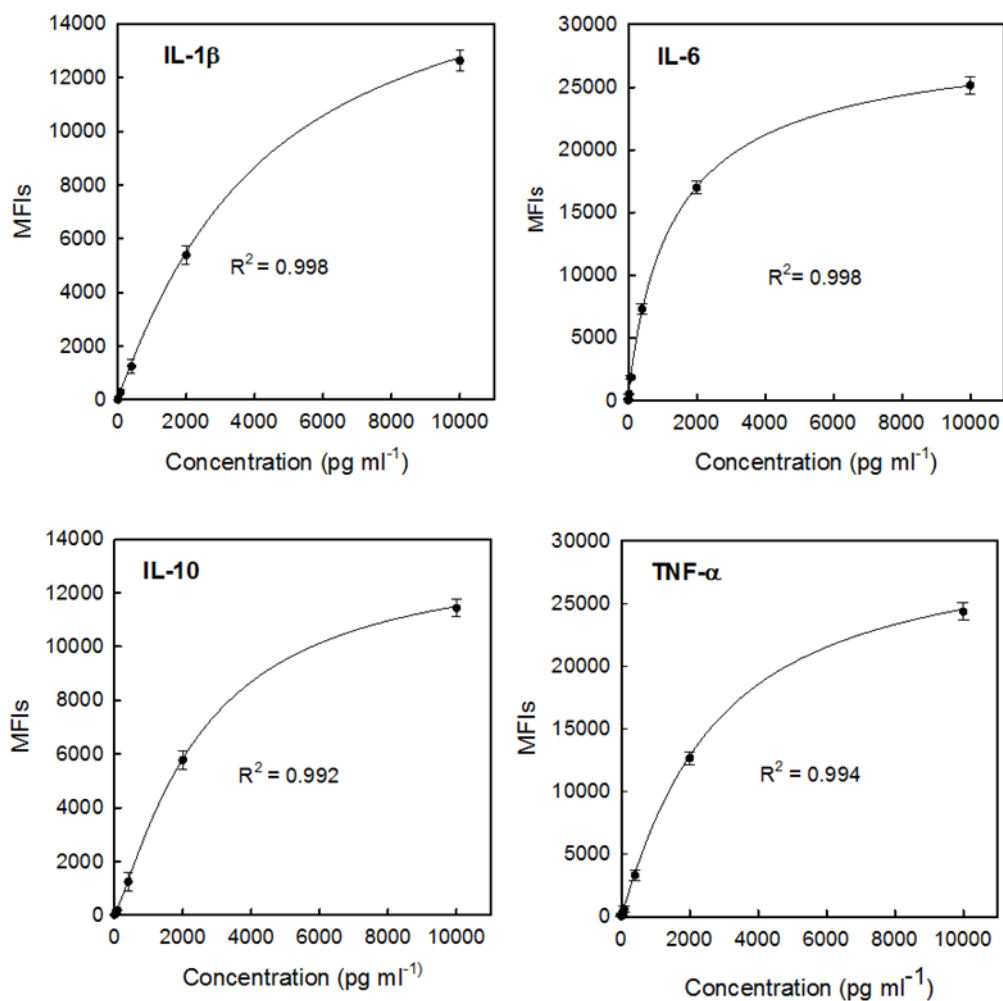


Figure 2.6 Standard curves of IL-1 β , IL-6, IL-10, and TNF- α . Cytokine concentrations (3.2-10000 pg ml⁻¹) were prepared in the multiplex assays with standard buffer and data points are the mean of triplicates \pm SEM. a is estimated response at zero concentration, b is slope factor, c is the mid-range concentration, d estimated response at infinite concentration and f is the asymmetry factor.

2.1.9 Neutrophil isolation

Neutrophils were separated from a sample of whole blood via density centrifugation using PolymorphprepTM (Axis-Shield, Kimbolton, UK), a commercially available solution, containing 13.8% sodium diatrizoate and 8.0% dextran-500 (Fig. 2.7).

Briefly, 5 ml of anticoagulated whole blood was layered over 5 ml of PolymorphprepTM (Axis-Shield, Kimbolton, UK) in a 15 ml centrifuge tube. The samples were centrifuged for $500 \times g$ for 35 min. The resulting layers of monocytes and neutrophils were removed separately from the solution using a plastic Pasteur pipette. Neutrophils were

then diluted in 15 ml HBSS solution. Following centrifugation at $200 \times g$ for 5 min, the pelleted neutrophils were resuspended in 5 ml H_2O for 30 s to hypotonically lyse erythrocytes, before 5 ml of 0.9% NaCl solution was added to restore osmolality. This step was repeated 2-3 times to ensure that all erythrocytes had been lysed. Neutrophils were then resuspended in 5 ml of RPMI-1640 and kept at room temperature until use (within 2 h).

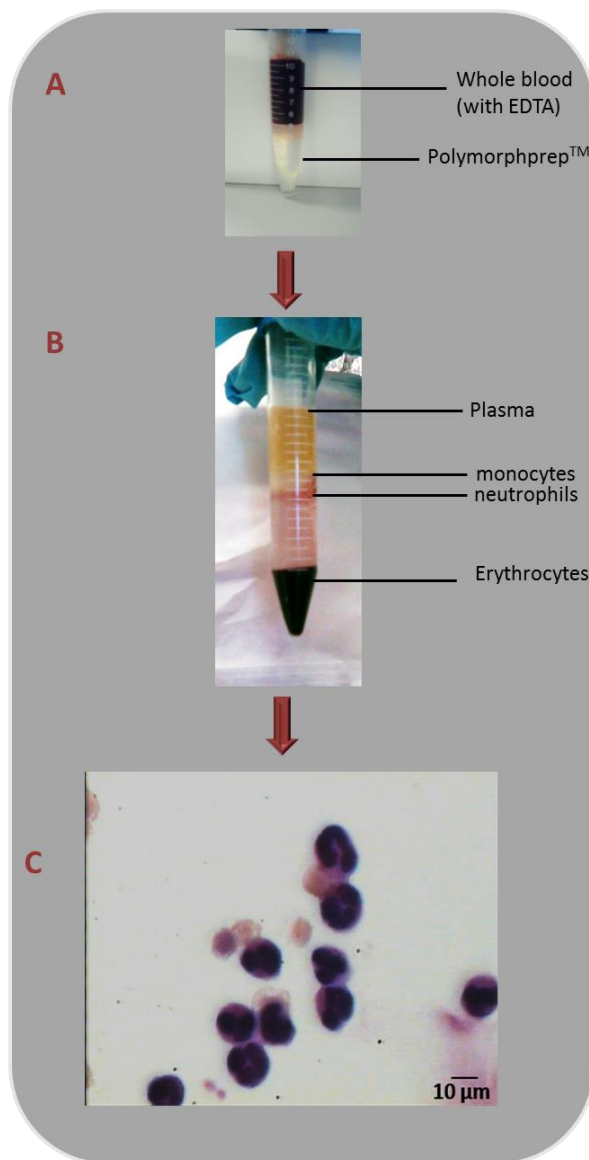


Fig. 2.7 Neutrophil isolation from human blood using Polymorphprep™. Density centrifugation (A) leads to pelleting of erythrocytes, and formation of both a monocyte cell band and a neutrophil cell band, which can be harvested and washed (B). Morphological study using modified Wright-Giemsa staining confirmed the neutrophil purity (C).

2.1.10 Statistical analysis

Data are expressed as means \pm SEM for three separate experiments ($n = 3$), unless otherwise stated, and analysed using Statgraphics Centurion XVI (Stat Point Technologies, Inc.). The Andersen-Darling test was used to check for normality ($P < 0.05$ indicates that the data are non-normal). Levene's test was performed to check that the variance of each group was not significantly different ($P < 0.05$ indicates a significant difference in variance between groups). In order to explore significant differences between reference and treatment groups one-way ANOVA or a non-parametric ranking (Kruskal-Wallis) were carried out as appropriate. In cases where there were two sets of treatments two-way ANOVA was used. In ANOVA, where the interaction was non-significant, *post hoc* analysis using Tukey's test was carried out, while for Kruskal-Wallis significant differences were determined by examining box and whisker plots with median notch. Where the interaction was significant, in order to understand the main effects, data were analysed further using pair-wise Mann-Whitney tests and/or one-way ANOVA. A P value of < 0.05 is considered significant throughout.

CHAPTER THREE

CHARACTERISATION OF

DIFFERENTIATED HL-60 CELLS AS A

NEUTROPHIL MODEL FOR *IN VITRO*

STUDIES

Abstract

Human leukaemic HL-60 cells are an established model for neutrophils for *in vitro* studies. DMSO and the retinoids, all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9-*cis* RA), are common regulators of HL-60 cell proliferation and differentiation. The aim of this study was to evaluate the effects of these agents on the proliferation and differentiation of HL-60 cells. The ability of HL-60 cells to undergo terminal differentiation into neutrophil like-cells was assessed via trypan blue, NBT reduction, and assessment of morphological changes. Complete inhibition of growth of HL-60 cells was seen with 1 μ M of ATRA (Kruskal-Wallis, $P < 0.05$). Exposure of HL-60 cells to ATRA (1 μ M) for only 3-5 days was sufficient to inhibit cell growth with most cells positive for NBT (one-way ANOVA, $P < 0.05$), and showing the features of banded and segmented neutrophils. However, DMSO was as effective as ATRA in inhibiting proliferation (Kruskal-Wallis, $P < 0.05$). Non-additive effects on HL-60 cell differentiation were observed upon combination of ATRA and 9-*cis* RA at 1 μ M. ATRA-differentiated HL-60 cells (1 μ M, 5 days differentiation) displayed functional characteristics commonly associated with peripheral blood neutrophils, including enhanced phagocytosis of *Staphylococcus aureus* (Student's *t*-test $P < 0.05$) in response to stimuli such as PMA and LPS, release of cytokines such as TNF- α , and induction of apoptosis.

3.1. Introduction

The human promyelocytic leukaemia cell line, HL-60, was isolated from an acute leukaemia patient and first grown in culture by Collins *et al.* (1978). These cultured cells display the typical features of leukaemia, namely an inability to respond to normal differentiation stimuli, unlimited proliferation capacity and decreased apoptosis. About 5-10% of these cells spontaneously differentiate into mature cell types in culture (Palis *et al.*, 1988; Fig. 3.1).

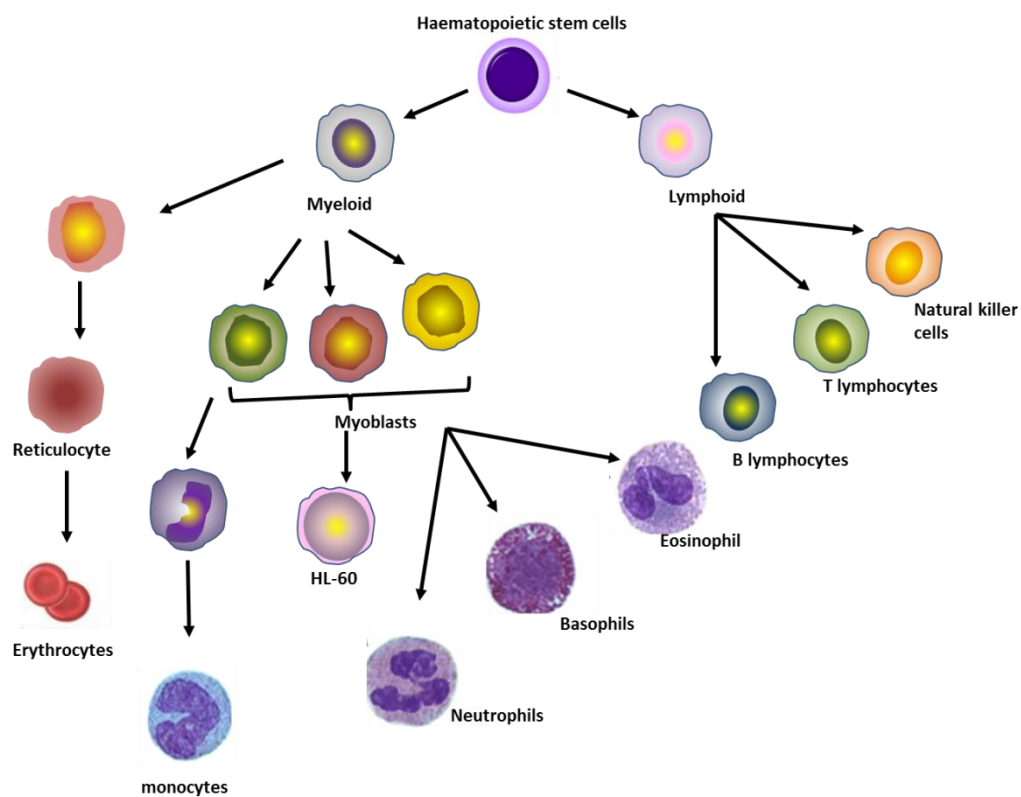


Fig. 3.1 A general model of haematopoiesis. Blood cells are produced from haematopoietic stem cells (HSC), which can undergo either self-renewal or differentiation after stimulation with specific cytokines and growth factors into progenitor cells committed to multiple lineages. These include common lymphoid and myeloid progenitors. The myeloid progenitors further divide to give rise to granulocytes, macrophages and erythroid cells, while the lymphoid progenitors are committed to T cells, natural killer cells and B cells. HL-60 cells are transformed promyelocytes. A variety of agents can induce these cells to differentiate into monocyte-like or neutrophil-like cells (Cooper and Hausman, 2000).

Various differentiation-inducing agents have been shown to induce differentiation of HL-60 cells into neutrophil-like cells, monocyte-like cells or eosinophil-like cells. These agents mediate their differentiation effects through different mechanisms

including remodelling of chromatin, interaction with respective receptors, alteration of DNA, inhibition of histone deacetylase, interference with DNA and RNA synthesis and disturbance of signal transduction (Tsiftoglou *et al.*, 2003). These agents vary not only in their mode of action but also in the timing of differentiation marker expression and differentiated phenotype which result. Chemical agents including retinoic acid (RA), which is commonly used to treat dermatological conditions as well as acute promyelocytic leukaemia (APML), and dimethyl sulphoxide (DMSO), are known to induce differentiation of HL-60 cells into cells with the morphological features of neutrophils (Breitman *et al.*, 1980, Collins *et al.*, 1978). These cells show functionality and markers that are typical of mature neutrophils including changes in cell shape, nuclear condensation, superoxide ($O_2^{\bullet-}$) production, increased ability to reduce NBT, chemotaxis, phagocytosis and expression of antigens specific to granulocytes (Collins and Foster, 1983, Newburger *et al.*, 1979). Exposure to vitamin D₃ results in differentiation of HL-60 cells into monocyte-like cells, as the cells begin to clump and acquire pseudopodia, and show increases in both non-specific esterase activity and levels of lysozyme (Birnie, 1988). Many factors can affect optimal differentiation: the concentration of inducing agent, the time of exposure and the proportion of cells in different stages of the cell cycle (Collins, 1987, Collins *et al.*, 1978). Recent studies have addressed the differentiation of HL-60 cells into neutrophil-like cells (Table 3.1). It is clearly important to standardize and optimize differentiation conditions in order to provide neutrophil-like cells suitable for use in *in vitro* experiments.

Table 3.1 A summary of inducing agents and the various outcomes of their use in differentiating HL-60 cells.

| Differentiating agent | Concentration | Outcome | Study |
|---|---|---|-------------------------------------|
| DMSO | 1.25% | Induced superoxide production with NBT dye reduction in DMSO-treated cells after stimulation with PMA; response to chemoattractants and development of complement receptor. | (Collins <i>et al.</i> , 1979) |
| all- <i>trans</i> retinoic acid (ATRA) | 1 pM-1 μ M | Maximal differentiation occurs at 1 μ M ATRA as indicated by nitro blue reduction. The percentage of mature cells is directly related to the length of exposure. | (Breitman <i>et al.</i> , 1980) |
| cAMP | 500 μ M | Treated cells exhibited a decrease in proliferation, expressed complement receptors, reduced NBT, adhered to substrate and showed chemotaxis. | (Chaplinski and Niedel, 1982) |
| Combination of ATRA, DMSO and/or 5-aza-2'-deoxycytidine (AzadCyD) | 1 μ M ATRA 1 μ M AzadCyD 1% DMSO | RA + DMSO + AzadCyD caused maximal NBT reduction after 72 to 96 h of incubation, with complete differentiation in HL-60 cells. | (Schwartzmann <i>et al.</i> , 1987) |
| DMSO or ATRA | 1.25% DMSO, 1 μ M ATRA | Increase in the proportion of cells with mature neutrophil morphology and apoptosis. | (Martin <i>et al.</i> , 1990) |
| ATRA + 9- <i>cis</i> RA | 1 μ M each | Inhibition of HL-60 cells proliferation, expression of myeloid-specific differentiation antigens (CD11b and CD14) with NBT reduction. | (Kizaki <i>et al.</i> , 1994) |
| combination of ATRA or 9- <i>cis</i> RA and vitamin D | 0.1 μ M ATRA 0.1 μ M 9- <i>cis</i> RA 0.01 μ M vit. D | Exerts additive effects on inhibition of proliferation and induction of HL-60 cells differentiation. | (Verstuyf <i>et al.</i> , 1995) |
| ATRA | 1 μ M | ATRA induces HL-60 cell differentiation with increased percentage of NBT positive cells and CD11b expression after 1, 2 and 3 days | (Jian <i>et al.</i> , 2011) |

The main aim of this study was to choose an effective agent for the induction of differentiation of HL-60 cells into neutrophil-like cells for use in *in vitro* experiments. A further aim was to examine the response of differentiated cells to stimuli that induce neutrophil activation or apoptosis. Such observations would provide information as to whether this cell line is a suitable model for mature granulocytes.

3.2 Materials and methods

The majority of cell culture reagents used, including fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, RPMI-1640 medium and trypan blue were obtained from Lonza (Slough, UK). Sterile PBS and Hank's balanced salt solution (HBBS) were obtained from Invitrogen (Paisley, UK). Tissue culture flasks, serological pipettes and other tissue culture plastic were obtained from Bibby Sterilin Ltd (Staffordshire, UK). Recombinant human TNF- α was obtained from Insight Biotechnology (Wembley, UK). TNF- α and IL-10 ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, USA). ATRA, 9-*cis* RA, DMSO, superoxide dismutase (SOD), ferricytochrome *c* (from horse heart) and dipotassium ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Aldrich (Poole, UK).

3.2.1 Cell culture

HL-60 cells (passage 6) were acquired from the European Collection of Cell Cultures (ECCAC, 98070106) and were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin in vented 75 cm² tissue culture flasks. Cells were maintained at 37 °C in a humidified atmosphere under 5% CO₂ (balance air). Passages 20 through to 45 were

used for experiments. However, for individual measurements passages that were closer in number (range of 4-8 passages) were used for replicate experiments.

3.2.2 Experimental design

ATRA and 9-*cis* RA stock solutions (10 mM) were prepared in 99% (v/v) ethanol and stored at -80 °C. Dilutions of stock solutions were made in growth medium to concentrations ranging from 1 pM to 1 μM so that the final concentration of ethanol in cultures was no higher than 0.1%. DMSO was diluted with RPMI-1640 to a concentration of 1.25% (final concentration) to induce differentiation.

Differentiation of HL-60 cells was induced by incubation of 2.5×10^5 cells ml⁻¹ with ATRA (1 pM-1 μM), 9-*cis* RA (1 pM-1 μM), ATRA + 9-*cis* RA (1 μM) or DMSO (1.25%) for 5 days in a humidified atmosphere at 5% CO₂ (balance air) at 37 °C. The cells were then washed twice in RPMI-1640, and subsequently counted and assayed as described below. Two controls were used in this experiment: in one, control cells were cultured in RPMI-1640 medium containing 0.1% absolute ethanol and in the other HL-60 cells were cultured in RPMI-1640 medium alone (Fig. 3.2).

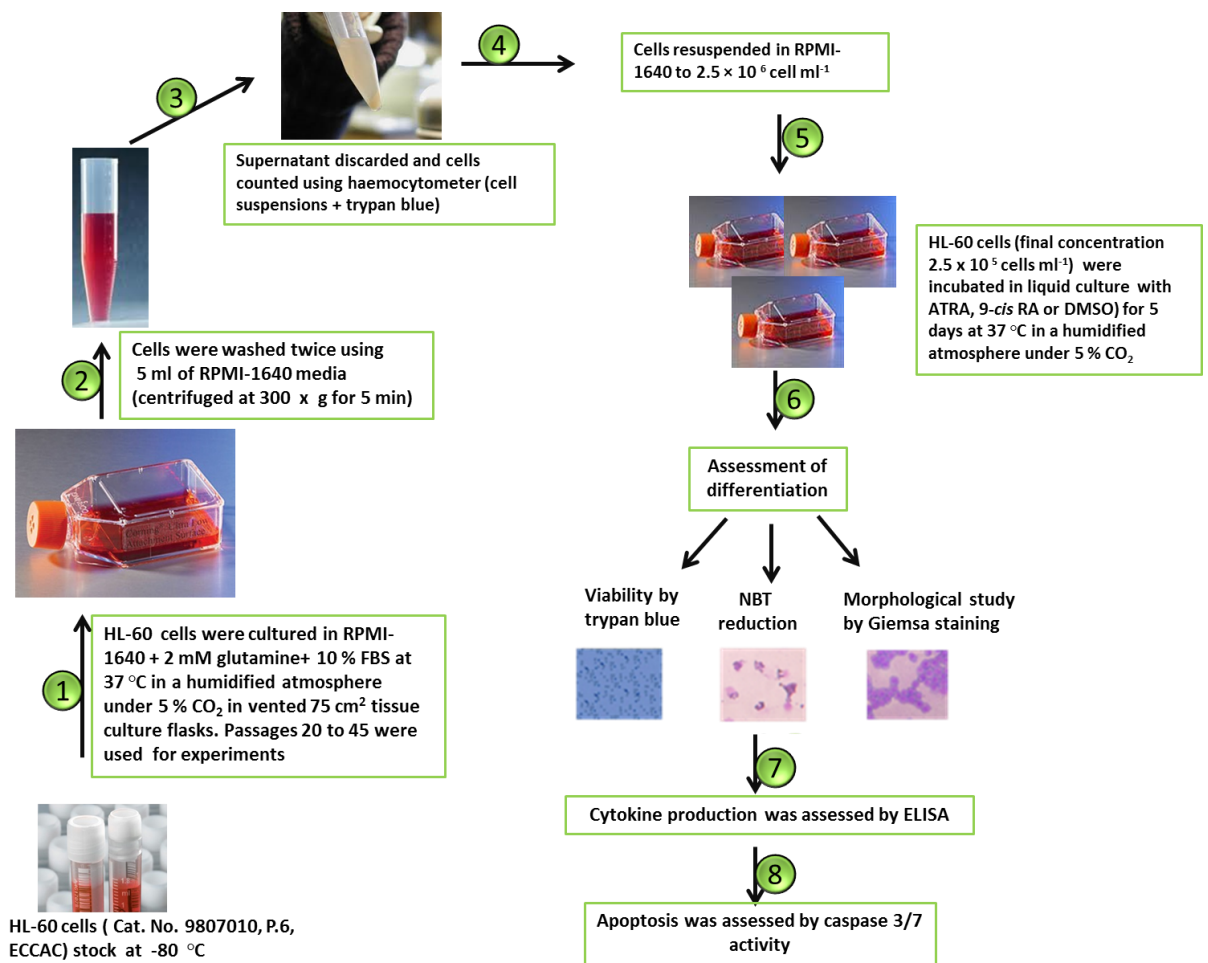


Fig. 3.2 HL-60 cells culture and differentiation. HL-60 cells (2.5×10^5 cells ml⁻¹) were incubated with different inducing agents (ATRA, 9-*cis* RA and DMSO) for 5 days at 37 °C in 5% CO₂, and then washed, counted and assayed for further analysis. Three separate experiments with passage numbers (22, 24 and 26) were performed.

3.2.3 Assessment of differentiation

The viability of cells was assessed by trypan blue exclusion assay. Cells were treated with differentiating agents and were collected at 0, 1, 2, 3, 4 and 5 days for viability analysis (section 2.1.5, Fig. 3.3).

The respiratory burst activity of neutrophil-like cells obtained by differentiating HL-60 cells was assessed by measuring NBT reduction (section 2.1.2). Cells were collected at 0, 3 and 5 days after differentiation (Fig. 3.3).

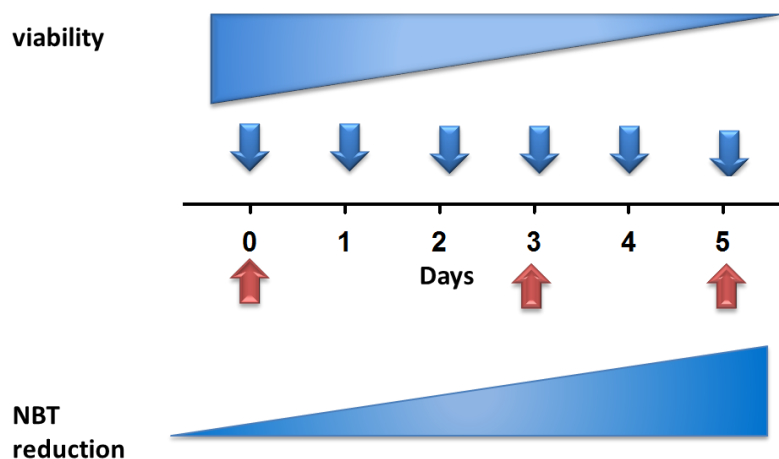


Fig. 3.3 Timing of cell viability and NBT reduction assays. Samples were collected for viability analysis using trypan blue at the indicated time points (0, 1, 2, 3, 4 and 5 days, blue arrows) and for NBT reduction assay at the indicated time points (0, 3 and 5 days, red arrows) after inducing HL-60 cell differentiation with different agents.

Cytospin slides of neutrophil-like cell suspensions (1×10^6 cells ml^{-1}) were prepared after 5 days differentiation using a Thermo Shandon Cytospin 4 centrifuge (Cheshire, UK; $400 \times g$ for 4 min). The slides were stained with Wright-Giemsa stain modified (section 2.1.4), and at least 500 cells were scored using a light microscope (equipped with a $\times 100$ objective; Olympus, Japan). Mature granulocytes were identified by the multi-lobed structure of the nucleus (Verstuyf *et al.*, 1995). One of the most common species of bacteria that can cause infection at wound sites is *Staphylococcus aureus* (Tillotson *et al.*, 2008). Therefore, the phagocytosis of *Staphylococcus aureus* NCIMB 6571 by ATRA-differentiated HL-60 cells was investigated in this chapter (section 2.1.4).

3.2.4 Quantification of TNF- α and IL-10 by ELISA

Tumour necrosis factor alpha (TNF- α) and interleukin-10 (IL-10) are produced by neutrophils in response to stimulation and are important for their recruitment into the inflamed site. A convenient method to measure TNF- α is via Enzyme-Linked Immuno

Sorbance Assay (ELISA). Cell suspensions ($100 \mu\text{l}$ of 1×10^5 cells ml^{-1}) of either HL-60 cells differentiated using ATRA or 9-*cis* RA, or human neutrophils (section 2.1.9) were stimulated with $100 \mu\text{l}$ of PMA, LPS, PGN or FMLP in 96-well plates for 18 h at 37°C under 5% CO_2 balance air. Samples were measured in triplicate. NuncMaxisorp® flat-bottom 96-well plates (eBiosciences, Ltd, Hatfield, UK) were coated with $50 \mu\text{l}$ of $0.5 \mu\text{g ml}^{-1}$ mouse anti-human TNF- α or IL-10 monoclonal antibody (capture antibody) and incubated overnight at 4°C . The plates were then washed 3 times with PBS containing 0.01% v/v Tween (PBST). Wells were blocked by adding $50 \mu\text{l}$ of PBST containing 2% (v/v) FCS for 4 h at room temperature. Standards (0 to 10000 pg ml^{-1}) of human TNF- α and IL-10 were prepared and $50 \mu\text{l}$ of each were added to the 96-well plate in duplicate (Fig. 3.4). After this, $50 \mu\text{l}$ of cell supernatants were added to the same plate and incubated overnight at 4°C . After three further washes with PBST, $50 \mu\text{l}$ of biotinylated mouse anti-human TNF- α monoclonal antibody was added and the plate incubated for 4 h at room temperature. Following three further washes with PBST, $50 \mu\text{l}$ of horseradish peroxidase (HRP)-streptavidin (1/250 dilution in PBS containing 1% BSA) was added and the plate incubated for 2 h at room temperature. After three more washes with PBST, $100 \mu\text{l}$ of tetramethylbenzidine (TMB) reagent was added, as a substrate for the HRP, and the plate incubated for 30 min at room temperature. The reaction was stopped with addition of $50 \mu\text{l}$ per well of 2 M sulphuric acid and plates read at 450 nm in a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA).

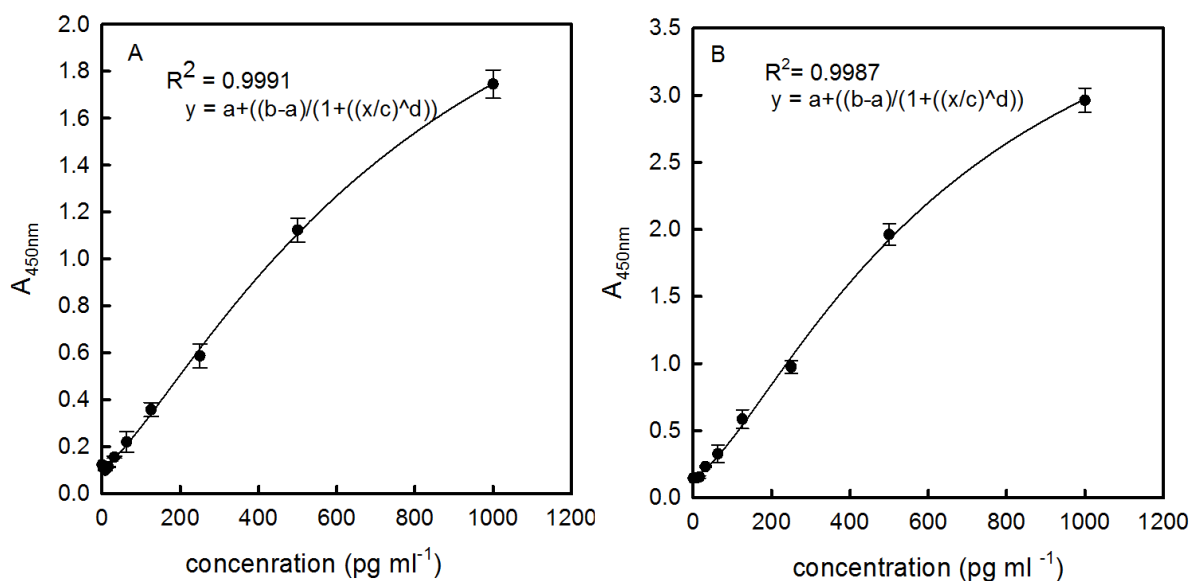


Fig. 3.4 Standard curves for TNF- α and IL-10. Cytokine standards (0-1000 pg ml^{-1}) for TNF- α (A) and IL-10 (B) were prepared in buffer and data points are the mean of duplicate measurements \pm SEM; a is the estimated response at zero concentration, b is slope factor, c is the mid-range concentration, d is the estimated response at infinite concentration.

3.2.5 Induction of apoptosis

Members of the caspase family of cysteine proteases are the central mediators of the proteolytic cascade leading to cell death and elimination of compromised cells. As such, the caspases are tightly regulated both transcriptionally and by endogenous anti-apoptotic polypeptides, which block productive activation (Earnshaw *et al.*, 1999). Furthermore, the enzymes involved in this process indicate distinct pathways and demonstrate specialized functions consistent with their primary biological roles. Assays that directly measure caspase activity can provide valuable information about the mechanism of death in dying cells.

Various agents have been described that induce apoptosis. The aim of this work was to investigate which of the available agents could induce apoptosis in neutrophil-like cells and thus would be suitable for use as a positive control. HL-60 cells differentiated using ATRA were resuspended at 1×10^6 cells ml^{-1} in RPMI-1640 medium supplemented

with 10% fetal calf serum and 2 mM L-glutamine, and incubated at 37 °C in the presence or absence (control) of 10-100 ng ml⁻¹ of TNF- α or H₂O₂ (33-132 μ M) for 3 h at 5% CO₂ (balance air).

Caspase 3/7 activity was measured using a fluorometric substrate, rhodamine 110 *bis*-(N-CBZ-L-aspartyl-L-glutamyl-valyl-L-aspartic acid amide, Z-DEVD-R110; Promega, Southampton, UK). Neutrophil-like cells (50 μ l of 2 \times 10⁵ cells ml⁻¹) were incubated with 50 μ l of the caspase 3/7 fluorometric substrate in a 96-well plate for up to 18 h, and the fluorescence was measured using a Cytofluor II fluorescence plate reader (PerSeptive Biosystems, Framingham, MA) with excitation wavelength 490 nm and emission wavelength 521 nm. Caspase 3/7 activity was expressed in terms of relative fluorescence units (RFU).

3.2.6 Statistical analysis

All data were analysed using Statgraphics Centurion XVI (StatPoint Technologies, Inc.). Data are expressed as means \pm SEM for three separate experiments in triplicate, unless otherwise stated. The relationships between the effects of differentiation agents and time were determined using linear or non-linear regression as appropriate. Regressions (curve fitting) were carried out using Sigma Plot 12.0. Data were also analysed using ANOVA or Kruskal-Wallis as appropriate following the Andersen-Darling test and Levene's test. In one case (Fig. 3.12) data were log₁₀ transformed before two-way ANOVA. For one-way ANOVA, Tukey's test was used *post hoc*. A *P* value of < 0.05 is considered significant throughout.

3.3 Results

3.3.1 Cell proliferation in response to ATRA, 9-*cis* RA and DMSO

There was a clear relationship between the cell density and time after treatment with differentiation-inducing agents (Figs 3.5 and 3.6). A significant increase in cell density was found in ATRA-treated cells at low concentrations of 1 nM (linear regression, $P = 0.0004$) and 1 pM (linear regression, $P = 0.0021$) compared to undifferentiated cells on day 0 (control; Fig. 3.5A). However, treatment with 1 μ M ATRA caused a slight but significant decrease in cell density (linear regression; $P = 0.0002$; Fig. 3.5A). Similarly, cells treated with 9-*cis* RA showed an increase in cell density in a time-dependent manner at 1 nM (linear regression, $P < 0.0001$) and 1 pM (linear regression, $P = 0.0017$; Fig. 3.5B), while no change in cell density was observed after treatment with 1 μ M 9-*cis* RA (linear regression, $P = 0.251$). A combination of 1 μ M of both retinoids, or treatment with 1.25% DMSO, caused a slight but significant decrease in the cell density over time compared to the control on day 0 (linear regression, $P = 0.009$ in both cases; Fig. 3.6). The regression analysis was confirmed by using the Kruskal-Wallis test. There was a significant decrease in cell density after 3, 4 and 5 days of incubation with 1 μ M ATRA, 1 μ M 9-*cis* RA and 1.25% DMSO (Kruskal-Wallis, $P = 0.015$, 0.001 and 0.0009, respectively) compared to undifferentiated cells (Fig. 3.7).

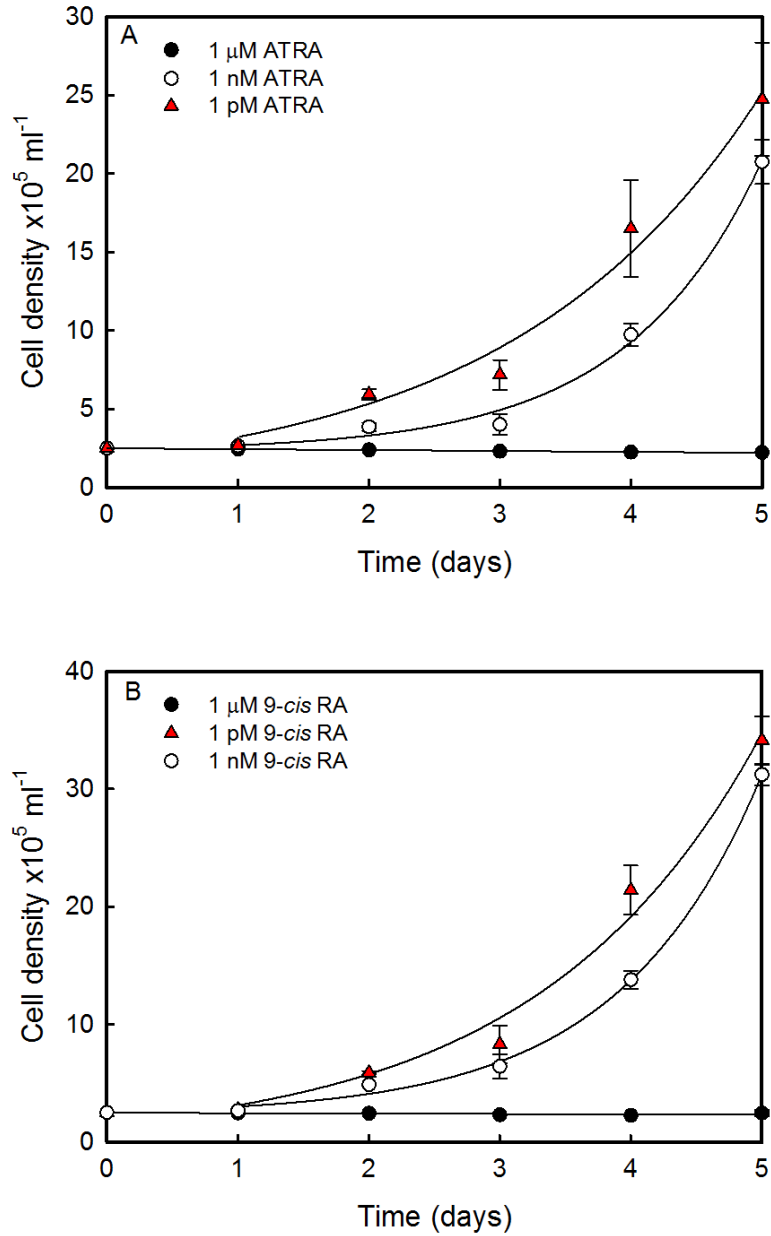


Fig. 3.5 Regression analysis showing the relationship between cell density and time of treatment with differentiation inducing agents. HL-60 cells (2.5×10^5 cells ml^{-1}) were incubated for 5 days in the absence or presence of (A) ATRA or (B) 9-*cis* RA at concentrations ranging from 1 pM to 1 μM to induce differentiation. Data are expressed as means \pm SEM for three separate experiments (passage numbers 22, 24 and 26; $n = 3$) with triplicate measurements. R^2 values are 0.984, 0.995 and 0.977 for 1 μM , nM and pM of ATRA, respectively and 0.986, 0.999 and 0.310 for 1 μM , nM and pM of 9-*cis* RA, respectively).

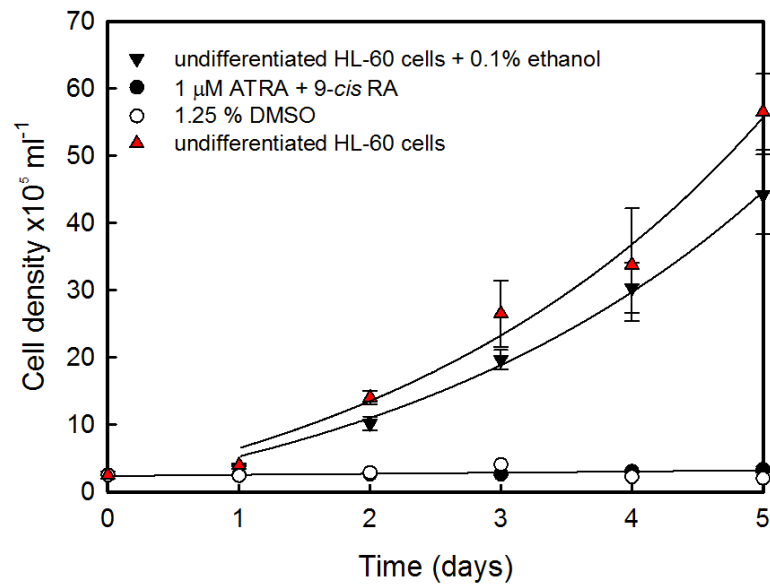


Fig. 3.6 Regression analysis showing the relationships between cell density and time of treatment with differentiation-inducing agents. HL-60 cells (2.5×10^5 cells ml^{-1}) were incubated for 5 days in the absence or presence of (A) a combination of ATRA + 9-*cis*- RA (1 μM) or (B) 1.25% DMSO to induce differentiation. Viability was assayed by trypan blue dye exclusion. Data are expressed as means \pm SEM for three separate experiments (passage numbers 22, 24 and 26; $n = 3$) with triplicate measurements. R^2 values are 0.987, 0.995 and 0.849 for undifferentiated cells + ethanol, 1 μM ATRA + 9-*cis* RA, 1.25% DMSO and undifferentiated cells, respectively.

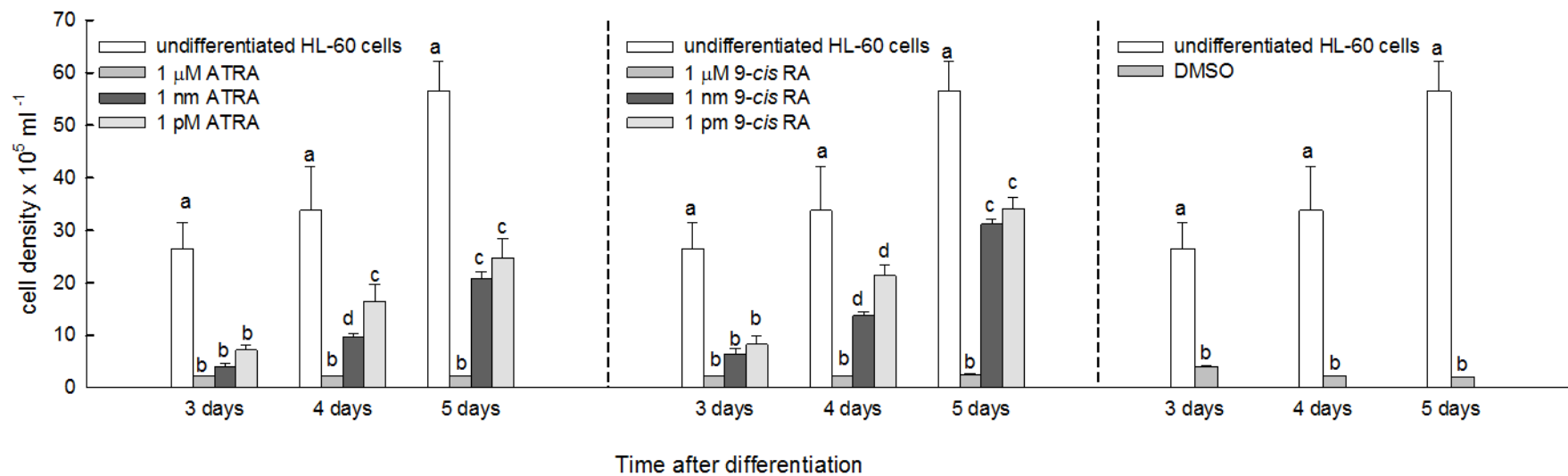


Fig. 3.7 Cell density at 3, 4 and 5 days after differentiation with various differentiation-inducing agents. HL-60 cells (2.5×10^5 cells ml^{-1}) were incubated for 5 days in the presence or absence of ATRA (1 μM), 9-*cis*- RA (1 μM) or 1.25% DMSO to induce differentiation. Viability of cells was assayed by trypan blue dye exclusion. Data are expressed as means \pm SEM for three separate experiments (passage numbers 22, 24 and 26; $n = 3$) with triplicate measurements. For each treatment on each day, bars with different lower case letter are significantly different (Kruskal-Wallis, $P < 0.05$).

3.3.2 NBT reduction during differentiation of HL-60 cells by retinoids and DMSO

The effectiveness of differentiation of HL-60 cells into neutrophil-like cells was demonstrated by measuring the percentage of NBT reduction (Fig. 3.8). In the case of unstimulated cells, by day 3 all cells exposed to differentiating agents (ATRA, 9-*cis* RA and DMSO) showed a higher degree of NBT reduction (one-way ANOVA, $P = 0.0091$) than undifferentiated HL-60 cells (Fig. 3.8A). By day 5 of incubation, both the 1 μM ATRA and DMSO treatments showed a significantly higher percentage of cells that reduced NBT (one-way ANOVA, $P = 0.0085$) compared to the control. Treatment with 1 μM 9-*cis* RA did not show a significant difference in the percentage of NBT-reducing cells in unstimulated cells (one-way ANOVA, $P > 0.05$).

A significantly higher % NBT stained cells was found at both day 3 and day 5 in PMA-stimulated cells after differentiation with 1 μM ATRA, 1 μM 9-*cis* RA and 1.25% DMSO (one-way ANOVA, $P = 0.001$ and 0.0009, respectively) compared to non-exposed control HL-60 cells (Fig. 3.8B).

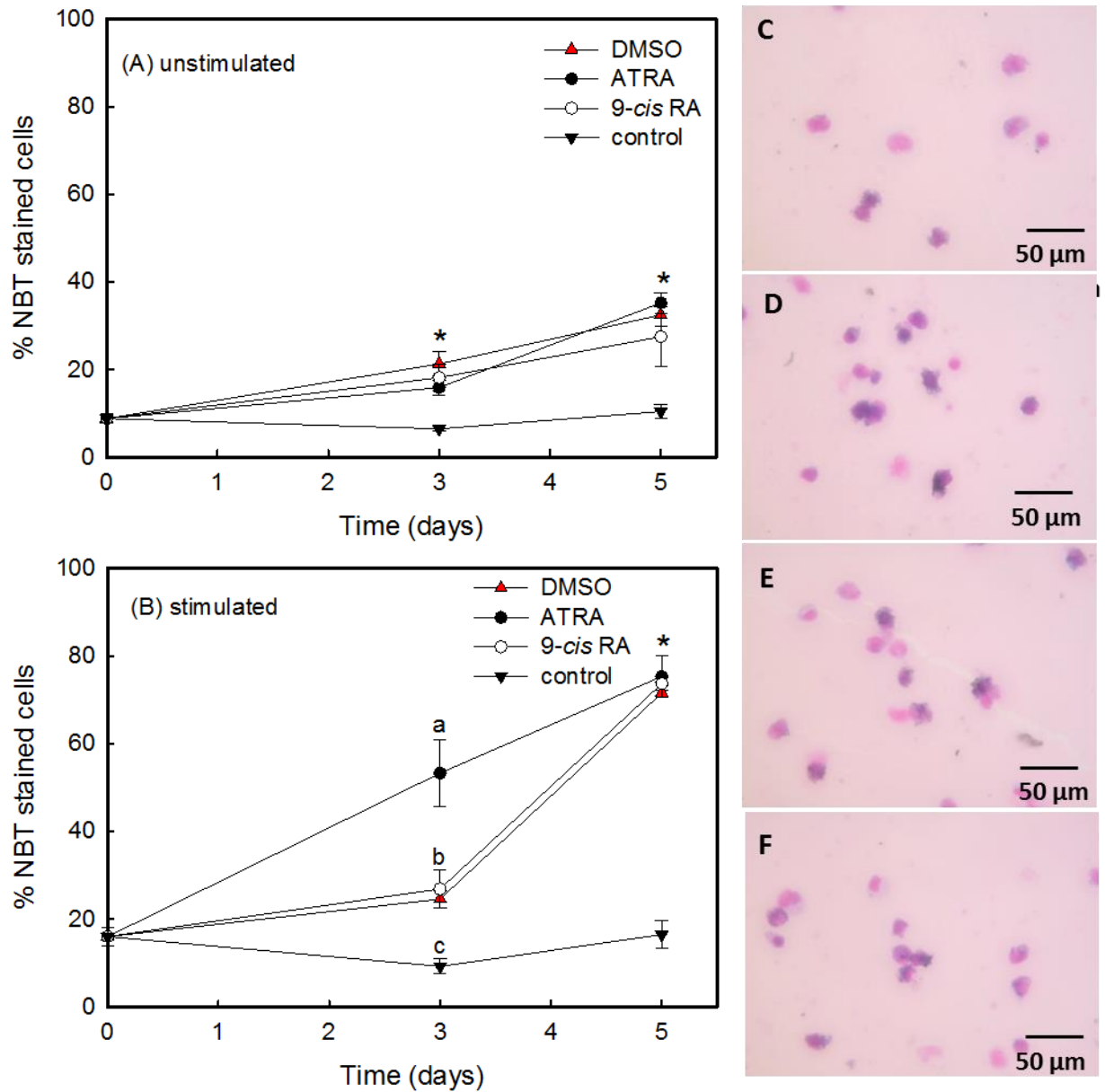


Fig. 3.8 NBT reduction by HL-60 cells differentiated using different differentiating agents. HL-60 cells (1×10^6 cells ml^{-1}) differentiated with ATRA, 9-cis RA and DMSO were incubated for 30 min with NBT (5 mg ml^{-1}) in the absence (A) or presence (B) of PMA (100 ng ml^{-1}). Data are means \pm SEM for three separate experiments (passage numbers 22, 24 and 26; $n = 3$) with triplicate measurements. * indicates significant differences from undifferentiated HL-60 cells on the same day (control), bars with different letters indicate a significant difference between treatment (one-way ANOVA, $P < 0.05$). Examples of cytopsin slides that were prepared and stained with Giemsa stain: (C) HL-60 cells; (D) ATRA; (E) 9-cis RA; and (F) DMSO. Original magnification $\times 40$.

3.3.3 Morphological alterations during HL-60 cells differentiation

In the absence of a differentiating agent, HL-60 cells were predominantly typical promyelocytes (Fig. 3.9A) with large round nuclei, dispersed nuclear chromatin and a relatively high nuclear:cytoplasmic ratio. Incubation with either ATRA or *9-cis* RA (1 μ M), or DMSO (1.25%) induced the majority of cells to undergo morphological changes characteristic of terminal differentiation of myeloid cells. The induced cells exhibited the following changes: small size, decrease in nuclear:cytoplasmic ratio and a marked reduction and segmentation of the nuclei (Fig. 3.9B, C and D).

Cells treated with 1 μ M ATRA exhibited morphological changes characteristic of mature cells in the granulocyte pathway by day 3; cells of mature neutrophil morphologies came to prominence on day 4 and remained the predominant cell type until termination of these cultures by day 5 as cells differentiated into banded segmented neutrophils (with nuclei with focal areas that are distinctly narrower than the width of the widest points and usually with irregular nuclear outlines; Fig. 3.9B). Treatment with *9-cis* RA gave the same results (Fig. 3.9C). However, DMSO-treated cells displayed little tendency to mature morphologically beyond the myelocyte stage of development (Fig. 3.9D).

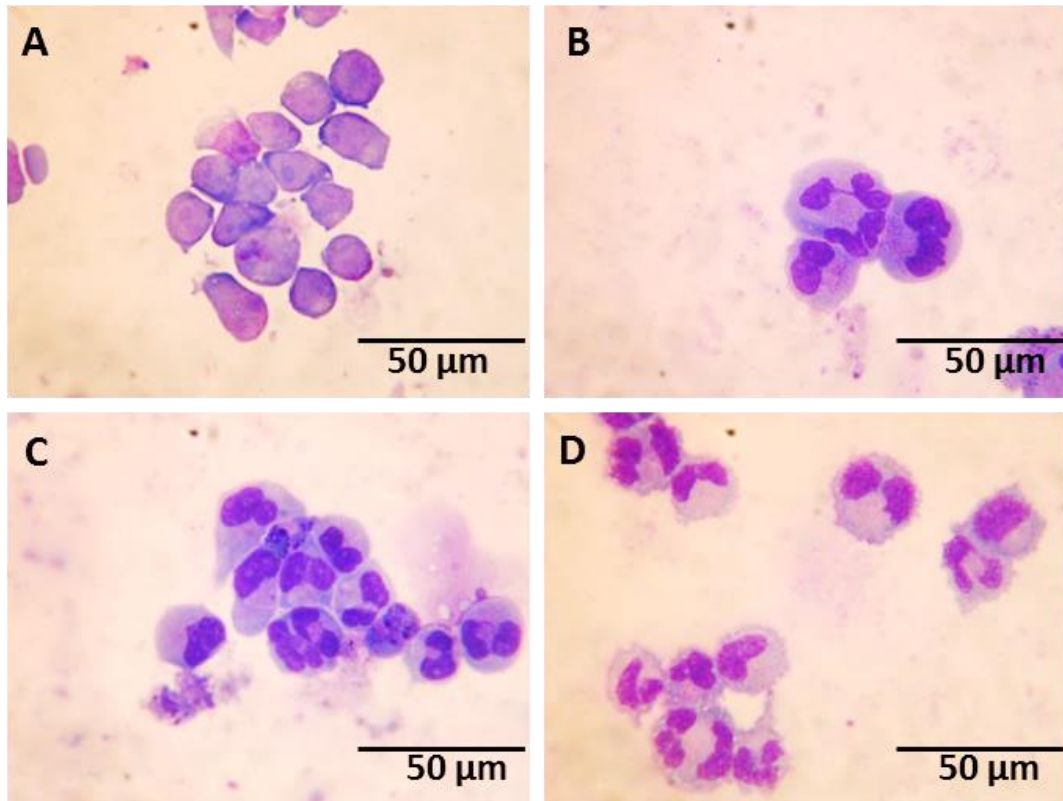


Fig. 3.9 Morphological changes induced in HL-60 cells after differentiation. Light microscopy images, produced using modified Wright-Giemsa staining showing morphology of (A) undifferentiated HL-60 cells, showing promyelocytes with characteristic large round nuclei and cytoplasmic granules; (B) ATRA-treated cells and (C) 9-*cis* RA-treated cells, showing maturation to banded segmented neutrophils with large round nuclei, each containing 2-4 nucleoli; and (D) DMSO-treated cells showing metamyelocytes and neutrophilic bands rather than segmented neutrophils. Cytospin slides of cell suspensions were prepared and stained with Wright Giemsa stain to characterise the morphological changes (original magnification $\times 100$).

3.3.4 Phagocytosis of *Staphylococcus aureus* NCIMB 6571 by differentiated HL-60

cells

Phagocytosis of *Staphylococcus aureus* NCIMB 6571 by undifferentiated and HL-60 cells differentiated with 1 μM ATRA is shown in Fig. 3.10A. After incubation with ATRA for 5 days, 76% of cultured cells phagocytosed *Staphylococcus aureus* NCIMB 6571 (Student's *t*-test, $P = 0.00002$), while less than 12% of undifferentiated HL-60 cells were capable of phagocytosing bacteria. Light micrographs of modified Wright Giemsa-stained, ATRA-treated cells incubated with *Staphylococcus aureus* NCIMB 6571 showed more evidence of phagocytosis of bacteria (Fig. 3.10C) compared to undifferentiated cells (Fig. 3.10B).

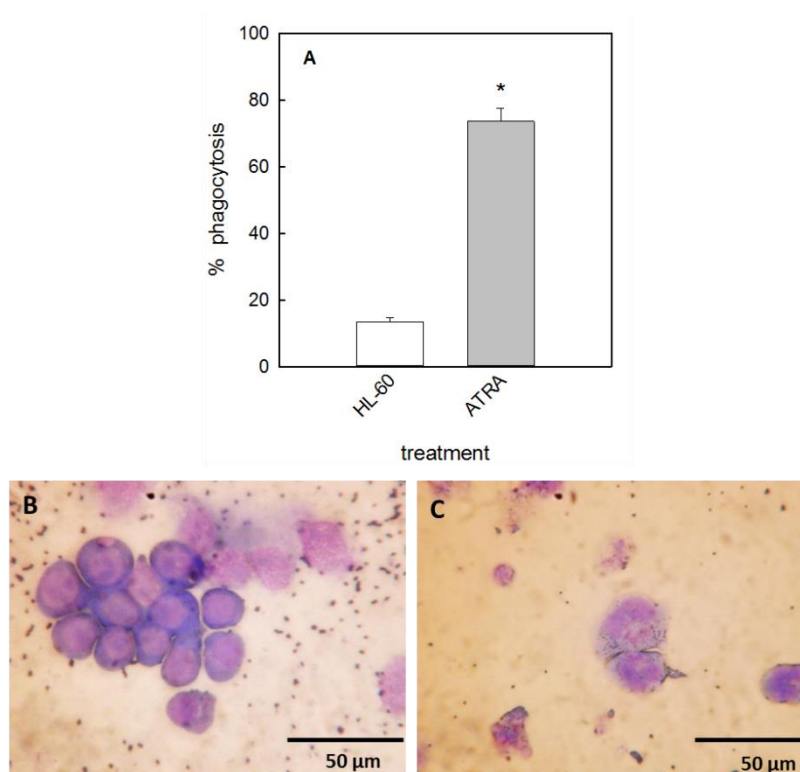


Fig. 3.10 Phagocytosis of *Staphylococcus aureus* NCIMB 6571 by undifferentiated and ATRA-differentiated HL-60 cells. (A) HL-60 cells or ATRA-treated cells ($1 \times 10^6 \text{ ml}^{-1}$) were incubated with *Staphylococcus aureus* NCIMB 6571 ($1 \times 10^7 \text{ ml}^{-1}$) for 30 min at 37 $^{\circ}\text{C}$, then cystospin slides were prepared, stained with Giemsa stain and cells counted to indicate the % of phagocytic cells. Data are expressed as means \pm SEM for three separate experiments (passage numbers 26, 28 and 30; $n = 3$) with triplicate measurements. * indicates a significant difference versus undifferentiated HL-60 cells (control; Student's *t*-test, $P = 0.00002$). Light microscopy study of phagocytosis of *Staphylococcus aureus* NCIMB 6571 by (B) HL-60 cells and (C) ATRA-differentiated cells (original magnification $\times 100$).

3.3.5 TNF- α production by neutrophil-like cells produced by differentiation of HL-60 cells

TNF- α production by differentiated HL-60 cells, both ATRA (Fig. 3.11A) and 9-*cis* RA treated (Fig. 3.11B; 1 μ M for 5 days), and human neutrophils isolated from blood (Fig. 3.11C) following stimulation with PMA, LPS, PGN or FMLP was quantified. Stimulation with PMA (100 ng ml⁻¹), LPS (100 ng ml⁻¹), PGN (10 μ g ml⁻¹), or FMLP (100 ng ml⁻¹) resulted in significant increases in TNF- α levels in ATRA-differentiated HL-60 cells (Kruskal-Wallis, $P = 0.0034$). The highest levels were observed in LPS-stimulated cells in comparison to control (unstimulated cells; Fig. 3.11A). PMA (25 ng ml⁻¹) and FMLP (1 μ g ml⁻¹ and 10 ng ml⁻¹) did not show an increase in TNF- α level compared to the control. Only 100 ng ml⁻¹ PMA and LPS were found to produce a significant increase in TNF- α in 9-*cis* RA-differentiated HL-60 cells (Kruskal-Wallis, $P = 0.00269$) compared to the control (Fig. 3.11B). However, stimulation of human neutrophils (primary cells) with all stimuli increased TNF- α release (Kruskal-Wallis, $P = 0.0139$; Fig. 3.11C). The data presented were from a single experimental run due to the high reagent costs involved. However, based on these preliminary results two stimuli, LPS and PMA were further investigated with triplicate experiments.

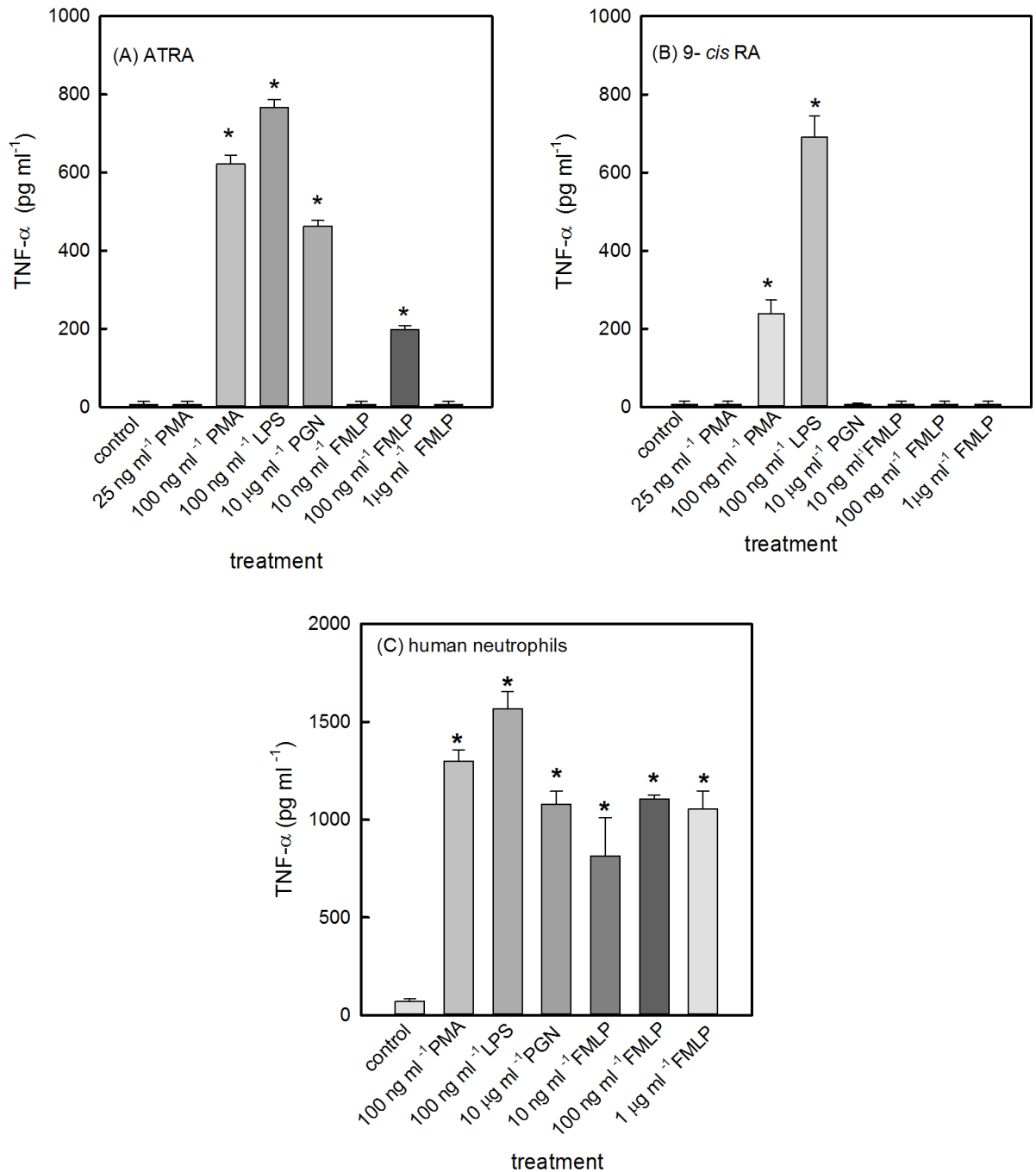


Fig. 3.11 **TNF- α production by differentiated HL-60 cells and human neutrophils.** HL-60 cells were differentiated using (A) ATRA or (B) 9-*cis* RA. (C) shows the results using human neutrophils. Cells (1×10^6 ml⁻¹) were incubated with different stimuli for 18 h under 5% CO₂ and 37 °C. The supernatant was collected and assayed for TNF- α by ELISA. Data are expressed as means \pm SEM for a single experiment (passage number 30; $n = 1$) with triplicate measurements. * indicates significant differences within this experiment only compared to the appropriate control in each case (Kruskal-Wallis, $P < 0.05$).

3.3.6 TNF- α and IL-10 production in response to oxygen exposure at different concentrations

In a second experiment to explore the effects of oxygen exposure on cytokine production, neutrophil-like cells obtained by differentiation of HL-60 cells (1 μ M ATRA for 5 days) were pre-treated with hyperoxia (95% O₂) or hypoxia (5% O₂) for 90 min and then stimulated with 100 ng ml⁻¹ PMA or LPS for 18 h. A non-significant interaction was found between the effects of treatment and stimulus on TNF- α and IL-10 release from the neutrophil-like cells (two-way ANOVA, $P > 0.05$). There was a significant increase in TNF- α and IL-10 production in ATRA-treated cells in response to PMA and LPS stimulation compared to unstimulated cells (two way ANOVA, $P < 0.00005$; Fig. 3.12A and B). Exposure to hyperoxia and hypoxia for 90 min at 20 °C, caused a significant decrease in TNF- α released by LPS and PMA-stimulated cells (two way ANOVA, $P < 0.00005$) compared to the normoxia (Fig. 3.12A).

ATRA-differentiated HL-60 cells showed low levels of anti-inflammatory IL-10 production (Fig. 3.12B). A significant decrease in IL-10 production in LPS and PMA-stimulated cells was observed post hyperoxia treatment (two way ANOVA, $P < 0.00005$) compared to normoxia (Fig. 3.9B). No change was observed in IL-10 levels post hypoxia treatment in LPS- or PMA-stimulated cells (76.8 ± 2.6 and 60.2 ± 7.7 pg ml⁻¹, respectively) compared with normoxia (66.6 ± 6.8 and 45.2 ± 4.9 pg ml⁻¹ for LPS and PMA-stimulated cells, respectively).

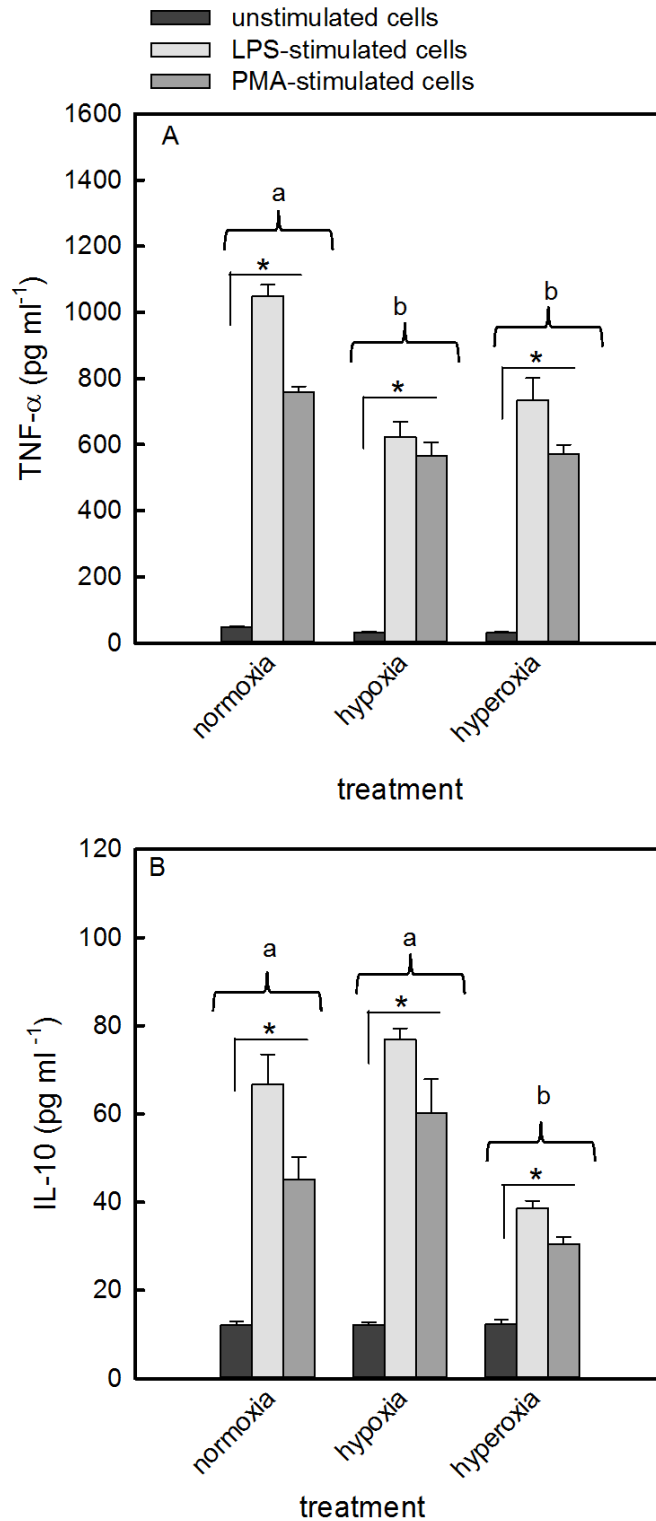


Fig. 3.12 TNF- α and IL-10 production by differentiated HL-60 cells following exposure to normoxia, hypoxia or hyperoxia. ATRA-differentiated HL-60 cells ($1 \times 10^6 \text{ ml}^{-1}$) were pre-treated with hyperoxia, hypoxia and normoxia (section 2.1.1) then incubated with different stimuli for 18 h under 5% CO₂ and 37 °C. The supernatant was collected and assayed for TNF- α and IL-10 by ELISA (section 3.2.4). Data are expressed as means \pm SEM for three separate experiments (passage numbers 28, 30 and 33; $n = 3$) with triplicate measurements. * indicates significant differences from unstimulated cells, for each treatment, bars with different lower case letter are significantly different (two-way ANOVA, $P < 0.05$).

3.3.7 Induction of apoptosis in differentiated HL-60 cells

Neutrophil-like cells obtained by differentiation of HL-60 cells were incubated with and without TNF- α and H₂O₂ as stimuli to induce apoptosis. Treatment with 10 ng ml⁻¹ TNF- α led to a statistically significant decrease in cell viability as measured by trypan blue uptake (one-way ANOVA, $P = 0.0016$). There was no change in cell viability with 50 and 100 ng ml⁻¹ TNF- α (one way ANOVA, $P > 0.05$) compared to the control (Fig. 3.13A). Cell apoptosis was confirmed by measurement of caspase 3/7 in neutrophil-like cells (Fig. 3.14A). TNF- α (10 ng ml⁻¹) caused a significant increase in caspase 3/7 activity (Kruskal-Wallis, $P = 0.0264$) compared to the control. Fig. 3.15 shows examples of time courses of the fluorescence intensity in caspase 3/7 assays. At 50 and 100 ng ml⁻¹ TNF- α , a significant increase in caspase 3/7 activity was also found in neutrophil-like cells compared to the control (Fig. 3.14A). In common with treatment with 10 ng ml⁻¹ TNF- α , treatment with H₂O₂ at a concentration of 33 μ M significantly decreased cell viability (one-way ANOVA, $P = 0.001$; Fig. 3.13B), and significantly increased caspase 3/7 activity (Kruskal-Wallis, $P = 0.0268$), but less than was observed with TNF- α (Fig. 3.14B). At high concentrations (66 and 132 μ M) of H₂O₂ cell viability was also significantly decreased but was accompanied by a significant decrease in caspase 3/7 activity compared to the control (Fig. 3.14B). Again, the data presented were from a single experiment due to the high reagent cost involved. However, from these preliminary results, the effect TNF- α (10 ng ml⁻¹) on inducing apoptosis was further investigated with triplicate experiments (Fig. 3.15).

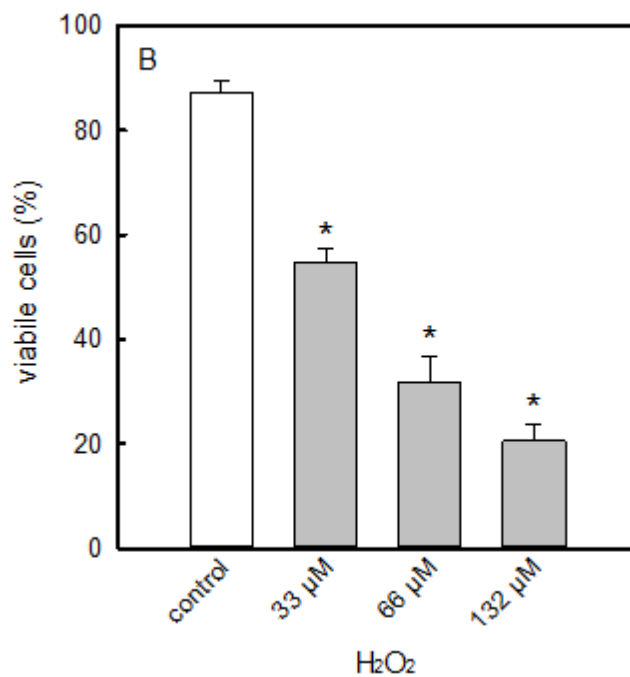
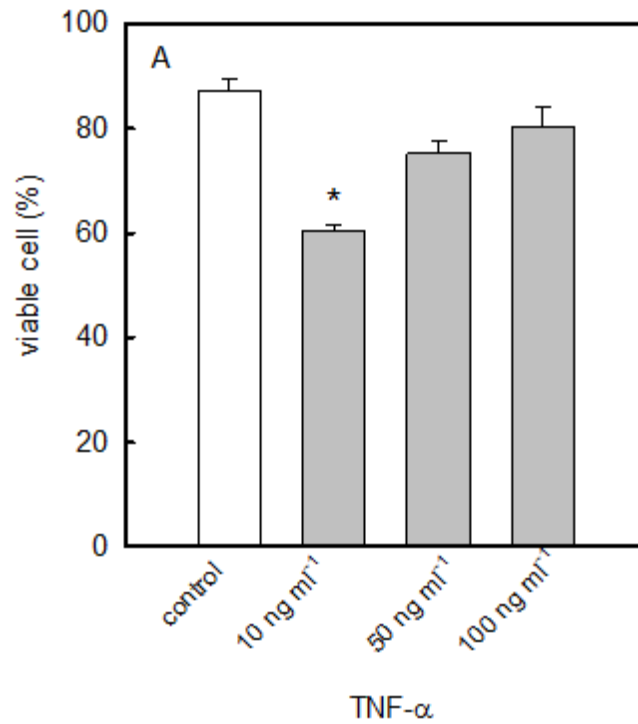


Fig. 3.13 Effects of H₂O₂ and TNF- α on the viability of neutrophil-like cells. Neutrophil-like cells were incubated with H₂O₂ (A) or TNF- α (B) to induce apoptosis for 3 h at 37 °C; viability was assayed by trypan blue dye exclusion (section 2.1.5). Data are expressed as mean \pm SEM for a single experiment (passage number 35; $n = 1$) with triplicate measurements. * indicates a significant difference within this experiment only compared to the control (one-way ANOVA, $P < 0.05$).

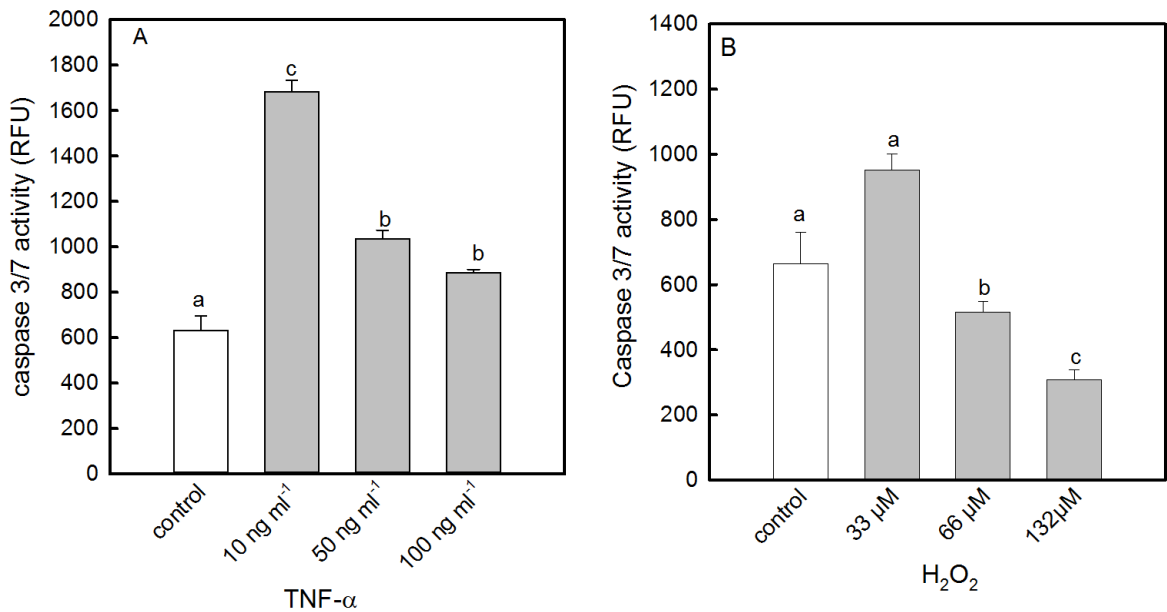


Fig. 3.14 Effects of H₂O₂ and TNF- α on caspase 3/7 activity in neutrophil-like cells. Neutrophil-like cells were incubated with H₂O₂ (A) or TNF- α (B) to induce apoptosis for 3 h at 37 °C after which caspase 3/7 activity was measured over 18 h (section 3.2.5). Data are expressed as mean \pm SEM for a single experiment (passage number 35; $n = 1$) with triplicate measurements, bars with different letters are significantly different (ANOVA or Kruskal-Wallis, $P < 0.05$).

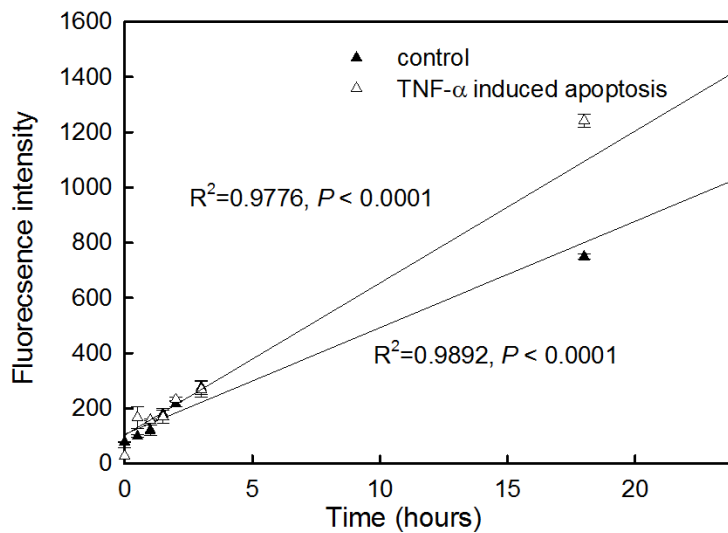


Fig. 3.15 Regression analysis illustrating the relationship between time and the production of the fluorescent product of reaction catalysed by caspase 3/7 in TNF- α induced apoptosis. Neutrophil-like cells were incubated with 10 ng ml⁻¹ TNF- α to induce apoptosis for 3 h at 37 °C after which caspase 3/7 activity was measured (section 3.2.5.1). Data are expressed as mean \pm SEM for three separate experiments (passage numbers 36, 38 and 40; $n = 3$) with triplicate measurements.

3.4 Discussion

Pre-treatment of HL-60 cells with 1 μ M ATRA demonstrated a maximal differentiation as indicated by a number of measurable physiological and biochemical changes, which included a decrease in cell density, increased superoxide production and alterations in cell morphology compared to undifferentiated cells. The other retinoid, 9-*cis* RA (at 1 μ M), and 1.25% DMSO also significantly inhibited cell proliferation. When a combination of ATRA and 9-*cis* RA at 1 μ M was added, there was no synergistic effect on the inhibition of cell proliferation. Possible explanations for this might be (1) that the two in combination may be mutually inhibitory, by modulating retinoid binding to the retinoid X receptors (RXRs) which play a key role in retinoid signalling and retinoid target genes (Mangelsdorf *et al.*, 1992, Leblanc and Stunnenberg, 1995), or (2) due to differences in the metabolism of each retinoid as different retinoid-specific enzymes are involved. Another possible explanation is that the investigation of the effect of the retinoids was limited to 1 μ M. Therefore future experiments using either of the individual retinoids or both in combination (at a concentration lower than 1 μ M) may be potentially useful. Cell proliferation was decreased within about 2 days from the incubation with ATRA, suggesting that exposure for 2 days is a minimal requirement to promote terminal differentiation in a significant fraction of HL-60 cells; this may be explained by the sensitivity of cells to the inducer only during a particular phase of the cell division cycle (Tarella *et al.*, 1982). The mechanisms responsible for ATRA-induced HL-60 cells granulocyte differentiation are not well defined. It has been suggested that the endogenous retinoic acid receptor alpha (RAR-alpha) plays an important role (Collins *et al.*, 1990). Moreover, some factors are known to be upregulated after ATRA exposure including genes associated with cell-cycle arrest, the ubiquitin/proteasome system, and the JAK/STAT, cAMP/PKA, PKC and calmodulin signalling pathways (Zhang *et al.*, 2000).

Based on these results, the decision was made to use ATRA at 1 μ M, so initial experiments were aimed at testing the function of these cells as a good model for neutrophils in *in vitro* studies. Cells differentiated with ATRA demonstrated a high percentage of NBT reduction. This was supported by an increase in their ability to phagocytose *Staphylococcus aureus* NCIMB 6571. This led to the conclusion that neutrophil-like cells have phagosomal respiratory burst activity. Differentiated HL-60 cells have been found to generate superoxide in response to PMA and zymosan at early stages during differentiation; this occurred prior to morphological changes being observed, although the superoxide levels never reached those observed in primary neutrophils (Newburger *et al.*, 1979). This may be explained by a low level of expression of the components required for activity, dysfunction of components or that only a percentage of the total cell population is responsive to the differentiation-inducing agent. In addition, Nordenfelt *et al.* (2009) demonstrated that ATRA-differentiated HL-60 cells possess azurophilic granules, while lacking the specific secondary granules with their associated oxidase components, therefore these cells develop an ability to mount a plasma membrane oxidative burst in the vicinity of forming phagosomes.

The reasons for using HL-60 cells as a model for neutrophils are as follows: they are multipotent; the cells have the ability to undergo spontaneous differentiation in culture; they exhibit features which have also been identified in neutrophils; and they provide a continuous source of neutrophil-like cells. This is critically important for assays that require relatively large numbers of cells. However, HL-60 cells are a leukaemic cell line which is defined by their abnormalities in proliferation, differentiation and apoptosis. Therefore, this model may not be suitable for some experiments of terminal differentiation events. It is also obvious that a terminally differentiated HL-60 cell does not exhibit the exact phenotype of its normal counterpart (Tagliafico *et al.*, 2002), which can make data

interpretation difficult. Additionally, these cells lose differentiation capacity with increasing passage number, for example 50 passages (Palis *et al.*, 1988). Consequently, if they are cultured for extended periods of time, the data may be non-consistent.

Enhanced respiratory burst activity and phagocytosis are not the only functions that can be exhibited by neutrophil-like cells. A preliminary study was carried out to test a range of possible stimuli; the results of this were broadly consistent with effects already reported in the literature (Kubin *et al.*, 1994, Grande *et al.*, 1995). From this PMA and LPS were selected as being suitable stimuli for further investigation. Stimulation of ATRA- or 9-*cis* differentiated cells with PMA and LPS at 100 ng ml⁻¹ caused a significant increase in TNF- α production but less so than in human neutrophils. Consistent with previous studies, the present study provides further evidence that neutrophil-like cells display complete functional activation (De Gentile *et al.*, 1994, Dubois *et al.*, 1994). Grande *et al.* (1995) reported the expression of IL-1 α , IL-1 β , IL-3, IL-6 and TNF- α from ATRA-differentiated cells and LPS has been shown to be a potent stimulator (Bemelmans *et al.*, 1994, Leeuwenberg *et al.*, 1994). Differentiated HL-60 cells produce IL-1 β and IL-8 in response to stimuli such as LPS (Shuto *et al.*, 2007). There are two possibilities as to why LPS could lead to activation of neutrophils: (a) via prolonged neutrophil life span and delayed apoptosis, and (b) via enhanced protein kinase C activity that is responsible for priming effects in neutrophils (Wightman and Raetz, 1984). This study focused on the effects of stimulation of neutrophil-like cells under normoxic conditions, but there is also a potential effect of elevated oxygen concentration on stimulus-induced cytokine release. In common with previous results, pre-treatment of neutrophil-like cells with LPS or PMA (100 ng ml⁻¹) after exposure to hyperoxia or hypoxia resulted in a decrease in pro-inflammatory TNF- α , while anti-inflammatory IL-10 levels decreased only after hyperoxia treatment. It has been shown that pre-treatment with hyperoxia decreases cytokine levels (for example TNF- α)

compared to normoxia as elevated oxygen concentrations inhibit stimulus-induced signal transcription, or translation (Granowitz *et al.*, 2002), or enhance sensitivity of the cells to hyperoxia-mediated apoptosis. A decrease in TNF- α levels was also found post hypoxia, these results raised the possibility that hypoxia modulates transcription factors for example NF- κ B or HIF-1 α which may affect cytokines levels, depending on the cell type, and duration of hypoxic exposure (Matuschak *et al.*, 2010). This is in agreement with Fritzenwanger *et al.* (2011) who demonstrated that short-term hypoxia decreased TNF- α concentrations in monocytes and INF- γ in CD4⁺ T lymphocytes (Zagórska and Dulak, 2004). These findings explain the regulatory roles of HIF-1 under hypoxic conditions and its association with decreased levels of pro-inflammatory cytokines, and support the association between HIF and NF- κ B signalling in short term hypoxia.

A wide range of stimuli have been used to induce apoptosis, some of which are components of pathological inflammatory diseases, such as TNF- α (Windsor *et al.*, 1993) or chemical agents such as H₂O₂ (Lundqvist-Gustafsson and Bengtsson, 1999). A preliminary study was carried out to test if possible stimuli induce apoptosis. From this, TNF- α (10 ng ml⁻¹) was selected as being a suitable stimulus for further investigation. TNF- α (10 ng ml⁻¹) treatment led to a decrease in viability and an increase in caspase 3/7 activity in neutrophil-like cells. These results are consistent with the effects of TNF- α previously demonstrated in neutrophils (Takeda *et al.*, 1993). TNF- α -mediated regulation of neutrophil apoptosis has been shown to be concentration-dependent; with high concentrations, TNF- α induces apoptosis via initiation of death signals via the TNF- α receptor, and activation of the apoptotic caspase cascade with degradation of the anti-apoptotic protein, Mcl-2 (Van Den Berg *et al.*, 2001). TNF- α at low concentrations enhances cell survival and activity through activation of NF- κ B (Liu *et al.*, 1996b) and expression of the anti-apoptotic protein Bcl-2 (Cross *et al.*, 2008). TNF- α also initiates a caspase-independent pathway in neutrophils (Maianski *et al.*, 2003). In the present

experiments, addition of 33 μM H_2O_2 to neutrophil-like cells also increased caspase 3/7 activity. However, with higher concentrations there was a decrease in activity perhaps as necrotic effects become more apparent than apoptotic effects with the increase of oxidative stress (Takeda *et al.*, 1999).

3.4.1 Conclusion

ATRA induced differentiation of HL-60 cells into neutrophil-like cells. HL-60 cell differentiation is marked by a number of measurable biochemical changes which include a decrease in proliferation, increased superoxide production and alterations in cell morphology. ATRA-differentiated cells displayed multiple functional responses upon differentiation similar to those observed in mature neutrophils. This suggests that this cell line is a useful model system for *in vitro* studies of neutrophils, as a large number of cells can be easily obtained, although there are limitations to this model system such as the abnormalities in proliferation and differentiation which are associated with HL-60 cells being a leukaemic cell line.

CHAPTER FOUR

THE EFFECT OF HBO ON

ANTIMICROBIAL DEFENCE

MECHANISMS IN NEUTROPHILS

Abstract

The persistent presence of neutrophils is a feature of chronic wounds. The detrimental effects of neutrophils may be due to released oxidants, ROS and destructive enzymes. Hyperbaric oxygen (HBO) therapy, the intermittent inhalation of 100% oxygen at pressures greater than atmospheric, is an effective treatment for various inflammatory conditions, including chronic wounds, but the mechanisms involved are not well understood particularly with respect to neutrophils. In this study, HL-60 cells differentiated by ATRA were used as an *in vitro* neutrophil model to explore the effects of a single 90 min exposure to normoxia, hypoxia, normobaric hyperoxia, hyperbaric normoxia and hyperbaric oxygen on antimicrobial functions and degranulation. Differentiated HL-60 cells were treated with PMA to stimulate the respiratory burst activity post-exposure to the different treatments. A single 90 min HBO exposure caused a significant increase in the oxidative burst activity of neutrophil-like cells as shown by superoxide ($O_2^{\cdot-}$) release (cytochrome *c* reduction; Kruskal-Wallis, $P = 0.019$), NBT staining (Kruskal-Wallis, $P = 0.011$) and H_2O_2 production (Kruskal-Wallis, $P = 0.016$). In addition, phagocytosis of *Staphylococcus aureus* was increased (one-way ANOVA, $P < 0.05$). Antimicrobial effects of HBO were also accompanied by a significant increase in MPO production (Kruskal-Wallis, $P = 0.015$). Although no consistent pattern was observed, both hyperoxia and pressure alone seemed to contribute to the increase in antimicrobial activity induced by HBO in these neutrophil-like cells. In conclusion, this data may go some way to explaining the effectiveness of HBO in the treatment of chronic wounds

4.1 Introduction

Neutrophils consume oxygen to maintain the NADPH oxidase-driven respiratory burst which generates ROS (Hampton *et al.*, 1998). Hypoxia is a feature of chronic wounds, where impaired microcirculation in wound area and increased oxygen consumption by cells such as leukocytes result in low oxygen levels especially at the centre of the wound. With a K_m for O_2 of 40-80 mm Hg, NADPH oxidase displays a relative resistance to hypoxia (Gabig *et al.*, 1979). Even at infected sites where the PO_2 is approximately 25 mm Hg (equivalent to 3% O_2), neutrophils are still able to activate the respiratory burst to kill microorganisms but with limited capacity (Gordillo and Sen, 2003, Hopf and Rollins, 2007). Superoxide production at 1% O_2 has been shown to be more than 75% of the level produced at 21% O_2 (Gabig *et al.*, 1979). This suggests that at these oxygen levels, NADPH oxidase is still able to function although with reduced activity. Furthermore, in chronic wound conditions, inflammatory cytokines (for example TNF- α) and infection, may affect neutrophil oxidative burst activity (see section 1.4.2). *In vitro*, LPS enhances oxidative stress produced by neutrophils in a dose-dependent manner (Bohmer *et al.*, 1992) with a significant effect at 1 ng ml⁻¹, which is much higher than the levels present in chronic wound. TNF- α is only able to activate oxidative burst activity in neutrophils *in vitro* at concentrations of 250 ng ml⁻¹ or above (Berkow *et al.*, 1987); again, this concentration is much higher than the levels found in chronic wounds. Priming of neutrophils with TNF- α at a concentration of 215 ng ml⁻¹ has been shown to significantly enhance the FMLP or LPS-induced respiratory burst activity, resulting in increased production of hydrogen peroxide (Wiemer *et al.*, 2010). Excessive neutrophil recruitment to the chronic wound site and high levels of inflammatory stimuli such as LPS and TNF- α , result in high levels of neutrophil degranulation leading to prolonged inflammation (Lee *et al.*, 1993). Higher levels of

elastase, collagenase (MMP-8) and gelatinase (MMP-9) were observed in chronic wounds compared to acute wounds (Nwomeh *et al.*, 1999). These MMPs cleave collagen types I, III, IV, V, VII, and destroy the growth factors necessary for wound healing such as bFGF and VEGF, thus impairing the wound healing process (Ai *et al.*, 2007).

Investigations examining the effects of HBO on the respiratory burst of neutrophils have yielded conflicting results (Thom *et al.*, 2008, Labrousche *et al.*, 1999, Kalani *et al.*, 2002, Jüttner *et al.*, 2003). For example, HBO has been demonstrated to enhance neutrophil function in some infections through the correction of tissue hypoxia and providing the optimal oxygen tension for the oxidative burst (Labrousche *et al.*, 1999, Mader *et al.*, 1980). HBO enhances respiratory burst activity in neutrophils by increasing NADPH oxidase enzymatic activity, the major enzymatic source of ROS (Acker and Acker, 2004). NADPH oxidase is a complex enzyme composed of at least five components, the membrane-bound components being the flavocytochrome b_{558} and $p22^{\text{phox}}$, which provide binding sites for cytosolic NADPH oxidase (Segal, 2005). The cytosolic components are $p67^{\text{phox}}$, $p47^{\text{phox}}$ and $p40^{\text{phox}}$. Upon neutrophil activation, the cytosolic components translocate to the plasma membrane, to form the active oxidase (Roos *et al.*, 2003). Recent evidence suggests that NADPH oxidase is involved in wound healing by modulating cell proliferation (survival), apoptosis, angiogenesis and fibrosis. After tissue injury, factors involved in haemostasis, coagulation (e.g. thrombin and PAF), and inflammation (e.g. TNF- α , IL-1 β) may provide a local stress environment that trigger NADPH oxidase dependent ROS production (Holland *et al.*, 1998, Monaco and Lawrence, 2003). Moreover, inhibiting NADPH oxidase activation or down-regulating NADPH oxidase expression suppressed thrombin-induced mitogenic effects on vascular smooth muscle cells (Patterson *et al.*, 1999). Exposure to hyperoxia can activate NADPH oxidase; Brueckl *et al.* (2006) demonstrated that

hyperoxia induced a rapid activation of NADPH oxidase in lung capillary endothelial cells, through intracellular Ca^{2+} elevation and Rac1 activation. Investigations of the effect of HBO on the NADPH oxidase complex are limited. In the pancreas of diabetic rats, exposure to HBO induces further ROS production and apoptosis through up regulation of expression of genes of subunits related to the NADPH oxidase complex (p47^{phox} mRNA expression; Matsunami *et al.*, 2011). However, a study in a rat subarachnoid haemorrhage model demonstrated that HBO decreased NADPH oxidase expression (gp91^{phox} subunit), activity and the level of oxidative stress at 24 h after subarachnoid haemorrhage (Liu *et al.*, 2007). This may be explained by the effects of HBO on the regulation of NADPH oxidase activators, including protein kinase C, cytokines and angiotensin II (Vignais, 2002) or oxygen sensing agents such as hypoxia-inducible factor-1, HIF-1 α (Huang *et al.*, 1998; Fig.4.1).

There is limited work examining neutrophil degranulation following HBO treatment. In a study using rat neutrophils, HBO (3 ATA for 45 min) had no effect on elastase release and MPO activity, suggesting no effect on primary granule release (Thom, 1993). Another study by Kang *et al* (2002) demonstrated that HBO increased the myeloperoxidase content in the lung, and this may be attributed to an increase in neutrophil infiltration into lung tissue.

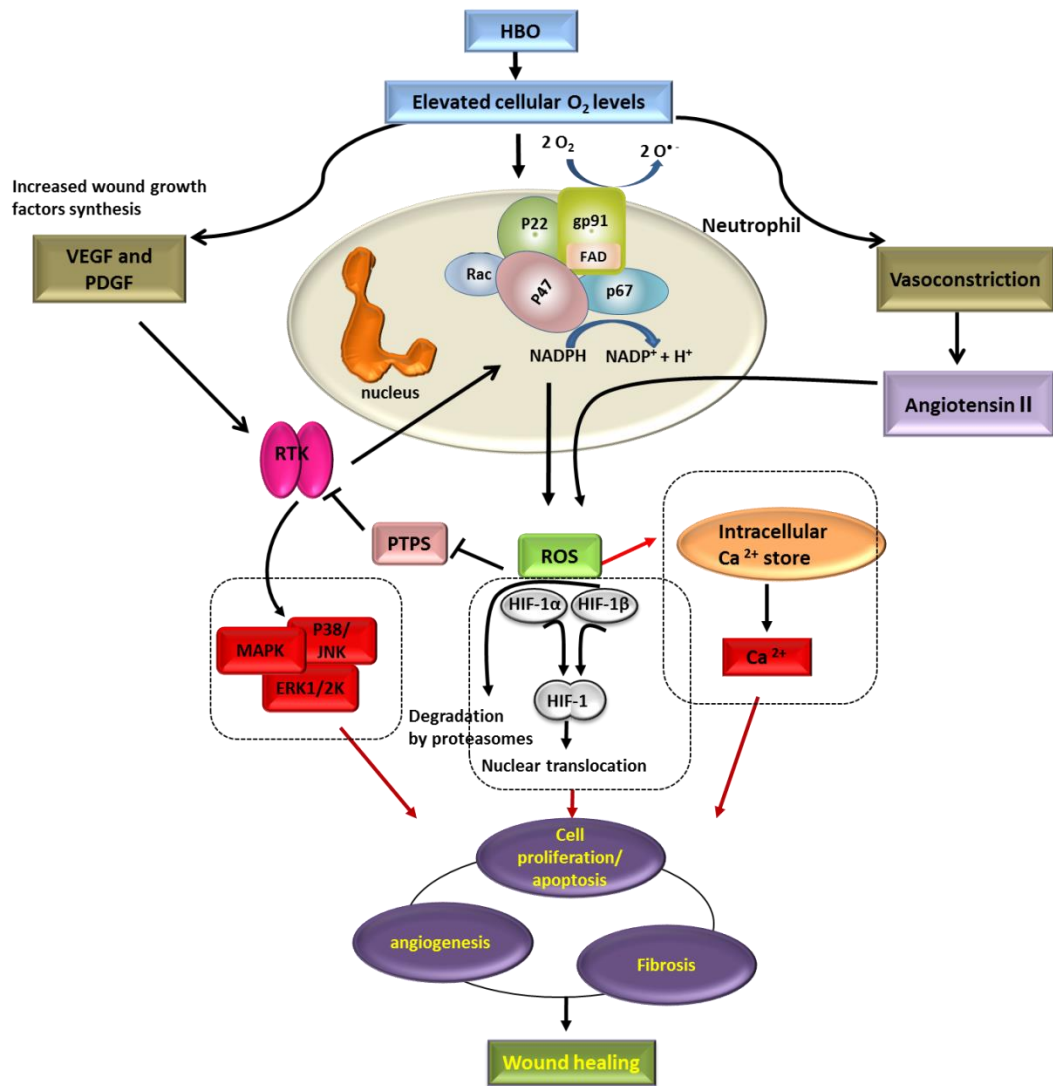


Fig. 4.1 The effect of HBO on NADPH oxidase. Increased tissue oxygenation enhances growth factor levels (e.g. VEGF and PDGF) and vasoconstriction. Upon binding of these factors to their receptor tyrosine kinases (RTK) or stimulation by G-protein coupled receptor (GPCR) agonists such as angiotensin II, NADPH oxidase is activated. ROS oxidize the essential cysteine residues of protein tyrosine phosphatases (PTPS), thereby inactivating these enzymes, and leading to enhanced activation of mitogen-activated protein kinases such as ERK1/2, MAPK and P38/JNK. NADPH oxidase-derived ROS may also elevate intracellular Ca²⁺ by enhancing the Ca²⁺ entry through the cell membrane or promoting Ca²⁺ release from the intracellular stores or activating HIF degradation; all these mechanisms may be involved in promoting cell proliferation, fibrosis and angiogenesis, which are critical components of tissue in healing processes (Ushio-Fukai, 2006, Sauer and Wartenberg, 2005, Datla *et al.*, 2007, Gorin *et al.*, 2004).

The effect of HBO on neutrophil oxidative burst activity has been examined in several studies but there is no agreement on its effect, while the investigation of the effects of HBO on degranulation is limited. This study aimed to examine the effect of exposure to HBO (90 min at 2.4 ATA) on both respiratory activity and degranulation in neutrophil-like cells.

4.2 Materials and methods

4.2.1 Experimental design

To investigate neutrophil function this study was divided into two parts. The first was to explore the effects of a 90-min exposure to HBO, pressure, hyperoxia, and hypoxia in respiratory function of neutrophil-like cells *in vitro*. Assays included quantification of superoxide production; NBT reduction (section 2.1.2); extracellular H₂O₂ levels (section 2.1.3); measurement of *Staphylococcus aureus* NCIMB 6571 phagocytosis (section 2.1.4); and nitrite (NO₂⁻); and nitrate (NO₃⁻; quantification as detailed below). The second part aimed to explore the effect of a 90-min exposure to 5 different treatments on MPO to determine the degranulation of neutrophil-like cells *in vitro*. The total glutathione (GSH) in neutrophil-like cells was also measured to assess redox status. The measured endpoints (in triplicate) for three separate experiments with a different passage number of HL-60 cells were used. HL-60 cells were cultured (section 3.2.2) in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), containing 2 mM glutamine, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin in Corning polystyrene vented 75 cm² tissue culture flasks. Cells were maintained at 37 °C in 5% CO₂ (balance air); passages 20 through to 45 were used for experiments. Differentiation of HL-60 cells was induced by incubation of 2.5 × 10⁵ cells ml⁻¹ with ATRA (1 µM) for 5 days after which they were harvested for further assays (section 3.2.2). Cells were added to 96-well plates and divided into 5 different treatment groups. The normoxia treatment (control) was exposed to 21% O₂ at 1 ATA; the hypoxia group was exposed to 5% oxygen at 1 ATA, the hyperoxia treatment was exposed to 95% O₂ at 1 ATA; the pressure group was exposed to 8.8 % O₂ at 2.4 ATA and HBO treatment was exposed to 97.9% O₂ at 2.4 ATA. Cells were treated for 90 min at room temperature (section 2.1.1). For the measurements of respiratory burst activity, cells were stimulated immediately

post-treatment with phorbyl myristate acetate (PMA) Sigma (Poole, UK; Fig. 4.2). The PMA solution was prepared by dilution with HBSS of a stock solution of 1 mg ml^{-1} in DMSO.

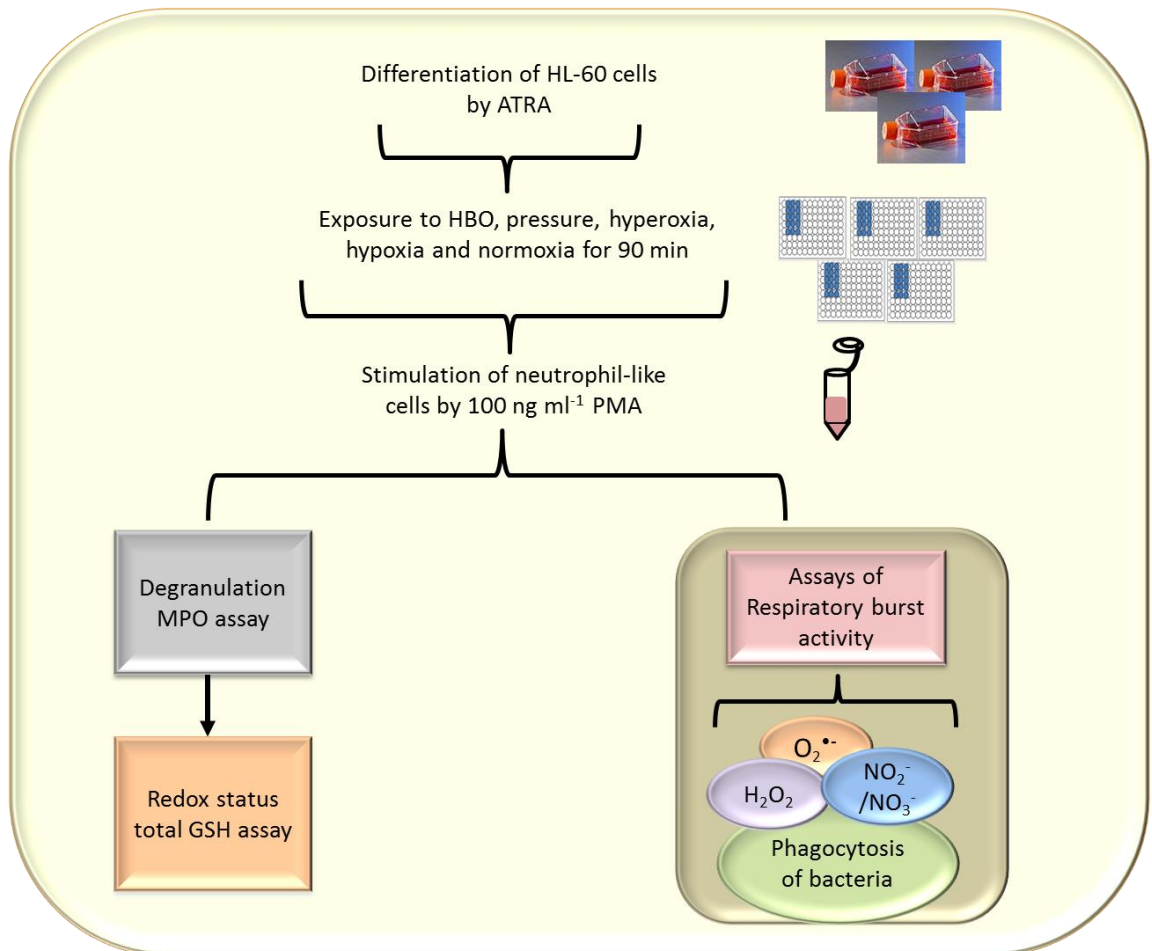


Fig. 4.2 Diagram showing the experimental design. ATRA-differentiated HL-60 cells were exposed to different treatments, stimulated with 100 ng ml^{-1} PMA, and assayed for antimicrobial functions. Time of exposure, stimulus and end points are indicated.

4.2.2 Measurement of superoxide production via cytochrome *c* reduction

The reduction of ferricytochrome *c* (Fe^{3+} cytochrome *c*) to ferrocyanochrome *c* (Fe^{2+} cytochrome *c*) by $\text{O}_2^{\bullet-}$ produced by neutrophil-like cells can be monitored at 550 nm, in the presence and absence of superoxide dismutase (SOD) as a negative control. The absorbance of samples with SOD present is subtracted from the absorbance of samples with no SOD present to determine the proportion of cytochrome *c* reduction that is SOD-inhibitable, and therefore $\text{O}_2^{\bullet-}$ -dependent (Edwards, 1996).

Ferricytochrome *c* (5 μl of 2 mM dissolved in potassium phosphate buffer; Sigma C-7752) was added to each well in a 96-well plate followed by 50 μl of neutrophil-like cell suspension (10^6 cells ml^{-1} in HBSS), 145 μl of HBSS, and 10 μl of 100 ng ml^{-1} PMA. The PMA solution was prepared by dilution with HBSS of a stock solution of 1 mg ml^{-1} in DMSO. Upon addition of the PMA, the absorbance at 550 nm was monitored in a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA) for 45 min at 37 °C. Two controls were used, one in which the stimulus was replaced with 10 μl of HBSS, and the other in which the stimulus was replaced with 2 μl of 3000 U ml^{-1} SOD in ammonium sulphate suspension and 8 μl of HBSS (Sigma S-3409). The reduction of cytochrome *c* was calculated using an extinction coefficient of 21.1 $\text{mM}^{-1} \text{cm}^{-1}$ and a path length of 0.66 cm.

4.2.3 Assay of $\text{NO}_2^-/\text{NO}_3^-$

Griess reagent together with nitrate reductase is widely used for determining nitrate and nitrite (Schmidt and Kelm, 1996.). It involves the reaction of NO_2^- at low pH with sulphanilamide (SAN). The resulting diazonium ion then reacts with a coupling reagent such as *N*-(1-naphtyl)-ethylenediamine (NED) to form an azo dye. The absorbance of the azo dye can be measured at 550 nm. Subsequently, the NO_2^- can be converted to NO_3^- by the use of NADPH-dependent nitrate reductase (NR). NO_3^- can also be

measured using Griess reagent. Aliquots (100 μl) of neutrophil like-cells in a suspension containing 1×10^6 cells ml^{-1} in phenol red-free HBSS were added to the wells of a 96-well plate and stimulated with PMA (100 ng ml^{-1}) for 30 min at 37 $^{\circ}\text{C}$. The plate was centrifuged at $200 \times g$ to remove cells and the supernatant from each well was transferred to a new plate. The assay mixture was prepared by mixing (per ml) 0.72 ml of 50 mM sodium phosphate buffer (pH 7.4), 0.08 ml of 1.6 U ml^{-1} nitrate reductase (NR, Sigma N7265), and 0.2 ml of 1 mM NADPH (prepared fresh in phosphate buffer each day, stored on ice during use). Fifty microlitres of the NO_3^- standard (up to 20 μM) or supernatant were mixed with 50 μl of the assay mixture to give a final concentration of 0.1 U ml^{-1} NR and 0.2 mM NADPH in the wells of a 96-well plate, and then the plate was incubated for 30 min at 20 $^{\circ}\text{C}$ to allow completely reduction of NO_3^- to NO_2^- . The diazotization reaction was started by adding 100 μl of sulphanilamide (1% [w/v] in 1 M HCl), followed by 100 μl of 0.1% NED 30-90 s later. The absorbance at 550 nm was measured a few minutes later in a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA; Moody and Shaw, 2006). A standard curve was plotted for the NO_3^- and NO_2^- (0-20 μM) to calculate the nitrite and nitrate concentration in the sample (Fig. 4.3).

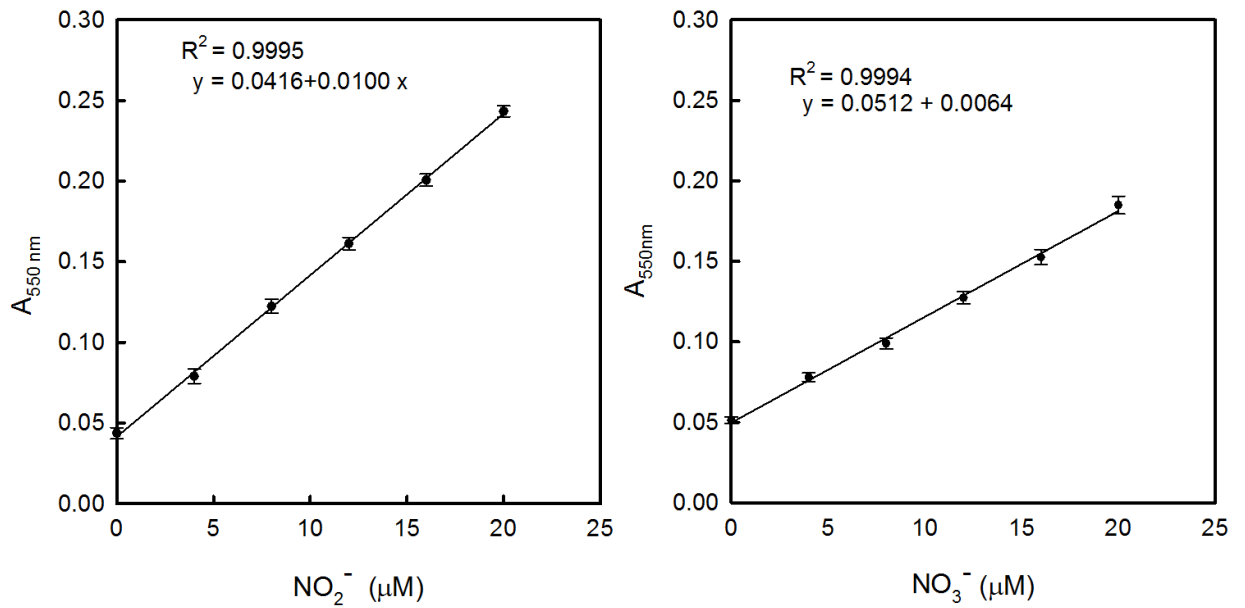


Fig 4.3 Nitrite and nitrate standard curves. Nitrite (NO₂⁻) and nitrate (NO₃⁻) standards (0.5 to 20 μM) were prepared in dH₂O. The data points are the means of triplicate measurements ± SEM.

4.2.4 Myeloperoxidase (MPO) assay

MPO is stored in the primary granules of neutrophils and catalyses the conversion of H₂O₂ and Cl⁻ into the potent oxidant hypochlorous acid (HOCl), which can be quantitated by trapping the generated species with the β-amino acid, taurine. The resultant stable product, taurine chloramine, can be quantitated by the ability of iodide to catalyse its oxidation reactions with suitable chromophores such as tetramethylbenzidine (TMB), and absorbance can be measured at 650 nm (Dyrbukt *et al.*, 2005, Weiss *et al.*, 1982).

MPO activity was measured essentially as described by Dyrbukt *et al.* (2005). Neutrophil-like cells were re-suspended in 10 mM phosphate buffer (pH 7.4), containing 140 mM sodium chloride and 10 mM potassium chloride. Taurine was then added to the cells (25 μl of 5 mM to 100 μl of 2 × 10⁶ cells ml⁻¹ in 1.5 ml microfuge tubes) and pre-incubated for 15 min at 37 °C. Finally, 125 μl of 100 ng ml⁻¹ PMA, the

stimulus to release MPO, was added and the mixture incubated for 30 min at 37 °C. At the end of the incubation period, catalase (10 µl of 40 U ml⁻¹) was added and the tubes placed on ice, after which they were centrifuged at 13,000 × g for 2 min to remove cells. The supernatants from each tube were transferred to a new plate. Taurine chloramine was detected by rapidly mixing 200 µl of supernatant or standard with 50 µl of developing reagent which was composed of 2 mM TMB in 400 mM sodium acetate buffer, pH 5.4, containing 10% dimethylformamide (DMF) and 100 µM sodium iodide. This solution was prepared by dissolving TMB in 100% DMF, diluting with acetate buffer to obtain the desired final concentration of TMB, and then adding sodium iodide. Formation of a blue product indicated the presence of chloramine. Five minutes after mixing the absorbance was recorded at 650 nm. A standard curve was plotted for the initial concentrations of hypochlorous acid (10-100 µM) versus the recorded increase in absorbance of the blue product to calculate the hypochlorous acid concentration in the samples (Fig. 4.4).

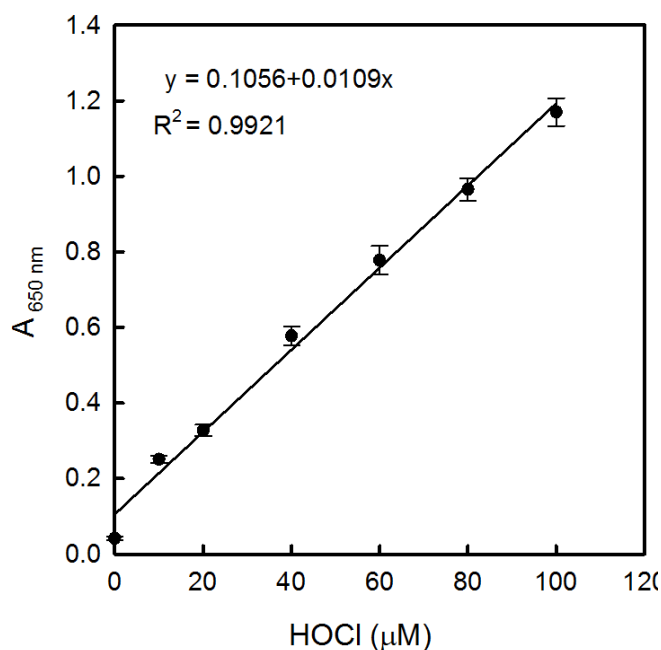


Fig 4.4. Standard curve for the detection of MPO using HOCl. Standards (10 to 100 µM HOCl) were prepared in dH₂O. The data points are the means of triplicate measurements ± SEM.

4.2.5 Measurement of total glutathione

The usual procedure for total glutathione measurements in biological sample involves homogenization (if necessary), deproteination, and non-specific or specific measurement of thiol content, the latter being used for cases in which precise knowledge of the reduced glutathione (GSH) content is required (Kosower and Kosower, 1987). The thiol group analysis is based on the use of 5, 5-dithiobis-2-nitrobenzoic acid (DTNB). In this assay, oxidized glutathione (GSSG) is reduced to GSH by NADPH in the presence of glutathione reductase (GR) and the total GSH is sequentially oxidised by DTNB. The rate of 2-nitro-5-thiobenzoic acid formation is monitored at 412 nm.

Aliquots of neutrophil-like cell suspension (100 μ l), containing 1×10^6 cells ml^{-1} were added to the wells of a 96-well plate after treatment with HBO, pressure control, hyperoxia, hypoxia or normoxia for 90 min. The cells were stimulated by the addition of 100 ng ml^{-1} PMA for 30 min; the total level of glutathione in the cell lysate (obtained by repeated freezing/thawing cells) was determined by the glutathione reductase enzyme recycling method as described by Adams *et al.* (1983). The reagent volumes were optimized to conduct this assay in 96-well plates. Samples or standard solutions of GSH (up to 20 μ M) were mixed in a 1:1 ratio with buffered DTNB (10 mM DTNB in 100 mM potassium phosphate, pH 7.5, containing 5 mM potassium ethylenediaminetetraacetate, EDTA). Buffer containing glutathione reductase (210 μ l containing 0.6 U, Sigma G3664) and 40 μ l of DTNB- treated samples were mixed in the wells of a 96 well plate. After equilibration for 1 min the reaction was started by adding 60 μ l of 1 mM NADPH, and the absorbance at 412 nm was measured for 10 min using a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). In this method the rate of increase of A_{412} is proportional to the concentration of total glutathione (i.e. GSSG and GSH).

4.2.6 Statistical analysis

All data were analysed using Statgraphics Centurion XVI software (Stat Point Technologies, Inc.). Data are expressed as means \pm SEM for 3 separate experiments. The Andersen-Darling test for normality was used followed by Levene's test to check that there were no significant differences in variance between the groups. Where appropriate, data were \log_{10} transformed. Data were analysed using ANOVA (one-way or two-way ANOVA) or Kruskal-Wallis. After ANOVA, *post hoc* analysis using Tukey's test was carried out. *P* value of < 0.05 was considered significant throughout.

4.3 Results

4.3.1 HBO enhances respiratory burst activity by differentiated HL-60 cells

ATRA-differentiated HL-60 cells were exposed to hyperbaric hyperoxia (97.9% O₂, 2.1% CO₂ at 2.4 ATA), hyperbaric normoxia (8.8% O₂ at 2.4 ATA), normobaric hyperoxia (95% O₂, 5% CO₂ at 1 ATA) or normobaric normoxia (21% O₂, 5% CO₂ at 1 ATA) for 90 min at 20 °C. After exposure respiratory burst activity was assessed by measuring superoxide (O₂^{•-}) production using both cytochrome *c* reduction by quantifying the percentage of NBT-stained cells and by measuring H₂O₂ production using the horseradish peroxidase/homovanillic acid (HRP/HVA) method. In all cases, PMA was used to stimulate the neutrophil-like cells.

Addition of PMA caused a significant increase in production of O₂^{•-}, indicating the successful differentiation to neutrophil-like cells compared to unstimulated cells (pair-wise Mann-Whitney, *P* < 0.05 ; Fig 4.5A). For superoxide production, no treatment effect (Kruskal-Wallis, *P* > 0.05) was observed on the O₂^{•-} production in unstimulated cells (0.32 ± 0.06 , 0.28 ± 0.11 , 0.26 ± 0.14 and 0.27 ± 0.13 nmol per 10⁶ cells for HBO, pressure, hyperoxia and hypoxia, respectively) compared with normoxia (0.23 ± 0.13).

Exposure of PMA-stimulated cells to HBO (90 min at 2.4 ATA, at 20 °C) caused a significant increase in $O_2^{\cdot-}$ production while hypoxia produced a significant decrease compared to normoxia (Kruskal-Wallis, $P = 0.019$; Fig. 4.5A). Pressure alone or hyperoxia did not lead to a change in PMA-induced $O_2^{\cdot-}$ production (2.19 ± 0.25 and 2.28 ± 0.04 nmol per 10^6 cells, respectively) compared with normoxia (1.95 ± 0.10 ; Fig. 4.5A).

Stimulation of cells with PMA also resulted in an increase in the percentage of cells stained with NBT compared to unstimulated cells (pair-wise Mann-Whitney, $P < 0.05$). A significant increase in the percentage of cells stained with NBT was found after exposure to HBO, pressure or hyperoxia compared to the normoxia (Fig. 4.5B; Kruskal-Wallis, $P = 0.011$). However, there was no effect on the percentage of cells stained with NBT after hypoxia ($38.8 \pm 5.5\%$ for stimulated cells) compared with normoxia ($48.4 \pm 3.2\%$ for stimulated). Similar to $O_2^{\cdot-}$ production, no treatment effect (Kruskal-Wallis, $P > 0.05$) was observed on the percentage of unstimulated cells stained with NBT (34.3 ± 9.3 , 33.9 ± 5.5 , 35.9 ± 3.9 , and $18.5 \pm 1.6\%$ for HBO, pressure, hyperoxia and hypoxia, respectively) compared with normoxia ($24.2 \pm 2.1\%$).

Respiratory burst activity was further assayed by measuring H_2O_2 production by neutrophil-like cells. Significantly higher H_2O_2 production by stimulated differentiated HL-60 cells was observed compared to unstimulated cells (pair-wise Mann-Whitney, $P < 0.05$; Fig. 4.6), as measured by oxidation of HVA. No significant effect was found on H_2O_2 production by unstimulated cells after different treatments (0.9 ± 0.1 , 1.5 ± 0.1 , 0.8 ± 0.1 and 0.5 ± 0.1 nmol per 10^6 cells for HBO, pressure, hyperoxia, and hypoxia, respectively; Fig. 4.6) compared with normoxia (0.8 ± 0.1 nmol per 10^6 cells). Post-HBO, there was a significant increase in the H_2O_2 release from PMA-stimulated cells; the same was also seen post exposure to pressure alone compared to normoxia (Kruskal-

Wallis, $P = 0.016$; Fig. 4.6). However, there were no effects on the H_2O_2 release from PMA-stimulated cells after hyperoxia (5.5 ± 0.3 nmol per 10^6 cells) compared to normoxia (5.6 ± 0.6 nmol per 10^6 cells). Similar to superoxide production, H_2O_2 levels were significantly decreased after hypoxia in PMA-stimulated cells to (1.8 ± 0.5 nmol per 10^6 cells).

Phagocytic activity with *Staphylococcus aureus* NCIMB 6571 was measured in differentiated HL-60 cells (section 2.1.4). A significant increase in the percentage phagocytosis was seen post-HBO, pressure and hyperoxia (one-way ANOVA, $P = 0.0002$), and phagocytic index post HBO and hyperoxia (one-way ANOVA, $P < 0.00005$), but not post-pressure (68.1 ± 1.1 , phagocytosis index). NO change was observed in the percentage phagocytosis and phagocytosis index post hypoxia (19.4 ± 3.2 , % phagocytosis and 49.4 ± 6.1 , phagocytosis index; Fig. 4.7A and B) compared with normoxia (24.8 ± 1.3 , % phagocytosis and 56.2 ± 5.3 , phagocytosis index).

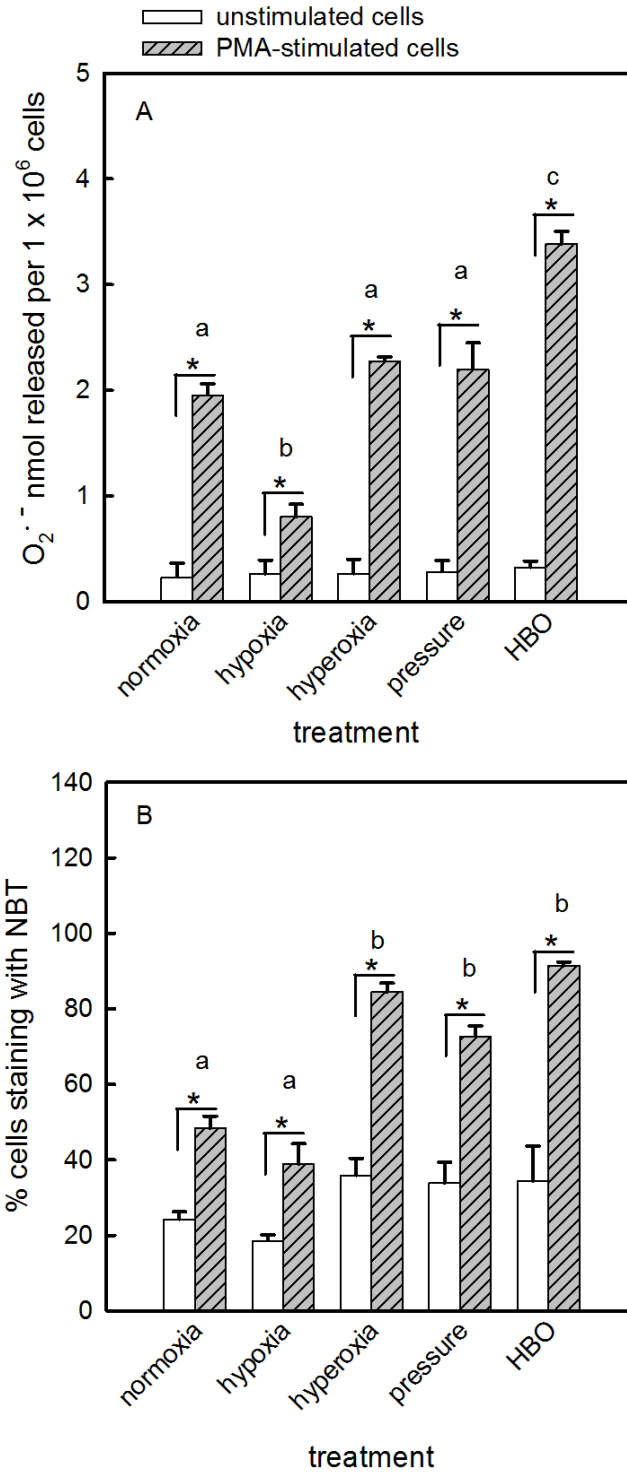


Fig. 4.5: $O_2^{\cdot -}$ release and % NBT reduction by neutrophil-like cells exposed to normoxia, hypoxia, hyperoxia, pressure or HBO. ATRA-differentiated cells were exposed to a range of conditions for 90 min (section 2.1.1) after which superoxide production was detected either using cytochrome *c* (A) (as described in section 4.2.2) or NBT (B) (as described in section 2.1.2) in the presence or absence of PMA. Data are means \pm SEM for three separate experiments (passage numbers 20, 24 and 28; $n = 3$) with triplicate measurements. * indicate significant differences from unstimulated cells (Mann-Whitney, $P < 0.05$), bars with different lower case letters indicate significant differences between different treatments for stimulated cells (Kruskal-Wallis, $P < 0.05$).

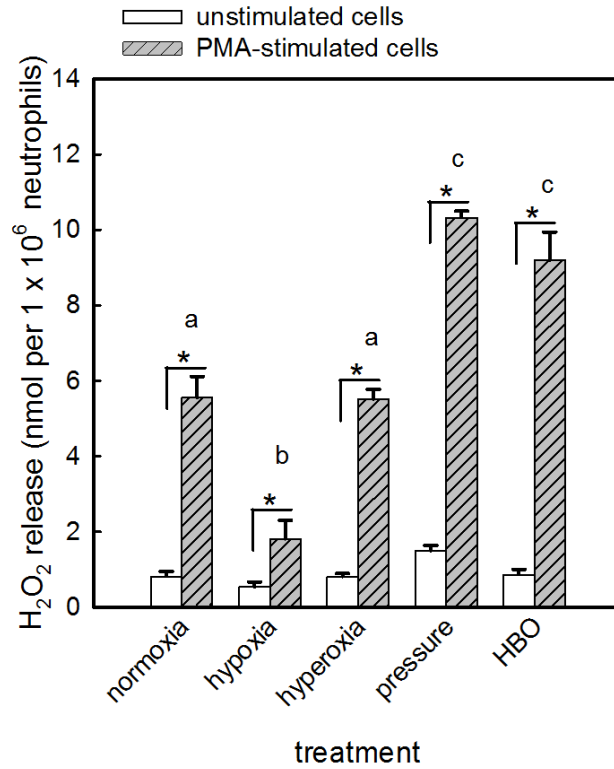


Fig 4.6 H_2O_2 production by neutrophil-like cells following exposure to normoxia, hypoxia, hyperoxia, pressure or HBO. ATRA-differentiated HL-60 cells were exposed to a range of conditions for 90 min (section 2.1.1). After exposure, cells were washed, stimulated with PMA and H_2O_2 levels in the medium were determined in both stimulated and unstimulated cells using HRP/HVA (section 2.1.3). Data are expressed as the amount of H_2O_2 produced per 10^6 cells. Data are means \pm SEM for three separate experiments (passage numbers 30, 32 and 34; $n = 3$) with triplicate measurements. * indicate significant differences from unstimulated cells (Mann-Whitney, $P < 0.05$), bars with different lower case letters indicate significant differences between different treatments for stimulated cells (Kruskal-Wallis, $P < 0.05$).

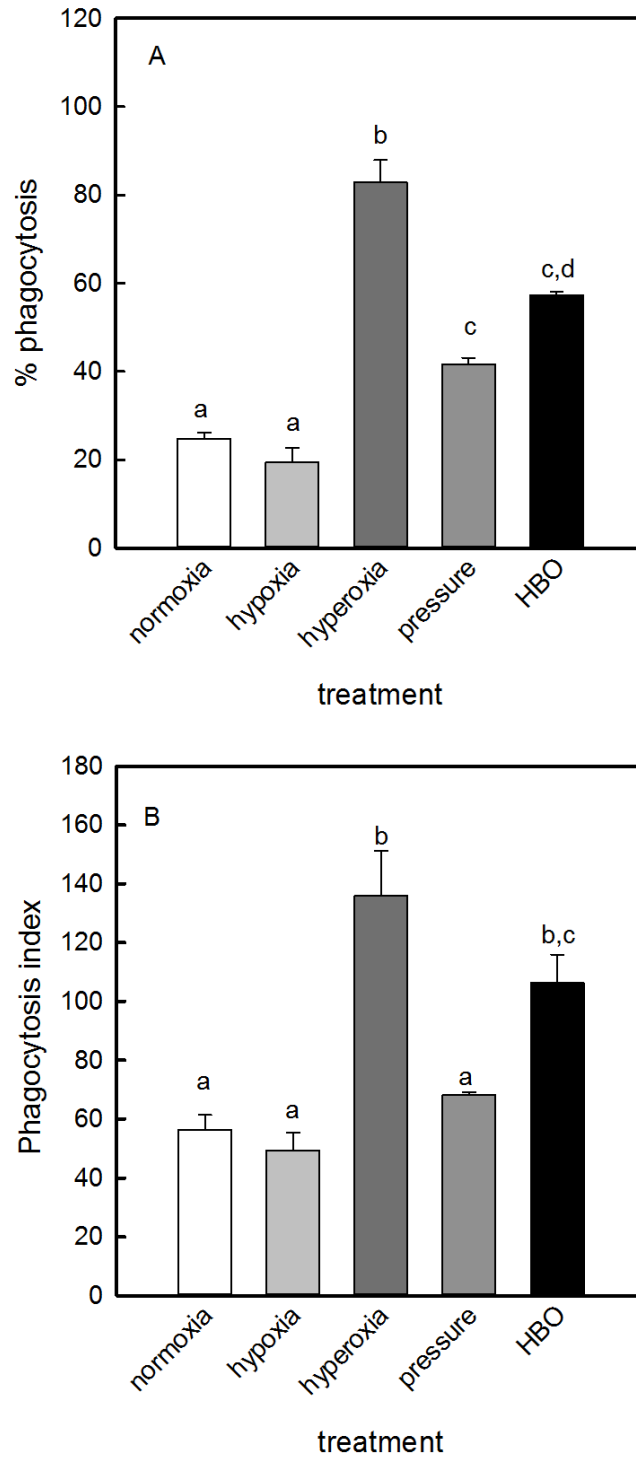


Fig 4.7 Phagocytosis of *Staphylococcus aureus* NCIMB 6571 by neutrophil-like cells following exposure to normoxia, hypoxia, hyperoxia, pressure or HBO. ATRA-differentiated cells were exposed to a range of conditions for 90 min (section 2.1.1), after which they were incubated for 30 min with *Staphylococcus aureus* NCIMB 6571 and % phagocytosis (A) and phagocytic index (B) were assessed (section 2.1.4). Data are means \pm SEM for three separate experiments (passage numbers 39, 42 and 45; $n = 3$) with triplicate measurements. Bars with different lower case letters indicate significant differences between different treatments (one-way ANOVA, $P < 0.05$).

4.3.2 Effect of HBO on NO_x production from differentiated HL-60 cells

Nitric oxide (NO) production in neutrophil-like cells was evaluated by measuring the concentrations of its direct oxidation products, nitrite and nitrate (NO₂⁻ + NO₃⁻), in the media. Pre-treatment with 100 ng ml⁻¹ PMA caused a significant increase in NO₂⁻, NO₃⁻ and NO_x levels in neutrophil-like cells compared to unstimulated cells (pair-wise Mann-Whitney, *P* < 0.05; Figs 4.8A, B and C). No treatment effects were found for unstimulated cells (Kruskal-Wallis, *P* = 0.65; Fig. 4.8A). Post-HBO, pressure and hyperoxia treatments, NO₂⁻ levels increased significantly in PMA-stimulated cells (Kruskal-Wallis, *P* = 0.02) compared to normoxia (Fig. 4.8A). No-treatment effects were found on NO₂⁻ level after exposure to hypoxia (0.054 ± 0.007 nmol per 10⁶ cells) compared to normoxia (0.052 ± 0.005 nmol per 10⁶ cells).

Interestingly, the supernatant from PMA-stimulated cells contained much more NO₃⁻ than NO₂⁻ levels as shown in Fig. 4.8B. No change was found on NO₃⁻ levels (Kruskal-Wallis, *P* = 0.11) in unstimulated cells after different treatments (0.34 ± 0.02, 0.33 ± 0.02, 0.40 ± 0.03 and 0.32 ± 0.01 nmol per 10⁶ cells for HBO, pressure, hyperoxia and hypoxia, respectively; Fig. 4.8B) compared to normoxia (0.34 ± 0.02 nmol per 10⁶ cells). There was a statistically significant effect on NO₃⁻ levels from PMA-stimulated cells after HBO and hypoxia exposure (Kruskal-Wallis, *P* = 0.02) compared to normoxia (Fig. 4.8B). There was no effect following treatment with pressure and hyperoxia (0.59 ± 0.05 and 0.73 ± 0.39 nmol per 10⁶ cells) compared to normoxia (0.62 ± 0.04; Fig. 4.8B).

No change was found on NO_x (NO₂⁻ + NO₃⁻) levels in unstimulated cells after different treatments (Kruskal-Wallis, *P* = 0.13; 0.34 ± 0.01, 0.33 ± 0.01, 0.40 ± 0.02, 0.341 ± 0.004 nmol per 10⁶ cells for HBO, pressure, hyperoxia and hypoxia, respectively; Fig. 4.8C). NO_x levels showed a significant increase in PMA-stimulated cells after HBO and

hyperoxia exposure compared to normoxia (Kruskal-Wallis, $P = 0.03$). No significant effects were found on NO_x levels in PMA-stimulated cells after exposure to pressure alone and hypoxia (0.68 ± 0.05 and 0.53 ± 0.02 nmol per 10^6 cells, respectively; Fig. 4.8C) compared to normoxia (0.62 ± 0.03 nmol per 10^6 cells).

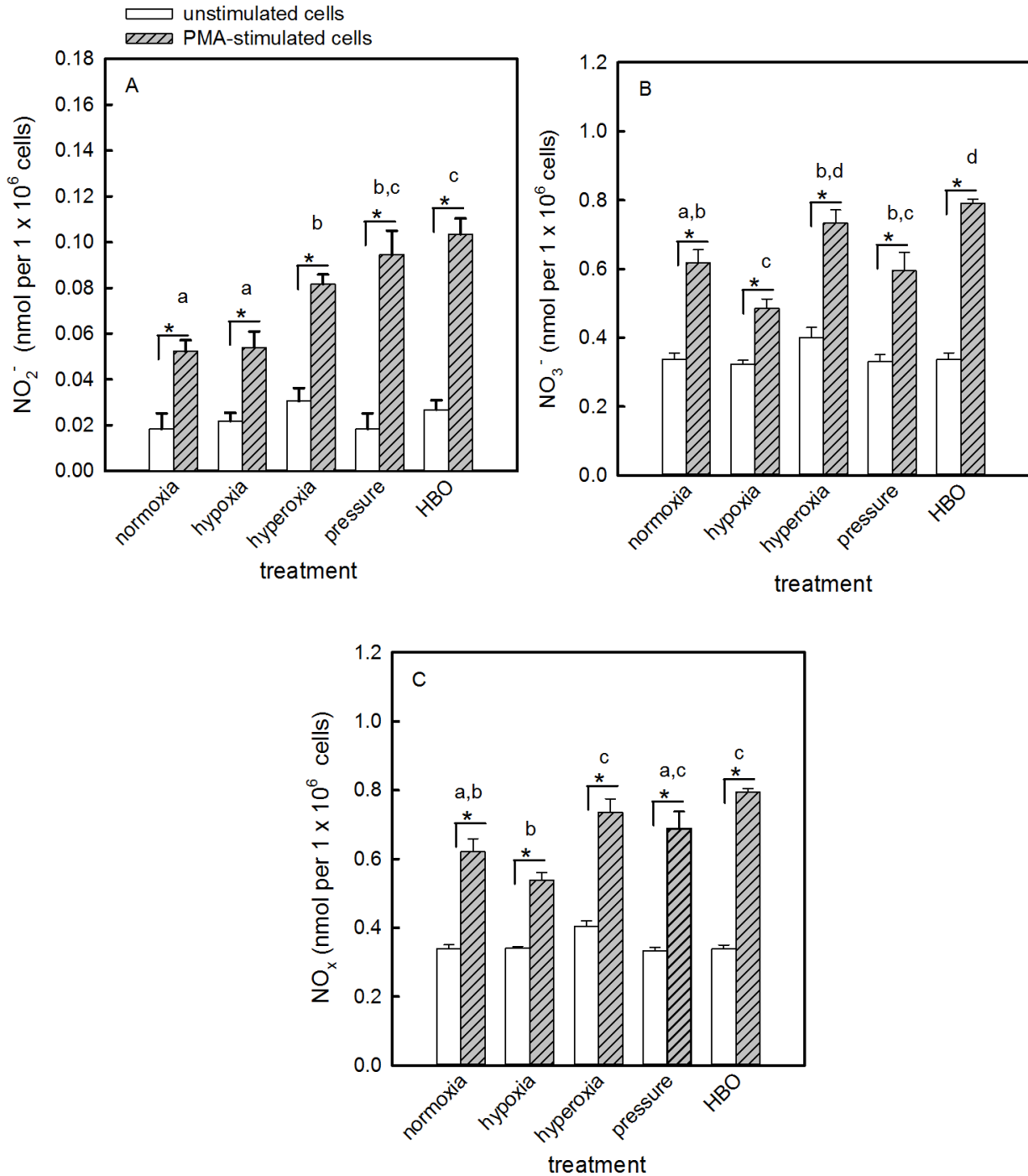


Fig. 4.8 NO₂⁻, NO₃⁻ and NO_x production by neutrophil-like cells following exposure to normoxia, hypoxia, hyperoxia, pressure or HBO. ATRA-differentiated HL-60 cells were exposed to a range of conditions for 90 min (section 2.1.1). After exposure, cells were washed, stimulated with PMA and NO_x levels in the medium were determined by measurement of NO₂⁻ and NO₃⁻ (section 4.2.3). Data are expressed as the amount of NO_x produced in nmol per 10⁶ cells. Data are means ± SEM for three separate experiments (passage numbers 36, 38 and 40; *n* = 3) with triplicate measurements. * indicate significant differences from unstimulated cells (Mann-Whitney, *P* < 0.05), bars with different lower case letters indicate significant differences between different treatments for stimulated cells (Kruskal-Wallis, *P* < 0.05).

4.3.3 Effects of HBO on degranulation of differentiated HL-60 cells

Degranulation was assessed by measuring myeloperoxidase (MPO) release. Stimulation with PMA produced significantly higher MPO activity in neutrophil-like cells compared to unstimulated cells (pair-wise Mann-Whitney, $P < 0.05$; Fig. 4.9). Pre-treatment with HBO significantly increased MPO activity in unstimulated cells (Kruskal-Wallis, $P = 0.005$), while pre-treatment with pressure, hyperoxia and hypoxia had no effect (0.23 ± 0.07 , 0.16 ± 0.02 and 0.08 ± 0.03 , respectively). There were significant increases in PMA-induced MPO release following HBO, pressure alone and hyperoxia (Kruskal-Wallis, $P = 0.015$) with the greatest increases observed following pressure and HBO. Exposure to hypoxia had no significant effect on degranulation of PMA-stimulated neutrophil-like cells (0.48 ± 0.09 nmol per 10^6 cells, respectively; Fig. 4.9) compared to normoxia (0.9 ± 0.18 nmol per 10^6 cells for stimulated cells).

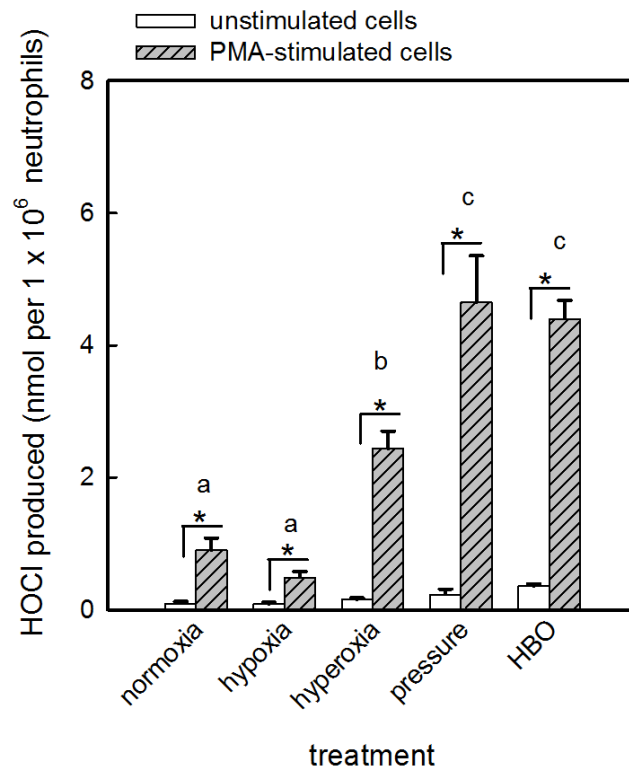


Fig. 4.9 MPO activity from neutrophil-like cells following exposure to normoxia, hypoxia, hyperoxia, pressure or HBO. ATRA-differentiated cells were exposed to a range of conditions for 90 min (section 2.1.1). After exposure cells were washed, stimulated with PMA and MPO activity in the supernatant was measured (section 4.2.4). Data are expressed as the amount of MPO produced per 10^6 cells. Data are means \pm SEM for three separate experiments (passage numbers 19, 22 and 25; $n = 3$) with triplicate measurements. * indicate significant differences from unstimulated cells (Mann-Whitney, $P < 0.05$), bars with different lower case letters indicate significant differences between different treatments for stimulated cells (Kruskal-Wallis, $P < 0.05$).

4.3.4 Effect of HBO on redox status

A significant interaction between treatment and stimulation was found on total GSH levels in neutrophil-like cells (two-way ANOVA, $P = 0.0001$). In general stimulation with PMA caused a decrease in total glutathione (two-way ANOVA, $P = 0.032$), except for the case of pre-exposure to hypoxia, which explains the interaction. Because of the interaction unstimulated and stimulated data were further analysed separately using one-way ANOVA. Exposure to HBO and pressure alone did not affect the glutathione level in both PMA-stimulated cells (0.42 ± 0.08 and 0.39 ± 0.07 , respectively) and

unstimulated cells (0.82 ± 0.09 and 0.81 ± 0.08 , respectively). A significant effect on total glutathione levels was found after treatment with hypoxia and hyperoxia in unstimulated cells (one-way ANOVA, $P = 0.006$) compared with normoxia (0.89 ± 0.12) and after hyperoxia in stimulated cells (one-way ANOVA, $P = 0.01$) compared with normoxia (0.49 ± 0.05 ; Fig. 4.10.).

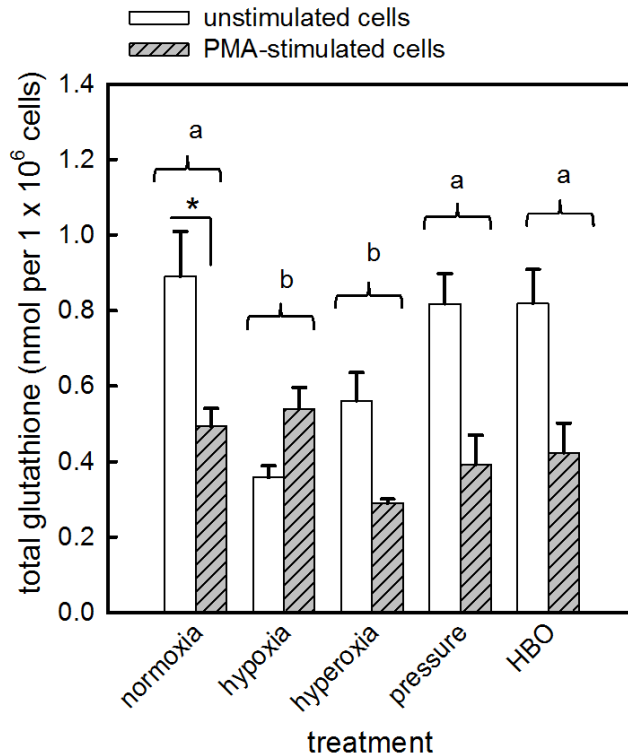


Fig. 4.10 Total glutathione production by neutrophil-like cells following exposure to normoxia, hypoxia, hyperoxia, pressure, or HBO. ATRA-differentiated cells were exposed to a range of conditions for 90 min (section 2.1.1). After exposure, cells were washed, stimulated with PMA and total glutathione was measured in the cell lysate in absence or presence of PMA (section 4.2.5). Data are expressed as the amount of GSH produced in nmol per 10^6 cells. Data are means \pm SEM for three separate experiments (passage numbers 19, 22 and 25; $n = 3$) with triplicate measurements. Bars with different lower case letters indicate significant differences between different treatments for unstimulated and stimulated, respectively (two-way ANOVA, $P < 0.05$).

4.4 Discussion

Pre-treatment with HBO enhanced the respiratory burst activity of neutrophil-like cells as shown by increased $O_2^{\cdot-}$ and H_2O_2 production compared to normoxia-treated cells. These results are consistent with the work of Labrousche *et al* (1999) who found that circulating neutrophils isolated from volunteer divers who had intermittently inhaled 100% O_2 for 135 min at 1.8 ATA showed increased bacteria-induced phagocytosis and respiratory burst activity. Similar results were obtained in a study on neutrophils isolated from patients receiving HBO therapy for treatment of diabetic foot infections (Top *et al.*, 2007). However, other studies are not consistent with these findings. For example, Jüttner *et al.* (2003) found no effect on respiratory burst or phagocytic activity of neutrophils isolated from healthy volunteers given repetitive HBO exposures. Kaln *et al.* (2002) found HBO treatment decreased opsonized zymosan-stimulated respiratory burst and had no effect on PMA-induced respiratory burst in neutrophils from healthy donors pre-exposed to HBO.

Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor that has been widely implicated in the regulation of the antimicrobial activity of granulocytes, including neutrophils (Cramer *et al.*, 2003, Peyssonnaud *et al.*, 2005, Walmsley *et al.*, 2005). Steady state levels of the HIF-1 α subunit are regulated in an oxygen-dependent manner, but there is debate over the precise mechanism(s) by which oxygen affects levels of this protein. An obvious possibility is the oxygen-dependent hydroxylation of HIF-1 α , which is the starting point for the sequence of events that lead to it being targeted for degradation by proteasomes (Epstein *et al.*, 2001). It has been suggested that oxygen consumption by mitochondria may modulate intracellular oxygen levels and hence influence the hydroxylation of HIF-1 α (Hagen *et al.*, 2003, Doege *et al.*, 2005). HL-60 cells have fully functional mitochondria, but after differentiation to neutrophil-like cells they show the morphological (shift from punctate to tubular

structure) and functional (decreased expression of cytochrome *c*) changes to their mitochondria that are characteristic of neutrophils (Maianski *et al.*, 2004b). Hence it seems unlikely that mitochondria in differentiated HL-60 cells would significantly affect intracellular oxygen levels.

An alternative view of the role of mitochondria in HIF-1 α signalling is that this is based on their production of ROS (Schroedl *et al.*, 2002). This seems to be particularly pertinent in neutrophils where mitochondria, as a result of localized deficiencies in one or more of the electron transfer complexes, may be potent ROS producers (Fossati *et al.*, 2003). These two models for HIF-1 α give rise to opposite expectations in neutrophil-like cells under normobaric or hyperbaric hyperoxia; under these conditions any limited ability of the mitochondria to maintain an intracellular oxygen gradient would be expected to be overwhelmed (hence no stabilisation of HIF-1 α), whereas mitochondrial ROS production would be expected to increase (hence stabilisation of HIF-1 α). The present data on the respiratory burst activity of neutrophil-like cells are consistent with the stabilisation of HIF-1 α via elevated mitochondrial ROS production under hyperoxic conditions. However, neither HIF-1 α activity nor protein levels were measured in this study. Further investigation including measurement of HIF-1 α , as well as another subunit, HIF-1 β , following treatments could provide information as to whether the expression of HIF-1 is active, and whether it is also affected by HBO.

Consistent with this, exposure to hypoxia significantly decreased O₂^{•-} and H₂O₂ levels with impaired phagocytosis of bacteria in PMA-stimulated neutrophil-like cells. Neutrophils are capable of respiratory burst that depends on the activity of NADPH oxidase that uses oxygen to form O₂^{•-}. Mitochondria have been demonstrated as possible oxygen sensors (Tan and Ratcliffe, 1991). A recent study has implicated mitochondria in the O₂ sensing that underlies the functional responses to hypoxia (Chandel *et al.*, 1996). Mitochondria generate ROS at ubisemiquinone sites (Turrens *et al.*, 1985), and a

change in mitochondrial redox status during hypoxia could alter ROS production, which is known to participate in the activation of transcriptional responses for example the HIF-1 activation pathway (Schreck *et al.*, 1991). Respiratory burst activity of leukocytes either stimulated with PMA or unstimulated was decreased after acute hypoxia, but was not affected by 24 h of hypoxia (Faoro *et al.*, 2011). This explanation is supported by the inhibition of MPO in PMA-stimulated cells post hypoxia. It has been demonstrated that ROS are required for full protease function within the phagosome (Reeves *et al.*, 2002). Therefore, the protective bactericidal effects may be impaired under these conditions. Our finding of impaired antimicrobial activity in neutrophil-like cells after hypoxia treatment is consistent with previous studies (Jonsson *et al.*, 1988, Ahn and Mustoe, 1990, Kivisaari and Niinikoski, 1975, Knighton *et al.*, 1986).

However, previous studies are not in agreement about the effects of HBO on neutrophil respiratory burst activity. Research on HBO has been shown to reduce, increase, or have no effect on ROS production in neutrophils (Jüttner *et al.*, 2003, Kaln *et al.*, 2002, Labrousche *et al.*, 1999, Thom *et al.*, 2008). The present study supports the argument for enhanced respiratory burst activity following HBO, which has been presented by both Labrousche *et al* (1999) and Thom *et al* (2008) as described previously.

Pre-treatment with HBO enhanced the phagocytic activity of neutrophil-like cells. This was supported by an HBO-induced increase in myeloperoxidase (MPO) activity, i.e. evidence of degranulation (exocytosis). Phagocytosis requires a controlled rearrangement of the actin cytoskeleton (May and Machesky, 2001). The same is also true of exocytosis. For example, Mitchell *et al.* (2008) found that depolymerisation of cortical F actin is essential for exocytosis of primary granules, which contain MPO. It has been shown that HBO inhibits adhesion of circulating neutrophils via inhibition of B₂ integrins (Thom, 1993, Thom *et al.*, 2008) and evidence has been presented that this

is mediated via nitrosylation of actin, which is ultimately dependent on an HBO-induced increase in NO production by stimulating isoforms of NOS (Thom *et al.*, 2008). iNOS and MPO co-localize within neutrophil primary granules and are responsible for excessive S-nitrosylation of β -actin, when neutrophils are subjected to hyperoxia (Thom *et al.*, 2008). The nitrosylation of actin was shown to influence the polymerisation of actin. Hence, this could be the basis of an explanation for the enhancement of phagocytic activity following HBO pre-treatment seen in this study.

The present study also demonstrated that HBO effects are not only due to elevated oxygen concentration, but that there is also potential for HBO-induced effects to be mediated by pressure alone (Grim *et al.*, 1990). There was no consistent pattern of the effect of pressure alone on the antimicrobial activity of neutrophil-like cells. On one hand, the increase in H₂O₂ production following HBO pre-treatment could be due to pressure alone, since hyperoxia under normobaric conditions had no significant effect. On the other hand, it appears that pressure might have an inhibitory effect on phagocytosis since a greater increase in phagocytic activity was seen after treatment with normobaric hyperoxia compared to HBO, and pressure alone had significant effect on % phagocytosis but not on phagocytosis index. Two possible general mechanisms by which pressure alone could influence the activity of neutrophils are (a) via deformation during the pressure treatment and (b) via the formation of microbubbles during the decompression, both of which could lead to the activation of mechanoreceptors (Makino *et al.*, 2006).

Inflammatory factors in chronic wound conditions such as hypoxia and high levels of TNF- α or LPS cause an increase in neutrophil degranulation (Lee *et al.*, 1993). However, in the present study, treatment with hypoxia failed to enhance respiratory burst activity and increase degranulation as was expected. A possible explanation for this is that the hypoxic conditions used in the present study (5% O₂ for 90 min) were at

a higher concentration than hypoxic conditions in chronic wounds (2% O₂). Additionally, other chronic wound conditions, such as TNF- α or LPS, were not applied in the present study and this may have resulted in insufficient production in neutrophil degranulation. In this study the changes in NO_x, which are the products of oxidation of NO, were investigated. The results provide additional evidence of increased NO production in PMA-stimulated cells in response to HBO treatments, suggesting that HBO-treated cells appeared to show higher levels of NOS activity. Inducible NOS (iNOS)-derived NO in neutrophils has been proposed to be key mediator of some effects of HBO on neutrophil function. HBO-induced increases in eNOS activity levels are thought to result from accelerated cycling of ferrous haem in the active site of the enzyme to ferric haem via oxidation due to increased availability of oxygen, which increases the activity of the NOS ten-fold (Thom *et al.*, 2003). Another possible mechanism of increased NOS activity following HBO treatment includes increased phosphorylation associated with increased heat shock protein (HSP-90; Cabigas *et al.*, 2006, Buras *et al.*, 2000). However, in this study iNOS enzyme activity and expression were not investigated; further investigations of this kind would be helpful in order to determine whether and how the enzyme activity and expression are affected by HBO. Exposure to pressure alone showed a significant effect in NO₂⁻ levels but not in NO₃⁻ levels in PMA-stimulated neutrophil-like cells. Thom *et al.* (2003) observed no changes in perivascular NO production in the aorta following treatment with a pressure control (7.46% O₂ at 2.8 ATA for 45 min). The possible explanation is that NO produced under pressure treatment was oxidized to NO₂⁻ but not further to NO₃⁻. NO could react with other ROS, for example O₂^{•-}, to form ONOO⁻. This in turn could react with tyrosine, forming 3-nitrotyrosine (nitration), or thiols, forming S-nitrosothiols (S-nitrosylation; Halliwell and Gutteridge, 2007, Gaston *et al.*, 2003). These reactions would decrease the likelihood of NO undergoing oxidation to NO₂⁻ and NO₃⁻.

GSH is oxidised to GSSG which is then reduced back to GSH in the cell to maintain the intracellular redox state. Glutathione redox cycle balance is important for cell integrity and function. Exposure to high oxidative stress results in cell damage and/or cell death and the subsequent release of GSH and other intracellular contents that are associated with harmful oxidative stress (Aoki *et al.*, 1996). In the present results no change was observed in total glutathione levels in unstimulated and PMA-stimulated cells after HBO and pressure compared to the control. Studies to date on the effects of HBO on total glutathione levels indicate that HBO increases total glutathione in both rats and mice (Yuan *et al.*, 2009, Kudchodkar *et al.*, 2007, Wang *et al.*, 2012). This might be explained as ROS and lipid peroxidation mediating the induction of anti-oxidant production as has been demonstrated for glutathione, GPx and catalase (Ishii *et al.*, 2002). In another study, HBO treatment was found to decrease total glutathione in free skin grafts in rats (Lemarie *et al.*, 1998). In the present study, exposure to hyperoxia led to a decrease in total glutathione compared to normoxia in both unstimulated and PMA-stimulated cells. A possible explanation of this might be that post hyperoxia there is an increase in production of ROS such as H₂O₂, which produces oxidative stress. The ROS may oxidize GSH through an enzymatic mechanism involving glutathione peroxidase, leading to the rapid transport of cytosolic GSSG out of the cell as a response to oxidative stress. Since mitochondria are a major source of ROS due to their consumption of molecular oxygen, redox control mechanisms are more limited in mitochondria than cytosol. Mitochondria are unable to export GSSG and must depend on NADPH reducing equivalents and glutathione reductase enzyme activity to resynthesize GSH (Olafsdottir and Reed, 1988). A decreased capacity to control endogenous and exogenous ROS causes mitochondria to be more susceptible to oxidative stress/dysfunction which can increase ROS generation; this could explain a decrease in total glutathione in PMA-stimulated cells. However, the present results

indicate that total glutathione is decreased by hypoxia in unstimulated cells. The present findings confirm a previous report showing hypoxic inhibition of glutathione synthesis in human fibroblasts (Bannai *et al.*, 1989) and identify mitochondrial ROS as the intracellular signal mediating this effect, although this may depend on cell type and local environment. Overall, this study did not show any significant decrease in total glutathione following a 90-min exposure to HBO or pressure alone; this was inconsistent with the H₂O₂ results. However, therapeutic application of HBO *in vivo* never exceeds 3 ATA or 90 min, which could keep the ROS generation under control and allow the antioxidant system to work efficiently to control oxidative stress.

4.4.1 Conclusions

Exposure of HL-60 cells differentiated with ATRA (neutrophil-like cells) to HBO enhanced the antimicrobial activity of the cells. Both the hyperoxia and pressure components of HBO contributed to the effects on neutrophil-like cells. These observations support the hypothesis that this could be responsible for some of the reduction in the infection observed in chronic wounds over the course of HBO treatment, the effects of which are only partially understood. The data are consistent with the possibility that HBO-mediated ROS may result in cytoskeletal changes and inhibition of B₂ integrin, which in turn are responsible for the therapeutic action of HBO. In terms of inflammation, this could be detrimental to wound healing because it enhances a pro-inflammatory environment. However, neutrophil apoptosis, regulated in part by ROS, is a key process both in resolving inflammation during wound healing and in the prevention of the release of intracellular contents that may cause tissue damage.

CHAPTER FIVE

THE EFFECTS OF HBO ON

NEUTROPHIL VIABILITY AND

APOPTOSIS

Abstract

Chronic wounds are characterised by extensive neutrophil infiltration and their prolonged survival. Neutrophil apoptosis is essential for the successful resolution of inflammation. Delayed apoptosis and/or clearance of neutrophils prolong tissue injury. Recent evidence has shown that exposure to HBO (elevated oxygen fraction administrated at > 1 ATA) enhances apoptosis of various cell lines. Therefore to analyse the mechanism by which HBO induces neutrophil apoptosis, neutrophil-like cells from differentiated HL-60 cells by ATRA were exposed to normoxia (21% O₂ plus 5% CO₂), normobaric hyperoxia (95% O₂ and 5% CO₂), hyperbaric normoxia (8.8% O₂, 2.1% CO₂, balance N₂ at 2.4 ATA) and hyperbaric oxygen (97.9% O₂, 2.1% CO₂ at 2.4 ATA). Several assays such as quantification of caspase 3/7 activity and a morphological study were used to identify apoptosis. A single 90-min exposure to HBO was shown to have a pro-apoptotic effect as demonstrated by decreased cell viability (one-way ANOVA, $P = 0.0004$), increased caspase 3/7 activity in both untreated and TNF- α -treated cells (Kruskal-Wallis, $P < 0.05$) and apoptosis-associated morphological changes using Giemsa (one-way ANOVA, $P = 0.0006$) or SYBR[®] Safe staining of cells (Kruskal-Wallis, $P = 0.03$). Additionally, HBO treatment demonstrated suppression of LPS-induced TNF- α and IL-1 β production (two-way ANOVA, $P < 0.05$) while IL-10 was increased (two-way ANOVA, $P = 0.002$) suggesting that HBO-induced apoptosis is associated with a suppressive inflammatory response. Both pressure and hyperoxia seemed to contribute to increased apoptosis induced by HBO. However, hypoxia treatment had either the opposite effect to HBO or a non-significant effect. In conclusion, a single exposure to HBO enhanced spontaneous and stimulus-induced apoptosis in neutrophil-like cells and this seems to be associated with an alteration in inflammatory mediators such as cytokines.

5.1 Introduction

Tissue injury and inflammation result in a decrease in oxygen tension at the wound site (Niinikoski *et al.*, 1972, Hunt *et al.*, 2004). Hypoxia has been shown to induce apoptosis in different cell types such as endothelial cells both *in vitro* (Matsushita *et al.*, 2000) and *in vivo* (Stempien-Otero *et al.*, 1999, Lee *et al.*, 2005), and in fibroblasts (Saed and Diamond, 2002) as well as in macrophages *in vitro* (Yun *et al.*, 1997). However, neutrophil apoptosis has been shown to be impaired by hypoxia (Hannah *et al.*, 1995, Walmsley *et al.*, 2005, Leuenroth *et al.*, 2000). It is thought that in low oxygen areas such as in arthritic joints (Cross *et al.*, 2006), neutrophils can function and survive for an extended length of time. Hypoxic inhibition of neutrophil apoptosis is regulated directly by the HIF-1 α hydroxylase oxygen-sensing pathway and NF- κ B expression (Walmsley *et al.*, 2005), and indirectly by the release of anti-apoptotic factors such as Mac-1 and MAPK (Leuenroth *et al.*, 2000).

In chronic wounds, apoptosis of cells involved in the wound healing process is also affected by inflammatory cytokines and growth factors. TNF- α is the predominant inflammatory cytokine in chronic wound fluids, and seems to mediate apoptotic pathways in a number of cell types, including fibroblasts (Alikhani *et al.*, 2004). However, in neutrophils, the effect of TNF- α on apoptosis is dose-dependent, at low concentrations ($\leq 1 \text{ ng ml}^{-1}$) impairing apoptosis via activation of survival signals by NF- κ B, (Van Antwerp *et al.*, 1998) whereas inducing apoptosis at high concentrations ($\geq 10 \text{ ng ml}^{-1}$). In chronic wound fluids, the TNF- α concentration is around 1 ng ml^{-1} , so neutrophil apoptosis is impaired, and inflammation prolonged. Other cytokines such as granulocyte-macrophage-colony stimulating factor (GM-CSF), IFN- γ and IL-3 have also been shown to protect neutrophils from apoptosis through reduction in the levels of

pro-apoptotic Bax protein (Dibbert *et al.*, 1999). IL-8 has also been shown to have anti-apoptotic effect on neutrophils (Hirata *et al.*, 2008).

Bacterial infection in chronic wounds can induce apoptosis in different cell types involved in the healing process such as fibroblasts, natural killer cells, macrophages and epithelial cells (Zhang *et al.*, 2008, Pan *et al.*, 2004, Chung *et al.*, 2009). LPS, a major component of the cell membrane of Gram-negative bacteria, induces apoptosis in endothelial cells (Zeng *et al.*, 2005, Koide *et al.*, 2007) and in macrophages (Munshi *et al.*, 2002) via the extrinsic pathway. In contrast, bacterial infection by *Escherichia coli*, *Staphylococcus aureus* or *Pseudomonas aeruginosa* has been shown to enhance neutrophil survival (Lee *et al.*, 1993, Nishimaki *et al.*, 2007), in which LPS, and the chemotactic peptide, C5a, stimulate GM-CSF, which in turn exerts inhibitory effects on neutrophil apoptosis (Lee *et al.*, 1993).

Few studies have investigated the effects of HBO on apoptosis. A single HBO treatment (100%, 3 ATA) for 1 h reduced apoptosis in brain tissue following ischaemic/reperfusion (I/R) injury (Calvert *et al.*, 2003) via a reduction in caspase-3 activation. Also, treatment with HBO reduced cell apoptosis in ischaemic wounds, by reducing the expression of pro-apoptotic proteins such as p53, and increasing Bcl-2 expression (Zhang *et al.*, 2008). However, in this study tissue homogenates were used and the effects of HBO on individual cell types were not indicated, although overall results demonstrated a reduction in apoptosis.

In leukocytes, HBO treatment causes DNA damage immediately after exposure, which is quickly repaired (Speit *et al.*, 1998), while in Jurkat cells and a T-lymphocyte cell line, HBO has been shown to induce apoptosis via loss of mitochondrial membrane potential and increased caspase-9 activation (Weber *et al.*, 2009a). Increased pressure showed the same effects in these cells (Chen *et al.*, 2007b). Gunguly *et al.* (2002), in a further study using Jurkat cells, demonstrated that Fas-induced apoptosis was enhanced

by HBO (97.9% O₂ and 2.2% CO₂ at 2.4 ATA). In the same study, apoptosis of granulocytic HL-60 cells also increased following 3 h of HBO treatment, but there was no effect after 90 min, as applied in patient protocols. HBO-induced apoptosis was reduced upon incubation with the antioxidant ascorbate, suggesting that oxidative stress is involved in the apoptotic effects of HBO. Furthermore, HBO also increased apoptosis in NCI-H929, an immortal B-cell line, but also under conditions not representative of patient treatment (Chen *et al.*, 2007b). The exposure to HBO at 2.5 ATA for 120 min increased apoptosis of cultured fibroblasts, while short term exposure to HBO, 30-60 min at 2.5 ATA enhanced cell growth (Conconi *et al.*, 2003).

HBO may enhance wound healing by accelerating the death of inflammatory cells that are found in wounds. Thus, the aim of this study was to investigate the effects of HBO on the apoptosis of neutrophil-like cells obtained by differentiation of HL-60 cells. The results presented in Chapter 4 demonstrated that oxidative stress caused by HBO exposure in neutrophil-like cells induces ROS production. ROS production is associated with neutrophil apoptosis, so the hypothesis in the present study is that HBO may exert a beneficial effect by inducing apoptosis in neutrophils and limiting release of their injurious contents into surrounding tissue.

5.2 Material and methods

5.2.1 Experimental design

Neutrophil-like cells, obtained by differentiation of HL-60 cells using ATRA (1 μM for 5 days), were used as an *in vitro* model for neutrophils (section 3.2.2). Neutrophil-like cells were exposed to different treatments: normoxia (21% O₂ plus 5% CO₂), hypoxia (5% O₂ plus 5% CO₂, balance N₂), normobaric hyperoxia (95% O₂ and 5% CO₂), hyperbaric normoxia (8.8% O₂, 2.1% CO₂, balance N₂ at 2.4 ATA) and hyperbaric

oxygen (97.9% O₂, 2.1% CO₂, balance N₂ at 2.4 ATA) for 90 min at 20 °C (Fig. 5.1). After treatment, washed neutrophil-like cells were incubated at 37 °C for 3 h in the presence or absence of 10 ng ml⁻¹ TNF-α (final concentration; Insight Biotechnology, Wembley, UK) to induce cell apoptosis (90 μl of 10⁶ cells ml⁻¹ in PBS plus either 10 μl of 100 ng ml⁻¹ TNF-α or 10 μl of PBS). Additionally, to test whether the accumulation of H₂O₂ was involved in HBO-induced apoptosis, catalase (200 U ml⁻¹ final concentration; Sigma, Poole, UK) was added to neutrophil-like cells and incubated at 37 °C for 3 h to remove extracellular H₂O₂ (90 μl of 10⁶ cells ml⁻¹ in PBS plus 10 μl of 2000 U ml⁻¹ catalase). Caspase inhibitor (10 μM final concentration zVAD-FMK; Promega, Southampton, UK) was added to neutrophil-like cells (90 μl of 10⁶ cells ml⁻¹ in PBS plus 1 μl of 1 mM zVAD and 9 μl of PBS) as a negative control. The measured end points were cell viability (trypan blue and MTT; see sections 2.1.5 and 2.1.6); cell apoptosis (caspase-3/7 activity; see section 3.2.5); apoptosis-associated morphological changes using Wright-Giemsa and SYBR[®] Safe staining (as detailed below); and quantification of anti-apoptotic NF-κB and Bcl-2 gene expression via qPCR. For qPCR experiments, cells (1 × 10⁶ cells ml⁻¹) were washed with PBS, treated with TNF-α (10 ng ml⁻¹) and incubated for 3 h at 37 °C at 5% CO₂ (balance air). RNA was extracted and expression of both NF-κB and Bcl-2 was analysed using qPCR (section 2.1.7.5). NF-κB protein was determined by Western blotting (as detailed below). Inflammatory response measurement was also investigated by using the Luminex technique (i.e. measurement of pro- and anti-inflammatory cytokines; section 2.1.8).

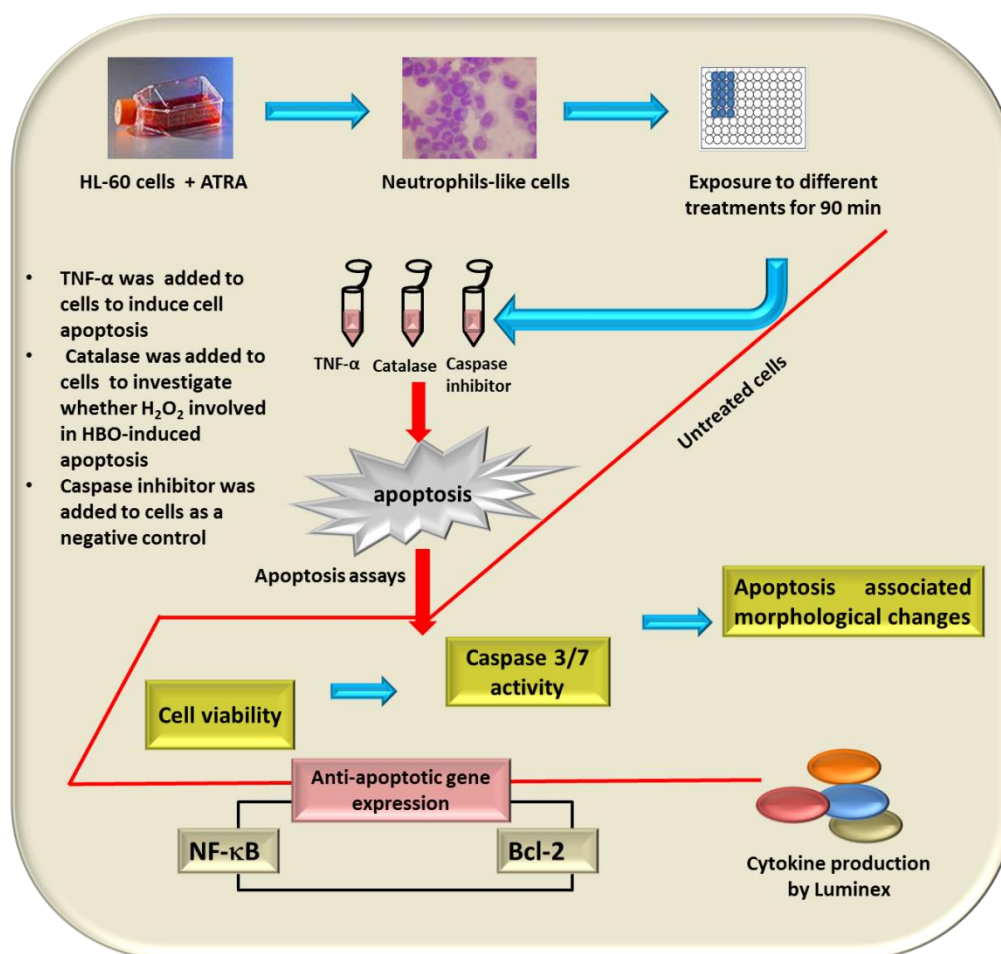


Fig. 5.1 Experimental design. Neutrophil-like cells obtained from ATRA-differentiated HL-60 cells were exposed to different oxygen treatments, after which cells were washed and incubated with 10 ng ml^{-1} TNF- α for 3 h at $37 \text{ }^\circ\text{C}$ to induce apoptosis. Different assays were conducted to determine cell viability (trypan blue and MTT), cell apoptosis (caspase 3/7 activity and apoptosis associated morphological changes). Pro- and anti-inflammatory cytokines as well as the expression of NF- κ B and Bcl-2 were also measured.

5.2.2 Morphological assessment of cell apoptosis

Following exposure to the five different treatments cells were washed in PBS. Cyto centrifuge preparations of 10^6 neutrophil-like cells, made up to a volume of $100 \text{ } \mu\text{l}$ with supplemented RPMI-1640 medium, were prepared using a Shandon Cytospin centrifuge (Cheshire, UK; 400 g for 5 min). Cells were then stained using modified Wright-Giemsa stain (section 2.1.4). Morphological changes characteristic of apoptosis were assessed microscopically using a $\times 100$ objective (Olympus BH, Japan). At least 500 cells per slide were counted. Triplicate slides were prepared for each condition.

This method has been previously demonstrated to correlate closely with other parameters of apoptosis (Savill *et al.*, 1989). Apoptotic cells were characterized by condensed, pyknotic nucleus, while membrane integrity was maintained.

5.2.3 Assessment of apoptosis by fluorescence microscopy

Quantitation of apoptosis was performed using the fluorescent DNA-binding dye SYBR[®] Safe. With this method, fluorescence intensity was measured. Variations in intensity indicate the distribution of euchromatin and heterochromatin in non-apoptotic cell nuclei, while apoptotic nuclei exhibit highly condensed chromatin that is uniformly stained by SYBR[®] Safe.

Cytocentrifuge preparations of 10^5 neutrophil-like cells, made up to a volume of 100 μ l with supplemented RPMI-1640 medium and 10 μ l of 1 \times SYBR[®] Safe (Sigma, Poole, UK), were prepared using a Shandon Cytospin centrifuge (400 *g* for 5 min). Morphological changes characteristic of apoptosis were assessed microscopically using a \times 100 objective on a fluorescence microscope (Leica HC, USA, excitation 488 nm and emission 530 nm). At least 500 cells per slide were examined and triplicate slides were prepared for each condition.

5.2.4 Preparation of nuclear protein extracts

Neutrophil-like cells were exposed to the different treatments for 90 min at room temperature, stimulated with 10 ng ml⁻¹ human recombinant TNF- α for 2 h then pelleted and resuspended in PBS at 5×10^6 cells ml⁻¹. Nuclear protein extracts were prepared in order to study the activation of NF- κ B. Cells were pelleted by centrifugation and washed with fresh PBS. After discarding the supernatants, an appropriate quantity of ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, pH 7.9, freshly added 0.5 mM DTT, 0.5 mM PMSF, 30 μ g ml⁻¹ leupeptin, 5 μ g ml⁻¹ aprotinin

and 5 $\mu\text{g ml}^{-1}$ pepstatin; Sigma, Poole, UK) was added (50-100 μl per 1×10^6 cells). After incubation on ice for 10 min, nuclei were pelleted by centrifugation (13,000 g for 5 min at 4 $^{\circ}\text{C}$). The clear lysates were then removed and the nuclei were washed once in a hypotonic lysis buffer to remove contaminating cytosolic proteins. They were then resuspended in hypertonic extraction buffer (5 mM HEPES, 1 mM MgCl_2 0.5 M NaCl, 25% glycerol, pH 7.0, freshly added: 0.5 mM DTT, 0.5 mM PMSF, 30 $\mu\text{g ml}^{-1}$ leupeptin, 5 $\mu\text{g ml}^{-1}$ aprotinin and 5 $\mu\text{g ml}^{-1}$ pepstatin) for 1-2 h at 4 $^{\circ}\text{C}$ under agitation. Hypotonic lysis buffer prevents leaching of proteins out of the nucleus during lysis, whereas the hypertonic extraction buffer makes the nuclear membrane porous, allowing nuclear proteins to escape into solution. After centrifugation (13,000 g for 10 min at 4 $^{\circ}\text{C}$), supernatants containing the nuclear protein were removed to fresh tubes and stored at -80 $^{\circ}\text{C}$ (Whiteside *et al.*, 1992).

5.2.5 Protein assay

Bradford reagent was used to measure protein concentrations. This is a dye-binding assay, involving the use of Coomassie Blue dissolved in phosphoric acid solutions. In the acidic environment of the reagent, protein binds to the Coomassie dye. This results in a spectral shift from the brown form of dye to a blue form that absorbs light at 590 nm (Bradford, 1976).

Protein concentrations were determined using commercially-available Bradford reagent (Sigma, Poole, UK) according to the manufacturer's instructions. Assays were performed in 96-well plates; standard curves were generated using dilutions of a bovine serum albumin protein standard ranging from 0-1 mg ml^{-1} in PBS. Reagent (200 μl) was added to 20 μl of sample or standard, and the plate was incubated at room temperature for 5 minutes after which the absorbance at 595 nm was determined for all wells using a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). Both samples and

standards were measured in triplicate and mean value calculated. The concentration of protein in each sample was then interpolated from the standard curve.

5.2.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The separation of cellular proteins (polypeptides) according to their molecular mass was achieved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The SDS in the sample buffer and running buffer denatures the polypeptides in the sample, and applies a negative charge dependent on the mass of the polypeptide, removing any influence of the charge on the electrophoretic mobility, thus allowing separation of polypeptides depending on the size. The separated polypeptides can be stained with Coomassie Blue or transferred to a membrane that can be probed with antibodies as in western blotting. Polyacrylamide gels were made according to the protocol in Table 5.2. Briefly, the resolving gel was made by combining 30% (w/v) bis/acrylamide (Bio-Rad, Hemel Hempstead, UK), ddH₂O and 1.5 M Tris pH 8.8, then freshly-made 10% (w/v) ammonium persulphate (APS) solution, 20% (w/v) SDS and tetramethylethylenediamine (TEMED; Sigma, Poole, UK) were added to act as polymerising agents.

Table 5.1: Solutions used in SDS-PAGE.

| Solutions | Concentration |
|-------------------|--|
| Solution A | 30% acrylamide, 0.8% bis-acrylamide |
| Solution B | 1.5 M Tris-chloride, pH 6.8 0.4% SDS |
| Solution C | 0.5 M Tris-chloride pH 6.8 10% SDS |
| Running buffer 5× | 190 mM Tris-base 1.92 M glycine 1% (w/v) SDS |
| Transfer buffer | 250 mM Tris-base 1.92 M glycine 20% methanol |
| Sample buffer | 10 M Tris-chloride, pH 6.8 20% (w/v) glycerol 4% (w/v) SDS 0.01% (w/v) bromophenol blue 5% (v/v) β-mercaptoethanol |

Table 5.2: Composition of resolving and stacking gels.

| Reagent | Gel percentage | | Stacking gel |
|--|----------------|-------|--------------|
| | 10% | 12.5% | |
| Solution A (30% acrylamide/bis-acrylamide) | 3.3ml | 4ml | 0.67 ml |
| Solution B (1.5 M Tris-chloride, 0.4% SDS, pH 6.8) | 2.5ml | 2.5ml | 1 ml |
| H ₂ O | 4.2ml | 3.5ml | 2.3 ml |
| 10% ammonium persulphate (APS) (0.1 g in 1 ml) | 50 μl | 50 μl | 30 μl |
| TEMED | 5 μl | 5 μl | 5 μl |

The solution was swirled to mix and immediately pipetted between glass plates using a plastic Pasteur pipette. The plates were filled until approximately 2 cm below the top of the shorter glass front plate, in order to allow room for the stacking gel. A few drops of propan-2-ol were then layered onto the gel to ensure an even surface and to exclude

oxygen which inhibits polymerisation of the gel. The resolving gel was allowed to polymerise for 30-40 min, after which, the propan-2-ol was poured off and the gel was rinsed with ddH₂O. The stacking gel was made according to the protocol in Table 5.2; 30% (w/v) bis/acrylamide, ddH₂O and 1 M Tris-chloride, pH 6.8, were combined, and then the polymerising agents 20% (w/v) SDS, 10% (w/v) APS and TEMED were added. The mixture was quickly pipetted onto the resolving gel until the glass plates were full. A plastic comb consisting of either 10 or 15 teeth 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 polymerised (30-40 min). Protein samples derived from cell extracts were denatured by heating at 95 °C for 5 min in 4× SDS sample buffer (Tris-chloride, pH 6.8, 40% (w/v), glycerol and 10% (w/v) bromophenol blue, combined and made up to 5 ml with ddH₂O, Table 5.1) with or without DTT depending on the form of the antigen recognised by the antibody being used and then cooled immediately on ice. Running buffer (Table 5.1) was poured into the tank, covering the gel. The comb was removed from the gel, taking care not to disturb the wells formed, before 20 µl samples were loaded onto the gel with 10 µl biotinylated protein marker (New England Biolabs, Hertfordshire, UK) in a separate lane to allow sizing of the polypeptides. Gels were run at 90 V in running buffer (Table 5.1) whilst the samples moved through stacking gel, after which the voltage was increased to 120 V. The run was stopped after approximately 45 min when the dye front approached the bottom of the gel; the gel then was removed from the gel apparatus and from between the glass plates, and rinsed in dH₂O. After electrophoresis, the polypeptides were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) for Western blotting.

5.2.7 Western blotting

Electrophoretically-separated polypeptides were transferred from the gel to a PVDF membrane, so that they could be probed with a primary antibody against a specific polypeptide, and then probed with a secondary antibody conjugated with horseradish peroxidase (HPR), which allowed for detection via chemiluminescence of the quantity of a specific polypeptide in the sample.

Following SDS-PAGE, the separated polypeptides were transferred to a PVDF membrane by semi-dry blotting, according to the manufacturer's instructions. The transfer process took 1 h at a constant voltage of 60 V with stirring by magnetic stirrer.

The blot membrane was removed and washed three times (5 min each time) in PBS-T (PBS with 0.5% Tween 20; Sigma, Poole, UK), and then blocked using 5% BSA in 1× TBS containing 0.05% Tween 20 for 1 h at room temperature to prevent non-specific binding of antibodies. The membrane was then washed three times (5 min each time) in PBS-T, and incubated overnight with the specific primary antibody (polyclonal rabbit anti-NF- κ B p65 (RelA); Enzo Life Sciences, Exeter, UK; 50 μ g diluted 1/1000 in 1× TBS containing 0.05% Tween 20) at 4 °C with gentle agitation. Following incubation with the primary antibody, the membrane was washed three times (5 min each time) in PBS-T. The membrane was then incubated with an HRP-conjugated goat anti-rabbit-IgG antibody (R&D Systems, Minneapolis, USA, 1 mg diluted 1/1000 in 1× TBS containing 0.05% Tween 20) for 1 h at room temperature. After another three washes (5 min each time) in PBS-T, the bound proteins were detected by using enhanced chemiluminescence (Amersham ECL reagent plus; GE Healthcare, Buckinghamshire, UK). According to the manufacturer's instructions, solutions A and B were mixed in a ratio of 40:1 and added to the membrane in Saran WrapTM (Dow chemical Company, UK). After 3 min, excess ECL reagent was removed by blotting the membrane with filter paper in Saran Wrap and incubating for 1 min at room temperature. Finally, the

chemiluminescent images were acquired every 5 s for 10 min using an EC imaging system (Ultra-Violet Products Ltd, Cambridge, UK).

5.2.8 Statistical analysis

All data were analysed using Statgraphics Centurion XVI software (Stat Point Technologies, Inc.). Data are expressed as means \pm SEM for 3 separate experiments. ANOVA (one-way or two-way ANOVA) or Kruskal-Wallis test were performed as appropriate to determine significant differences between treatments. After ANOVA, *post hoc* analysis using Tukey's test was carried out. A *P* value of < 0.05 was considered significant throughout.

5.3 Results

5.3.1 Effects of treatments on neutrophil-like cell viability

Cell viability was assessed using the trypan blue exclusion and MTT reduction assays (Fig. 5.2A and B). Under normoxia, the percentage viability of neutrophil-like cells was 80.3 ± 1.1 using trypan blue. Immediately after HBO, pressure and hyperoxia, the viability of neutrophil like cells was significantly decreased (one-way ANOVA, $P = 0.0004$) compared to normoxia (Fig. 5.2A), whereas no treatment effect was found after exposure to hypoxia ($78.6 \pm 1.8\%$). The changes in cell viability were confirmed by using the MTT reduction assay (based on reduction of MTT to an insoluble purple formazan by intracellular enzymes). The results were similar to those observed in the trypan blue assay in that HBO, pressure and hyperoxia caused significant decreases in MTT reduction (Kruskal-Wallis, $P = 0.0229$; Fig. 5.2B). Hypoxia treatment also did not significantly affect MTT reduction (0.42 ± 0.06).

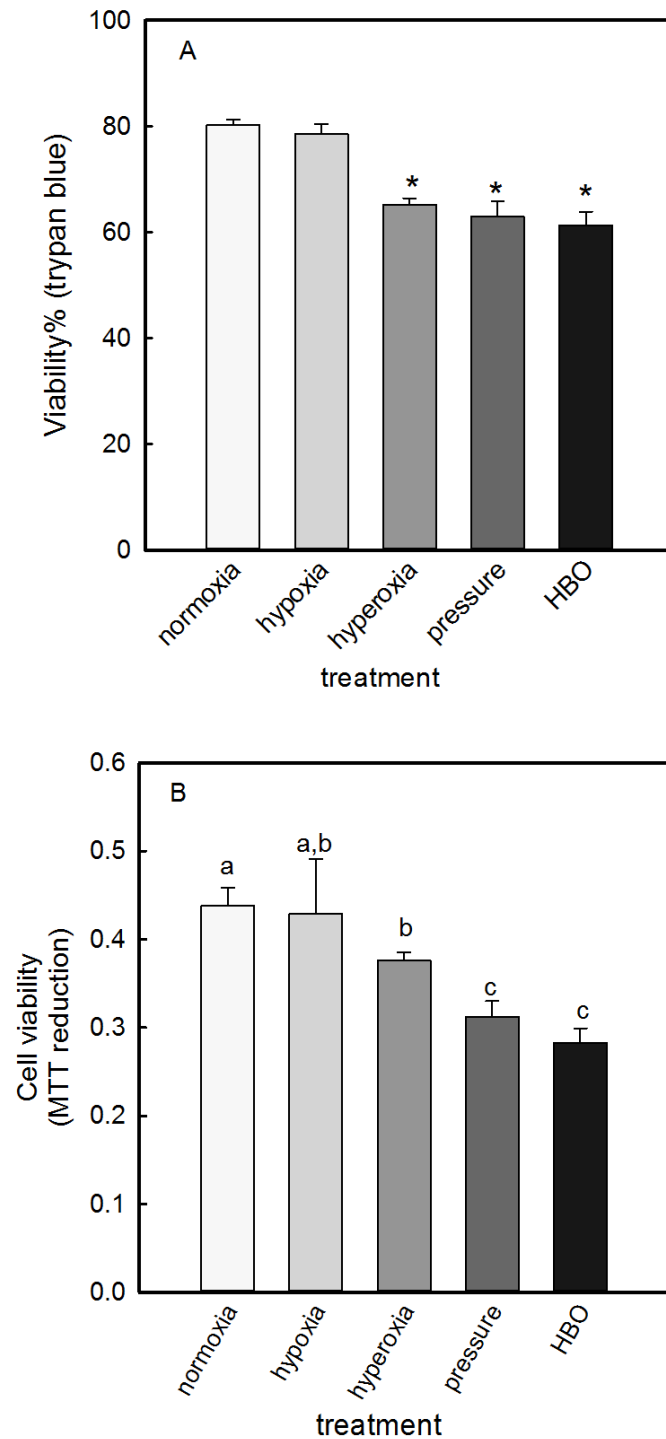


Fig. 5.2 Effects of treatments on cell viability in neutrophil-like cells. ATRA-differentiated HL-60 cells were exposed to a range of conditions for 90 min (section 2.1.1), after which cells were washed. Cell viability was assessed using trypan blue (A) and MTT reduction (B) (sections 2.1.5 and 2.1.6). Data are means \pm SEM for three separate experiments (passages numbers 20, 24 and 28) with triplicate measurements ($n = 3$). * significant difference versus normoxia. For each treatment, bars with the different lower case letters are significantly different (one-way ANOVA, $P = 0.0004$ for A; Kruskal-Wallis, $P = 0.0229$ for B).

5.3.2 Effects of treatments on apoptosis of neutrophil-like cells

The effects of HBO on apoptosis of differentiated HL-60 cells were investigated by measuring caspase 3/7 activity. The cells were treated with or without TNF- α to induce apoptosis after exposure to different treatments. HBO, pressure and hyperoxia produced a significant increase in caspase 3/7 activity in cells treated with TNF- α compared to normoxia (Kruskal-Wallis, $P = 0.016$; Fig. 5.3, overleaf). Pressure and HBO also increased caspase 3/7 activity in untreated cells compared to normoxia (Kruskal-Wallis, $P = 0.02$). Exposure to hypoxia resulted in a decrease in caspase 3/7 activity in TNF- α treated cells, but no change in untreated cells (687 ± 63 RFU). Previous studies have shown a link between H₂O₂ levels and apoptosis in undifferentiated HL-60 cells (Ganguly *et al.*, 2002). To test whether extracellular H₂O₂ was involved in HBO-induced apoptosis, catalase was added after exposure to different treatments. Contrary to expectation and similar to TNF- α , caspase 3/7 activity increased when cells were exposed to HBO or pressure in the presence of catalase (Kruskal-Wallis, $P = 0.015$) compared to normoxia. No increase was seen in cells exposed to hyperoxia (1409 ± 75 RFU; Fig. 5.3) whereas hypoxia caused a decrease in caspase 3/7 activity (460 ± 0.9 RFU) compared to normoxia. In all cases, addition of a caspase inhibitor to neutrophil-like cells inhibited caspase 3/7 activity (Fig. 5.3).

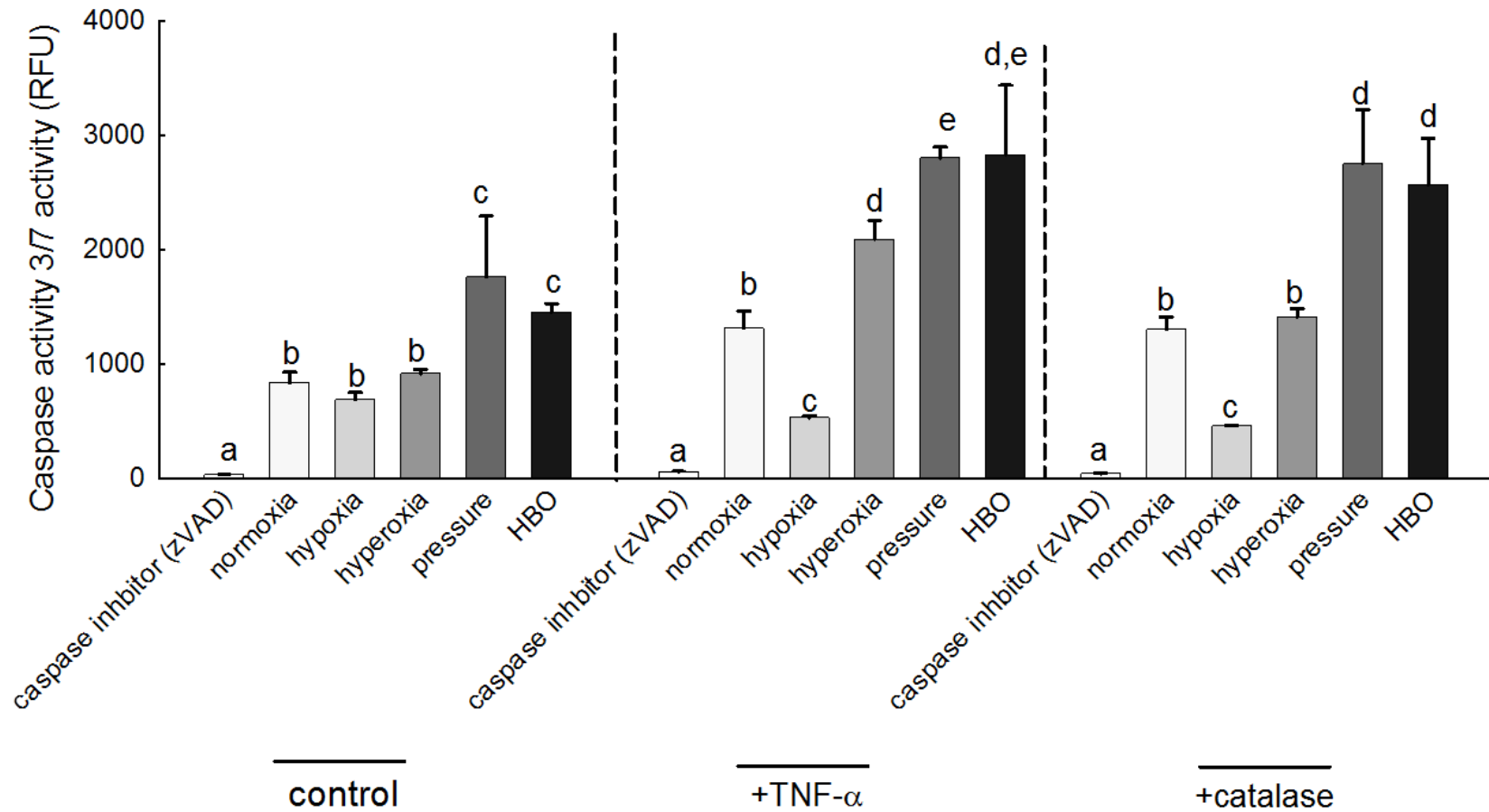


Fig. 5.3 Effects of treatments on cell death in neutrophil-like cells. ATRA-differentiated HL-60 cells were exposed to a range of conditions for 90 min (section 2.1.1), after which cells were washed and stimulated to undergo apoptosis by incubation with TNF- α , then caspase 3/7 activity was assayed (section 3.2.5). Catalase was also added to test whether H₂O₂ was involved in apoptosis. The caspase inhibitor, zVAD-FMK, was added as a negative control. Data are means \pm SEM for three separate experiments (passage numbers 33, 35 and 37, $n = 3$), with duplicate measurements. For each treatment, bars with the different lower case letters are significantly different (Kruskal-Wallis, $P < 0.05$).

5.3.3 Effects of HBO on morphological changes relating to cell apoptosis

Apoptosis was confirmed by morphological assessment using modified Wright-Giemsa (Fig. 5.4, overleaf) and SYBR[®] Safe staining (Fig. 5.5). Apoptotic cells showed nuclei with condensed chromatin but with plasma membranes still intact (Figs 5.4 and 5.5 B, C and D). Significant increases in cells showing morphological changes associated with apoptosis were seen following exposure to HBO and hyperoxia in Wright-Giemsa stained cells (one-way ANOVA, $P = 0.0006$) and post HBO, pressure and hyperoxia in SYBR[®] Safe stained cells (Kruskal-Wallis, $P = 0.03$) compared to normoxia (Figs 5.4F and 5.5F). However, there was no change in the apoptosis-associated morphological changes in neutrophil-like cells post hypoxia treatment (Wright-Giemsa and SYBR[®] Safe staining; $8.8 \pm 2.1\%$ and $20.6 \pm 1.8\%$, respectively) compared with normoxia (Wright-Giemsa and SYBR[®] Safe staining; 15.2 ± 1.9 and 21.3 ± 1.1 , respectively).

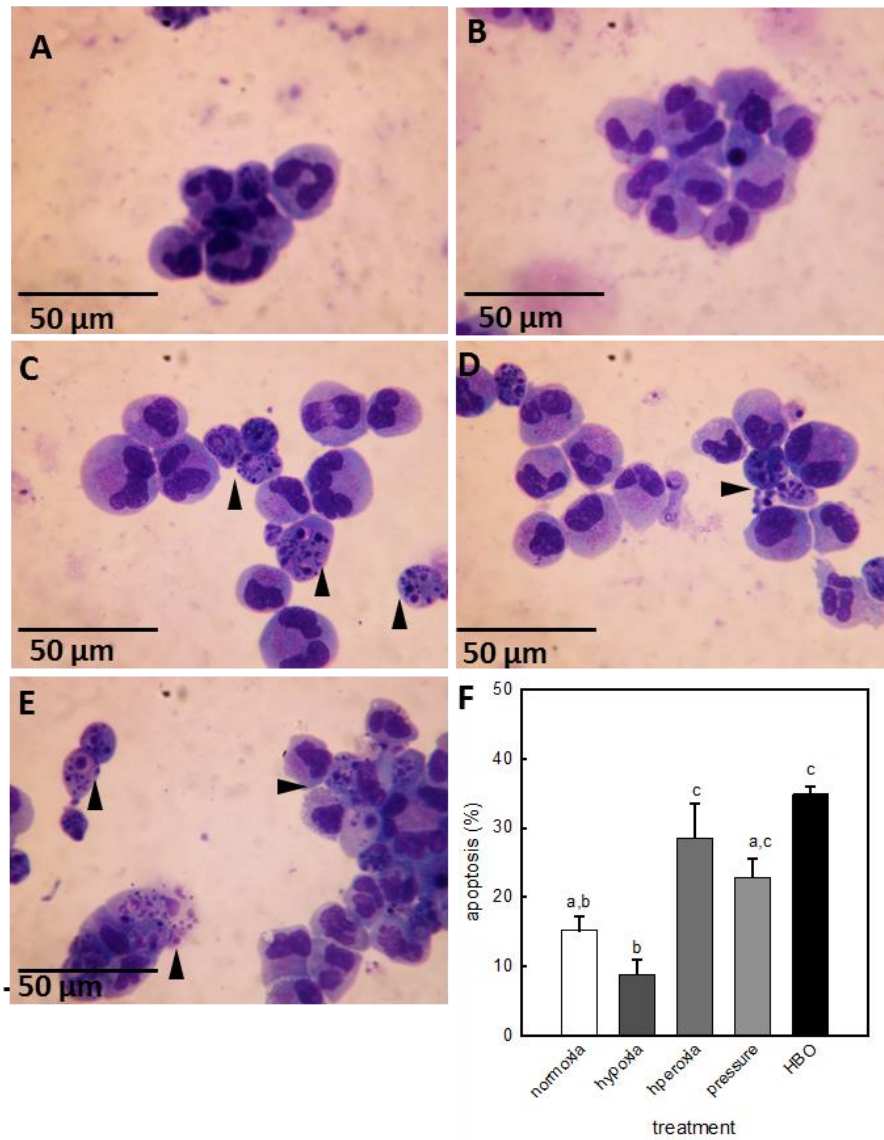


Fig. 5.4 Effects of treatments on morphological changes associated with apoptosis in neutrophil-like cells using modified Wright-Giemsa staining. ATRA-differentiated HL-60 cells were exposed to normoxia (A) hypoxia (B) hyperoxia (C) pressure (D) and HBO (E) for 90 min, after which cells were washed and morphological changes were assessed using Giemsa staining by light microscopy (section 5.2.2). The percentage of apoptosis was assessed morphologically in triplicate slides for each experiment (F). Data are means \pm SEM for three separate experiments (passages numbers 20, 24 and 28; $n = 3$). For each treatment bars with the different lower case letters are significantly different (one-way ANOVA, $P = 0.0006$). Black arrowheads indicate chromatin condensation and fragmentation.

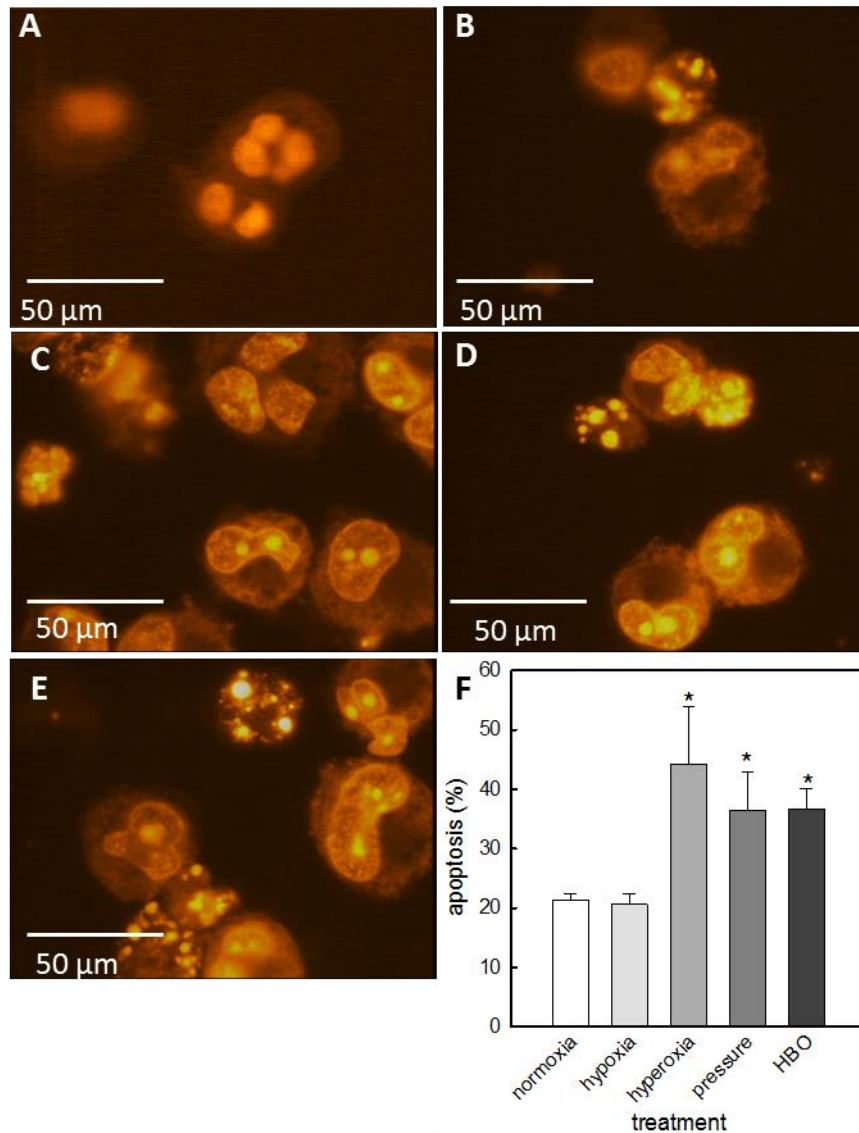


Fig. 5.5 Effects of treatments on morphological changes associated with apoptosis in neutrophil-like cells using SYBR Safe. ATRA-differentiated HL-60 cells were exposed to hypoxia (A), normoxia, (B) hyperoxia (C) pressure (D) and HBO (E) for 90 min, after which cells were washed and morphological changes were assessed using SYBR[®] Safe staining by fluorescence microscopy (section 5.2.3). The percentage of apoptosis was assessed morphologically for triplicate slides for each experiment (F). Data are means \pm SEM for three separate experiments (passages numbers 27, 29 and 32; $n = 3$). * significant difference versus normoxia (Kruskal-Wallis, $P = 0.03$).

5.3.4 Effects of oxygen treatments on NF- κ B and Bcl-2 gene expression in neutrophil-like cells

Expression of the anti-apoptotic factors NF- κ B and Bcl-2 was quantified in neutrophil-like cells following exposure to HBO, pressure, hyperoxia, normoxia and hypoxia by using qPCR (Fig. 5.6A and C). Neutrophil-like cells were treated with or without 10 ng ml⁻¹ TNF- α after exposure to different conditions. For both genes, there was no significant interaction between the treatment and stimulation (two-way ANOVA, $P > 0.05$). The addition of TNF- α up regulated the expression of NF- κ B and Bcl-2 in neutrophil-like cells when compared to the control cells (two-way ANOVA, $P = 0.0002$ and 0.002 , respectively). Exposure to HBO, pressure, hyperoxia and hypoxia significantly decreased the NF- κ B expression (two-way ANOVA, $P < 0.00005$) compared to normoxia (Fig. 5.6A, overleaf). Expression of Bcl-2 was significantly decreased following HBO, pressure and hyperoxia treatments (two-way ANOVA, $P = 0.004$) compared with normoxia. No change was observed following exposure to hypoxia (1.1 ± 0.2 and 1.5 ± 0.3) compared to untreated cells following normoxia (Fig. 5.6C). The gene expression results for both NF- κ B and Bcl-2 were confirmed by electrophoresis of PCR products on agarose gels (NF- κ B, Fig. 5.6B; Bcl-2, Fig. 5.6D).

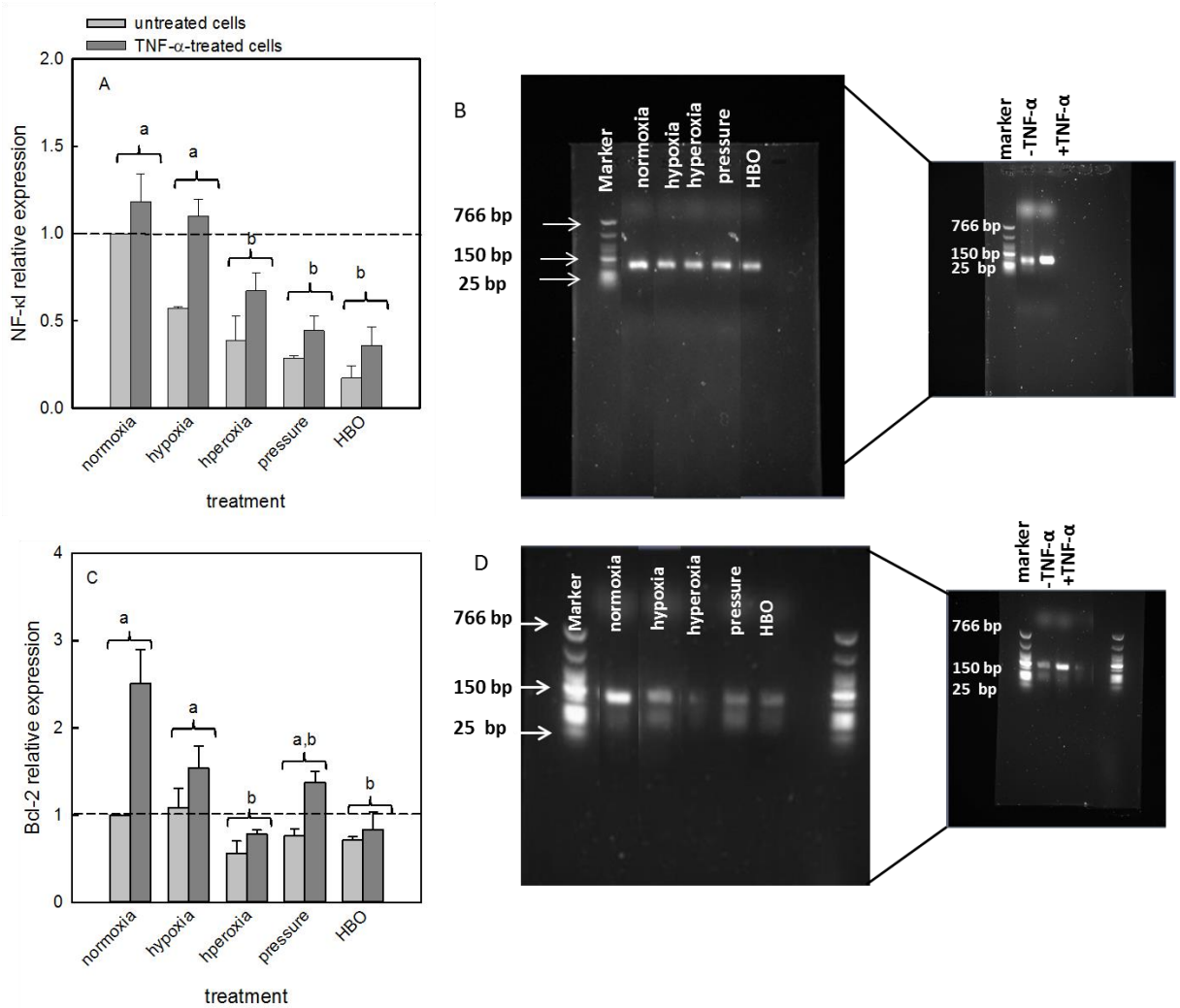


Fig.5.6 Effects of treatments on gene expression in neutrophil-like cells. ATRA-differentiated HL-60 cells were exposed to a range of conditions for 90 min (section 2.1.1), after which cells were washed and cDNA was synthesised from extracted RNA. qPCR was used to determine expression of NF-κB (A) and Bcl-2 (C) (section 2.1.7.5). Results were normalised against unstimulated cells under normoxia (dotted line). Data are means ± SEM for three separate experiments (passage numbers 20, 24 and 28; $n = 3$). For each treatment bars with the different lower case letters are significantly different (two-way ANOVA, $P < 0.05$). Photograph of PCR products showing NF-κB (B) and Bcl-2 (D) in TNF-α stimulated cells after different treatments as well as in unstimulated cells under normoxic conditions in 1% agarose gels.

5.3.5 Effects of oxygen treatments on NF- κ B protein expression

NF- κ B (p65 RelA) levels in the nuclear fraction in neutrophil-like cells treated with or without TNF- α after exposure to different treatments was analysed by western blotting (Fig. 5.7A and B). No significant effects were found on NF- κ B (p65, RelA) expression in untreated cells after exposure to different treatments (Fig. 5.7A). Immediately after exposure to HBO, pressure and hyperoxia there was a decrease in the expression of NF- κ B (p65, RelA) in TNF- α –treated cells compared to normoxia, while hypoxia treatment did not significantly affect expression (Fig. 5.7B).

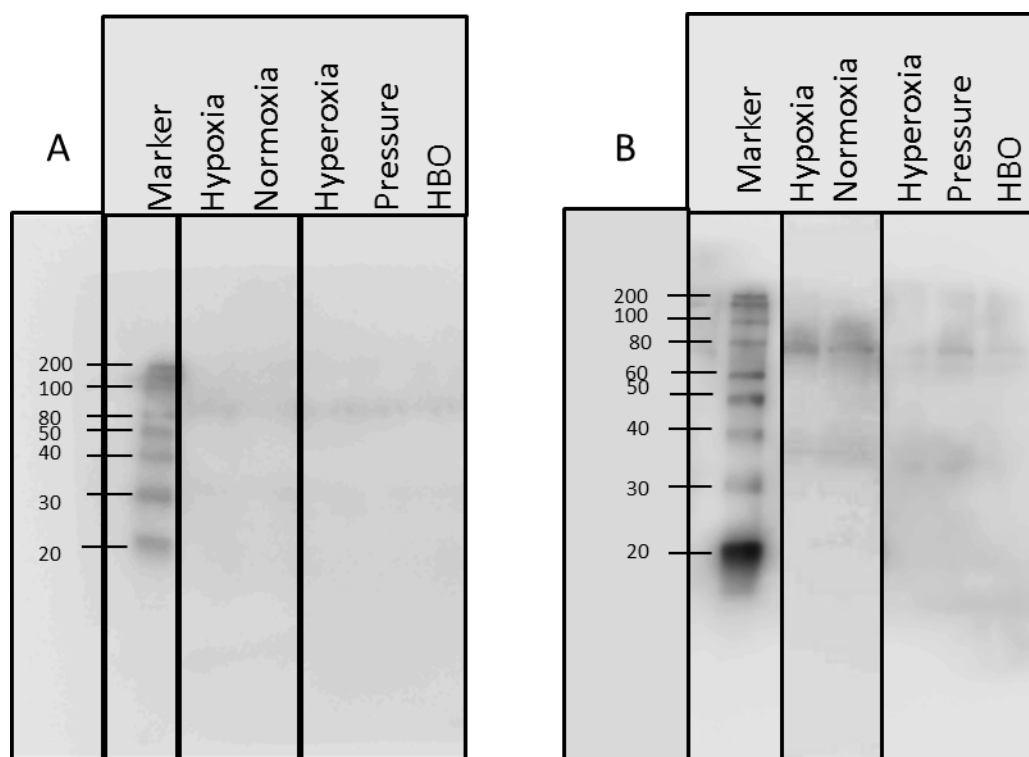


Fig. 5.7 Effects of treatments on western blot analysis of NF- κ B protein in the nuclear fraction of neutrophil-like cells. ATRA-differentiated HL-60 cells were exposed to a range of conditions for 90 min (section 2.1.1), after which cells were washed and untreated (A) or treated with TNF- α (B). The nuclei were then isolated and levels of NF- κ B (p65, RelA) quantified by Western blotting (section 5.2.7).

5.3.6 Effects of oxygen treatments on pro- and anti-inflammatory cytokine release

The levels of cytokines released from neutrophil-like cells were determined using the Lumindex[®] technique (see section 2.1.8). IL-6 levels were less than threshold values and hence were not detectable. A significant interaction was found between the treatment and stimulation (two-way ANOVA, $P = 0.0001$, 0.0189 and 0.0016 for TNF- α , IL-1 β and IL-10, respectively). Stimulation with 100 ng ml^{-1} LPS caused an increase in levels of TNF- α , IL-1 β and IL-10 released from neutrophil-like cells compared to unstimulated cells (two-way ANOVA, $P < 0.00005$; Figs 5.8A, B and C). No effects were found on TNF- α levels in unstimulated cells (one-way ANOVA, $P = 0.112$). TNF- α released from LPS-stimulated neutrophil-like cells was found to significantly decrease after HBO, pressure, hyperoxia and hypoxia pre-treatments compared with normoxia (one-way ANOVA, $P = 0.005$).

Post HBO treatment there appeared to be a decrease in IL-1 β released from LPS-stimulated cells (two-way ANOVA, $P = 0.03$). However, because of the interaction, unstimulated and stimulated data were analysed separately using one-way ANOVA to further investigate the main effect of treatment. No effect was found on IL-1 β levels in unstimulated cells (one-way ANOVA, $P = 0.126$; Fig. 5.8B). A significant decrease in IL-1 β levels was found following HBO and pressure treatment (one-way ANOVA, $P = 0.043$). No difference was found in IL-1 β levels following hyperoxia and hypoxia treatments for LPS-stimulated cells (97.5 ± 18.3 and $105.5 \pm 13.3 \text{ pg ml}^{-1}$, respectively) compared with normoxia ($114.4 \pm 17.8 \text{ pg ml}^{-1}$). Treatment with HBO and pressure significantly decreased IL-10 levels (one-way ANOVA, $P = 0.002$) in unstimulated cells compared with normoxia ($18.8 \pm 0.6 \text{ pg ml}^{-1}$). No effects was found on IL-10 levels in unstimulated cells following hyperoxia and hypoxia (17.7 ± 1.4 and $18.7 \pm 1.7 \text{ pg ml}^{-1}$, respectively) compared with normoxia (18.8 ± 0.6). In contrast, the anti-inflammatory cytokine, IL-10 showed a significant increase in HBO-treated neutrophil-

like cells compared to normoxia (two-way ANOVA, $P = 0.002$; Fig. 5.8C). Again, because of the interaction, unstimulated and stimulated data were further analysed separately using one-way ANOVA. A significant increase in IL-10 levels was found following HBO treatment for LPS-stimulated cells (one-way ANOVA, $P = 0.003$) but the differences in IL-10 values following pressure, hyperoxia and hypoxia treatment were not significant (74.9 ± 5.4 , 44.9 ± 9.3 and 73.8 ± 6.3 pg ml^{-1} , respectively) compared with normoxia (65.6 ± 5.0 pg ml^{-1} ; Fig. 5.8C).

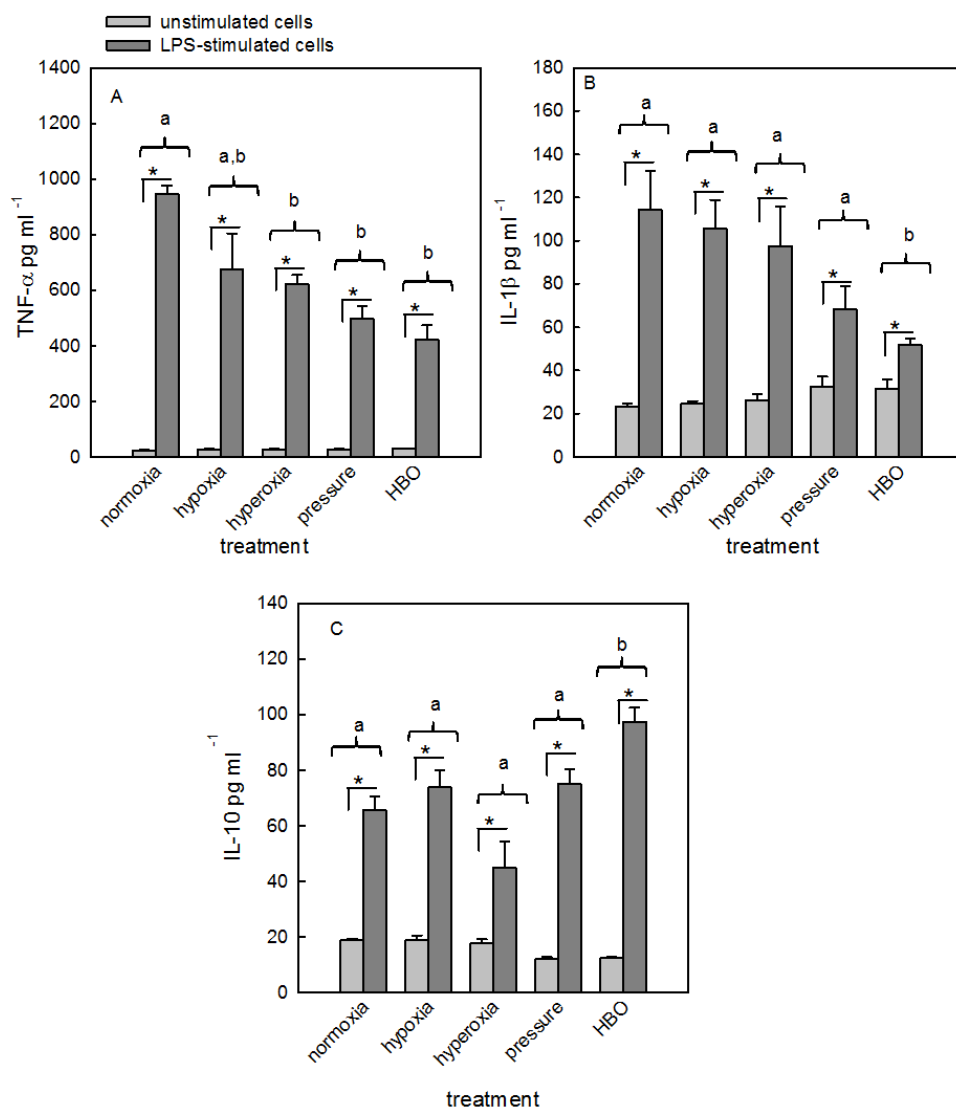


Fig. 5.8 Effects of treatments on cytokine production in neutrophil-like cells. ATRA-differentiated HL-60 cells were exposed to a range of conditions for 90 min (section 2.1.1), after which cells were washed and stimulated by LPS. Cytokine production was measured by Luminex[®] assay (section 2.1.8). Data are means \pm SEM for three separate experiments (passages numbers 28, 30 and 32; $n = 3$) with duplicate measurements. * indicate significant differences from unstimulated cells. For each treatment bars with the different lower case letters are significantly different (ANOVA, $P < 0.05$).

5.4 Discussion

Enhancement of neutrophil anti-microbial activity seems to be of benefit for the resolution of inflammation in chronic wounds. It has been demonstrated that antimicrobial activity of neutrophils is associated with their apoptosis (Kobayashi *et al.*, 2003, Nakazato *et al.*, 2007, Watson *et al.*, 1996). Increased neutrophil apoptosis and their clearance is essential for the resolution of inflammation, since chronic wounds are characterised by the persistence of an inflammatory and hypoxic environment in which there is prolonged neutrophil life at the wound site (Yager and Nwomeh, 1999). In the present study, pre-treatment with HBO treatment led to a decrease in viability (trypan blue and MTT) of neutrophil-like cells, and an increase in apoptosis as assessed by caspase 3/7 activity, Giemsa staining and fluorescence staining using SYBR[®] Safe.

These results are consistent with the effects of HBO previously demonstrated in a range of haematopoietic cells including undifferentiated HL-60 cells, Jurkat cells and murine thymocytes (Ganguly *et al.*, 2002). For example, in HL-60 cells, HBO increased both spontaneous and γ irradiation-induced apoptosis, but normobaric hyperoxia or pressure alone had little effect (Ganguly *et al.*, 2002). Hyperoxia increases apoptosis of other cells of hematopoietic origin such as thymocytes (Stefanelli *et al.*, 1995), thymoma cell lines, lymphoma cell lines (Muschel *et al.*, 1995) macrophage cell lines (Petrache *et al.*, 1999), as well as myocytes (Absher *et al.*, 1994) and prostate carcinoma cells lines (Kalns *et al.*, 1998). There is evidence that the increase in apoptosis associated with antimicrobial activity in neutrophils is a consequence of the increased ROS production by the cells themselves (Watson *et al.*, 1996, Nakazato *et al.*, 2007). Furthermore, mitochondria are likely to have a role in both ROS production and in the intrinsic apoptotic pathway (Maianski *et al.*, 2004b). The results presented in Chapter 4 showed that there was an increase in ROS production and degranulation post-HBO treatment. It

has also been shown that ROS can activate death receptor signalling thereby promoting neutrophil apoptosis via the extrinsic pathway (Scheel-Toellner *et al.*, 2004).

Since exogenous H₂O₂ can cause cell apoptosis (Lin *et al.*, 1997), the hypothesis in this study was that HBO induced apoptosis by increasing intracellular H₂O₂ levels. Therefore, catalase, a H₂O₂ scavenging enzyme, was added to the cells which were pre-exposed to a range of oxygen conditions. Exogenous catalase did not inhibit HBO-induced apoptosis in neutrophil-like cells and did not result in a significant difference in caspase 3/7 activity. This suggests that exogenous catalase does not decrease the H₂O₂ levels, because it cannot penetrate the cell membrane due to its size and can only directly affect intercellular H₂O₂ levels by catalysing the degradation of extracellular H₂O₂ to O₂ and H₂O. However, diffusion of H₂O₂ from the cytoplasm into the medium is possible and this could eventually lead to a lowering of the intracellular H₂O₂ in the presence of extracellular catalase.

The present study also demonstrated an increase in neutrophil-like cell apoptosis after treatment with pressure alone. This provides evidence that increased pressure can modify cell function and susceptibility to undergo apoptosis. A prior study by Oh *et al.* (2010) indicated that apoptosis of human H460 lung cancer cells can be initiated by increasing pressure to 2 ATA, which provides a mechanical and physiological stimulation. Previous research findings demonstrated that exposure to HBO or pressure alone inhibited both benign and malignant human mammary epithelial cell proliferation but did not induce apoptosis (Maianski *et al.*, 2004b, Granowitz *et al.*, 2005). The apoptotic effects of pressure can be explained by conformational changes and mechanical effects after pressure treatment that may affect G-protein-coupled receptors (GPCRs); these are trans-membrane receptors that activate G protein signalling pathways when bound by ligands and act as mechanoreceptors. They have been identified in a range of cells such as vascular smooth muscle cells (VSMCs), human

embryonic kidney (HEK) cells and neutrophils (Makino *et al.*, 2006, Zou *et al.*, 2004, Yasuda *et al.*, 2008, Voets and Nilius, 2009). Mechanoreceptors are responsible for the response to fluid shear stress and initiate multiple receptor cellular pathways that have been implicated in apoptosis.

Hyperoxia-induced death is well documented in many studies (Barazzone and White, 2000, Ganguly *et al.*, 2002). Hyperoxia enhances ROS formation and accumulation, which are present in many cells triggering apoptosis. ROS are primarily generated in mitochondria via mitochondrial respiration which initiates the signal pathways in the cells and often converges upon the mitochondria to promote release of cytochrome *c* to the cytoplasm and activate procaspase-9, which in turn activates the apoptotic cascade (Li *et al.*, 1997, Reed, 1997). Additionally, ROS are involved in death receptor-initiated signalling pathways, especially in the TNF- α receptor 1 (TNFR1) pathway which initiates the caspase cascade (Shen and Pervaiz, 2006). The present results are consistent with these studies, as caspase 3/7 activity and morphological changes associated with apoptosis increased in neutrophil-like cells exposed to hyperoxia.

HBO not only induced spontaneous apoptosis in neutrophil-like cells, but also enhanced apoptosis in the presence of TNF- α . A study by Ganguly *et al.* (2002) examined the effect of HBO in Jurkat and HL-60 cells induced to undergo apoptosis by CH-11 anti-Fas antibody or γ -radiation, respectively. The study demonstrated no direct effect of HBO on apoptosis, although Fas-induced apoptosis was enhanced by HBO. Granulocytic differentiated HL-60 cells showed no change in apoptosis after 90 min with HBO treatment, a condition representative of patient treatment, but HBO increased apoptosis after 3 h. In this study, TNF- α was used to induce death in neutrophil-like cells after treatment with different oxygen conditions. TNF- α -induced death signalling is initiated by the activation of TNFR1. ROS are potent activators of TNFR1 that induce Jun N-terminal kinase (JNK) activation, a member of the mitogen-activated protein

kinases (MAPK) and its activation is one of key events in TNF- α induced cell death (Hongbo and Fabien, 2008). High concentrations of TNF- α (10-100 ng ml⁻¹) induce spontaneous neutrophil apoptosis which is characterized by caspase activation, internucleosomal DNA, and morphological features of apoptosis, the increase in which is ROS-dependent (Van Den Berg *et al.*, 2001). Consistent with these studies, in the present investigation, the caspase activity in neutrophil-like cells induced to die by TNF- α , was higher post HBO and pressure compared to normoxia. This suggests that HBO mediates its effects on TNF- α -induced apoptosis through increased ROS production. Previously it has been shown in rat alveolar Type II cells that the initiation phase of pulmonary oxygen toxicity causes an increase in TNF- α and its receptor TNFRI leading to activation of caspases 3 and 8 (Guthmann *et al.*, 2005). However, two studies have shown that stimulation of neutrophils by TNF- α involves not only the classical caspase-dependent pathway, but also caspase-independent cell death, and it was found that the death of neutrophils treated with a combination of TNF- α and the caspase inhibitor zVAD was dependent on mitochondrial-derived ROS (Maianski *et al.*, 2004b, Liu *et al.*, 2003). In support of this, a single exposure to HBO induced apoptosis in Jurkat-T-cells via mitochondria-dependent mechanisms and ROS production, which could have enhanced the formation of pro-apoptotic substances in cells such as the pro-apoptotic members of the Bcl-2 family, Bik and Bax (Weber *et al.*, 2009a).

Hypoxia pre-treatment in this study resulted in no change in apoptosis of neutrophil-like cells compared with normoxia conditions. This agrees with a study by Hannah *et al.* (1995) that indicated prolonged neutrophil survival under hypoxic conditions. The anti-apoptotic effect of hypoxia is dependent on continuing protein synthesis, associated with upregulation of HIF-1 α levels. HIF-induction inhibits neutrophil apoptosis and radiation-induced apoptosis in Jurkat cells (Vissers and Wilkie, 2007, Weinmann *et al.*, 2004).

Since mitochondrial outer membrane permeability is controlled by Bcl-2 (Green and Kroemer, 2005) and mitochondria may have a role in HBO induced apoptosis, in the present study the gene expression of Bcl-2 and its association with NF- κ B was investigated. Interestingly, the results demonstrated a down regulation in the expression of Bcl-2 after pre-treatment with HBO, pressure and hyperoxia in TNF- α treated cells, and NF- κ B at the transcription and protein levels after pre-treatment with HBO, pressure or hyperoxia in both TNF- α treated and untreated cells. TNF- α is known to trigger activation of NF- κ B, which requires phosphorylation and degradation of I κ B α . Following activation, NF- κ B translocates to the nucleus to control the expression of survival genes such as Bcl-2. Thus, the decline in the expression of NF- κ B in neutrophil-like cells is associated with a decrease in Bcl-2 expression. These results may be explained by a number of different factors. Firstly, that down regulation of Bcl-2 expression occurs in response to apoptotic stress induced by HBO. Therefore, mitochondrial mechanisms dependent on Bcl-2 may be involved in the HBO-induced apoptosis. Bcl-2 down regulation may initiate lymphocytes to undergo apoptosis (Hotchkiss *et al.*, 2005). A study by Li *et al.* (2005) has demonstrated the effects of HBO on apoptotic genes in rat brains after global ischaemia-hypotension; increased expression of caspase-8 and a decrease of Bcl-2 and HIF-1 α expression was found following HBO treatment (3 ATA, 2 h). Release of mitochondrial pro-apoptotic factors is likely to be accompanied by a breakdown of mitochondrial membrane potential. Therefore, further experiments are needed to investigate the relevance of the Bcl-2 down regulation and breakdown of mitochondrial membrane potential. The second factor which may explain these results is that Bcl-2 action is controlled by NF- κ B, which plays an important role in the regulation of granulocyte apoptosis (Ward *et al.*, 2004). Inhibition of NF- κ B enhanced the pro-apoptotic effects of TNF- α in neutrophils

and mediated its effects as a powerful inducer of eosinophil apoptosis (Ward *et al.*, 1999, Fujihara *et al.*, 2002).

Cell apoptosis is kept in balance by the presence of pro-and anti-apoptotic proteins. NF- κ B affects this balance by up regulating expression of proteins that prevent apoptosis (Karin and Lin, 2002). In support of this, the present work found that HBO-induced apoptosis was associated with decreased NF- κ B expression and down regulation of the anti-apoptotic gene Bcl-2. Blocking NF- κ B activation results in Bax protein expression (Bentires-Alj *et al.*, 2001) and down regulation of Bcl-2 (De Moissac *et al.*, 1999). Thus HBO-inhibited NF- κ B activation shifts the balance towards apoptosis. The present results suggested that increased accumulation of I κ B α in the nucleus of neutrophil-like cells might be responsible of down regulation of NF- κ B by masking its nuclear localization sequence and inhibiting the ability of NF- κ B to bind to DNA (Beg *et al.*, 1992, Didonato *et al.*, 1996). This may be identified as one of the underling mechanisms of HBO-induced apoptosis (Fig. 5.9).

From the present findings, hypoxia pre-treatment showed no change in Bcl-2 expression in untreated and TNF- α cells compared with normoxia. The majority of gene expression after hypoxia is regulated by HIF-1. Under normoxic conditions, HIF-1 is degraded by proteasome function, while under hypoxic conditions HIF-1 is stabilised and acts as transcription factor for various genes involved in angiogenesis, energy metabolism, cell proliferation and viability, as well as apoptosis (Zagórska and Dulak, 2004). Additionally, an association between HIF and NF- κ B has previously been identified (Görlach and Bonello, 2008, Taylor and Cummins, 2009, Zagórska and Dulak, 2004). NF- κ B activation is required the phosphorylation and degradation of I κ B (Chen *et al.*, 2007a). Subsequent phosphorylation and dimerization of NF- κ B subunits p50/p65 permits NF- κ B translocation to the nucleus. However, this prevents HIF-1 α degradation

and leads to an increase in HIF-1 α concentration. The present study found a significant decrease in NF- κ B at the transcription levels in unstimulated and TNF- α stimulated cells following hypoxia, which may be explained by the cross talk between NF- κ B and HIF-1 signalling in short term hypoxia. Examination of changes in HIF-1 α activity and protein expression could provide some information on the pathways involved.

Neutrophil apoptosis exerts anti-inflammatory effects through limiting the release of toxic contents that could cause injury to the tissue. The present study provided further evidence of the immunosuppressive effects of HBO-induced apoptosis via its effect on cytokine production. HBO treatment led to a significant decrease in the release of pro-inflammatory TNF- α and IL-1 β , while a significant increase in the anti-inflammatory IL-10 release by LPS-stimulated cells was observed following HBO. Exposure to normobaric hyperoxia or pressure decreased only TNF- α levels and had no effect on the other cytokines. This suggests that HBO-induced inhibition of inflammation seems to be a function of both pressure and hyperoxia. The present results are in agreement with other studies that demonstrated that HBO inhibits LPS-induced IL-1 and TNF- α release in splenic macrophages (Inamoto *et al.*, 1991), circulating monocytes/macrophages (Lahat *et al.*, 1995) and human peripheral blood mononuclear cells (Granowitz *et al.*, 2002). However, there is no study to date on the effects of HBO on cytokine release from neutrophils. A possible mechanism for impaired pro-inflammatory cytokine production was the induction of apoptosis in the cytokine-producing cells. For example, HBO induced apoptosis in neutrophil-like cells as shown by increased caspase 3/7 activity and a decrease in cell viability. Neutrophil apoptosis is down regulated by pro-inflammatory mediators such as TNF- α , IFN- γ , G-CSF, GM-CSF and IL-2 (Colotta *et al.*, 1992, Brach *et al.*, 1992, Lee *et al.*, 1993); this inhibition of neutrophil apoptosis not only increases the life span of cultured neutrophils but also prolongs their functional longevity (Lee *et al.*, 1993), whereas IL-10 enhances neutrophil apoptosis and their

clearance by macrophages (Michlewska *et al.*, 2009). In line with previous studies, HBO treatment caused a significant increase in IL-10 in neutrophil-like cells. Another mechanism by which HBO might suppress pro-inflammatory cytokine production is via its effects on signal transduction, transcription, and/or translation. For example, HBO inhibits cytokine production by down regulation of PGE₂ production (Inamoto *et al.*, 1991), inhibition of cox-2 expression (Yin *et al.*, 2002) and by inducing the formation of heat shock protein, HSP (Xie *et al.*, 1999). HBO has been shown to increase ROS generation (Benedetti *et al.*, 2004, Conconi *et al.*, 2003) and this may play a role in inducing expression of HSPs, for example HSP-70, in response to oxidative stress. The intracellular HSP concentration has been demonstrated to be correlated to antioxidant enzymes under conditions of oxidative stress to attenuate ROS-dependent cellular and tissue damage (Currie *et al.*, 1988, Mocanu *et al.*, 1993). HSP-70 synthesis also correlates with a decrease in cytokine levels through transcriptional pathways; this is an example of a protective measure conferred by an HSP (Grunenfelder *et al.*, 2001).

Hypoxia pre-treatment resulted in no change in the pro-inflammatory cytokines TNF- α and IL-1 β and anti-inflammatory IL-10 production by neutrophil-like cells. Studies on hypoxia have been shown to decrease (Fritzenwanger *et al.*, 2011), increase (Ghezzi *et al.*, 1991) or have no effect (Burki and Tetenta, 2013) on pro-inflammatory cytokines levels. However, the cellular response to hypoxia is highly regulated by HIF-1 α which plays a distinct role in regulating pro-inflammatory cytokines levels, in part by direct binding to the promoters of cytokine genes (Zagórska and Dulak, 2004).

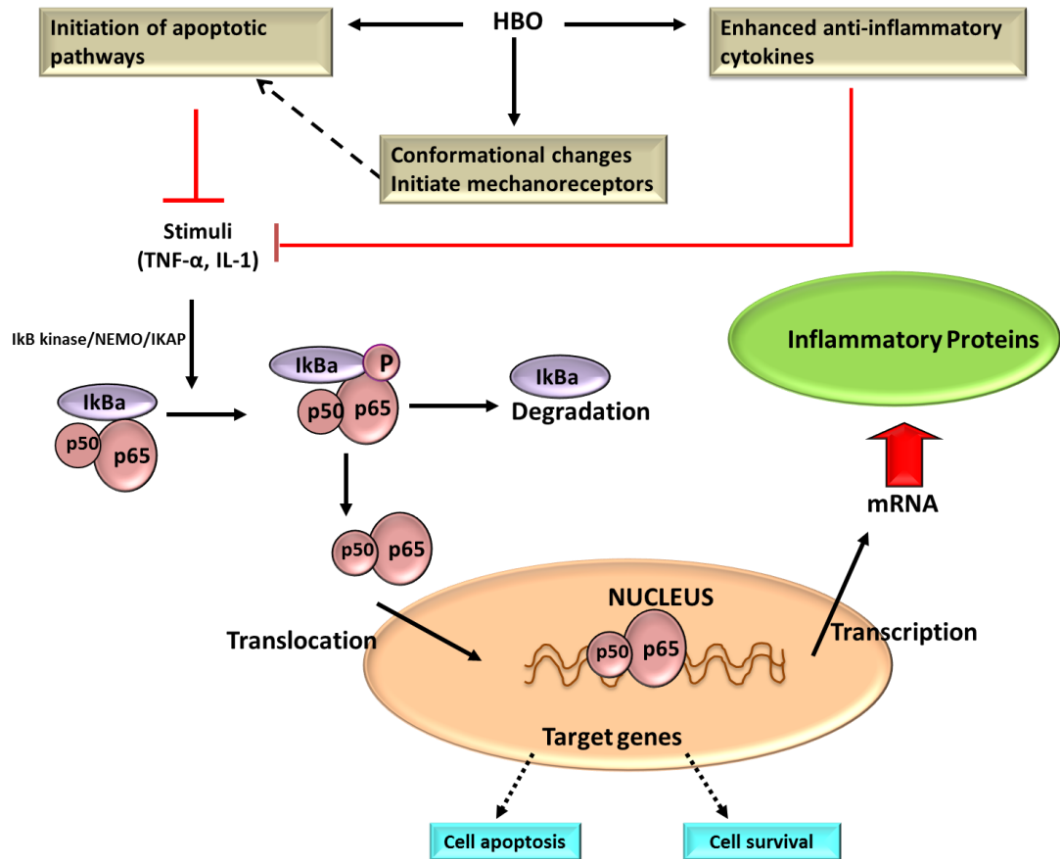


Fig. 5.9 Proposed mechanisms by which HBO may affect transduction or transcription signals by HIF-1 and NF-κB in neutrophil-like cells. Observations from the present study suggest that HBO-induced apoptosis in neutrophil-like cells is associated with mitochondrial function and ROS production. HBO also leads to enhanced IL-10 production, and taken together results in reduction of pro-inflammatory cytokines TNF-α and IL-1β. Since TNF-α is a potent stimulus for NF-κB activation, a decreased TNF-α level results in a reduction of IκBα phosphorylation and its degradation, down regulation of NF-κB activity, and the subsequent transcription and expression of inflammatory proteins such as apoptotic protein B1-2. Additional effects of HBO are pressure dependent, such as conformational changes which could lead to the activation of mechanoreceptors that are implicated in apoptosis.

5.4 1 Conclusion

Neutrophil apoptosis is of key importance in the resolution of inflammation. A single exposure to HBO enhances spontaneous and TNF- α -induced apoptosis in neutrophil-like cells via oxidative stress that may potentially result in the formation of pro-apoptotic substances in the cells or in the medium. The hyperoxia and pressure components of HBO both contribute to effects on neutrophil-like cells. Apoptotic effects of HBO are associated with an anti-inflammatory response. These findings confirm the immunomodulatory effect of HBO. Further studies are needed to address the exact mechanisms as to how HBO mediates oxidative stress and activates apoptotic pathways.

CHAPTER SIX

THE EFFECTS OF HBO ON

NEUTROPHIL CLEARANCE BY

MONOCYTE-DERIVED MACROPHAGES

Abstract

A critical step in the resolution of inflammation is neutrophil apoptosis and their subsequent clearance by macrophages. Early findings indicated that HBO induced neutrophil-like cell apoptosis. However, the effects of HBO leading to the clearance of neutrophils by macrophages are still unclear. In the current study *in vitro* co-culture experiments with bovine neutrophils and monocyte-derived macrophages (MDM Φ) were carried out to clarify the role of HBO, pressure, hyperoxia, hypoxia and normoxia in this process. Both fresh and 22 h-aged neutrophils were used as targets for phagocytosis. The results showed that phagocytosis of fresh and 22 h-aged neutrophils by MDM Φ significantly increased after HBO treatment as assessed using flow cytometry and light microscopy (two-way ANOVA, $P < 0.00005$). The efficient recognition of apoptotic cells is associated with neutrophil respiratory burst activity, and pro-/anti-inflammatory cytokine secretion. Enhanced the clearance of neutrophils (fresh or 22 h old) was accompanied by a significant increase in H₂O₂ levels following HBO treatment (two-way ANOVA, $P < 0.05$ respectively), with up regulation of IL-10 (anti-inflammatory cytokine) mRNA gene expression in MDM Φ that ingested fresh or 22 h-aged neutrophils. The gene expression of TNF- α (pro-inflammatory cytokine) mRNA did not change in LPS-stimulated MDM Φ that ingested fresh or 22 h-aged neutrophils after HBO, pressure and hyperoxia (two way-ANOVA, $P > 0.05$). These findings suggest that HBO activated MDM Φ participate in the clearance of apoptotic cells, and that this process is ROS-dependent. In addition, uptake of apoptotic neutrophils by MDM Φ that are exposed to HBO may contribute to the resolution of inflammation, because HBO-induced apoptosis up-regulated IL-10 mRNA expression.

6.1 Introduction

Phagocytic clearance of neutrophils undergoing apoptosis acts a key point in the inflammatory response, and probably for the subsequent progression of wound healing. Apoptotic cells undergo specific surface changes that allow phagocytes to recognise and ingest them (Brouckaert *et al.*, 2004). Exposure of phosphatidyl serine (PS) on the outer leaflet of the plasma membrane, due to loss of plasma membrane asymmetry, is essential for the uptake of apoptotic cells by macrophages (Fadok *et al.*, 2001b, Fadok *et al.*, 1992). Oxidation of PS is an integral feature of programmed cell death, and exposure of oxidised PS may also serve to enhance the clearance of apoptotic cells (Kagan *et al.*, 2002). It is known that the oxidative burst via NADPH oxidase activity is critical for PS exposure in PMA-stimulated cells and macrophage uptake (Engelich *et al.*, 2001).

In chronic wounds, previous studies have demonstrated that inefficient clearance of apoptotic cells leads to chronic inflammation (Kaneto *et al.*, 1999). While neutrophil recruitment to the wound site is required to remove bacterial infection, macrophages are essential for resolving inflammation. Although monocytes are recruited from peripheral blood to the wound site later, tissue macrophages are already present within the tissue to phagocytose cell debris and contaminating bacteria (Haney, 2000, Heinrich *et al.*, 2003). Depletion of macrophages, but not neutrophils, from wound sites delays wound healing (Leibovich and Ross, 1975). Diabetic wounds are characterised by elevated load of apoptotic cells (Darby *et al.*, 1997) which may contribute in part to impaired apoptotic clearance activity of the macrophages at the diabetic wound site. This is supported by a study by Khan *et al.* (2010) that demonstrated that the impairment of macrophage phagocytosis in diabetic wounds resulted in an increase of apoptotic cells at the wound site which in turn prolonged the inflammatory phase and the progress of

wound healing. At the wound site, successful phagocytosis of apoptotic cells results in suppression of pro-inflammatory cytokines and the progression of wound healing (Lee *et al.*, 1993, Khanna *et al.*, 2010). Increased levels of the pro-inflammatory cytokines TNF- α and IL-6, together with decreased IL-10, are characteristic of non-healing wounds such as diabetic wounds (Werner and Grose, 2003). IL-10 is well known to suppress the inflammatory response (Moore *et al.*, 2001). A major source of the cytokines in wounds is macrophages (Werner and Grose, 2003) which in chronic wounds produce higher levels of pro-inflammatory cytokines than those in acute wounds. This leads to the suggestion that the inflammatory response in chronic wounds is due in part to increased pro-inflammatory cytokine production by wound macrophages.

Also, the presence of bacterial products at the chronic wound site may affect the ability of phagocytes to ingest apoptotic cells. Chronic wounds are characterised by the presence of high levels of LPS (section 1.3.1). LPS induces TNF- α production and initiates the inflammatory response which in turn can influence apoptosis of inflammatory cells (Lee *et al.*, 1993, Murray *et al.*, 1997). A previous study by Michlewska *et al.* (2009), reported that LPS inhibited phagocytosis of apoptotic neutrophils that contributed in part to the LPS-induced TNF- α production (Feng *et al.*, 2010).

HBO can exert profound effects on immune function (section 1.6.4.1). HBO treatment has been found to increase apoptosis in HL-60 cells, Jurkat cells and lymphocytes (Weber *et al.*, 2009a, Ganguly *et al.*, 2002). Moreover, HBO has been shown to reduce cytokine expression by monocytes and macrophages collected from rats or from human peripheral blood after stimulation with LPS (Lahat *et al.*, 1995, Benson *et al.*, 2003). Due to the immunosuppressive effects of HBO, the contribution of the clearance of apoptotic cells in terms of cytokine production remains unknown. Therefore, the effects

HBO on clearance of apoptotic cells by macrophages may be potentially important in the resolution of inflammation and hence on the progression of wound healing. There has been no work to date examining the effect of HBO on neutrophil phagocytosis by macrophages. Previous studies have shown that mechanical stimuli such as pressure can modulate cell morphology and function in other cell types (Basson *et al.*, 2000, Thamilselvan and Basson, 2004). Recent evidence suggests that physical forces, for example pressure and repetitive strain, may alter macrophage functions. Monocyte migration and receptor expression in macrophages increases with pressure (40-130 mmHg; Sakamoto *et al.*, 2001). Additionally, in response to very high cyclic pressure or high pressure combined with endotoxin stimulation, macrophages produce pro-inflammatory cytokines (Mattana *et al.*, 1996, Miyazaki and Hayashi, 2001). The mechanisms responsible for these effects are still not defined. Therefore, the current study aimed to determine whether HBO-induced apoptosis was able to enhance neutrophil clearance by MDM Φ in a non-inflammatory response. In order to elucidate the effects of HBO on resolution of inflammation and the potential involvement of ROS-mediated apoptosis in this process, monocyte-derived macrophages (MDM Φ) from bovine blood were used as a macrophage model to phagocytose apoptotic neutrophils and to determine any associated up regulation in pro- and anti-inflammatory cytokine gene expression by MDM Φ that had ingested apoptotic cells. Whilst bovine neutrophil apoptosis has been studied, there has been no work examining apoptosis and phagocytosis of these cells and the effects of HBO. Therefore this study was interested in addressing this process, as well as the mechanisms of the clearance of apoptotic neutrophils by MDM Φ and their safe removal at the site of inflammation.

6.2 Materials and methods

6.2.1 Tissue culture solutions

Dulbecco's Modified Eagle Medium (DMEM) and Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} were obtained from Gibco Ligo Technologies (Paisley, UK). Fetal calf serum was purchased from Lonza (Wokingham, UK) and was heat inactivated ($56\text{ }^{\circ}\text{C}$, 1 h) before addition to the media (10%). All tissue culture solutions were stored at $4\text{ }^{\circ}\text{C}$ and warmed to $37\text{ }^{\circ}\text{C}$ prior to use.

6.2.2 Experimental design

The work described in this chapter is split into consecutive parts. This was essential in order to explore the effects of HBO, pressure alone, hyperoxia, hypoxia and normoxia on phagocytosis of apoptotic neutrophils by MDM Φ . Neutrophils isolated from bovine blood were used in apoptosis and phagocytosis assays either immediately after isolation from blood (fresh) or after incubation at $37\text{ }^{\circ}\text{C}$ at 5% CO_2 balanced with air for 22 h (aged). The first experiment was conducted to explore the effects of a 90-min exposure to different treatments on neutrophil apoptosis. This was achieved by measurements of viability (trypan blue and MTT, sections 2.1.5 and 2.1.6), annexin V/PI staining, DNA fragmentation measurement and morphological study of Wright-Giemsa stained fresh and aged neutrophils (section 2.1.4). Neutrophils from at least three animals were used for each experiment and each assay was carried out in triplicate. Neutrophils from an additional three animals were used for morphological studies of apoptosis post exposure to different oxygen conditions (Fig. 6.1).

The second experiment aimed to test the phagocytosis of neutrophils by MDM Φ . The experiment was achieved by co-culture of fresh or 22 h-aged neutrophils (following 90 min treatment, section 2.1.1) with MDM Φ for 1 h at $37\text{ }^{\circ}\text{C}$ under 5% CO_2 (balance air).

Phagocytosis was assessed using either flow cytometry or histochemical detection of MPO activity. The expression of genes for both pro- and anti-inflammatory cytokines was measured before and after co-culture of fresh and aged neutrophils with MDMΦ (Fig. 6.1). The extracellular production of H₂O₂ was also measured (section 2.1.3) as a marker of respiratory burst activity of neutrophils before and after co-culture with MDMΦ.

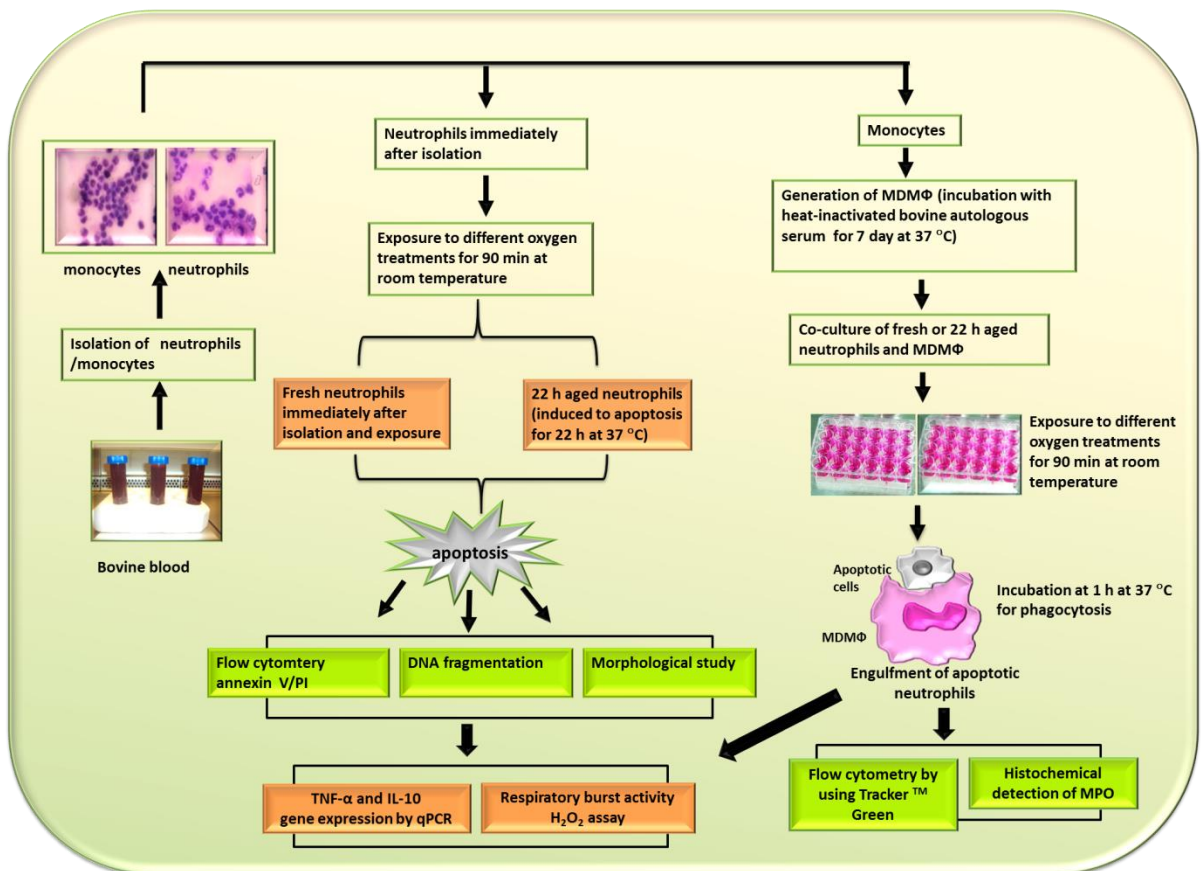


Fig. 6.1 Experimental design. Neutrophils and monocytes were isolated from bovine blood and were used for apoptosis and phagocytosis assays either immediately after neutrophil isolation (fresh) or after 22 h incubation at 37 °C under 5% CO₂, balance air (aged). Firstly, neutrophils were exposed to different treatments for 90 min then assessed immediately or after 22 for apoptosis. This was achieved by (trypan blue/MTT) viability assay, annexin V/PI staining, DNA fragmentation and morphological study by Wright-Giemsa staining for signs of apoptosis. Secondly, phagocytosis of neutrophils was investigated by co-culture of fresh or 22 h-aged neutrophils (following exposure to different oxygen treatments for 90 min with MDMΦ for 1 h at 37 °C under 5% CO₂ balance 95% air). Phagocytosis was assessed using either flow cytometry or histochemical detection of MPO.

6.2.3 Bovine blood

Samples of blood were obtained from twenty Holstein cows (age 220 ± 60 d, 12 female, 8 male) slaughtered at Gage's Abattoir (Gage's Farm, Ashburton, Devon, UK). The animals had no clinical symptoms of disease.

6.2.4 Cell isolation and culture

Both monocytes and neutrophils were isolated from bovine blood as previously described (Quinn *et al.*, 2007). The method involved lysis of contaminating red blood cells (RBC) with density centrifugation in Histopaque[®] 1077/1119 to obtain pure suspensions of neutrophils and monocytes. Briefly, 50 ml of blood was collected into Vacutainer[™] tubes containing EDTA. Whole blood was centrifuged at $500 \times g$ for 10 min at 4°C , and then Platelet Rich Plasma (PRP) was removed. The buffy coat, found at the plasma-red blood cell interface was collected, washed and re-suspended in DMEM at a concentration of 4×10^6 cells ml^{-1} (Fig. 6.2). Cell suspensions (monocytes/lymphocytes, 0.5 ml per well) were plated into individual wells in 24 well plates and incubated for 1 h (37°C , 5% CO_2 balance air) to allow monocyte adherence. Following 1 h incubation, tissue culture medium was removed and non-adherent cells including contaminating lymphocytes were removed by washing twice in DMEM.

The remaining RBC and leucocytes (mostly neutrophils) were split between three 50 ml centrifuge tubes, diluted with 5 ml of cold H_2O and gently mixed for about 2 min in order to lyse the RBC. Immediately after this period 2.5 ml of 0.2% NaCl was added to restore isotonicity. The suspension was centrifuged at $200 \times g$ for 5 min at 4°C , after which the supernatant was removed. These steps were repeated twice if necessary to lyse remaining RBCs. To obtain highly purified and functional neutrophils, the crude neutrophil suspension was layered above 10 ml of Histopaque[®] 1077/1119 mixture (Sigma-Aldrich, Poole, UK) and centrifuged at $440 \times g$ for 25 min at room temperature.

The pelleted neutrophils and any contaminating mononuclear cells remained at the sample/medium interface. The neutrophil pellet was washed in 50 ml HBSS and resuspended in DMEM medium (Fig. 6.2).

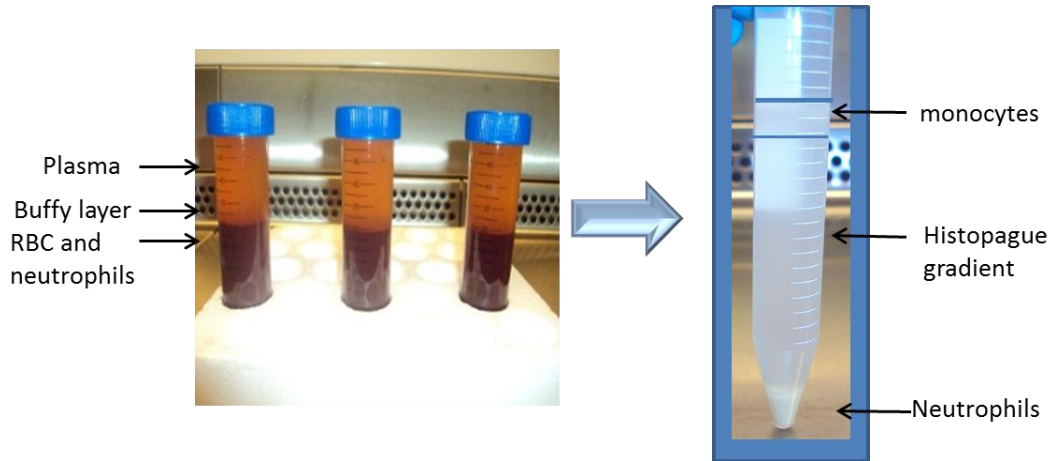


Fig. 6.2 Leukocyte isolation from freshly drawn bovine blood. Density centrifugation over Histopaque® 1077/1119 leads to pelleting of neutrophils and formation a monocyte cell band which can be harvested and washed for further analysis.

6.2.5 Generation of monocyte-derived macrophages (MDMΦ)

Monocytes differentiated into MDMΦ using fresh heat-inactivated autologous serum, is a good model for the *in vitro* study of phagocytosis of apoptotic neutrophils by macrophages. Monocytes (4×10^6 cells ml^{-1}) were cultured in DMEM (containing 2 mM glutamine, 100 U ml^{-1} penicillin and 100 $\mu\text{g ml}^{-1}$ streptomycin) and 10% (v/v) heat-inactivated bovine autologous serum (derived from platelet-rich plasma by recalcification with 20 mM CaCl_2 for 1 h at 37 °C in glass tubes) and plated (0.5 ml per well) in 24 well plates. The cells were incubated under 5% CO_2 (balance air). The medium was replaced every three days and the cells were used for experiments within seven days of plating.

6.2.6 Induction of apoptosis

Following isolation from peripheral blood using Histopaque[®] (section 6.2.4), neutrophils were cultured at 37 °C under 5% CO₂ (balance air) at a concentration of 5 × 10⁶ cells ml⁻¹ in DMEM. After treatment with different oxygen conditions (section 2.1.1), neutrophils were either used fresh or aged by transferring 0.5 ml aliquots of cell suspension in DMEM supplemented with 10% (v/v) autologous serum to each well of a 24-well plate and incubating for 22 h, at 37 °C under 5% CO₂ (balance air), during which time a proportion of the cells underwent apoptosis (Savill *et al.*, 1989). Cells were harvested at the indicated times and resuspended in DMEM (free serum) and analysed immediately.

6.2.7 Assay of neutrophil apoptosis by annexin V binding and propidium iodide with flow cytometry

The annexin V/PI cell apoptosis assay is dependent upon the exposure of PS on the surface of apoptotic cells. One of the major features of apoptosis is the loss of membrane asymmetry, which results in the translocation of PS (normally located on the inner membrane leaflet) to the outer membrane leaflet. The PS can be quantified by using a specific phospholipid binding protein, annexin V and flow cytometry (Van Engeland *et al.*, 1998). The conjugation with fluorescein isothiocyanate (FITC) allows identification of annexin V binding by flow cytometry, and is often used in conjunction with another stain such as propidium iodide (PI). Propidium iodide is a stain that will only penetrate the damaged membrane of dead cells and bind to their DNA, and hence will identify dead cells from viable and early apoptotic cells (Zhang *et al.*, 1999). Necrotic cells will stain with PI, while both of these stains will fail to stain live cells. In the early stages of apoptosis, cells will express PS and so will stain positive for annexin V, but will still have intact membranes, and so will exclude the PI stain. Neutrophils

were pelleted by centrifugation at $400 \times g$ for 5 min. This was done either immediately after isolation or after aging. Cells were then resuspended in DMEM, after which the percentage of apoptotic neutrophils was determined by flow cytometry. Cells were washed in cold PBS and resuspended at a concentration $1-2 \times 10^6$ cells in annexin V binding buffer. The cells in 100 μ l of annexin V binding buffer were pelleted, before addition of FITC-conjugated annexin V (5 μ l; eBioscience, Hatfield, UK). The tubes were incubated on ice in the dark for 15 min after which the cells were washed and resuspended in 100 μ l of annexin V binding buffer, after which PI (1 μ g) was added (5 μ l). Flow cytometric analysis of cell suspensions was performed by using an ArialTM II FACS (excitation 520 nm and emission 620 nm, Becton-Dickinson, San Jose, USA) within 1 h of staining. Data for 10,000 events was analysed for each sample using Becton-Dickinson FACS Diva software. Neutrophils were initially gated by their characteristic forward scatter (FSC) and side scatter (SSC) profiles. Cell debris was gated out before analysis, on the basis of FSC and SSC properties. Controls included to set up compensation and quadrants were (1) cells stained with FITC-labelled annexin-V alone and (2) cells stained with PI alone. Cells were defined as apoptotic (AV^+/PI^-), late apoptotic (AV^+/PI^+), dead (AV^-/PI^+), or viable (AV^-/PI^-). Each subpopulation was expressed as a percentage of the total population of neutrophils (Fig. 6.3).

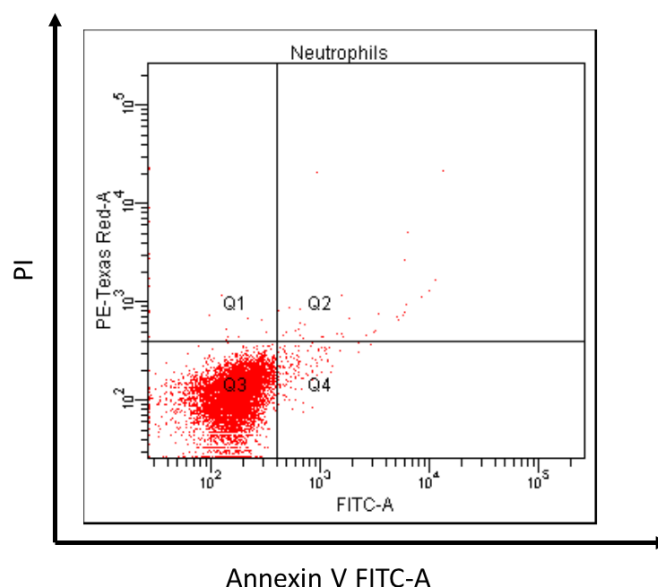


Fig. 6.3 Bovine neutrophils populations detected after staining with FITC-annexin V/PI. Neutrophils were stained with both FITC-annexin V and PI, and then analysed using flow cytometry. The plot of FITC-annexin V/PI fluorescence was then used to differentiate the cells in various stages of cell death. Neutrophils were initially gated by their characteristic forward scatter (FSC) and side scatter (SSC) profiles. Cell debris and residual macrophages were gated out before analysis on the basis of forward- and side-scatter properties. Controls included to set-up quadrants were: cells stained with FITC-labelled annexin-V alone and cells stained with PI alone. Dead cells were defined as Annexin V⁻ and PI⁺ (Q1); late apoptotic cells were annexin V⁺ and PI⁺ (Q2); viable cells were annexin V⁻ and PI⁻ (Q3); and early apoptotic cells were annexin V⁺ and PI⁻ (Q4).

6.2.8 Assay of DNA fragmentation in apoptotic neutrophils

During apoptosis, activated nucleases degrade the higher order chromatin structure of DNA into fragments (DNA laddering). This is considered to be an important marker of apoptotic cell death in many cell types. These fragments can be extracted from cells and visualized by agarose gel electrophoresis (Counis and Torriglia, 2000, Filipski *et al.*, 1990).

Determination of apoptotic cell death was confirmed by detection of DNA laddering using gel electrophoresis. Briefly, apoptotic neutrophils were collected and washed with PBS, and the DNA was extracted with a Wizard[®] genomic DNA purification kit (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Neutrophils were washed in PBS before addition of 150 µl of lysis buffer and subsequent mixing. Samples were then applied to the Wizard[®] SV mini-spin columns

(placed in collection tubes) and centrifuged for 3 min at $13,000 \times g$. The eluate was discarded and columns were transferred to fresh collection tubes. Wash solution (650 μ l) was then added to the columns/collection tubes and centrifugation carried out as before. The columns were again transferred to clean collection tubes and eluate discarded. Washing with 650 μ l of wash solution was repeated three times, giving a total of four washes of the minicolumns. To eliminate the possibility of wash solution carry over, collection tubes containing eluate were discarded and columns placed in fresh tubes for centrifugation at $13,000 \times g$ for 2 minutes. The Wizard[®] SV minicolumns were transferred to fresh 1.5 ml tubes and 250 μ l of room temperature, nuclease-free water added to each and incubated for 2 min before being centrifuged at $13,000 \times g$ for 1 min. DNA samples were stored at $-80 \text{ }^{\circ}\text{C}$ until use. DNA fragmentation in neutrophils was assessed qualitatively by agarose gel electrophoresis. Extracted DNA samples were loaded on to a 1% agarose gel, containing SYBR[®] Safe (1 \times) and 1 \times TAE (section 2.1.7.4) for 1 to 2 h at 90 V. The gel was visualized under UV a using a visualiser (UVitec Limited, England). Gel images were evaluated for low molecular weight DNA fragments separated in size by 180 to 200 base pairs, a hallmark of apoptosis (Wyllie *et al.*, 1980). DNA fragment sizes were determined by using DNA size marker (a mixture of fragments of known sizes; New England Biolabs, Hitchin, UK) providing molecular size markers of 0.5 to 12 kbp. A standard curve was plotted by measuring the distance migrated for each band on the gel (Fig. 6.4).

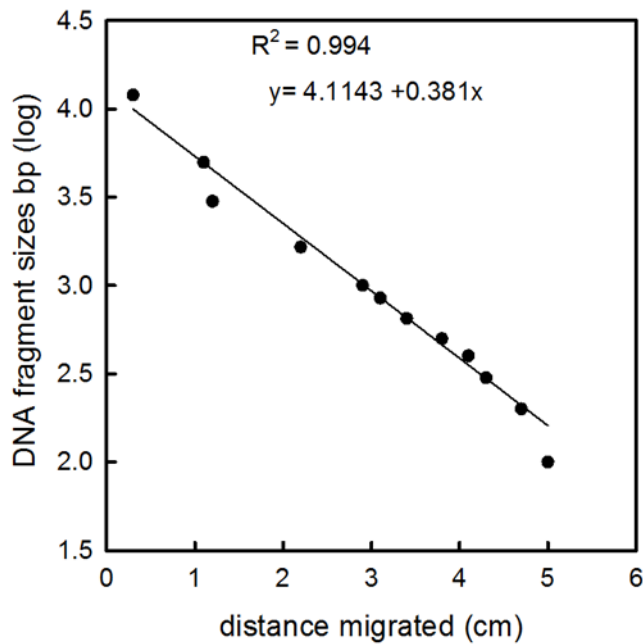


Fig. 6.4 DNA size marker was used to produce a standard curve. DNA size marker (0.5 to 12 kbp) was used to determine the DNA fragment sizes by measuring the distance migrated for each band on the gel.

6.2.9 Detection of phagocytosis of apoptotic cells by flow cytometry

For the flow cytometric phagocytosis assay, a previously described method was applied (Rossi *et al.*, 2006). Neutrophils were used as targets to assess MDM Φ phagocytosis by flow cytometry. Neutrophils were labelled with the fluorescent dye TrackerTM Green (5-chloromethyl fluorescein diacetate, CMFDA, 492 nm excitation and 516 nm emission, Invitrogen, Paisley, UK) at a final concentration of 2 $\mu\text{g ml}^{-1}$ and seeded in 24-well plates at 5×10^6 cells ml^{-1} for 15 min at 37 °C under 5% CO₂, balance air. Target cells were then collected for co-culture with phagocytes.

For co-culture, 0.5 ml of suspension containing 5×10^6 target cells was added to each MDM Φ -containing well (which had been washed with HBSS without Ca²⁺ and Mg²⁺ following 6-8 days differentiation, section 6.2.5), after which the cells were exposed to different conditions (section 2.1.1). Finally they were co-cultured at 37 °C under 5%

CO₂ (balance air) for 1 h. After incubation, non-ingested neutrophils and medium were removed from the wells by washing with PBS. The remaining cells, i.e. MDMΦ with ingested neutrophils, were detached from the plates using 300 μl of 0.25% trypsin/1 mM EDTA for 15 minutes at 37 °C followed by 15 minutes at 4 °C (to avoid any clumping of cells leading to blockage of the flow cytometer's sample intake nozzle), washed and resuspended in PBS and transferred to FACS tubes. In order to ensure that all the MDMΦ were removed from wells, plates were examined by light microscopy. Cells were analysed using an Arial™ II FACS (Becton-Dickinson San Jose, USA) using FACS Diva software[©] (Becton-Dickinson) with (excitation 492 nm/ emission 517 nm). All quadrants were set up according to matched controls including unstained MDMΦ and neutrophils stained with Tracker™ green alone.

Neutrophils and MDMΦ that had ingested apoptotic neutrophils were separated (gated) by FSC and SSC profile, and at least 10,000 events were counted in each sample within the MDMΦ gate. Uningested neutrophils were identified by the combination of their green fluorescence (FL-positive) due to Tracker™ green labelling and their relatively smaller size (FSC) when compared with the MDMΦ (Fig. 6.10). MDMΦ populations were gated based on FSC and SSC scatter characteristics, and MDMΦ demonstrating green fluorescence (FL-positive) were deemed to have ingested apoptotic neutrophils. The percentage of MDMΦ that had ingested neutrophils was calculated: the number of Tracker™ green positive events/total number of MDMΦ × 100 (Michlewska *et al.*, 2009). All results are reported as the percentage of total cells which were stained.

6.2.10 Detection of phagocytosis of apoptotic cells by light microscopy

This assay is based upon identification of MPO activity, a component of neutrophil primary granules. MDM Φ are fixed with glutaraldehyde and stained for MPO using H₂O₂ and dimethoxybenzidine as substrates. MPO activity leads to formation of a soluble yellow-brown product detectable by light microscopy (Hart *et al.*, 1997).

In order to assess phagocytosis by microscopy, MDM Φ were incubated with neutrophils as described in section 6.2.9. Following phagocytosis, medium was removed from each well and the wells were washed three times with ice-cold PBS to remove all non-engulfed neutrophils. Next, the cells were fixed with 2.5% glutaraldehyde in PBS for 10 minutes and stained for MPO activity using one part of 0.1 mg ml⁻¹ dimethoxybenzidine as substrate to one part of freshly prepared 0.03% (v/v) H₂O₂ in PBS. To achieve an appropriate level of staining, cells were incubated at 37 °C for 60 min at room temperature. Finally, uptake of apoptotic neutrophils by MDM Φ was assessed by light microscopy (Nikon, Japan). MDM Φ that had ingested neutrophils were MPO positive (yellow-brown reaction product), while MDM Φ which had not been MPO negative. Phagocytosis of apoptotic neutrophils was assessed by counting 500 cells per three replicate wells. The percentage of MDM Φ that had ingested different numbers of apoptotic neutrophils was expressed as phagocytosis percentage.

6.2.11 Measurement of TNF- α and IL-10 expression using qPCR

MDM Φ were cultured at 5×10^6 cells ml⁻¹ and stimulated with LPS (100 ng ml⁻¹) for 2 h before addition of neutrophils, and co-cultured for 1 h at 37 °C. They were then washed in PBS to remove non-ingested neutrophils. MDM Φ that had ingested apoptotic neutrophils were detached from plate (section 6.2.9), washed in PBS and pelleted. Total RNA was isolated, cDNA synthesised and qPCR for TNF- α and IL-10 genes was carried out (section 2.1.7.5). For gene expression measurements, neutrophils were

stimulated with LPS (100 ng ml⁻¹) for 2 h and total RNA was isolated and processed for qPCR as described above.

6.2.12 Statistical Analysis

Data are expressed as means \pm SEM. Statistical analysis was carried out using Statgraphics Centurion XVI software (Stat Point Technologies, Inc.). Following the Andersen-Darling and Levene's tests, data were analysed by two-way ANOVAs (parametric data) or Kruskal-Wallis (non-parametric). After two-way ANOVA, if the interaction was non-significant, Tukey's test was used. If the interaction was significant, the data for fresh and 22 h-aged neutrophils were further analysed separately by one-way ANOVA followed by Tukey's. A *P* value of < 0.05 is considered significant throughout.

6.3 Results

6.3.1 Cell purity after isolation

Isolated monocytes and neutrophils were assessed for purity using an Arial™ II FACS flow cytometer (Becton-Dickinson, San Jose, USA). Forward scatter (SSC-A) and side scatter (FSC-A) were used to identify cell populations based on size and granularity. Morphological analysis of Wright-Giemsa stained cytopsin preparations was also used (Figs 6.5A and B). Figure 6.5A shows the flow cytometer light scattering profiles of monocyte and neutrophil preparations, which were isolated using Histopaque® 1077/1119. Monocyte and neutrophil preparations were routinely above 95% purity. Figure 6.5B shows cytopsin preparations of monocytes and neutrophils visualized with modified Giemsa staining and light microscopy using a $\times 100$ oil immersion objective.

Neutrophils are characterized by the presence of multi-lobed nuclei and monocytes by large ‘folded’ nuclei.

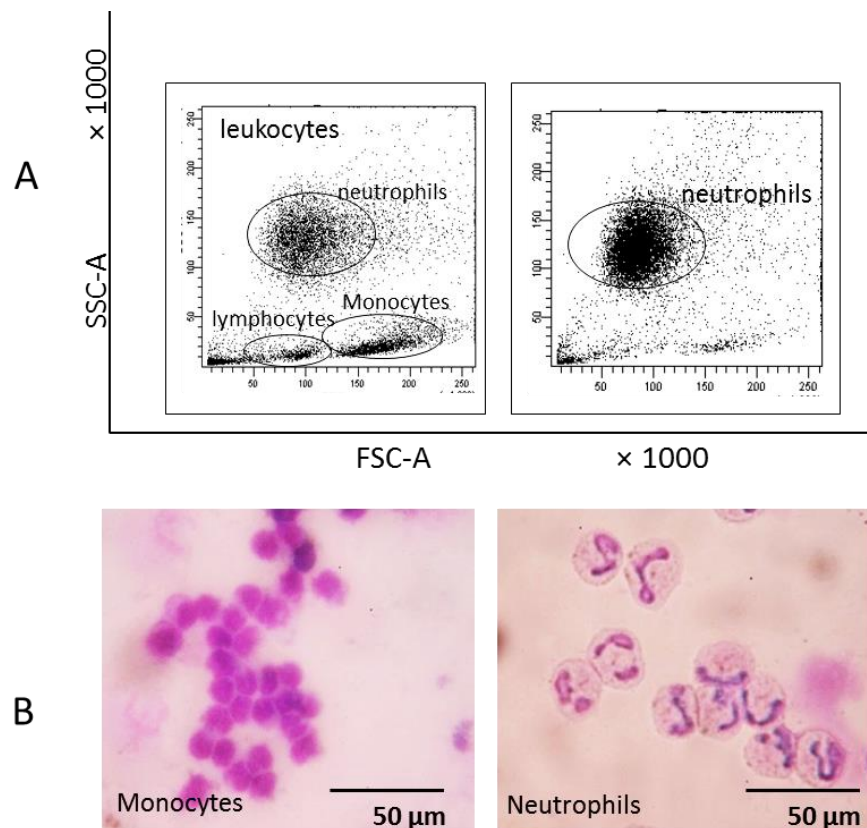


Fig. 6.5 Purity of monocytes and neutrophils. Monocytes and neutrophils were isolated from freshly drawn bovine blood by Histopaque[®] 1077/1119. Whole blood (left) and neutrophil (right) preparations were assessed for purity by flow cytometry (A) and cell morphology (B). Neutrophils are characterized by the presence of multi-lobed nuclei and monocytes by large ‘folded’ nuclei.

6.3.2 Effect of treatments on neutrophil viability

Cell viability using trypan blue and MTT assays was assessed in fresh neutrophils and 22 h-aged neutrophils and the results are presented in Figs. 6.6A and B. A significant interaction between time and treatment was found for trypan blue and MTT (two-way ANOVA, $P < 0.05$). There was a statistically significant decrease in the percentage cell viability in 22 h-aged neutrophils (two-way ANOVA, $P < 0.00005$ for trypan blue and MTT) compared with fresh neutrophils post different treatments. A significant decrease in viability was observed for both fresh and 22 h-age neutrophils pre-exposed to HBO, pressure, hyperoxia compared with normoxia (one-way ANOVA, $P < 0.05$ for trypan

blue and MTT). Hypoxia pre-treatment had no significant effect on the percentage cell viability in fresh and 22 h-aged neutrophils (98.8 ± 1.8 , $74.6 \pm 0.8\%$, trypan blue and 84.6 ± 1.9 , $64.1 \pm 5.0\%$, MTT for fresh and 22 h-aged neutrophils, respectively) compared to normoxia (Figs 6.6A and B).

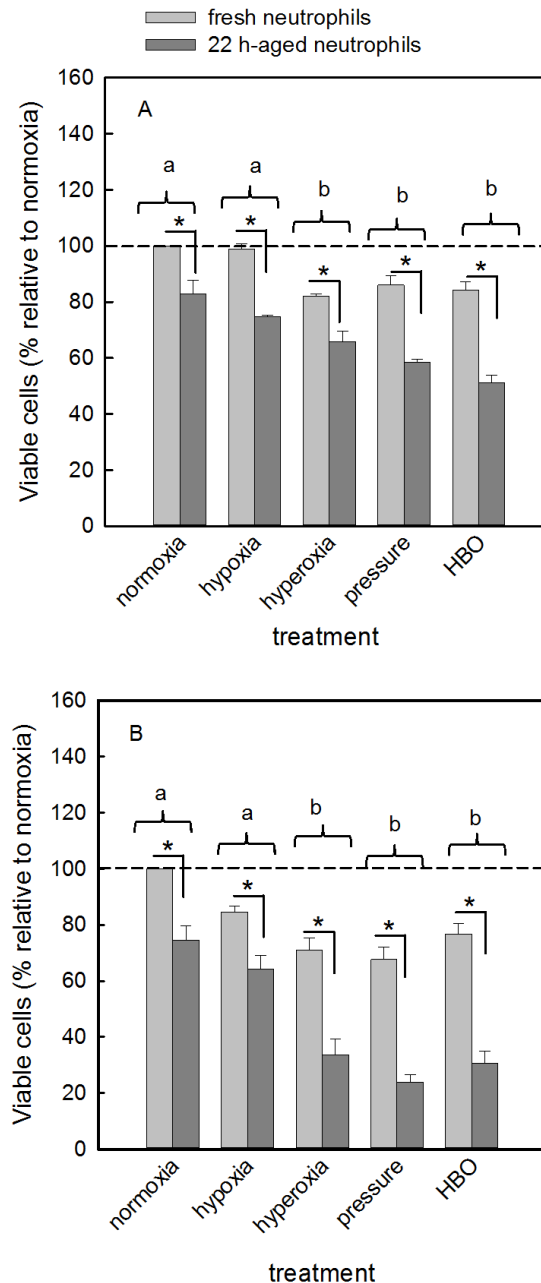


Fig. 6.6 Neutrophil cell viability (% relative to normoxia) after pre-treatment with hypoxia, hyperoxia, pressure and HBO. Neutrophils were isolated from bovine blood and exposed to a range of conditions for 90 min (section 2.1.1). After exposure, cells were washed, then immediately assayed or aged for 22 h to induce apoptosis. Cell viability was assessed using trypan blue (A) or MTT (B). Data are expressed as means \pm SEM for three separate experiments with triplicate measurements ($n = 3$). Results are normalised against viability of fresh neutrophils pre-treated with normoxia for both trypan blue and MTT assays; control values were ($86.5 \pm 19.5\%$ and 0.326 ± 0.057 , respectively) for both fresh and aged neutrophils. * indicate significant differences from fresh neutrophils. For each treatment bars with the different lower case letters are significantly different (ANOVA, $P < 0.05$).

6.3.3 Effect of treatments on apoptosis

The effects of different treatments on neutrophil apoptosis were assessed using several assays: FITC-annexin V/PI staining via flow cytometry, morphology using Giemsa staining and DNA fragmentation. Fig. 6.7A shows an example of neutrophils stained with FITC-annexin V/PI using fluorescence microscope after pre-treatment with normoxia (control). Cells staining with red fluorescence indicate late apoptosis, while cells staining with green fluorescence indicate early apoptosis. Fig. 6.7B shows the cell populations at different stages of death for both fresh and aged neutrophils after HBO treatment as measured using flow cytometric analysis. Following HBO treatment there was a significant increase in the cell population in late apoptotic (Q2) and early apoptotic (Q4).

Dual staining with FITC-labelled Annexin V and PI showed a significant decrease in the percentage of live and an increase in early apoptotic cells in fresh neutrophils assayed immediately after HBO or pressure treatments (one-way ANOVA, $P = 0.045$ and 0.02 , respectively) compared with normoxia (81.2 ± 3.4 and $10.4 \pm 1.5\%$ for live and early apoptosis, respectively; Fig. 6.8A). Both hyperoxia and hypoxia pre-treatments showed no significant effect on the levels of live cells (51.8 ± 12.7 and $84.5 \pm 3.5\%$, respectively) and early apoptotic cells (12.7 ± 1.8 and $8.0 \pm 2.1\%$, respectively). Also, there was no significant difference between treatments in the percentage of the cell population that was late apoptotic or dead compared to normoxia ($6.4 \pm 1.6\%$ late apoptotic cells and $2.7 \pm 1.4\%$ dead cells, Fig. 6.8A). The same pattern was seen with neutrophils aged for 22 h post different treatments. There was a significantly higher proportion of early apoptotic cells (Kruskal-Wallis, $P = 0.03$) and a corresponding decrease in live cells (Kruskal-Wallis, $P = 0.02$) post-HBO and pressure treatments compared with normoxia ($60.2 \pm 8.7\%$ live cells and $12 \pm 4.3\%$ early apoptotic cells, Fig. 6.8B), but not following hyperoxia ($40.6 \pm 9.0\%$ live cell and $20.3 \pm 5.7\%$ early

apoptotic cells) and hypoxia treatments ($70.4 \pm 11.5\%$ live cells and $11.8 \pm 4.2\%$ early apoptotic). Aged neutrophils also demonstrated no significant difference in the percentage of late apoptotic and dead cells (one-way ANOVA, $P > 0.05$) post treatments compared with normoxia ($12.0 \pm 2.1\%$ late apoptotic cells and $17.9 \pm 1.9\%$ for dead cells, respectively). To further assess the effects of different treatments on neutrophil apoptosis, morphological changes were analysed by light microscopy using modified Wright-Giemsa staining (Figs 6.9A and B). These changes include membrane blebbing but not loss of integrity, nuclear and cytoplasmic condensation and fragmentation, and formation of apoptotic bodies. There was a significant increase in morphological changes that are typically observed during apoptosis in 22 h-aged neutrophils compared to fresh neutrophils (two-way ANOVA, $P < 0.005$). A significant increase was observed in morphological changes relating to cell apoptosis in both fresh and 22 h-aged neutrophils after exposure to HBO, pressure and hyperoxia (two-way ANOVA, $P < 0.00005$; Fig. 6.9B), compared with normoxia ($15.1 \pm 1.2\%$, fresh neutrophils and $21.9 \pm 4.2\%$, aged neutrophils). As expected, the hypoxia treatment significantly decreased apoptosis of both fresh and 22 h-aged neutrophils compared with normoxia (Fig. 6.9B).

The cleavage of chromatin into multi-nucleosome sized fragments also indicates that cell death has occurred via apoptosis. Figs 6.10A and B show the DNA ladders observed on agarose gels for fresh and 22 h-aged neutrophils after treatments. The amount of fragmented DNA in 22 h-aged-neutrophils increased compared to fresh neutrophils. The only characteristic DNA profile detected in fresh neutrophils was immediately after HBO treatment confirming cell death via apoptosis (Fig. 6.10A). The degree of DNA fragmentation was more marked in 22 h-aged neutrophils after HBO, with less genomic DNA visible (Fig. 6.10B). However, lower but detectable DNA

fragmentation was observed in 22 h-aged neutrophils after pressure, hyperoxia and hypoxia treatments compared to normoxia (Fig. 6.10B).

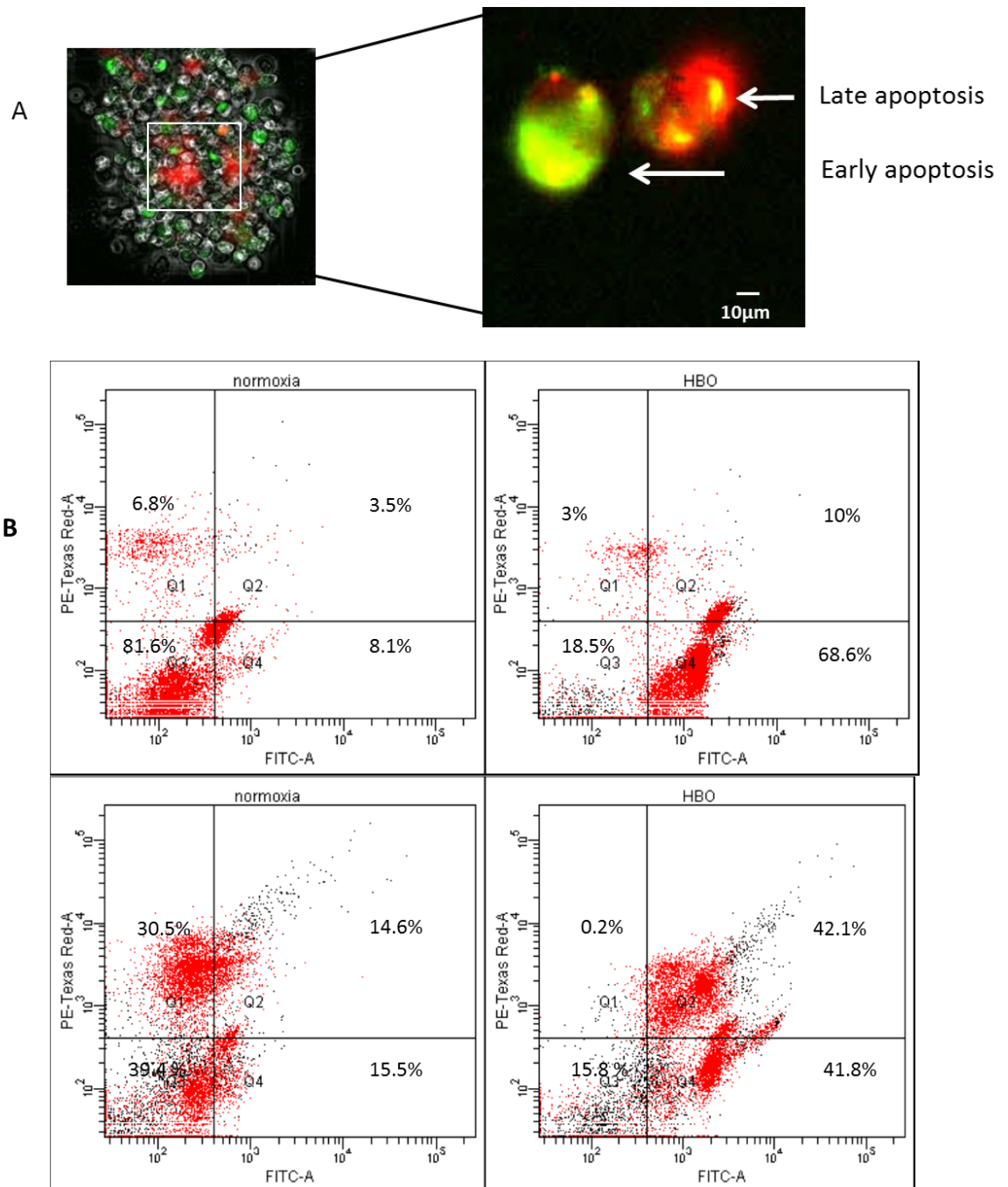


Fig. 6.7 Representative analysis of neutrophil morphology and cell populations assessed via flow cytometry. Neutrophil cell apoptosis was determined by staining of 22 h-aged neutrophils with FITC-annexin V (green) and PI (red) (section 6.2.8) using fluorescence microscopy (A) Neutrophils isolated from bovine blood were treated with normoxia or HBO or for 90 min. After exposure the cells were washed and then immediately assayed or aged for 22 h to induce apoptosis. Cells were stained with FITC-annexin V and PI, and analysed using flow cytometry (section 6.2.8) (B). Neutrophils were initially gated by their characteristic forward scatter (FSC) and side scatter (SSC) profiles. Cell debris and residual macrophages were gated out before analysis on the same basis. Controls included to determine quadrants were: cells stained with FITC-labelled annexin-V alone and cells stained with PI alone. Dead cells were defined as annexin V⁻ and PI⁺ (Q1); late apoptotic cells were annexin V⁺ and PI⁺ (Q2); viable cells were annexin V⁻ and PI⁻ (Q3); and early apoptotic cells were annexin V⁺ and PI⁻ (Q4).

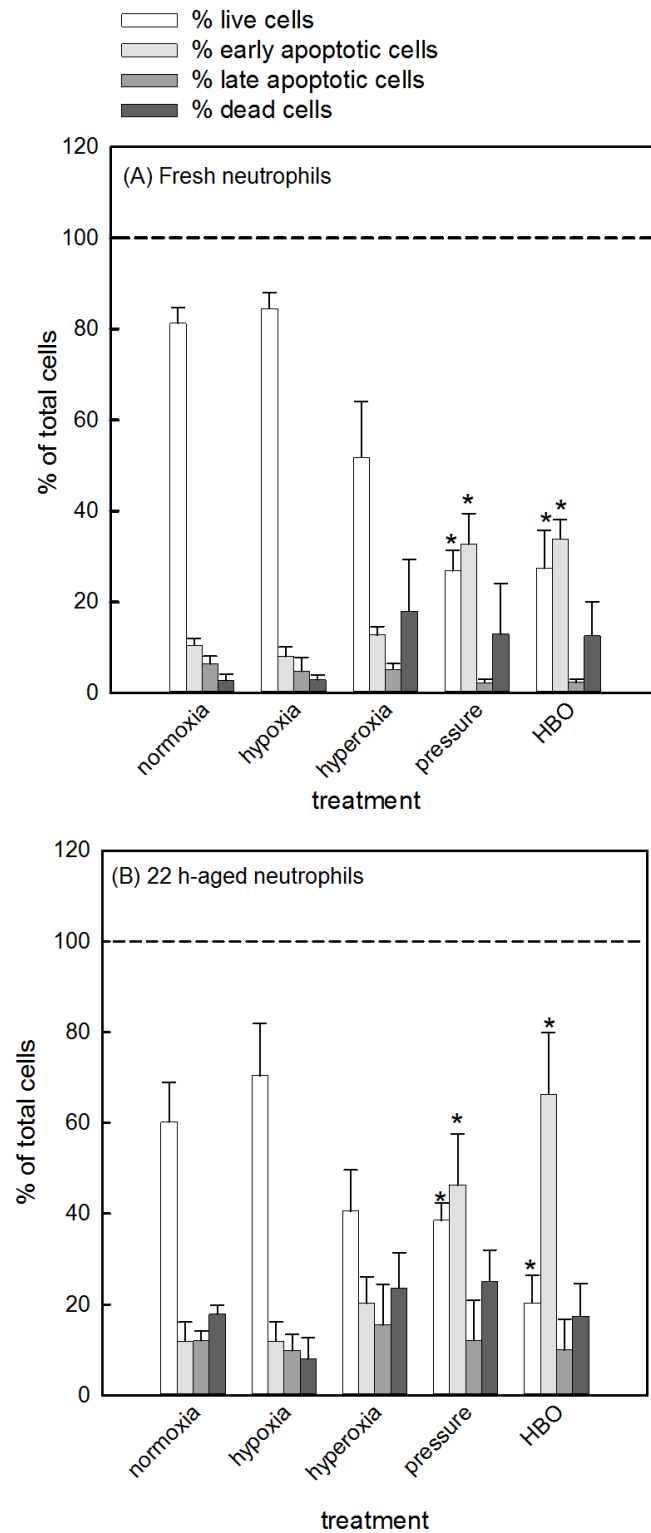


Fig. 6.8 Neutrophil populations after pre-treatment with normoxia, hypoxia, hyperoxia, pressure, and HBO. Neutrophils were isolated from bovine blood and exposed to a range of conditions for 90 min (section 2.1.1). After exposure, cells were washed, and then assayed immediately (fresh neutrophils) (A) or aged for 22 h to induce apoptosis (B). The proportions of dead, viable, late and early apoptotic neutrophils were assessed by flow cytometry after staining with both FITC-conjugated annexin V and PI (section 6.2.7). Data are expressed as means \pm SEM for three separate experiments ($n = 3$) with triplicate measurements. * significant difference versus normoxia (Kruskal-Wallis, $P < 0.05$).

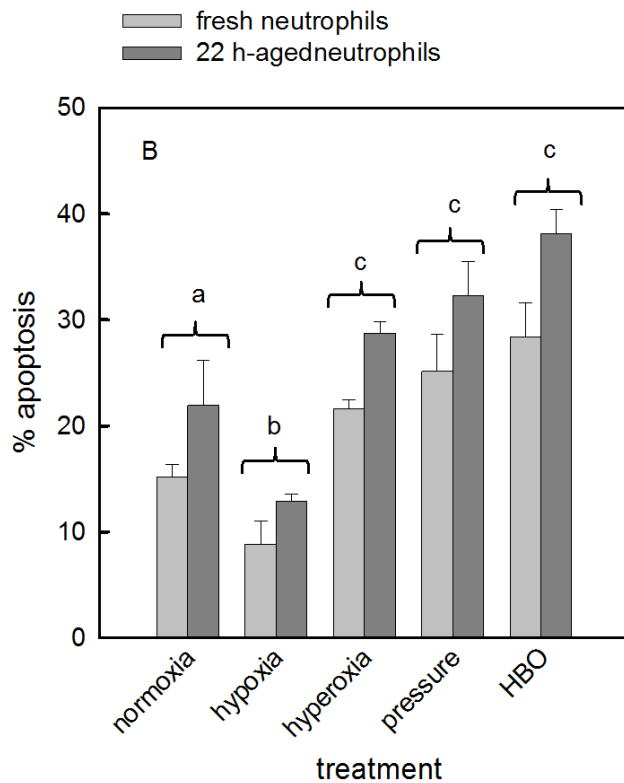
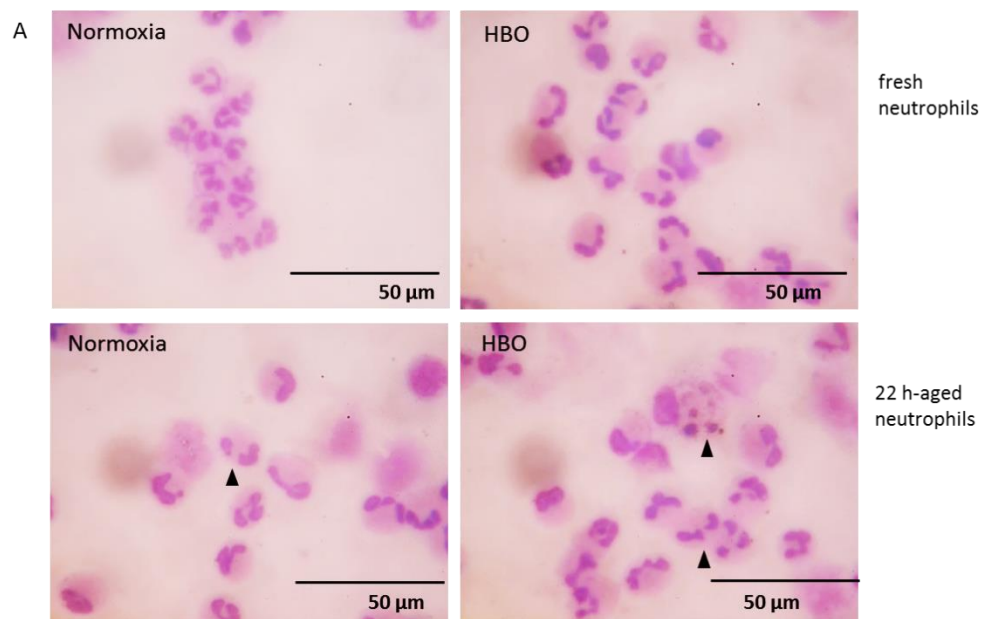


Fig. 6.9 Morphological analysis of apoptosis by light microscopy in neutrophils pre-treated with normoxia, hypoxia, hyperoxia, pressure and HBO. Neutrophils were isolated from bovine blood and exposed to a range of conditions for 90 min (section 2.1.1). After exposure, cells were washed, and then assayed immediately (fresh neutrophils) or aged for 22 h to induce apoptosis before assay. Cytospin preparations (500 g for 5 min) of neutrophils stained with Giemsa stain were used to analyse cell apoptosis morphology (A). Light microscopy of neutrophils (A) showing morphological changes in fresh and 22 h-aged neutrophils after exposure to normoxia and HBO. Neutrophils were characterised by their multi-lobed nuclei (upper left panel). Bold arrows indicate nuclear and cytoplasmic condensation and fragmentation. Cells were stained with Wright-Giemsa stain. In cells pre-treated with HBO there was a higher percentage of cells with typical features of apoptosis compared to those pre-treated with normoxia. Data are expressed as means \pm SEM for three separate experiments ($n = 3$) with triplicate measurements. For each treatment bars with the different lower case letters are significantly different (two-way ANOVA, $P < 0.05$; B).

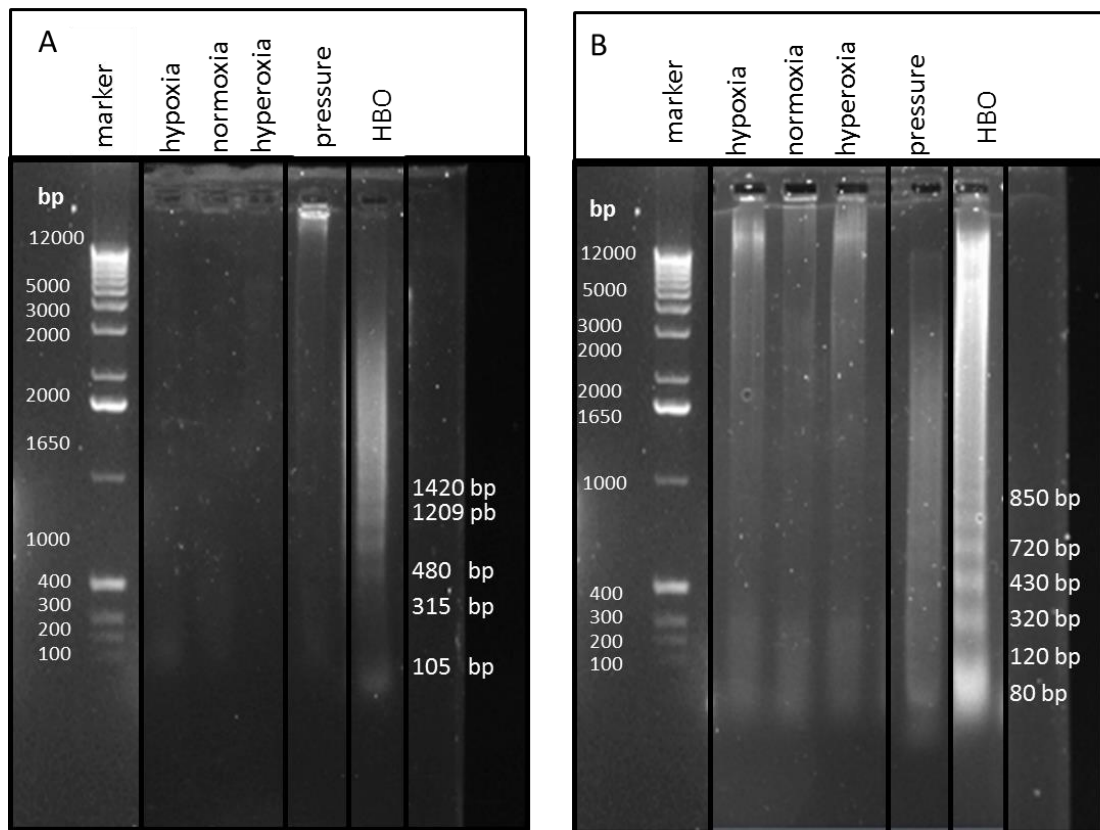


Fig. 6.10 An example of the electrophoretic ladder pattern of DNA fragmentation from neutrophils pre-treated with normoxia, hypoxia, hyperoxia, pressure and HBO. Neutrophils were isolated from bovine blood and exposed to a range of conditions for 90 min (section 2.1.1). After exposure, cells were washed, and then assayed immediately (fresh neutrophils) (A) or aged for 22 h to induce apoptosis before assay (B). The DNA was extracted from cells (section 6.2.8) run on 1% agarose gels containing SYBR[®] Safe (1×), and visualized under UV.

6.3.4 Effects of treatments on phagocytosis of neutrophils

Representative flow cytometric dot-plots demonstrating MDM Φ uptake of apoptotic neutrophils upon treatment with different treatments are presented in Fig. 6.11A. The populations of apoptotic neutrophils and MDM Φ that had ingested apoptotic neutrophils were gated into quadrants that match the controls (unstained MDM Φ and apoptotic neutrophils stained with TrackerTM green alone): TrackerTM green-stained apoptotic neutrophils (high fluorescence, low forward scatter, Q1); MDM Φ that had phagocytosed neutrophils (high fluorescence, high forward scatter, Q2); unstained neutrophils (low fluorescence, low forward scatter, Q3) and MDM Φ that had not ingested apoptotic neutrophils (low fluorescence, high forward scatter, Q4). Phagocytosis of fresh neutrophils was increased by HBO to 18.9% compared with normoxia (12.4%) and of aged neutrophils to 30.2% compared with normoxia (14.6%; Fig. 6.11A).

The percentage of MDM Φ that had ingested neutrophils labelled with TrackerTM green was assessed by flow cytometric analysis and the results are presented in Fig. 6.11B. As expected, phagocytosis of 22 h-aged neutrophils by MDM Φ was significantly increased (two-way ANOVA, $P < 0.005$) compared with fresh neutrophils. Immediately after HBO and pressure treatments, MDM Φ co-cultured with fresh and 22 h-aged neutrophils were characterized by a significantly increased uptake of neutrophils (two-way ANOVA, $P < 0.00005$) compared to normoxia ($6.8 \pm 0.8\%$ fresh neutrophils and $12.9 \pm 0.9\%$ 22 h-aged neutrophils; Fig. 6.11B). No differences in MDM Φ phagocytosis of fresh and 22 h-aged neutrophils were observed after hyperoxia ($10.9 \pm 3.3\%$ and $22.0 \pm 2.2\%$, respectively) and hypoxia ($8.3 \pm 1.0\%$ and 10.4 ± 1.7 , respectively) pre-treatments compared with normoxia ($6.8 \pm 0.9\%$ and $12.9 \pm 0.9\%$, respectively, Fig. 6.11B).

Phagocytosis of apoptotic cells by MDM Φ was further confirmed by light microscopic examination of cytocentrifuged preparations of MDM Φ after the phagocytosis assay had been performed. This analysis clearly showed the presence of MPO-positive cells inside MDM Φ (Fig. 6.12A). Quantification of % phagocytosis confirmed the FACS data in that MDM Φ were shown to be more able to uptake 22 h-aged neutrophils than fresh neutrophils following different treatments (two-way ANOVA, $P = 0.009$). In common with the flow cytometric results, MDM Φ exhibited a higher capacity to phagocytose fresh and 22 h-aged neutrophils immediately after HBO and pressure treatments (two-way ANOVA, $P < 0.00005$) compared with normoxia ($29.9 \pm 1.3\%$ and $34.6 \pm 2.4\%$ for fresh and 22 h-aged neutrophils, respectively). Exposure to hypoxia did not produce a significant change in percentage phagocytosis of neutrophils ($22.3 \pm 2.3\%$ and $29.2 \pm 1.6\%$ for fresh and 22 h-aged neutrophils, respectively) compared with normoxia (Figs 6.12A and B).

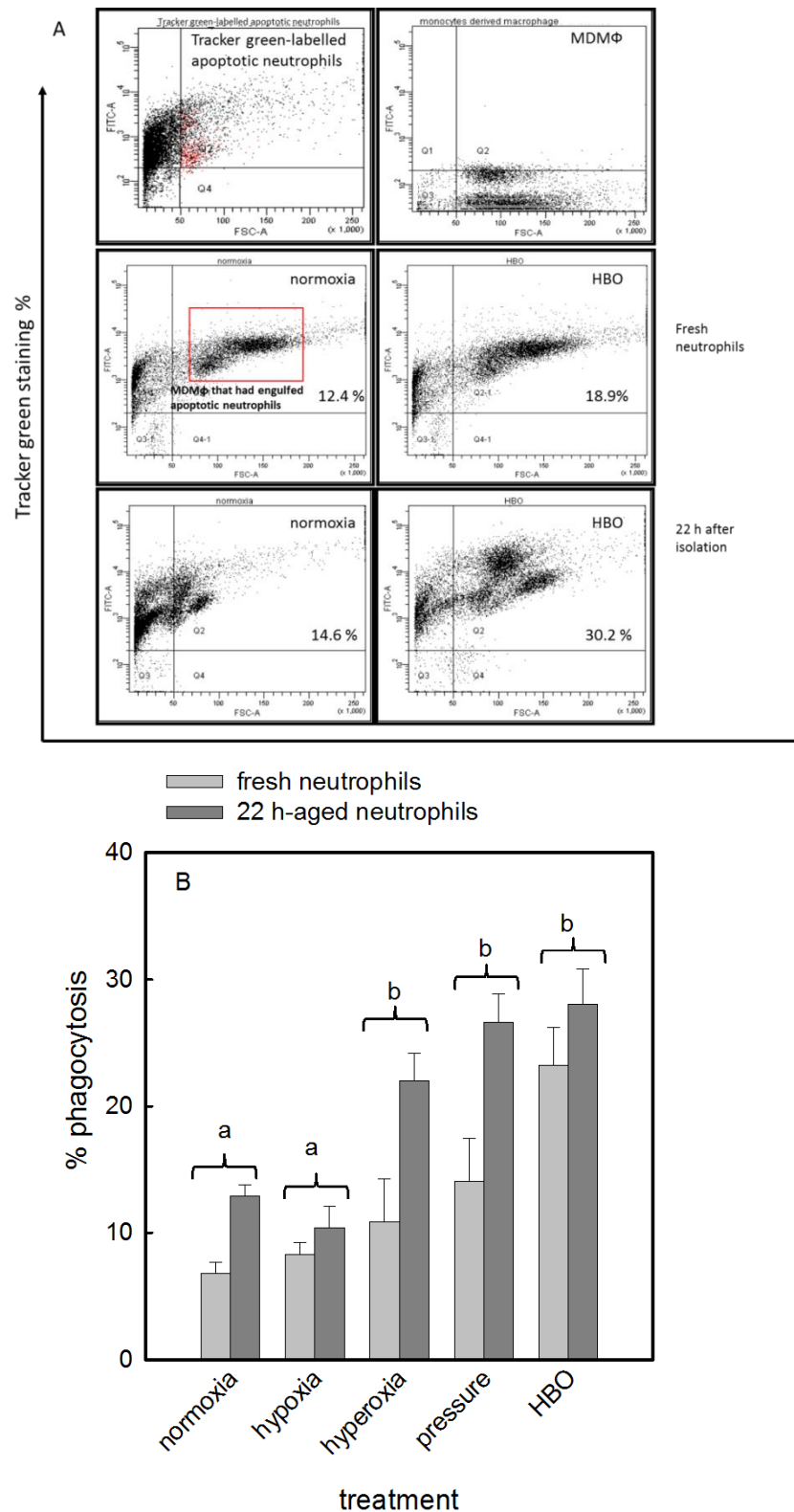


Fig. 6.11 Flow cytometry analysis of phagocytosis of neutrophils by MDMΦ after pre-treatment of the neutrophils with normoxia, hypoxia, hyperoxia, pressure and HBO. Neutrophils (either fresh or 22 h aged) were labelled with Tracker™ Green and co-cultured with MDMΦ, after which they were exposed to a range of conditions for 90 min (section 2.1.1). Cells were washed and analysed using flow cytometry. Examples of flow cytometry dot blots demonstrating MDMΦ that have or have not engulfed neutrophils for fresh and 22 h-aged neutrophils after exposure to HBO or normoxia (A). The results are presented as the percentage of MDMΦ that had ingested neutrophils (B). Data are expressed as means \pm SEM for three separate experiments ($n = 3$) with triplicate measurements. For each treatment bars with different lower case letters are significantly different (two-way ANOVA, $P < 0.05$).

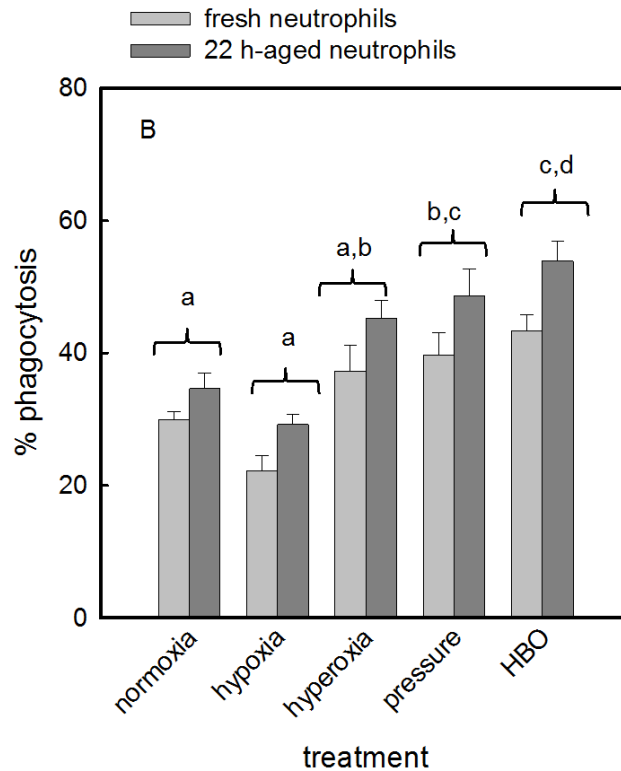
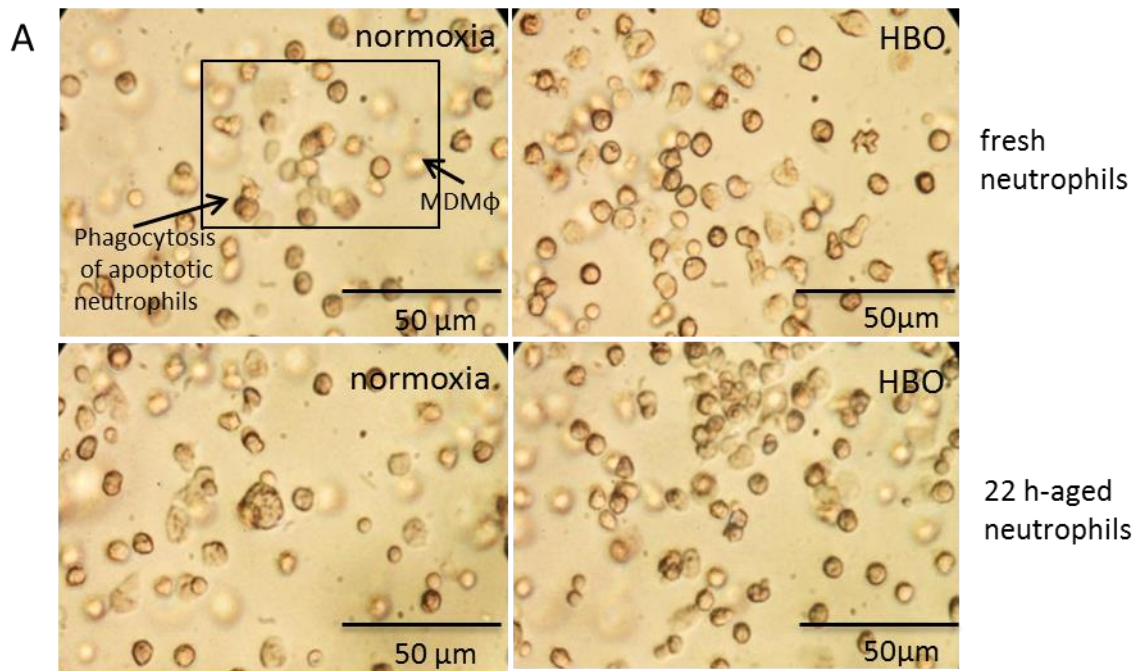


Fig. 6.12 Light microscopic detection of phagocytosis of neutrophils by MDMΦ after pre-treatment of the neutrophils with normoxia, hypoxia, hyperoxia, pressure and HBO. Fresh or 22 h-aged neutrophils were co-cultured with MDMΦ and exposed to a range of conditions for 90 min (section 2.1.1). After exposure, cells were washed, fixed and stained for MPO, and then were analysed using light microscopy. Light microscopic analysis demonstrating MDMΦ that had engulfed apoptotic neutrophils for both fresh and 22 h-aged neutrophils after exposure to HBO or normoxia. MDMΦ that had ingested neutrophils contain a yellow-brown reaction product (MPO-positive cells). MDMΦ that had not ingested neutrophils were MPO-negative (A). Results are presented as the percentage of MDMΦ that had ingested neutrophils. Data are expressed as means \pm SEM for three separate experiments ($n = 3$) with triplicate measurements. For each treatment bars with different lower case letters are significantly different (two-way ANOVA, $P < 0.05$; B).

6.3.5 Oxidants mediate neutrophil clearance by MDM Φ : possible effects of HBO

The H₂O₂ concentrations in the supernatants from PMA-stimulated fresh or 22 h-aged neutrophils cultured alone or after co-culture of these with MDM Φ are shown in Fig. 6.13A. H₂O₂ levels showed a significant increase in fresh neutrophils and 22 h-aged neutrophils post treatment with HBO, pressure and hyperoxia (two-way ANOVA, $P < 0.00005$) compared with normoxia (22.0 ± 0.5 and 18.7 ± 0.1 nmol per 10^6 cells for fresh and 22 h-aged neutrophils, respectively). In contrast, hypoxia pre-treatment had no effect on H₂O₂ levels in fresh and 22 h-aged neutrophils (26.3 ± 0.8 and 20.6 ± 2.3 nmol per 10^6 cells, respectively; Fig. 6.13A) compared with normoxia.

HBO and pressure pre-treatments gave a significant increase in H₂O₂ levels in MDM Φ that had ingested fresh or 22 h-aged neutrophils (two-way ANOVA, $P < 0.00005$) compared with normoxia (27.5 ± 0.6 and 35.1 ± 2.8 nmol per 10^6 cells in MDM Φ that had ingested fresh and 22 h-aged neutrophils, respectively). No treatment effects were observed on H₂O₂ levels in co-cultures of MDM Φ with fresh or 22 h-aged neutrophils after pre-treatment to hyperoxia (31.3 ± 0.7 and 41.5 ± 0.4 nmol per 10^6 cells, respectively) and hypoxia (26.0 ± 1.3 and 38.0 ± 0.2 nmol per 10^6 cells, respectively, Fig. 6.13B).

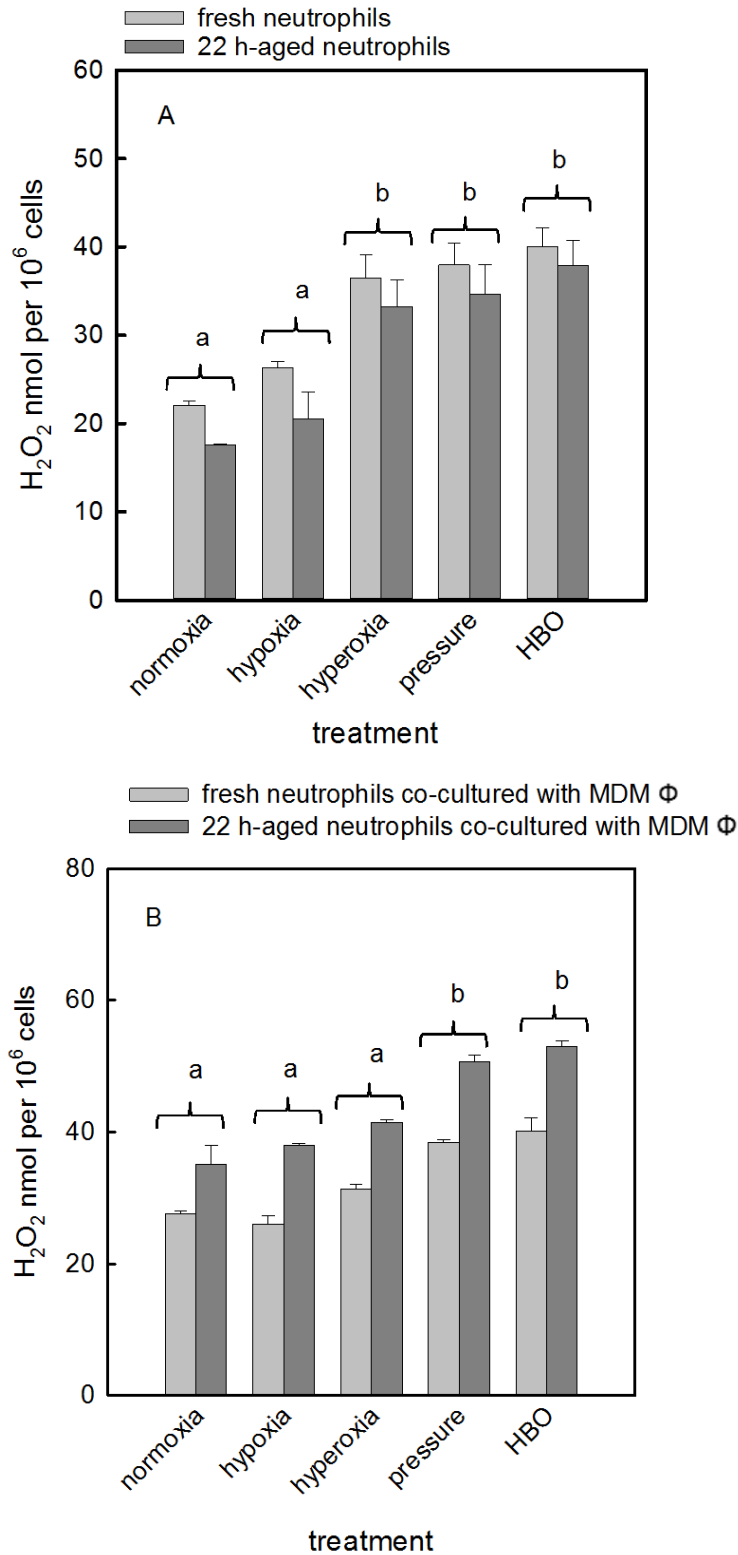


Fig. 6.13 H₂O₂ release by isolated neutrophils and MDMΦ that had ingested neutrophils after exposure to normoxia, hypoxia, hyperoxia, pressure and HBO. Neutrophils were isolated from bovine blood exposed to a range of conditions for 90 min (section 2.1.1). After exposure, fresh or 22 h-aged neutrophils were assayed after stimulation with 100 ng ml⁻¹ PMA (A). For co-culture experiments (B) fresh or 22 h-aged neutrophils were stimulated with PMA, incubated with MDMΦ and exposed to a range of conditions for 90 min. H₂O₂ levels in the medium were determined in both neutrophils and MDMΦ that had ingested neutrophils using HRP/homovanillic acid (section 2.1.3). These are expressed as the amount of H₂O₂ produced per 10⁶ cells. Data are means ± SEM for three separate experiments (*n* = 3) measured in triplicate. For each treatment bars with different lower case letters are significantly different (two-way ANOVA, *P* < 0.05).

6.3.6 Effects of HBO on TNF- α and IL-10 gene expression following phagocytosis of apoptotic neutrophils

It has been proposed that clearance of apoptotic neutrophils results in a non-inflammatory response (Fox *et al.*, 2010). Therefore the functional consequences of phagocytosis of apoptotic neutrophils by MDM Φ were investigated by measuring the cytokine gene expression (TNF- α and IL-10) of fresh neutrophils, 22 h-aged neutrophils and MDM Φ that had ingested neutrophils following different treatments and stimulation with 100 ng ml⁻¹ LPS using qPCR. For fresh and 22 h-aged neutrophils cultured alone, the data were normalised against fresh neutrophils under normoxia (Figs 6.14A and B) while co-culture data were normalised against MDM Φ cultured alone (Figs 6.14C and D). HBO, pressure and hyperoxia significantly up-regulated the gene expression of TNF- α in fresh and 22 h-aged neutrophils (two-way ANOVA, $P < 0.00005$; Fig. 6.14A) compared to normoxia (1.0 ± 0.0 and 1.5 ± 0.2 fold changes for fresh and 22 h-aged neutrophils, respectively). Hypoxia pre-treatment had no effect on gene expression of TNF- α in fresh and 22 h-aged neutrophils (1.3 ± 0.4 and 2.1 ± 1.1 fold changes, respectively).

A significant interaction was found between different treatments and time (fresh and 22-h aged neutrophils) for IL-10 expression (two-way ANOVA; $P < 0.009$). Because of the interaction, the data were further analysed using one-way ANOVA. The only significant increases in IL-10 gene expression were observed with fresh and 22 h-aged neutrophils after HBO treatment (one-way ANOVA, $P < 0.05$; Fig. 6.14B) compared with normoxia (1.0 and 0.29 ± 0.08 fold for fresh and 22 h-aged neutrophils, respectively).

A significant interaction was found between different treatments and time (fresh and 22h-aged neutrophils) for IL-10 in MDM Φ which had ingested fresh and 22 h-aged neutrophils (two-way ANOVA; $P = 0.008$). Following MDM Φ stimulation with 100 ng

ml⁻¹ LPS and co-culture with fresh neutrophils for 1 h at 37 °C for quantification of phagocytosis, no change was found in TNF- α gene expression in MDM Φ that had ingested fresh and aged neutrophils after different treatments (two-way ANOVA, $P < 0.05$; Fig. 6.14C) compared with normoxia (1.73 ± 0.34 for fresh neutrophils and 0.99 ± 0.02 for 22 h-aged neutrophils fold induction compared with MDM Φ).

There was a significant increase in IL-10 gene expression in MDM Φ that had ingested fresh and 22 h-aged neutrophils after pre-treatment with HBO, pressure and hyperoxia (two-way ANOVA, $P = 0.0008$; Fig. 6.14D) compared with normoxia (3.07 ± 1.17 for fresh neutrophils and 6.4 ± 1.2 for 22 h-aged neutrophils fold induction compared with MDM Φ). Because of the interaction, the data were further analysed using one-way ANOVA. The only significant increases in IL-10 gene expression were observed with fresh neutrophils following hypoxia (one-way ANOVA, $P = 0.047$). No change was found in IL-10 expression in MDM Φ that had ingested fresh neutrophils following HBO, pressure and hyperoxia (7.07 ± 2.41 , 0.94 ± 0.21 and 4.3 ± 1.4 fold induction compared with MDM Φ) compared with normoxia (3.07 ± 1.17 fold induction compared with MDM Φ). A significant increase in IL-10 was found in MDM Φ that had ingested 22 h aged neutrophils following exposure to HBO, pressure and hyperoxia (one-way ANOVA, $P = 0.0008$). Exposure to hypoxia had no effect on IL-10 gene expression in MDM Φ that had ingested 22 h-aged neutrophils (8.79 ± 2.41 fold induction compared with MDM Φ ; Fig. 6.14D) compared to normoxia (6.43 ± 1.23 fold induction compared with MDM Φ).

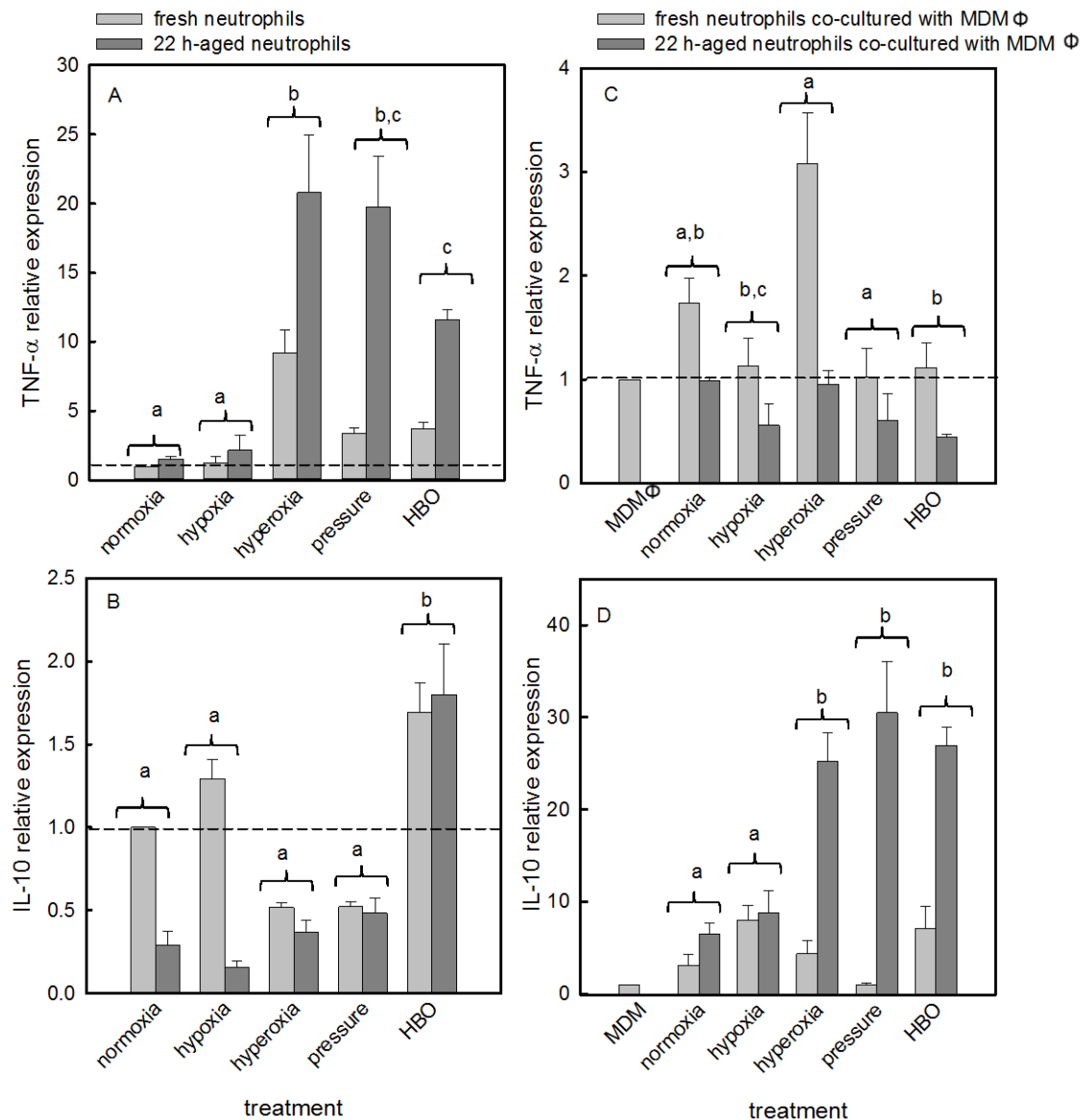


Fig. 6.14 TNF- α and IL-10 expression by neutrophils and MDM Φ that had ingested neutrophils after treatment with normoxia, hypoxia, hyperoxia, pressure and HBO. Neutrophils were isolated from bovine blood exposed to a range of conditions for 90 min (section 2.1.1). After exposure, cells were washed and either used immediately (fresh neutrophils) or after 22 h of aging. Cells were stimulated with 100 ng ml⁻¹ LPS and assayed (A and B) or co-cultured with MDM Φ (C and D). Neutrophils co-cultured with MDM Φ were exposed to a range of conditions for 90 min (section 2.1.1). qPCR was used to determine RNA expression under different treatments, and expression was normalised against endogenous controls. Gene expression under different treatments is expressed as a fold change in expression versus fresh neutrophil under normoxia (A and B) or MDM Φ cultured alone (C and D). Data are means \pm SEM for three separate experiments ($n = 3$) measured in triplicate. For each treatment bars with different lower case letters are significantly different (two-way ANOVA, $P < 0.05$).

6.4 Discussion

It was not clear from *in vitro* studies whether HBO could alter the ability of MDM Φ to phagocytose the apoptotic neutrophils, as this process is seen as an important step in the resolution of inflammation and the progress of wound healing. The hypothesis behind the present study was that oxidative stress contributes to the apoptosis of neutrophils and that this perhaps augments macrophage clearance mechanisms. Flow cytometric analysis using FITC-annexin V/PI, assessment of morphological changes, and DNA fragmentation assays demonstrated that HBO and pressure induced apoptosis in both fresh and 22 h-aged neutrophils after exposure. Studies of this type should be conducted with care as cells can display morphological features of apoptosis without DNA fragmentation and it is also possible that DNA fragmentation assays give false positive results during necrosis (Yasuhara *et al.*, 2003, Grasl-Kraupp *et al.*, 1995). Therefore, it is important that at least two different methods are used in the quantification of apoptosis, to confirm the data and to ensure the different stages of apoptosis are investigated. In this chapter, cell viability was assessed using trypan blue and MTT reduction, although these assays give only a crude indication of cell states, and are not able to indicate early apoptosis of cells. Hence, dual staining with annexin V and PI was used to identify cells that were undergoing early or late apoptosis, or necrosis, and so to give the whole progression of cell death after different treatments.

There was an increase in the proportion of neutrophils displaying morphological features characteristic of apoptosis including nuclear chromatin condensation (observed by light microscopy) and DNA fragmentation over 22 h of aging. Flow cytometric assays with fresh or 22 h-aged neutrophils demonstrated a high percentage of early apoptotic cells after HBO and pressure treatments. Despite differences in relative methods, all three assays showed a significant increase in the percentage of apoptotic

neutrophils after exposure to HBO with the greater apoptotic response detected in 22 h-aged neutrophils. These data are in agreement with the observations described in Chapter 5 with neutrophil-like cells. Previous studies have demonstrated that HBO is able to induce apoptosis *in vitro* in cultures of Jurkat-T cells (Ganguly *et al.*, 2002, Chen *et al.*, 2007b), HL-60 cells (Ganguly *et al.*, 2002), NCI-H929 cells (Chen *et al.*, 2007b) and murine thymocytes (Ganguly *et al.*, 2002).

The present work is the first to demonstrate the effects of HBO in inducing apoptosis in bovine neutrophils. Subsequently, several assays were conducted to investigate the mechanisms involved in HBO-induced apoptosis of neutrophils. One of the mechanisms proposed for the enhanced spontaneous neutrophil apoptosis in this study, is that neutrophil-induced apoptosis is a function of oxygen partial pressure. HBO has been shown to up-regulate NADPH oxidase activity and caspase-3/7 activity, which suggests that HBO-induced ROS production may enhance apoptosis, resulting in the formation of pro-apoptotic substances in the cells or in the medium (Matsunami *et al.*, 2011). It is well known that mitochondrial-generated ROS are involved in the release of cytochrome *c* and other pro-apoptotic proteins from mitochondria, therefore HBO-induced apoptosis could be via the mitochondrial pathway (Weber *et al.*, 2009a). Neutrophils have at least semi-functional mitochondria. This idea of semi-functionality is mainly based on the observation that mitochondrial poisons such as cyanide do not influence cellular functions in neutrophils (Borregaard and Herlin, 1982). Moreover, electron microscopy studies have identified hardly any mitochondria in neutrophils (Fossati *et al.*, 2003) with very low mitochondrial respiration in these cells (Peachman *et al.*, 2001). However, the mitochondria has been recently visualised as a tubular network by specific fluorescent dyes (Maianski *et al.*, 2002, Fossati *et al.*, 2003). Also, in neutrophils undergoing spontaneous or induced apoptosis, the mitochondria form clusters to where pro-apoptotic Bax protein is localized to produce permeabilization of

the outer mitochondrial membrane with subsequent release of additional pro-apoptotic proteins (Maianski *et al.*, 2002, Pryde *et al.*, 2000). Thus the exogenous H₂O₂ released by the activity of xanthine oxidase or glucose oxidase, induces apoptosis in neutrophils (Rollet-Labelle *et al.*, 1998). Also, endogenous production of ROS has been shown to be involved in the activation of apoptosis by Fas and TNF- α (Kasahara *et al.*, 1997, Scheel-Toellner *et al.*, 2004, Van Den Berg *et al.*, 2001). This agrees with the results presented here; HBO and pressure enhanced H₂O₂ production in both fresh and 22 h-aged neutrophils post exposure, which in turn could have enhanced the neutrophil apoptosis.

Other mechanisms could explain the results of the FITC-annexin V/PI assay. Annexin V binds to PS that has been externalised on the surface of cells undergoing apoptosis. Exposure to pressure could have caused deformation which leads to conformational changes that might enhance PS translocation to the outer leaflet of the cell membrane in order to be recognised by annexin V. In the case of pressure alone, the present results showed an increase in cell apoptosis and a decrease in cell viability in fresh neutrophils and 22 h-aged neutrophils after exposure. Neutrophils aged for 22 h showed a significant increase in apoptosis and a decrease in viability after exposure to HBO. In contrast, the study by Weber *et al.* (2009b) demonstrated that 24 h after exposure to HBO (30 min at 2.8 ATA), there was no increase in apoptosis in mononuclear cells. A possible explanation of the differences observed between this study and the current study may be due to differences in exposure time and cell type used, as the behaviour of neutrophils in terms of the effect of oxygen tension on apoptosis is rather different to that observed in other cells (Nathan, 2003).

Pre-treatment with hypoxia did not affect neutrophil apoptosis (fresh or 22 h-aged) and maintained cellular viability. These observations are in line with the previous studies using neutrophil-like cells as described in Chapter 5.

An important functional characteristic of macrophages is their ability to ingest apoptotic cells. Interestingly, the present data clearly indicate that HBO enhanced MDM Φ ingestion of apoptotic neutrophils. MDM Φ exhibited a higher capacity to ingest fresh neutrophils after HBO exposure, which confirmed the apoptotic effects of HBO that results in a high percentage of early apoptotic neutrophils that are targeted for phagocytosis. The morphological study showed the same pattern, although the differences in the percentages of phagocytotic cells observed in the flow cytometry assay and the morphological study was large. Several factors may have contributed to this difference, in particular that the data presented are from separate experiments with cells derived from different animals, and the assays were not performed in a side-by-side comparison. In addition, no studies to date have investigated the effects of HBO on neutrophil clearance. Shiratsuchi and Basson (2004) found that increasing extracellular pressure to 20 mm Hg above ambient pressure stimulated phagocytosis of serum-opsonized latex beads by PMA-stimulated THP-1 macrophages. These effects seemed to be due to inhibition of FAK-Y397 autophosphorylation and consequent inhibition of ERK activation. Similarly, the same authors demonstrated that exposure to pressure 20 mm Hg above ambient pressure for 2 h also increased the phagocytosis of latex beads by monocytes that were isolated from human blood or MDM Φ prepared by culturing with autologous unheated serum. These effects were mediated by p38 MAPKs which are activated by cellular stress and microbial phagocytosis in macrophages (Bertram *et al.*, 1997). In the present study, cells were treated with HBO or pressure alone at 2.2 ATA for 90 min. The flow cytometric analysis and light microscopy study have confirmed that HBO treatment affects MDM Φ phagocytosis. The pressure utilized during HBO may have contributed to the phagocytosis percentage increase because hyperoxia treatment did not show the same effects as HBO treatment.

In addition, it is possible that oxidants may be responsible for an increase in phagocytosis of apoptotic cells, due to ROS generation during HBO treatment. Oxidants have been implicated in apoptosis associated with ageing neutrophils (Oishi and Machida, 1997, Kasahara *et al.*, 1997). Therefore, treatments that stimulate oxidant release appear to accelerate this process (Kettritz *et al.*, 1997, Harper *et al.*, 2000). The present study suggests that functional NADPH oxidase may have a role in PS exposure in activated neutrophils as well as in their clearance by MDM Φ . In the present study, a significant increase in H₂O₂ was observed after HBO, pressure and hyperoxia exposure in fresh and 22 h-aged neutrophils. Furthermore, the increase was correlated with enhanced neutrophil apoptosis, the process necessary for clearance of neutrophils. These results are in agreement with the work reported in Chapters 4 and 5, that indicated an increase of ROS and apoptosis in neutrophil-like cells post a 90-min HBO exposure. Recent studies have identified the ability of H₂O₂ to induce apoptosis in neutrophils (Rollet-Labelle *et al.*, 1998, Lundqvist-Gustafsson and Bengtsson, 1999). Phosphatidyl serine exposure on apoptotic neutrophils can occur as a result of H₂O₂ production, enabling their uptake and clearance by macrophages (Hampton *et al.*, 2002). Arroyo *et al.* (2002) found that NADPH oxidase-induced oxidative stress in neutrophil-like cells initiated apoptosis and subsequent recognition and phagocytosis of these cells via pathways dependent on oxidation and externalization of PS. Therefore, the H₂O₂ generated by both fresh neutrophils and 22 h-aged neutrophils following exposure to HBO and pressure has two functions, firstly to serve as a substrate for MPO to generate other microbiocidal agents, and secondly to trigger pathways that are needed for the clearance of neutrophils. These observations were confirmed by a marked increase in H₂O₂ levels in MDM Φ that had ingested fresh and 22 h-aged neutrophils following HBO treatment. These findings are consistent with the previous suggestion of Fadeel *et al.* (1998) that PS externalisation in neutrophils may occur through two pathways for: a

caspase-dependent pathway activated during apoptosis and a ROS-dependent pathway that is involved in the clearance of activated cells. Patients with chronic granulomatous disease (CGD), whose neutrophils have a defective NADPH oxidase, are characterized by an accumulation of neutrophils at the wound site due to impaired neutrophil apoptosis and clearance (Amulic *et al.*, 2012). This study suggests that a functional NADPH oxidase has a role in neutrophils and macrophages as part of the process of apoptotic cell clearance.

The present study showed that exposure to hypoxia had no effect on MDM Φ phagocytosis of fresh and 22 h-aged neutrophils observed by flow cytometry using Tracker Green and light microscopic examination. Although previous studies did not specifically examine the effects of hypoxia on MDM Φ phagocytosis of apoptotic cells, such studies have clearly identified that neutrophil apoptosis was markedly inhibited under hypoxic conditions (Mecklenburgh *et al.*, 2002, Walmsley *et al.*, 2005). This effect involves a role of HIF-1 α that regulates the most aspects of responses under hypoxic conditions (Walmsley *et al.*, 2005).

However, due to the complex regulatory effects of HBO on phagocytosis and the limited information from previous research on HBO, further investigation is needed to determine the phagocytosis signalling pathway, as well as to define the effects of HBO on MDM Φ before co-culture with neutrophils in order to distinguish these effects from those after phagocytosis of apoptotic cells.

Chronic wounds have been shown to be related to defects in the resolution of inflammation (Serhan *et al.*, 2007). There are two main mechanisms involved in the resolution of inflammation: cell apoptosis and their subsequent clearance by macrophages (Gilroy *et al.*, 2004). Therefore, the hypothesis in the present study was that HBO treatment enhances the clearance of apoptotic neutrophils in a non-inflammatory process, which may involve effects on pro- and anti-inflammatory

mediators such as TNF- α and IL-10. Before ingestion by MDM Φ , TNF- α gene expression was significantly increased in fresh neutrophils and 22 h-aged neutrophils following exposure to HBO, pressure and hyperoxia treatments. The maximal IL-10 mRNA gene expression was observed in fresh and 22 h-aged neutrophils only after HBO treatment. This difference in the pattern of gene expression in 22 h-aged neutrophils is associated with higher percentages of dead and late apoptotic cells. The release of the cellular contents by dying cells leads to the development of a pro-inflammatory response (Haslett *et al.*, 1994, Haslett, 1992). In addition, *in vitro* culture of neutrophils could enhance TNF- α and IL- β release (Van Dervort *et al.*, 1994). However, it is interesting that neutrophils (fresh and 22 h-aged) showed an increase in IL-10 mRNA gene expression when exposed to HBO that suggests that HBO mediates anti-inflammatory effects. The immunosuppressive effects of HBO have been identified in various studies (Inamoto *et al.*, 1991, Satio *et al.*, 1991, Lahat *et al.*, 1995, Benson *et al.*, 2003). The present study also showed that HBO, pressure and hyperoxia-mediated phagocytosis of apoptotic cells is associated with an increase in IL-10 gene expression. Uptake of fresh 22 h-aged neutrophils by MDM Φ resulted an increase in IL-10 gene expression following HBO, pressure and hyperoxia treatments compared with normoxia, while no change was found in TNF- α levels in MDM Φ that had ingested fresh or neutrophils after different treatments. Previously published data has demonstrated the regulatory role of IL-10 in phagocytosis of apoptotic cells (Savill, 2000, Fadok *et al.*, 1998, Voll *et al.*, 1997). It was shown that the phagocytosis of apoptotic cells by macrophages is associated with suppression of the inflammatory response by decreasing the induction of pro-inflammatory cytokines such as TNF- α and IL-6 (Voll *et al.*, 1997; Fadok *et al.*, 1998; Savill, 2000). Previous studies have demonstrated that HBO inhibits LPS, lipid A and phytohaemagglutinin A-induced TNF- α and IL-1 β production, and PHA-induced production and mRNA gene expression

(mRNA) of IL-1 β by human blood-derived monocytes-macrophages (Benson *et al.*, 2003). Another study examined the effects of HBO on pro-inflammatory cytokines (TNF- α , IL-6 and IL-8) in whole blood from healthy individuals previously stimulated with LPS; exposure to HBO enhanced cytokine release (Fildissis *et al.*, 2004). IL-10 has been shown to inhibit TNF- α production (Xu *et al.*, 2006) and up-regulate phagocytosis (Ogden *et al.*, 2005). Phagocytosis of apoptotic neutrophils is dynamically regulated by the balance between pro- and anti-inflammatory cytokines, specifically TNF- α and IL-10 (Fox *et al.*, 2010). It is clear that exposure of cells to HBO enhanced apoptosis of neutrophils and this was associated with an increase in their clearance by MDM Φ . In fact, uptake mediated by the PS exposure and recognition of apoptotic neutrophils has been associated with an anti-inflammatory response, where incubation of macrophages with lysed neutrophils induced the production of IL-8, TNF- α and IL-10 by macrophages (Fadok *et al.*, 2001a). However, TNF- α production is highly controlled by a variety of complex mechanisms. TNF- α gene transcription involves a number of transcription factors such as red blooded (REB), LPS-induced TNF- α transcription factor (LITAF), activation transcription factor 2 (ATF-2), c-jun, early growth response 1 (Egr-1) and NF- κ B (Skinner *et al.*, 2008). For example, activation of NF- κ B by LPS induces the transcription of many pro-inflammatory cytokines (Baldwin, 1996). Therefore, suppression of NF- κ B has a key role in regulating inflammatory responses. Recent studies have identified NF- κ B as regulator of TNF gene activation (Hall *et al.*, 2005). The activation and translocation of NF- κ B to the nucleus results in transcription of various inflammatory genes including TNF- α (Meldrum, 1998). IL-10 inhibits the production of anti-inflammatory cytokines in human monocytes through the suppression of NF- κ B activation (Wang *et al.*, 1995), and enhancement of the expression of the NF- κ B inhibitor, I κ B (Lentsch *et al.*, 1997). Our recent findings are that HBO induced apoptosis and suppression of NF- κ B gene expression and protein in neutrophil-like

cells. Furthermore, HBO treatment enhanced anti-inflammatory cytokine IL-10 production. These observations suggest that HBO-enhanced clearance of apoptotic cells not only prevents the release of toxic intercellular contents, but also could stimulate the macrophages to produce anti-inflammatory or suppressive mediators, such as IL-10. Data obtained in the present study suggest the sequence of events shown in Fig. 6.15 which suggest that HBO mediates activation of NADPH oxidase and consequently ROS production. Oxidative stress induced by HBO initiates apoptosis pathways and hence enhanced recognition of apoptotic neutrophils by MDM Φ .

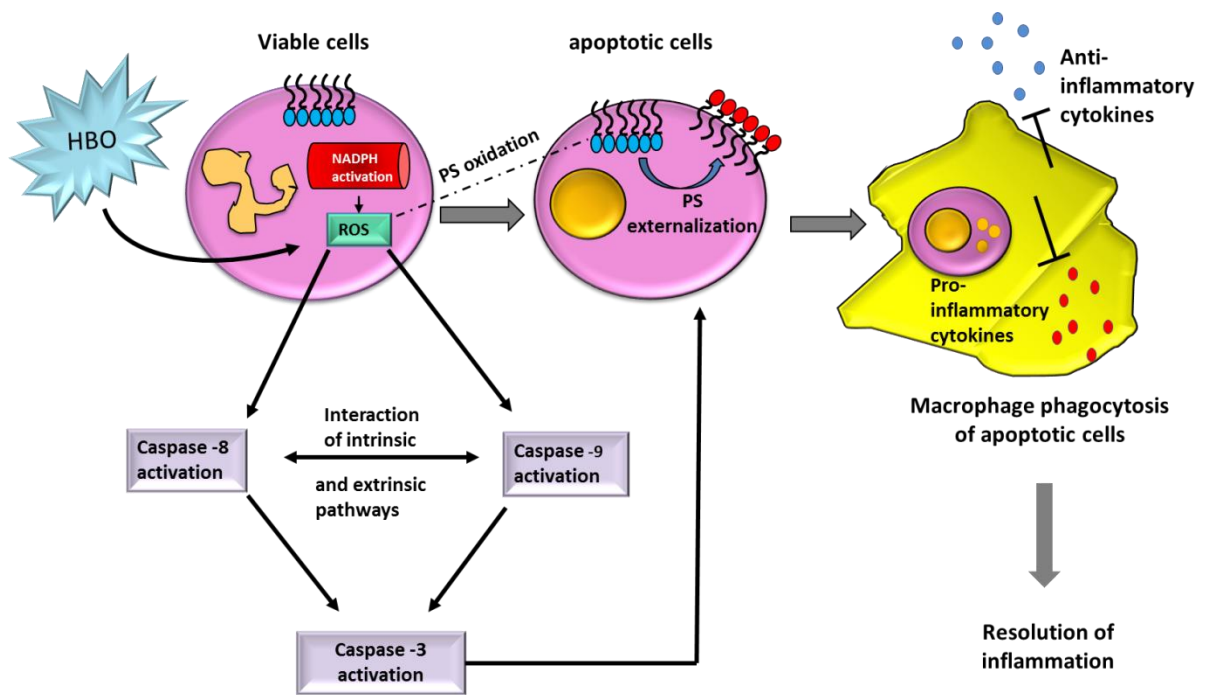


Fig 6.15 Diagram summarizing hypothetical effects of HBO on regulation of neutrophil clearance by macrophages. Exposure to HBO induces neutrophil apoptosis, followed by recognition and phagocytosis of intact apoptotic cells by macrophages. NADPH oxidase has a role in the process of macrophage elimination of activated neutrophils. HBO induces NADPH oxidase activation and ROS production which leads to initiation of the caspase cascade to induce neutrophil apoptosis via the intrinsic and extrinsic pathways, which in turn causes PS oxidation and its translocation from inner to outer leaflet of plasma membrane, acting as a main signal for macrophages to phagocytose these apoptotic cells. This process is also associated with anti-inflammatory effects on macrophages by decreasing the expression of pro-inflammatory cytokines and increasing anti-inflammatory cytokine production.

6.4.1 Conclusion

The findings presented here provide evidence that HBO enhances neutrophil engulfment by MDM Φ . It is likely that the HBO effects are mediated by the generation of ROS and induction of neutrophil apoptosis that in turn accelerate their uptake by MDM Φ . Furthermore, the data demonstrate another aspect in that HBO treatment is able to enhance the removal of apoptotic cells by MDM Φ in a non-inflammatory process through decreased gene expression of TNF- α and increased gene expression of IL-10. Overall, this study provides new insights into the role of HBO in the regulation of the phagocytic removal of apoptotic cells, which may prove useful in developing a therapeutic approach to the resolution of inflammation.

CHAPTER SEVEN

EFFECT OF LONG-TERM

REPETITIVE HBO EXPOSURE ON

PATIENTS WITH CHRONIC WOUNDS:

***AN IN VIVO* STUDY**

Abstract

Although its clinical benefits are clear, HBO has also been reported to enhance ROS production and cause oxidative stress in several tissues. Recent studies have shown that HBO-induced oxidative stress is directly related to both its exposure to pressure and duration. However, these studies were performed with a single HBO treatment, whereas its clinical use depends on long-term, repetitive exposure. The purpose of this study was to determine the effect of long-term repetitive HBO in patients with chronic wounds. Four patients with chronic wounds were involved in this study and exposed to repeated HBO sessions (up to 40 sessions at 2.4 ATA for 90 min). Blood samples were taken immediately after 20, 30 and 40 sessions. Plasma antioxidant enzyme activities (CAT, GPx and SOD) and total ascorbate were measured as indirect markers of oxidative stress. Levels of pro- and anti-inflammatory cytokines were also measured to investigate the inflammatory response following repeated HBO exposures. Additionally, neutrophils were isolated from blood to investigate the expression of the anti-apoptotic genes, NF- κ B and Bcl-2. The findings demonstrated that long term-exposure to HBO significantly decreased the antioxidant activity (CAT and SOD after 30 HBO sessions; one-way repeated measures ANOVA, $P = 0.005$ and 0.02 , respectively) and pro-inflammatory cytokines (TNF- α and IL-6 after 20 and 30 HBO sessions; one-way repeated measures ANOVA, $P < 0.05$) in plasma, with down regulation of NF- κ B and Bcl-2 in both unstimulated and stimulated cells after 20 and 30 HBO sessions (two-way repeated measures ANOVA, $P < 0.001$). In conclusion, it is clear that repetitive treatment with HBO causes oxidative stress. Despite possible injurious effects, it is accepted that HBO-mediated ROS are responsible for the HBO immunosuppressive effects.

7.1 Introduction

HBO therapy is used in the treatment of various clinical conditions, such as carbon monoxide poisoning, decompression sickness, osteomyelitis, and enhanced healing of selected problem wounds (Torbati *et al.*, 1992). However, HBO has the potential risk of oxygen toxicity because 100% oxygen is breathed during the session (Thom *et al.*, 2002). The side effects of HBO are associated with prolonged exposure to levels greater than those generally used in clinical conditions (Harabin *et al.*, 1990, Torbati *et al.*, 1992). It is assumed that hyperoxic injury is caused by the metabolic production of ROS at an increased rate that is dependent on oxygen tension, and which is in excess of the capacity of cell antioxidant defence mechanisms (Sies, 1997). *In vitro* studies have identified that $O_2^{\cdot-}$, H_2O_2 , and lipid peroxidation can be increased in homogenates, slices, and subcellular organelles of tissues such as lung and brain when the oxygen tension is increased (Freeman and Crapo, 1981, Jamieson, 1991). The clinically-approved maximum pressure and duration of exposure is 3 ATA and 120 min and the common clinical protocol for standard HBO therapy is 1.8-2.8 ATA for 60-90 min (Kleen and Messmer, 1999). It has been reported that HBO toxic effects correlate with high pressures and long exposure durations (Harabin *et al.*, 1990, Kleen and Messmer, 1999, Wada *et al.*, 2001). Furthermore, oxidative stress induced by HBO seems to be less of a concern at the 2.4 ATA pressure level often used clinically (Bitterman *et al.*, 1993). With repeated long-term exposure, HBO below 2 ATA can actually decrease oxidative stress (Yatsuzuka, 1991, Ozden *et al.*, 2004) by inhibiting lipid peroxidation (Kudchodkar *et al.*, 2000), and increasing the activity of the antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), which are critical to the cell's defences against oxidative stress-induced by ROS (Ozden *et al.*, 2004, Nie *et al.*, 2006).

Hyperbaric oxygen is well known to exert a significant influence on immune function in general and cytokine production in particular (Al-Waili *et al.*, 2006). It has been shown to inhibit the levels of pro-inflammatory cytokines (Hansbrough *et al.*, 1980, Inamoto *et al.*, 1991, Lahat *et al.*, 1995, Satio *et al.*, 1991), increase release of the anti-inflammatory cytokine IL-10 (Yatsuzuka, 1991) and induce HSP-90 expression (Ozden *et al.*, 2004). Hyperbaric oxygen also reduced both inflammation and pain in an animal model (Kudchodkar *et al.*, 2000), but the molecular mechanisms mediating these immunomodulatory actions are still unclear. This evidence suggests that HBO may have some regulatory function on immune responses. HBO effects are generally attributed to its separate components, namely increased oxygen concentration and increased pressure. The anti-inflammatory effects of HBO seem to be pressure-dependent. For example, in one animal study, hyperbaric pressure without the addition of oxygen was shown to decrease TNF- α level (Nie *et al.*, 2006). In another study in humans, HBO (100% O₂ at 2 ATA) and hyperbaric pressure (10.5% O₂ at 2 ATA) both resulted in a decrease in IFN- γ level in plasma, while exposure to 100% O₂ at 1 ATA demonstrated the opposite effect (Akin *et al.*, 2002, Luongo *et al.*, 1998, Granowitz *et al.*, 2002). Based on these findings, repeated exposures to HBO might help improve the resolution of inflammation in chronic wounds. HBO inhibits proliferation of the HL-60 granulocytic cell line (Mcintyre *et al.*, 1997), suppresses β -integrin-dependent adherence in both human and rat neutrophils (Thom, 1993, Thom *et al.*, 1997) and causes a decrease in circulating CD4⁺ T cells (Bitterman *et al.*, 1993, Bitterman *et al.*, 1994). HBO has been shown to induce cell apoptosis via oxidative stress and ROS production, these effects being time-dependent (Ganguly *et al.*, 2002), and contributes to both in terms of increased pressure and hyperoxia. Repeated exposure to HBO (2.4 ATA for 90 min, once daily for seven days) has been shown to induce further ROS

production and apoptosis via mitochondria in streptozotocin-induced diabetic rats (Matsunami *et al.*, 2009).

This study aimed to test whether long-term repetitive exposure to HBO would modify the antioxidant enzymes including CAT, GPx and SOD as indirect markers to evaluate oxidative stress following HBO exposure in patients with chronic wounds, and whether this is more potent than the influence induced by a single exposure to HBO *in vitro* (see Chapter 5). In addition, the immune response following a long-term repetitive exposure to HBO was also investigated, including whether it was modified during HBO treatments. This study may help to elucidate the mechanisms involved during HBO treatment in patients with chronic wounds.

7.2 Materials and methods

7.2.1 Ethical Considerations

All studies reported in this chapter were approved by the Devon and Torbay Research Ethics Committee (REC reference number: 08/H0202/149, Exeter, UK). All patients were recruited from the Plymouth Hyperbaric Medical Centre (Diving Disease Research Centre, DDRC, Tamar Science Park, Plymouth PL8 8BU, UK) and given a minimum of 24 h to consider participation before giving their consent to the DDRC.

7.2.2 Experimental design

Four patients with venous leg ulcers (male, $n = 3$ and female, $n = 1$; mean age of 65 ± 5.9 years) were enrolled and were accepted to undergo HBO treatment between March 2011 and November 2011. Patients who met the selection criteria (at least one chronic ulcer, wound present for six weeks or more, over 18 years of age and suitable for HBO therapy) were included in the study. All the patients were maintained on their medical

treatments such as insulin and antibiotics (*e.g.* flucloxacillin, fucidin, cefradine and co-amoxiclav) throughout the course of the HBO treatment.

Patients were exposed to HBO treatment (1 session per day) according to a routine therapy protocol. The multiplace chamber was pressurized with compressed air to 2.4 ATA while the patients breathed 100% O₂ through a mask for one period of 90 min, with 2 × 5 min intervals during which the patient breathed air. The patients were monitored after repeated exposure to HBO (20, 30 and 40 HBO sessions). A blood sample (20 ml) was taken from each patient before and after the 20, 30 and 40 HBO treatments using a 19 gauge needle to avoid mechanical stress, which can activate neutrophils. The blood was then immediately put in dipotassium-EDTA Vacutainer® tubes and kept at room temperature until analysis. Plasma samples were obtained by centrifuging the blood at 400 × *g* for 20-25 min at 4 °C. The supernatant was divided into 200 µl aliquots, and stored at -80 °C until analysis for antioxidant enzyme activities (as detailed below) and pro- and anti-inflammatory cytokines using a Luminex® assay (section 2.1.8).

Neutrophils were isolated from blood samples (section 2.1.9) for gene expression quantification using qPCR. Cell suspensions (1 × 10⁶ cells ml⁻¹) were washed with PBS, and treated with 10 ng ml⁻¹ TNF-α and incubated for 3 h at 37 °C at 5% CO₂ (balance air). RNA was extracted and expression of NF-κB and Bcl-2 genes was analysed using qPCR (section 2.1.7.5; Fig. 7.1). Based on the response to HBO treatment and the rate/quality of wound healing, just two patients continued through to the 40th HBO session, which decreased the power of the study.

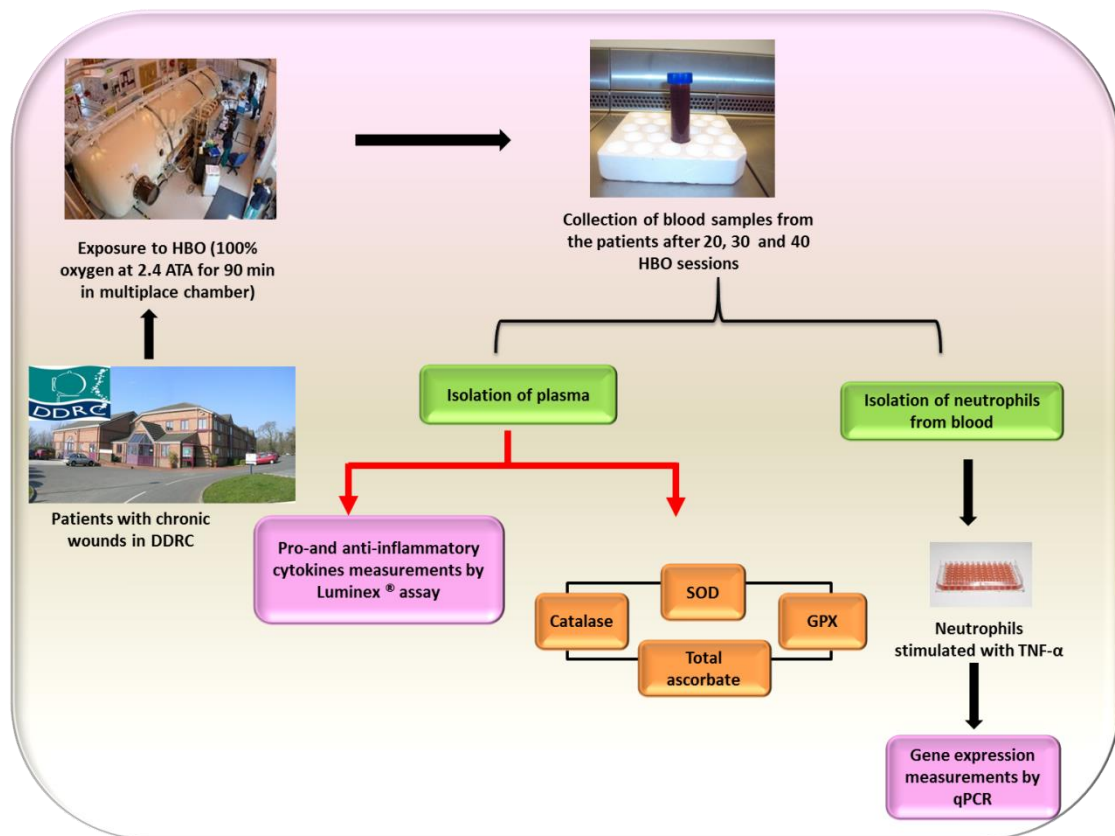


Fig. 7.1 Experimental design. Four patients ($n = 4$) with venous leg ulcers were enrolled in this study. Patients were exposed to HBO treatment (1 session per day) according to a routine therapy protocol in the multiplace chamber. Blood samples were taken from each patient after repeated exposure to HBO (20, 30 and 40 HBO treatments). Plasma samples were obtained by centrifuging the blood at $400 \times g$ for 20-25 min at 4°C and assessed for antioxidant enzyme activity, total ascorbate and pro and anti-inflammatory cytokines. Neutrophils were also isolated from blood for gene expression experiments.

7.2.3 Biochemical analysis

7.2.3.1 Catalase (CAT) assay

Catalase activity may be determined in two successive steps. Firstly, catalase catalyses the dismutation of a known concentration of H_2O_2 to produce water and oxygen (O_2), and secondly, Amplex Red reagent reacts with any unreacted H_2O_2 (catalysed by HRP) to produce the highly-fluorescent oxidation product, resorufin (Mueller *et al.*, 1997).

Following plasma sample preparation, $50\ \mu\text{l}$ aliquots of the sample were added to each well of a 96-well plate, and then $25\ \mu\text{l}$ of $40\ \mu\text{M}$ H_2O_2 was added. The plates were then incubated for 30 min at 37°C . After incubation, $50\ \mu\text{l}$ of reaction buffer containing $100\ \mu\text{M}$ Amplex Red reagent and $0.4\ \text{U ml}^{-1}$ of HRP (Sigma-Aldrich, Poole, UK) was added

to each well. The resulting fluorescence was measured using a CytoFluor II fluorescence microplate reader (PerSeptive Biosystems, Framingham, USA) with excitation 540 nm and emission 590 nm. The Amplex Red stock solution was prepared at 10 mM in DMSO and aliquots stored at -20 °C. Catalase activity was calculated from a standard curve prepared using purified catalase (0-2000 U ml⁻¹, Sigma, Poole, UK; Fig. 7.2).

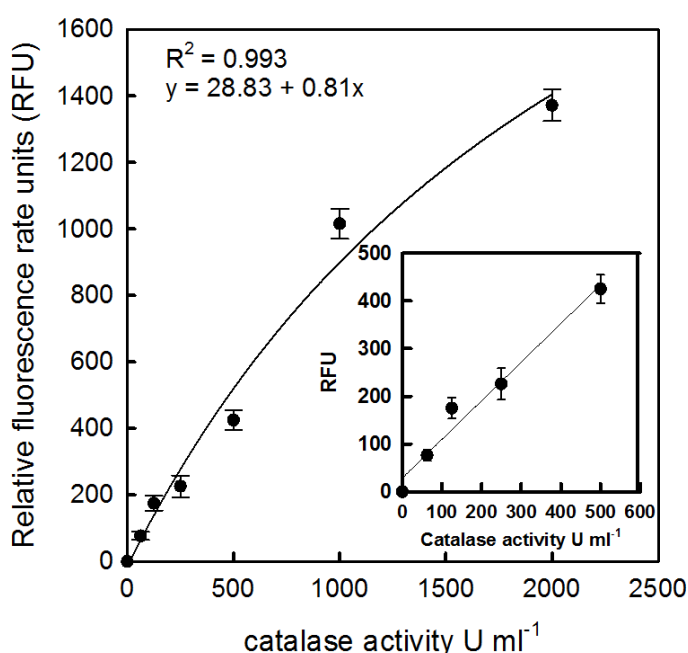


Fig.7.2 Standard curve of catalase assay. Catalase concentrations (0 - 2000 U ml⁻¹) were prepared in assay buffer and the data points are the mean of triplicates \pm SEM. A linear fit curve with $R^2 = 0.993$ over a more limited range of catalase activities was used to calculate catalase activity (insert).

7.2.3.2 *Glutathione peroxidase (GPx) assay*

This assay measures GPx indirectly using a coupled reaction catalysed by glutathione reductase (GR). Oxidized glutathione (GSSG) is produced upon reduction of H₂O₂ catalysed by GPx activity, and then recycled back to its reduced state (GSH) by NADPH (catalysed by GR). The oxidation of NADPH to NADP⁺ is accompanied by a

decrease in absorbance at 340 nm, the rate of decrease being directly proportional to the GPx activity in the sample (Mannervik, 1985).

Plasma from patients with venous leg ulcers was prepared as described in section 7.2.2. The assay mixture was prepared by mixing 9.2 ml of buffer (5 mM potassium HEPES, pH 7.5, containing 1 mM dipotassium EDTA) with 1 mM NADPH, followed by the addition of 100 μ l of 100 U ml⁻¹ glutathione reductase (GR; Sigma G3664), 50 μ l of 200 mM GSH, and 95 μ l of 10 mM potassium cyanide. The assay mixture (290 μ l) was added to each well of a 96 well plate, followed by 15 μ l of plasma before mixing. The reaction was started by adding 5 μ l of 0.042 % (w/w) H₂O₂ solution to each well. The rate of change of absorbance at 340 nm was measured for at least 10 min in a VersaMax plate reader (Molecular Devices, Sunnyvale, CA, USA). GPx activity was calculated in pmol ml⁻¹ min⁻¹.

7.2.3.3 Superoxide dismutase (SOD) assay

Superoxide dismutase activity can be measured indirectly through the reduction of cytochrome *c* by superoxide. The superoxide can be generated via the activity of xanthine oxidase (XO). The rate of reduction of cytochrome *c* (detected as the rate of increase of absorbance at 550 nm) is inhibited by SOD, thereby giving a measure of SOD activity (Mccord and Fridovich, 1969).

The assay mix consisted of three reagents (xanthine, xanthine oxidase and cytochrome *c*) dissolved in potassium buffer (pH 7.8) stored at room temperature during use and at 4 °C between uses. Xanthine (0.5 mM) was prepared fresh each day and stored at room temperature during use. Xanthine oxidase (5 μ l of Sigma X4500, diluted with 495 μ l of potassium phosphate buffer, final concentration, 0.32 U ml⁻¹) was prepared fresh each day and stored on ice during use. Cytochrome *c* (12.5 mg in 0.5 ml) was prepared in phosphate buffer (pH 7.8) and stored on ice during use and at -20 °C between uses. The

assay mixture was prepared from 8.85 ml potassium phosphate buffer, 1 ml xanthine and 50 μl cytochrome *c*. Superoxide dismutase was prepared fresh each day by diluting 10 μl of a commercial (Sigma S8409, from bovine erythrocytes) with 90 μl buffer (final concentration, 960 U ml^{-1}) and stored on ice during use. Assay mix (10 ml) was prepared just before use. This was sufficient for at least 32 wells. Plasma from patients with venous leg ulcers before and after HBO treatment was purified by centrifugation ($400 \times g$ for 5 min). Aliquots (50 μl) of plasma were added to the wells of a 96-well plate. Some wells were also used as controls either by replacing the sample with SOD or with buffer. Xanthine oxidase solution (100 μl) was added immediately before use to the assay mix, the wells were loaded as appropriate and readings started within 1 min of this addition. Assay mixture (250 μl) was pipetted into each well and mixed carefully. The plate was immediately transferred to the VersaMax plate reader (Molecular Devices, Sunnyvale, CA, USA), and the increase in absorbance monitored at 550 nm using a kinetic program for at least 5 min. All data are expressed as units ml^{-1} .

7.2.3.4 Determination of total ascorbic acid by high-performance liquid chromatography (HPLC)

The well-known method for measuring ascorbic acid is HPLC with ultraviolet (UV) or electrochemical detection (Levine *et al.*, 1999). For total ascorbic acid a sensitive HPLC electrochemical detector was used, which is based on a chemical reaction involving either oxidation or reduction of ascorbic acid, to produce an electron flow measurable as current, which may be correlated to the type and quantity of the ascorbic acid (Washko *et al.*, 1989).

Plasma or ascorbate standard (up to 200 μl) was mixed with 10% metaphosphoric acid (MPA; Sigma-Aldrich, Poole, UK) containing 2.0 mM EDTA to acidify the serum and stabilize the ascorbate. The tubes were capped and mixed well on a vortex mixer. The

mixture was centrifuged for 10 min at $20,000 \times g$ at $4\text{ }^{\circ}\text{C}$. The supernatant ($90\text{ }\mu\text{l}$) was transferred to another tube and $10\text{ }\mu\text{l}$ of 350 mM of Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) in 5% MPA containing 1 mM EDTA was added; the tubes were then capped and mixed well on a vortex mixer. The mixture was incubated for 20 min at room temperature to reduce oxidised dehydroascorbate back to ascorbate. The mixture was injected into a C-18 reversed-phase column and eluted with a mobile phase of 0.1 M of sodium acetate containing 0.54 mM EDTA and 1.5 mM octylamine. Quantitation was by measurement of peak area and is based on a standard curve generated using different concentrations of known standards of ascorbate ($5\text{-}50\text{ }\mu\text{M}$; Fig.7.3).

The chromatography system consisted of an LDC/Milton Roy ConstaMetric HPLC pump connected to a BAS LC-4L electrochemical detector (Bioanalytical Systems Inc., West Lafayette, IN, USA). The column was a Hichrom reversed-phase C18 column, $189 \times 4.8\text{ mm}$, particle size $4\text{ }\mu\text{m}$, operated at a flow rate $1\text{-}2\text{ ml min}^{-1}$.

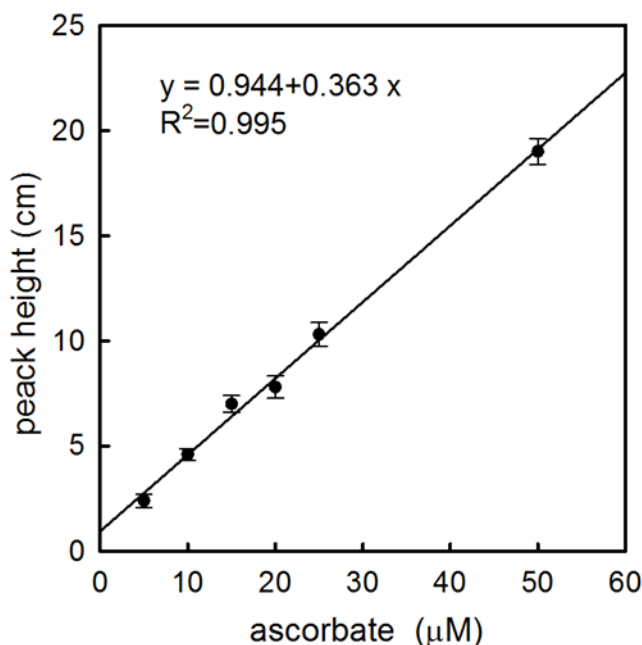


Fig. 7.3 Standard curve of ascorbate assay. Ascorbate concentrations ($5\text{-}50\text{ }\mu\text{M}$) were prepared in dH_2O and the data points are the mean of triplicates \pm SEM.

7.2.4 Statistical analysis

Data were analysed using Statgraphics Centurion XVI software (Stat Point Technologies, Inc.). Data are expressed as means \pm SEM ($n = 4$) for 0, 20 and 30 HBO sessions and were analysed using repeated measures ANOVA. A P value of < 0.05 is considered significant throughout.

7.3 Results

7.3.1 Effect of prolonged repeated HBO treatment on plasma enzymatic antioxidant defences

After completion of HBO sessions (20 to 40 sessions) a significant decrease was observed in wound area in patients with chronic wounds (paired t -test, $P = 0.032$) compared to before treatment (control; Fig. 7. 4A). The activities of SOD, CAT and GPx were measured in the plasma of patients with chronic wounds before and after repeated HBO sessions. Before HBO treatment the activity values in the plasma were 49.2 ± 3.6 U ml⁻¹ for CAT, 2.9 ± 0.2 pmol min⁻¹ ml⁻¹ for GPx and 27.0 ± 2.4 U ml⁻¹ for SOD. Exposure of the patients with chronic wounds to repeated HBO treatments resulted in a decrease in antioxidant enzyme activities. Following the 20 HBO sessions no significant decrease was observed in CAT, GPx and SOD activities (36.9 ± 4.0 U ml⁻¹, 2.7 ± 0.4 pmol min⁻¹ ml⁻¹ and 23.7 ± 1.3 U ml⁻¹ respectively, Figs 7.5A, B, and C). A significant decrease in CAT (one-way repeated measures ANOVA, $P = 0.005$) and SOD (one-way repeated measures ANOVA, $P = 0.02$) was observed following 30 HBO sessions compared to before HBO treatment, whereas there was no significant decrease in GPx activity (one-way repeated measures ANOVA, $P > 0.05$, Figs 7.5A, B and C).

After 40 HBO sessions only two patients remained in the study. The antioxidant enzyme activities were decreased to 20.6 and 20.9 U. ml⁻¹ for CAT, 1.8 and 1.7 pmol min⁻¹ ml⁻¹ for GPx and 20.8 and 16.3 U ml⁻¹ for SOD, but due to limited sample size (*n* = 2), it is uncertain whether this decline in antioxidant enzyme activity was significant (Figs 7.5 D, E and F).

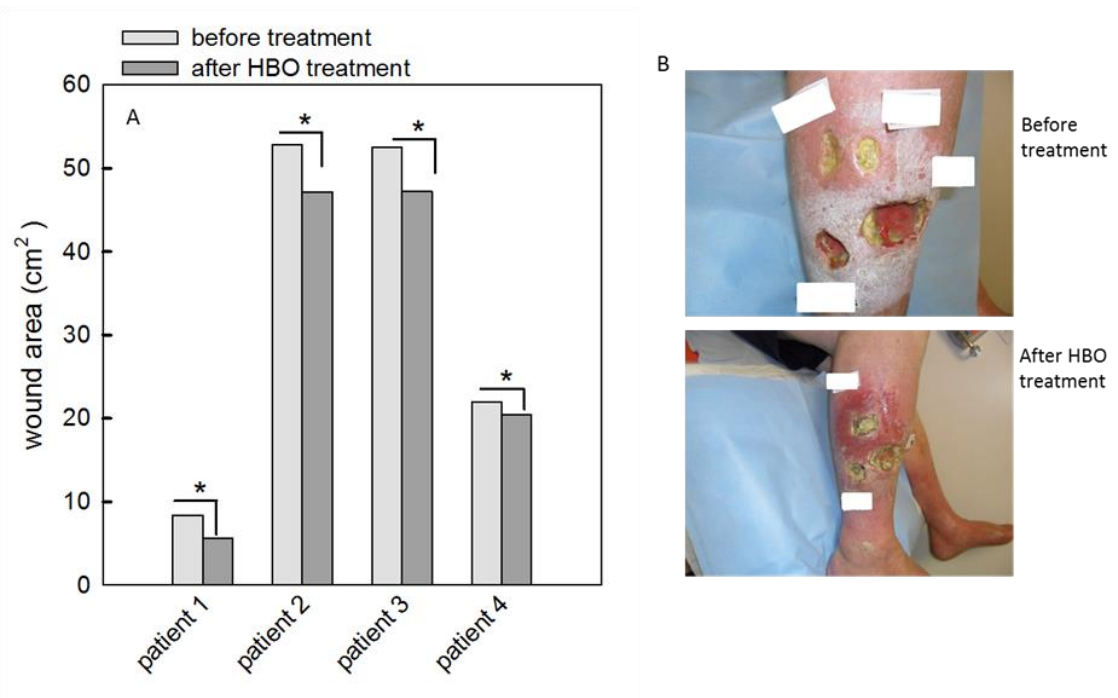


Fig.7.4 Wound area in the patients with chronic wounds after exposure to the repeated HBO sessions. (A) Wound area was measured in patients with chronic wounds before and at the end of long-term repetitive HBO treatments. * indicate significant differences from before HBO treatment. (B) Representative photo of a wound is shown before the initiation of HBO treatment, and after 30 HBO sessions.

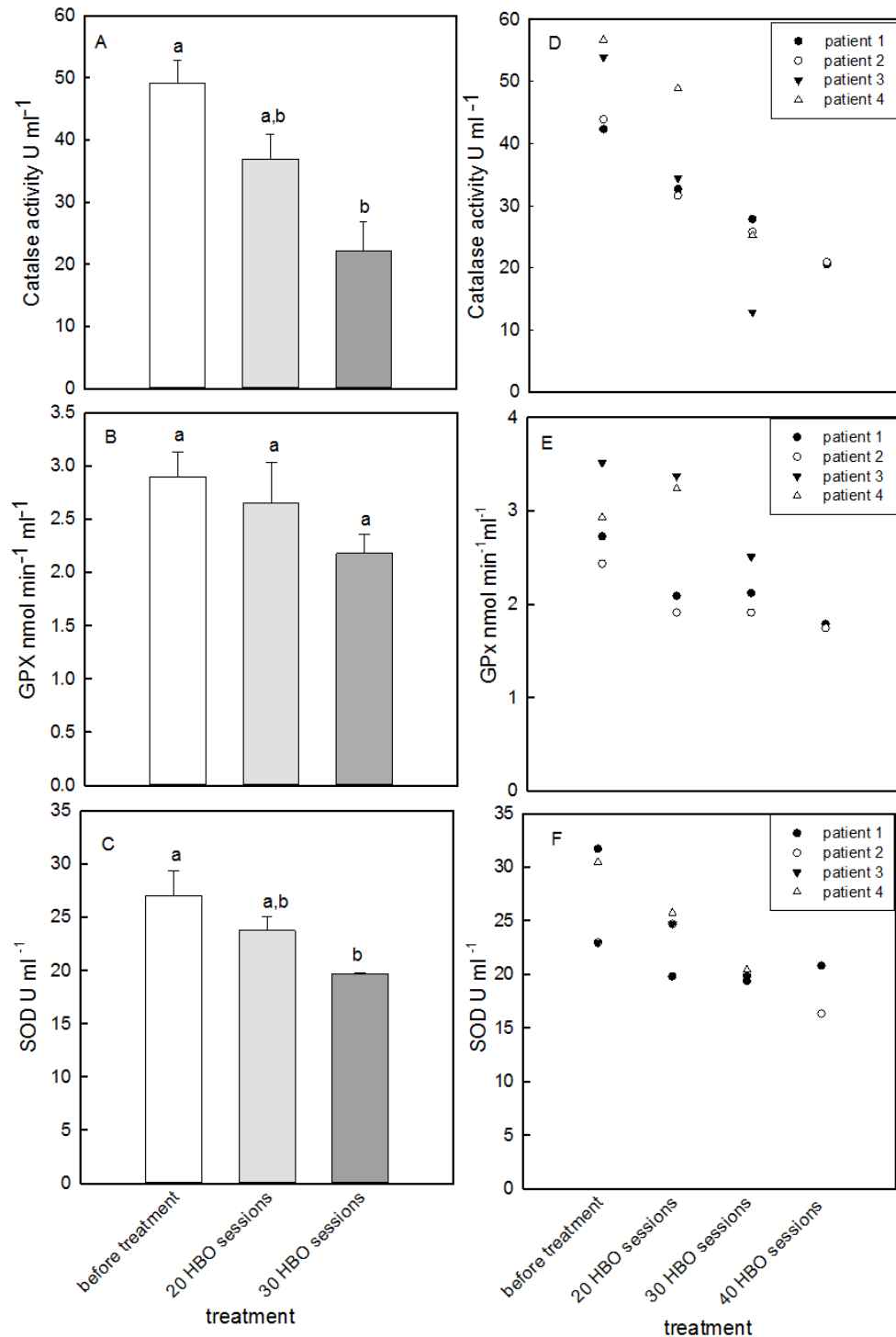


Fig. 7.5 Plasma antioxidant activities in patients with chronic wounds after exposure to repeated HBO sessions. Plasma samples were isolated from the blood of patients with chronic wounds before and after exposure to repeated HBO sessions. Catalase activity was assessed in the presence of Amplex Red and HRP (A). GPx activity was assessed in the presence of NADPH and GR (B) and SOD activity was assessed in the presence of cytochrome *c* and XO (sections 7.2.3.1, 2 and 3). Data are expressed as means \pm SEM, for four patients, $n = 4$ with triplicate measurements. Bars with the different lower case letters are significantly different (one-way repeated measures ANOVA, $P < 0.05$). Scatter plot of catalase, GPX and SOD activities for all patients before HBO, after repeated 20 and 30 HBO sessions ($n = 4$) and after 40 HBO sessions ($n = 2$; D, E and F).

7.3.2 Effect of prolonged repeated HBO treatment on non-enzymatic antioxidant defences

The levels of the non-enzymatic antioxidant ascorbic acid were measured before and after HBO treatment in the plasma of patients with chronic wounds. There were no significant changes in total ascorbate (ascorbate + dehydroascorbate) at the end of the 20 HBO sessions ($12.5 \pm 0.6 \text{ nmol ml}^{-1}$) and the 30th HBO session ($11.7 \pm 1.1 \text{ nmol ml}^{-1}$) compared to before treatment ($12.9 \pm 0.9 \text{ nmol ml}^{-1}$; Fig. 7.6A). Since only two patients with chronic wounds continued until the 40 HBO sessions, it was not possible to analyse these data due to a limited sample size ($n = 2$, 10.1 and 8.6 nmol ml^{-1} , Fig. 7.6 B).

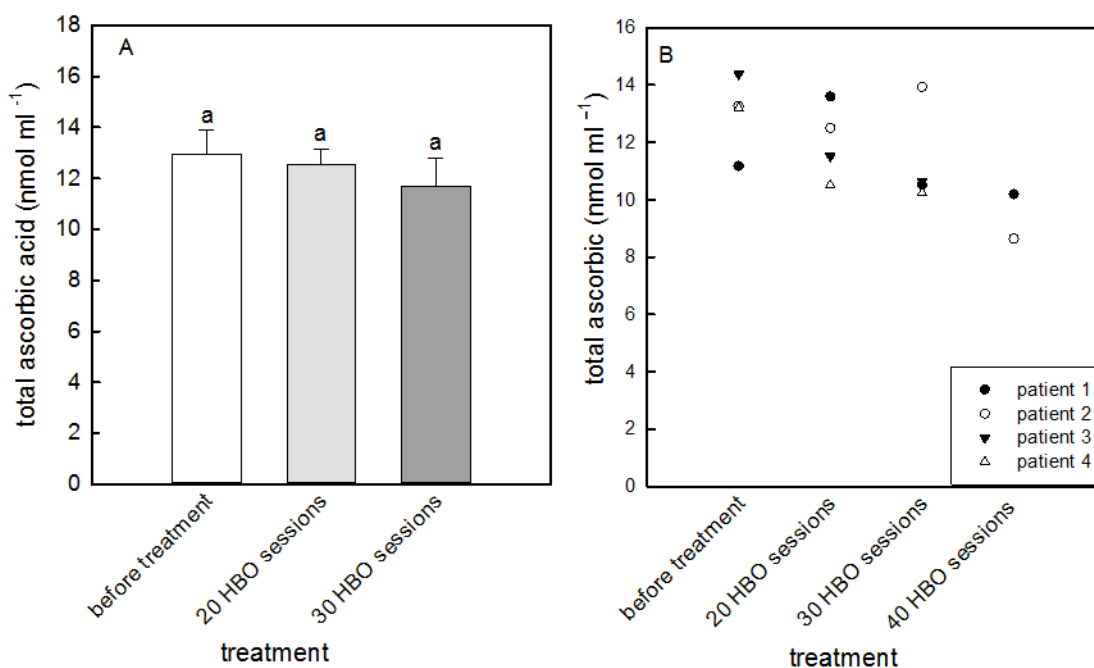


Fig 7.6 Plasma ascorbate levels in the patients with chronic wounds after exposure to repeated HBO sessions. Plasma samples were isolated from blood of patients with chronic wounds before and after exposure to repeated HBO sessions. Ascorbate was measured using HPLC and calculated against a standard curve (see section 7.2.4.4). Data are expressed as the means \pm SEM, for four patients, $n = 4$ with triplicate measurements. Bars with the same lower case letters are not significantly different (one-way repeated measures ANOVA, $P > 0.05$). Scatter plot of ascorbate levels for all patients before HBO, after 20 and 30 HBO sessions ($n = 4$) as well as after 40 HBO sessions ($n = 2$; B).

7.3.3 Effect of prolonged repeated HBO treatment on pro- and anti-inflammatory cytokines in plasma

The plasma levels of TNF- α , IL-1 β , IL-6 and IL-10 were measured in patients with chronic wounds using the multiplex Luminex[®] assay technique. Before HBO treatments, the values for TNF- α , IL-1 β , IL-6, and IL-10 were 57.3 ± 6.6 , 30.9 ± 4.9 , 45.6 ± 3.9 and 7.2 ± 1.2 pg ml⁻¹, respectively (Figs 7.7A, B, C and D). Following the 20 and 30 HBO sessions, TNF- α and IL-6 levels were significantly decreased in the plasma of chronic wound patients compared to before treatment (one-way repeated measures ANOVA, $P = 0.002$). IL-1 β production was not significantly changed following 20 and 30 HBO sessions (22.5 ± 3.6 and 20.3 ± 3.3 , respectively) while no change was observed in IL-10 plasma levels after 20 and 30 HBO treatments (4.90 ± 0.3 and 8.4 ± 0.3 pg ml⁻¹, respectively, Figs 7.7A, B, C and D).

Exposure to repeated 40 HBO sessions decreased the TNF- α to 29.8 and 25.6 pg ml⁻¹, IL1 β to 6.5 and 9.5 pg ml⁻¹ and IL-6 levels to 18.6 and 18.9 pg ml⁻¹, whereas IL-10 levels were increased to 17.7 ± 6.0 and 29.7 pg ml⁻¹. Again, it was not possible to analyse these data due to a limited sample size ($n = 2$; Figs 7.7 E, F, G and H).

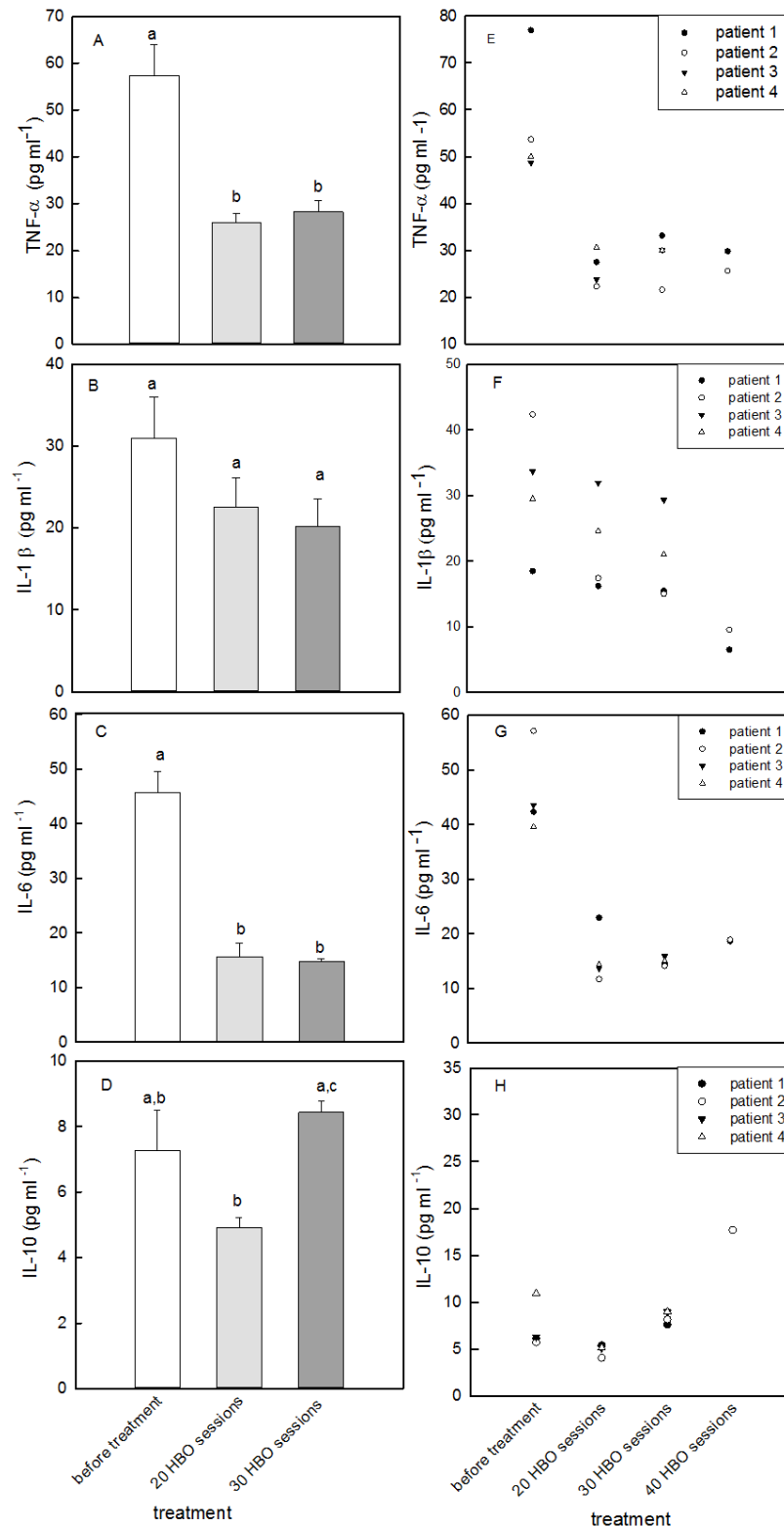


Fig. 7.7 Plasma pro- and anti-inflammatory cytokine levels in the patients with chronic wounds after exposure to the repeated HBO sessions. Plasma samples were isolated from blood from patients with chronic wounds before and after exposure to repeated HBO sessions. TNF- α , IL-1 β , IL-6, IL-10 levels were analysed by Luminex (section 2.1.8) and calculated against a standard curve. Data are expressed as the means \pm SEM, for four patients, $n = 4$ with triplicate measurements. Bars with the different lower case letters are significantly different (one-way repeated measures ANOVA, $P = 0.002$ for TNF- α and IL-6 following 20 and 30 HBO sessions, A, B, C and D). Scatter plot of cytokines for all patients before HBO, after repeated 20 and 30 HBO sessions ($n = 4$) as well as 40 HBO sessions ($n = 2$) (E, TNF- α) (F, IL-1 β) (G, IL-6) and (H, IL-10).

7.3.4 Effect of prolonged repeated HBO treatment on NF- κ B and Bcl-2 gene expression in neutrophils

Gene expression of both NF- κ B and Bcl-2 was analysed by qPCR (Figs 7.8A and 7.9A). The gene expression of NF- κ B was significantly decreased after 20 and 30 HBO sessions in untreated cells and TNF- α treated cells (two-way repeated measures ANOVA, $P < 0.001$ fold change compared to untreated cells before HBO treatment; Fig. 7.8A). The same pattern was observed with Bcl-2 expression following 20 and 30 HBO sessions in untreated and TNF- α treated cells (two-way repeated measures ANOVA, $P < 0.001$ fold induction compared to untreated cells before HBO treatment). Exposure to 40 HBO sessions decreased the expression of NF- κ B gene in TNF- α treated cells (0.24 and 0.15 fold induction compared to untreated cells before HBO treatment, respectively) and in untreated cells (0.08 and 0.04 fold induction compared to untreated cells before HBO treatment respectively). The same was also observed for Bcl-2 in TNF- α treated cells (0.21 and 0.13 fold induction compared to untreated cells before HBO treatment respectively) and in untreated cells (0.05 and 0.03 fold induction compared to untreated cells before HBO treatment respectively). Due to sample size ($n = 2$), it was not possible to analyse these data. The qPCR results for NF- κ B and Bcl-2 were confirmed by visualisation of the PCR product using 1% agarose gel electrophoresis (Figs 7.8B and 7.9B).

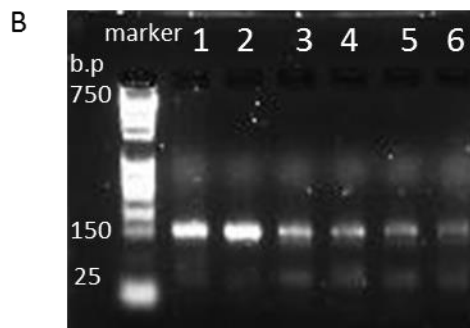
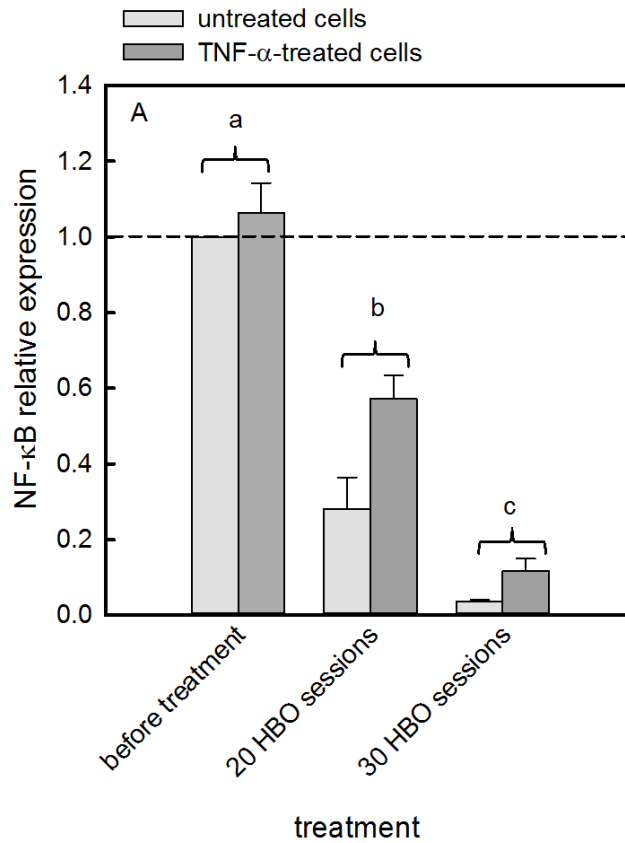


Fig. 7.8 NF- κ B gene expression in neutrophils from patients with chronic wounds after exposure to repeated HBO sessions. Neutrophils were isolated from blood from patients with chronic wounds before and after exposure to repeated HBO sessions, after which cells were washed and either treated with TNF- α or left untreated. cDNA was synthesised from extracted RNA. qPCR was used to determine expression of NF- κ B (section 2.1.7.5). Gene expression was normalised against the untreated cells before HBO exposure. Data are expressed as the means \pm SEM, for four patients, ($n = 4$) with triplicate measurements. For each HBO session, bars with the different lower case letters are significantly different (two-way repeated measures ANOVA, $P < 0.05$) (A). The PCR product for NF- κ B, before and after exposure to long-term repeated HBO sessions in both TNF- α treated and untreated cells in 1% agarose gel. Lanes: before HBO unstimulated cells (1), before HBO TNF- α stimulated cells (2), 20 HBO sessions unstimulated cells (3), 20 HBO sessions TNF- α stimulated cells (4) 30 HBO sessions unstimulated (5) and 30 HBO sessions TNF- α stimulated cells (6, B).

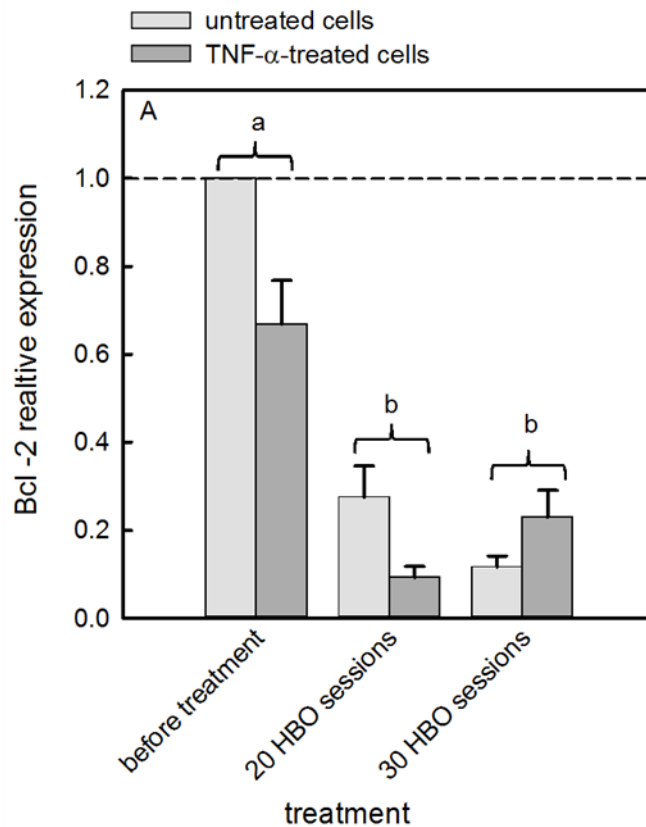


Fig. 7.9 Bcl-2 gene expression in neutrophils from patients with chronic wounds after exposure to repeated HBO sessions. Neutrophils were isolated from blood from patients with chronic wounds before and after exposure to repeated HBO sessions, after which cells were washed and treated with TNF- α or left untreated. cDNA was synthesised from extracted RNA. qPCR was used to determine expression of Bcl-2 (section 2.1.7.5). Gene expression was normalised against the untreated cells before HBO exposure. Data are expressed as the means \pm SEM, for four patients, ($n = 4$) with triplicate measurements. For each HBO session, bars with the different lower case letters are significantly different (two-way repeated measures ANOVA, $P < 0.05$, A). The PCR product for Bcl-2, before and after exposure to long-term repeated HBO sessions in both TNF- α treated and untreated cells in 1% agarose gel. Lanes: before HBO unstimulated cells (1), before HBO TNF- α stimulated cells (2), 20 HBO sessions unstimulated cells (3), 20 HBO sessions TNF- α stimulated cells (4) 30 HBO sessions unstimulated (5) and 30 HBO sessions TNF- α stimulated cells (6, B).

7.4 Discussion

Although sample size in this preliminary study was small and hence the results need to be treated with caution, nevertheless, the present work demonstrated a decrease in antioxidant enzyme activities after repeated exposure to HBO in patients with chronic wounds. This effect became apparent in patients after 20 HBO sessions, with significant effects observed after 30 HBO sessions as indicated by decreased antioxidant enzyme activity, particularly CAT, and SOD activities. Antioxidant enzyme levels, particularly SOD, have been shown to be related to increased oxidative stress and may be considered as potential indirect markers of it (Cherubini *et al.*, 2005). One explanation for the present results is that HBO induced an oxidative stress response in patients with chronic wounds, which could have led to decreased antioxidant enzyme activities, as a consequence of oxidative damage. An alternative explanation for the decrease in antioxidant enzyme activities is that HBO enhanced the resolution of inflammation in patients with chronic wounds which led to a reduction in oxidative stress and consequently also to reduced antioxidant enzyme activities. Hyperbaric oxygen has been demonstrated to exhibit anti-inflammatory effects by modifying growth factor and cytokine effects by regulating their levels or receptors (Zhao *et al.*, 1998, Benson *et al.*, 2003). The present results require further exploration and replication to confirm. A series of 15 HBO treatments has been shown to increase levels of reactive oxygen metabolites, malondialdehyde and decrease the activities of SOD and CAT in patients with pathological conditions related to hypoxia (Benedetti *et al.*, 2004). The results of the current study corroborate this earlier finding because they demonstrated a decrease in CAT and SOD activities after 20 and 30 HBO sessions with significant effects only after the latter, which suggests that the antioxidant enzymes could have been inactivated by singlet oxygen and peroxy radicals after prolonged oxidative stress (Narkowicz *et al.*, 1993). HBO has been identified to cause an increase in the production of ROS

(Narkowicz *et al.*, 1993) and consequently can lead to oxidative stress (Bearden *et al.*, 1999). This depends on the toxic nature of the hyperoxic component of HBO (Wispe and Roberts, 1987). In addition, a clear relation for elevated levels of oxidative stress with both increased exposure pressure and time was demonstrated in lung tissue (Ay *et al.*, 2007). However, an increased level of antioxidant enzyme activities in rat lung and erythrocytes was not found after a 90-min exposure (Ay *et al.*, 2007). These observations provide evidence for the safety of HBO administration within the clinically-approved limit. Since the antioxidant enzyme activities were measured immediately after HBO sessions, the decrease in antioxidant activities is of particular importance indicating an uncontrolled oxidative effect in cases of 30 HBO exposures or more. Interestingly, oxidative DNA damage has previously been detected after a single HBO exposure but not after further treatments under the same conditions (Speit and Bonzheim, 2003), demonstrating the induction of an adaptive response. However, these protective mechanisms were not detected in cases where HBO treatment was repeated more than twice (Speit *et al.* 2002). It seems that the results of the present study may be due to the fact that HBO treatment may cause uncontrolled oxidative stress in patients with chronic wounds when continued for more than 20 sessions as shown by a decrease of antioxidant enzyme activities (CAT and SOD) at the 30 HBO session but not after 20 sessions, in spite of the adaptive responses after a single session of HBO. Additionally, HBO might affect antioxidant enzyme transcription or have an indirect effect through superoxide production (Oury *et al.*, 2002) as the beneficial effects of HBO seems to be through superoxide and H₂O₂ (Hink and Jansen, 2001). Another possible explanation for a decrease in antioxidant enzyme activities is that HBO enhanced the resolution of inflammation in patients with chronic wound through its effects on cytokine levels, which caused a reduction in oxidative stress and consequently antioxidant enzyme activities. HBO has been demonstrated to exhibit anti-inflammatory effects by

modifying growth factor and cytokine effects by regulating their levels or receptors (Zhao *et al.*, 1998, Benson *et al.*, 2003). Further work should be conducted to investigate how HBO affects antioxidant enzymes (e.g. directly by gene transcription or indirectly through ROS production for example superoxide production) with varying durations of HBO exposure.

In a previous study, a single exposure to HBO decreased ascorbate levels and elevated lipid peroxidation in human volunteers (Bader *et al.*, 2007). Contrary to expectations, the current study did not find any change in ascorbate levels following repeated HBO treatment. One possible reason for this is that the depletion of antioxidants by oxidative stress activates signal transduction pathways leading to antioxidant mobilization in the body through the plasma (Elsayed, 2001). It should be noted that patients with chronic wounds generally have low ascorbate levels (Burns *et al.*, 2003) and that it is possible that this might be accentuated by ROS production following HBO leading to excessive oxidative stress that overwhelms the endogenous cellular antioxidant mechanisms. However, the present study does not indicate that this is the case. Nevertheless, supplementing with antioxidants, such as ascorbate, during HBO treatment could potentially enhance the therapeutic effect of HBO (Alleva *et al.*, 2005). As well as being an antioxidant, ascorbate has been shown to stimulate the rate of procollagen secretion in a cell culture system (Murad *et al.*, 1981, Blanck and Peterkofsky, 1975) via the formation of hydroxyproline that provides stability for the procollagen triple helix (Berg and Prockop, 1973), whereas ascorbate deficiency is associated with defective connective tissue (Peterkofsky, 1991), impaired wound healing, weaker scars and abnormal capillary formation (Burns *et al.*, 2003).

The levels of cytokines in plasma or serum are often low and these levels are influenced by factors such as the length of time between the drawing of the patient's blood to the separation of plasma, the temperature of storage, and repeated thawing and freezing

(Fletcher *et al.*, 2009). Therefore, the availability of sensitive multiplex technology, in this case the Luminex[®] assay, was useful in that it allowed the determination of multiple cytokines simultaneously in plasma samples as well as in control samples. Previous studies have examined *in vitro* effects of HBO on cytokine production (Van Den Blink *et al.*, 2002, Brenner *et al.*, 1999). These studies involved the measurement of cytokines in stimulated whole blood, mononuclear cells and the supernatants of cell culture *in vitro*, which may not be predictive of what occurs *in vivo*. In addition, they involved single exposures to HBO; while in reality patients often receive 20 or more HBO treatments. HBO treatment decreased plasma TNF- α in a murine zymosan-induced shock model (Luongo *et al.*, 1998). However, *in vivo* effects of repeated exposure to HBO on plasma cytokine levels of patients with chronic wounds are still unclear. The present results demonstrated that HBO significantly decreased plasma TNF- α and IL-6 after 20 and 30 HBO sessions compared to before HBO treatment. Although there was a trend for the levels of the anti-inflammatory cytokine IL-10 to increase after the repeated HBO sessions, this increase was not significant. Overall, the results suggest an immunosuppressive effect of HBO and that it has broad ranging effects on the adaptive immune response. HBO could be exerting its immunosuppressive effects by inducing apoptosis in cytokine-producing cells. For example, exposure to HBO can enhance apoptosis in murine thymocytes, lymphocytes, and granulocytic cell lines (Ganguly *et al.*, 2002) and result in a decrease in circulating CD4⁺ cells in rats (Bitterman *et al.*, 1994) and humans (Bitterman *et al.*, 1993). Neutrophil apoptosis may also contribute to the immunosuppressive effects of HBO treatment (Weber *et al.*, 2009a). Therefore, it is suspected that in chronic wounds, the effects of HBO may involve the induction of apoptosis in neutrophils. Interestingly, repeated exposure to HBO treatment decreased Bcl-2 expression after the 20 and 30 sessions. Bcl-2 is part of a signal transduction pathway that counterbalances oxidative stress effects and protects the cells from ROS-

mediated apoptosis (Hockenbery et al., 1993). A possible explanation for the present results is that ROS act to down-regulate endogenous Bcl-2 within cells, thus in different cell types, increases in ROS correlate with decrease in Bcl-2 and vice versa (Hildeman *et al.*, 2003). Since levels of Bcl-2 within cells are critical to antiapoptotic activity, down regulation of Bcl-2 could be a mechanisms to sensitize cells to apoptosis. Bcl-2 itself does not possess antioxidant activity, but it may act indirectly to increase antioxidants (e.g. glutathione or superoxide dismutase) within cells (Voehringer and Meyn, 2000, Lee *et al.*, 2001). Thus, decreased expression of Bcl-2 may allow cells to be affected by ROS, possibly by allowing a decrease in endogenous antioxidant enzymes. The current results relating to CAT and SOD levels after repeated exposure to HBO support this hypothesis as repeated exposure to HBO lead to a decrease in antioxidant enzyme activities and Bcl-2 expression. Down regulation of the Bcl-2 family is mediated by signal transduction involving transcription agents such as NF- κ B (Lee *et al.*, 1999). The present results demonstrated a down regulation of NF- κ B after repeated exposure to HBO treatment which may result in down regulation of Bcl-2.

It is widely accepted that HBO mediates its beneficial effect via ROS (Hink and Jansen, 2001). The HBO effects on pro-inflammatory cytokine production could also have a stress-related and hyperoxia-dependent component which may be at least partly responsible for these effects. Additionally, the decrease in cytokine production by repeated exposure to HBO appears to involve transduction signals such as NF- κ B, a prominent signal transduction factor in the inflammatory processes, for example in TNF- α production. Therefore, NF- κ B is involved in immunodulatory signals of increased pressure or oxygen. In support of this notion, repeated exposure to HBO caused a significant decrease in NF- κ B expression after 20 and 30 HBO treatments in neutrophils from patients with chronic wounds as well as in neutrophil-like cells, while a single exposure to HBO (2.4 ATA, 90 min) led to a decrease in both NF- κ B gene

expression and protein levels (see Chapter 5). Thus, signal transduction through NF- κ B may be involved in the effects of long term exposure to HBO. Apoptotic cell death can be prevented by the expression of nuclear NF- κ B (Beg and Baltimore, 1996, Wang *et al.*, 1996), which is responsible for controlling expression of genes involved in inflammation. This suggests that NF- κ B may also be a part of the signal transduction process that is involved in the cell survival mechanism (Barinaga, 1996). Previous studies have demonstrated an association between ROS production and NF- κ B activation (Sen and Packer, 1996, Shea *et al.*, 1996). A single exposure to HBO (2.8 ATA, 1 h) induced NF- κ B activation in isolated human peripheral-blood mononuclear cells (Madden *et al.*, 2011), in mouse lymphocytes (Shea *et al.*, 1996) and leukocytes (Dennog *et al.*, 1996) in response to increased ROS generation. NF- κ B activity was also enhanced following DNA damage (Beg and Baltimore, 1996, Wang *et al.*, 1996). Several factors can explain the difference between the results presented here with these studies. For example, the HBO exposure conditions used in this study may differ from those that patients might be exposed to in other studies. Another factor may be the type of cells used. For example, monocytes and lymphocytes were used in these studies as the cell viability was not affected by hyperoxia. However, in neutrophils the mechanism of HBO is different in that a single *in vitro* exposure to HBO was shown to induce apoptosis with increased ROS production in both neutrophil-like cells and neutrophils from bovine blood. In addition, in previous studies mentioned above, the NF- κ B activity was measured and not the NF- κ B mRNA gene expression as in the present study. Increased levels of mRNA do not necessarily result in similarly increased levels of NF- κ B activity; it is an estimate of the functional difference that occurs at the protein level. In a previous study, nuclear translocation and DNA-binding activity of NF- κ B failed to produce any significant difference in NF- κ B-dependent gene expression in

human monocytes exposed to a pulsed ultra-wideband electromagnetic field (Natarajan *et al.*, 2006). Furthermore, other signals such as mechanical pressure which can affect G-protein-coupled receptors (GPCRs) that act as mechanoreceptors have been identified on different cells, including neutrophils (Zou *et al.*, 2004) and may possibly affect NF- κ B activation. This suggests that HBO-mediated down regulation of NF- κ B could be partly due to a pressure effect.

However, it should be borne in mind that the presence or absence of cytokines in biological fluids such as plasma reflects a complex balance of enhancing and inhibitory signals acting on cells. Such signals regulate their production and catabolism, as well as their binding to target cells, and also the modulation of their receptors on the cell surface (Cavaillon *et al.*, 1992). Plasma cytokines might be regarded as a spill over from the tissue, and their levels do not necessarily parallel their activity. In patients with sepsis, measurements of pro- and anti-inflammatory cytokine levels in plasma only indirectly reflected tissue secretion, and combining them with other measurements such as NF- κ B activity provided a useful record of the overall pattern of immunological events in these patients (Arnalich *et al.*, 2000). Also, plasma cytokine levels poorly reflect the mRNA expression of pro- and anti-inflammatory cytokines in neonatal pigs (Brix-Christensen *et al.* (2003). Further *in vivo* studies may allow better investigation of the mechanisms of prolonged HBO treatment on cytokines and gene expression.

7.4.1 Conclusion

In conclusion, repetitive HBO sessions led to a condition of oxidative stress in patients with chronic wounds that appeared to affect the antioxidant defence system. A possible explanation for the decrease in antioxidant enzyme activities is that paradoxically HBO exposure may result in short-term oxidative stress that induces immunosuppressive effects (a decrease in the pro-inflammatory cytokines, TNF- α and IL-6) which ultimately lead to a decrease in oxidative stress (reflected in the lowered plasma antioxidant enzyme activities). However, the decrease in plasma antioxidant enzymes could also be explained as direct oxidative damage as a consequence of the HBO treatment, and so further studies should focus on whether the changes in enzyme activity result from chemical modifications caused by reactive oxygen species or from changes in expression of the proteins.

CHAPTER EIGHT

GENERAL DISCUSSION

8.1 General discussion

HBO is a medical treatment in which patients are placed in a chamber pressurized to more than one ATA breathing 100% pure oxygen. HBO as a treatment has been established for a number of clinical and surgical conditions; for example it has been used for a range of inflammatory conditions. However, the exact mechanism of its action is still the subject of some discussion.

HBO has been successfully prescribed as a treatment option for chronic wounds for at least 40 years (Kulonen and Niinikoski, 1968). Chronic wounds are common and persistent problems, with significant effects on quality of life with an additional economic cost (Kranke *et al.*, 2004). The application of HBO has been shown to aid the healing of problematic wounds and it has been demonstrated to reduce the risk of amputations (Thackham *et al.*, 2008). Wound healing is a highly regulated and interactive process involving three main phases, inflammation, proliferation and remodelling. There is a degree of overlap of these phases over time. There is a growing consensus that chronic wounds remain stuck in the inflammatory phase, with an overabundance and persistence of neutrophils (Agren *et al.*, 2000, Yager *et al.*, 1996). In fact, in chronic wounds the inflammatory response is characterized by continued neutrophil infiltration (Nwomeh *et al.*, 1998b, Yager and Nwomeh, 1999). Despite their beneficial role in wound healing as part of the host's defence against infection, neutrophils may have detrimental effects on wound healing by destroying growth factors and degrading ECM components via the production of proteolytic enzymes (Yager and Nwomeh, 1999, Agren *et al.*, 2000). Neutrophil apoptosis is important in the successful resolution of inflammation, leading to down regulation of pro-inflammatory functions and their safe clearance by macrophages. Local hypoxia predisposes wounds to infection due to limited neutrophil-mediated killing of bacteria by ROS. Exposure to HBO restores this defence against infection and improves the rate of healing. Although

these effects enhance the host defence, the overall effects of HBO appear to be immunosuppressive (Brenner *et al.*, 1999). The mechanisms by which these effects are produced remain largely unrecognised. Therefore, research is essential in providing sufficient scientific evidence for the therapeutic application of HBO and to elucidate the mechanisms involved in order to improve treatment protocols and the subsequent benefits to patients.

It is widely accepted that breathing oxygen at pressures greater than 1 ATA will increase production of ROS (Thom, 2011). This is critical for the therapeutic action of HBO. While oxidative stress caused by ROS may mediate damage to surrounding tissue and cells, ROS also have a role in signal transduction cascades, and can generate positive or negative effects depending on their concentrations and their intracellular localisation. Therefore, the present study focused on the effect of HBO on the inflammatory response of neutrophils and how significant modification of their action could affect the resolution of inflammation in patients after treatment.

Firstly, the effect of a single exposure to HBO was investigated on the antimicrobial activity of neutrophil-like cells and their apoptosis, obtained by differentiation of HL-60 cells using ATRA. The study also investigated with neutrophil-like cell apoptosis and their subsequent clearance by MDM Φ , measured after a single exposure to HBO, and investigated whether this is associated with HBO-induced ROS production. Changes in pro- and anti-inflammatory cytokine production by neutrophil-like cells were also investigated. An *in vivo* study was conducted to investigate the effect of prolonged exposure to HBO on patients with chronic wounds.

The research presented here has provided evidence of anti-inflammatory effects of HBO and their role in the resolution of the inflammatory response in the pathogenesis of chronic wounds. The most important evidence was the significant increase in neutrophil apoptosis and their clearance by macrophages following a single exposure to HBO (2.4

ATA, 90 min). These effects appear to be partly due to HBO-mediated ROS production. The study further supported the previous research and confirmed the immunosuppressive effects of single exposure to HBO through a decrease in pro-inflammatory cytokines and promotion of anti-inflammatory cytokines in neutrophil-like cells. In addition, this work has highlighted some interesting features of HBO, such as the effects of pressure which have not been fully explained (Fig. 8.1).

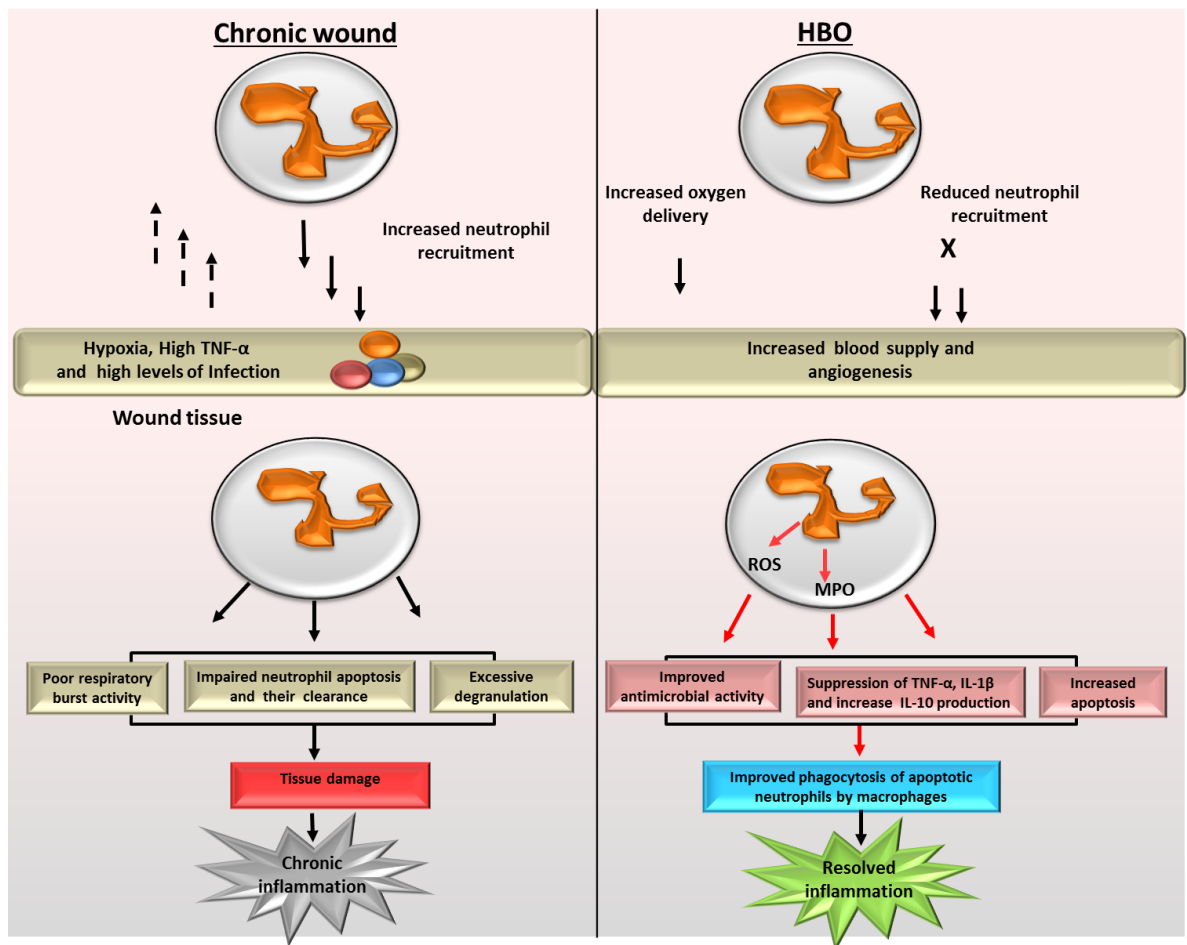


Fig. 8.1 Proposed mechanisms by which HBO affects neutrophil function and apoptosis based on the findings of the present study. Exposure to HBO increases oxygen delivery and enhances the respiratory burst activity and ROS production by neutrophils, which in turn improves antimicrobial activity. This, combined with improved neutrophil apoptosis and neutrophil clearance via phagocytosis by macrophages, reduces pro-inflammatory cytokine levels and increases levels of the anti-inflammatory cytokine, IL-10. This results in resolution of the inflammation. Red arrows highlight the findings of the present study.

The inflammatory response and subsequent wound healing are principally dependent on cellular immune elements (neutrophils and macrophages) leading to a recognizable physiological response. As well as encountering invading microorganisms, these cells also produce signalling molecules that actively participate in wound healing. Research to date regarding clinical treatments remains limited in terms of understanding the cellular events of tissue injury and inflammation. Although it is becoming clear that neutrophil apoptosis is critical for resolving inflammation, phagocytic activity of neutrophils is still required for proper macrophage function and wound healing. What is not yet clear is the degree to which the clinician's attempts to minimize the neutrophil-mediated tissue damage through accelerating apoptosis might interfere with neutrophil functions in wound healing. Elucidating this balance is a key target in understanding the resolution of inflammation and the wound healing progress. Clinical therapies may focus on modifying the cellular response at the tissue and immune cell levels, possibly to decrease the detrimental effects of inflammation and enhance wound healing (Butterfield *et al.*, 2006). Recently, ROS-mediated neutrophil apoptosis has been suggested as an essential step for the generation of neutrophil extracellular mediators that bind and kill invading microorganisms (Fuchs *et al.*, 2007).

Since therapeutic HBO treatment demonstrates the beneficial effects of resolving inflammation and chronic wound healing, the mechanisms involved suggest a strong relationship between HBO treatment and ROS. *In vitro* and *in vivo* studies suggest contradictory evidence on this subject (Hink and Jansen, 2001, Benson *et al.*, 2003, Thom *et al.*, 2008, Thom, 2009). However, previous research has identified the key HBO effect as being ROS formation. For a long time, ROS generation by HBO was considered to be a detrimental factor inducing unexpected damage to tissue and cells. However, more recent evidence has identified that ROS production is balanced with antioxidant defence systems, and that this balance is essential for the redox signalling

system. Therefore, ROS at moderate levels are able to regulate cell functions (Trachootham *et al.*, 2008).

The present study investigated the effects of a single HBO treatment on antimicrobial activity and apoptosis of neutrophils. HL-60 cells differentiated using ATRA were used as a well-defined neutrophil-like cell model that can be readily maintained in standard conditions. Initial experiments showed that incubation of HL-60 cells with 1 μ M ATRA for 5 days initiated their differentiation into neutrophil-like cells, whilst incubation with PMA as the stimulus demonstrated PMA's ability to enhance the respiratory burst activity in these neutrophil-like cells. Therefore, a comparative experiment was carried out whereby neutrophil-like cells were exposed to a single 90 min treatment of either HBO (2.4 ATA), pressure, hyperoxia or hypoxia. The biochemical assays confirmed that pre-treatment with HBO enhanced the respiratory burst activity of neutrophil-like cells. Exposure to HBO increased $O_2^{\bullet-}$ and H_2O_2 production compared to normoxia-treated cells.

ROS such as $O_2^{\bullet-}$ and H_2O_2 serve as signalling molecules in different pathways regulating production of various growth factors and cytokines (Allen and Balin, 1989, Calabrese *et al.*, 2007, Maulik, 2002). HBO treatment enhanced the phagocytic activity of neutrophil-like cells and increased MPO activity and evidence of degranulation (exocytosis). HBO-induced oxidative stress also regulates the production of NO production, which is known to react with $O_2^{\bullet-}$ to generate $ONOO^-$ and OH^{\bullet} . The present study showed that high levels of NO_x were observed following HBO as well as hyperoxia treatments. A possible mechanism by which HBO exerts its effects is via the impaired neutrophil adherence through the inhibition of β_2 integrin. A key target of HBO effects on neutrophil adhesion might be the cytoskeleton. The present findings are consistent with the idea that HBO may induce changes in the cytoskeleton that are manifested by increased actin depolymerisation that normally follows stimulus-induced

F-actin assembly. Actin depolymerisation plays a key role in a number of neutrophil functions including respiratory burst, phagocytosis and chemotaxis (Crawford and Eggleton, 1991, Cano *et al.*, 1991). This suggests that actin might be an important, functional target for HBO. Rac1, a member of the Rho family of GTPases, which contribute to the organization of the actin cytoskeleton and of the associated sites of cell adhesion to the extracellular matrix (Yager *et al.*, 1996), regulates exocytosis through the formation of F-actin, which is necessary for granule translocation to the sites of exocytosis, whilst depolymerisation of actin should occur concurrently with cytoplasmic F-actin formation to control actin remodelling during neutrophil activation.

Treatment with HBO causes an increase in NO production in cells as a consequence of enhanced NOS function (Thom, 1993). NO not only acts as a free radical, but also has a role in VEGF regulation and subsequently angiogenesis. Nitrite (NO_2^-), the major oxidation product of NO, can react with hypochlorous acid that is generated by MPO to yield nitryl chloride and nitrogen dioxide (NO_2). Under physiological conditions, NO_2^- can react with protein and result in an *S*-nitrosylation reaction that appears to be responsible for the impairment of β_2 integrin function observed in HBO-treated neutrophils (Thom *et al.*, 2008). This is also in accordance with earlier observations which have shown, for example, that HBO-mediated elevation of *S*-nitrosylated proteins was inhibited in neutrophils pre-treated with N^G -nitro-L-arginine methyl ester (L-NAME) or in neutrophils from MPO knock-out mice (Thom *et al.*, 2008). Therefore, HBO-induced reactive species formation by iNOS and MPO activity are responsible for excessive *S*-nitrosylation of β -actin. This commonly occurs in neutrophils due to the high abundance of MPO.

Oxygen levels have been found to regulate Ca^{2+} signals via mechanisms involving ROS (Ermak and Davies, 2002). Ca^{2+} release and storage may be directly regulated by the cytoskeleton, particularly actin depolymerisation. Ca^{2+} levels are reduced when cells are

exposed to dominant-negative constructs of Rac proteins and restored when exposed to constitutively active forms of Rac (Hong-Geller *et al.*, 2001). Therefore, Rac-mediated signalling in neutrophils may exploit Ca²⁺ signalling as a mode of primary granule translocation and exocytosis (Mitchell *et al.*, 2008).

Regarding the results in the present study, enhancement of antimicrobial activity of neutrophil-like cells by HBO seems to limit the resolution of inflammation in chronic wounds. However, antimicrobial activity in neutrophils is associated with apoptosis (Nagaoka *et al.*, 2012). Neutrophil apoptosis is clearly essential for wound healing, since chronic wounds are characterized by the persistence of inflammatory and hypoxic conditions in which neutrophil longevity is increased (Khanna *et al.*, 2010). Meanwhile, HBO treatment led to a decrease in the viability of neutrophil-like cells, an increase in apoptosis as assessed using caspase 3/7 activity and morphological changes associated with apoptosis. The increase in apoptosis of the neutrophil-like cells seems to be a consequence of increased ROS production by the cell itself that activates death receptor signalling thereby promoting neutrophil apoptosis via caspase cascade activation. In addition, there is the possibility of a role for mitochondria both in ROS production and in the initiation of the intrinsic apoptotic pathway. The HBO-induced oxidative stress is linked with a mitochondrial pathway of apoptosis (Weber *et al.*, 2009a).

Although neutrophils possess mitochondria that are deficient in certain life-maintaining functions, a growing body of research has reported that mitochondria are involved in neutrophil apoptosis. Release of cytochrome *c* appears to play a major role, since it is released from mitochondria upon an apoptotic insult and initiates the extrinsic pathway of apoptosis, promoting oligomerization of Apaf-1 and consequently caspase-9 activation (Liu *et al.*, 1996a, Adams and Cory, 2002). Neutrophils are thought to express a low amount cytochrome *c* which perhaps leads to the accumulation of electrons in the respiratory chain and subsequent ROS formation, when other

respiratory complexes, which do not need cytochrome *c*, are still active. The low content of cytochrome *c* seen in neutrophils leads to conditions that promote caspase-9 activation (Maianski *et al.*, 2004a). Pro- and anti-apoptotic members of the Bcl-2 family of proteins control the release of cytochrome *c*, Apaf-1 and caspase-9 (Hengartner, 2000, Nicholson *et al.*, 1995). While Bcl-2 and Bcl-x1 exert anti-apoptotic effects, the pro-apoptotic member of the Bcl-2 family, Bax, can directly cause the release of cytochrome *c* and cause apoptotic effects. HBO treatment caused a down regulation of the gene expression of the anti-apoptotic factor Bcl-2 in untreated and TNF- α treated neutrophil-like cells. These results suggest the possibility of HBO-induced apoptosis via the mitochondrial pathway. Due to the fact that HBO treatment combines the dual elements of hyperoxia and high pressure, it is possible that the apoptosis increase observed could be due to either of these factors. Down regulation of the Bcl-2 gene was exhibited in untreated and TNF- α treated cells following exposure to hyperoxia. The molecular control of neutrophil apoptosis is still unclear; studies have suggested that apoptosis is triggered in the absence of survival factors such as HIF and NF- κ B. For instance, TNF-induced apoptosis inactivated the NF- κ B pathway via the inactivation of a typical protein kinase C that regulated the IKK enzymatic activity (Diaz-Meco *et al.*, 1999) and factors that activate NF- κ B blocked the apoptosis. LPS blocked TNF-induced apoptosis in myeloid cells through activation of NF- κ B (Manna and Aggarwal, 1999). The findings in the present study also suggest a role for NF- κ B as an anti-apoptotic agent. HBO, pressure and hyperoxia treatment decreased the expression of NF- κ B and protein levels in the nuclear fraction in neutrophil-like cells that were treated with TNF- α . HBO may affect several genes that may play a role in inhibiting apoptosis and whose expression is regulated by NF- κ B; these include cellular inhibitors of apoptosis such as cIAP-1, cIAP-2 and TRAF2 (Stehlik *et al.*, 1998, Chu *et al.*, 1997). The Bcl-2 homologue Bfl-1/A1 is another gene whose transcription is regulated by NF- κ B and

which inhibits apoptosis (Wang *et al.*, 1999, Zong *et al.*, 1999). Bcl-2 activates NF- κ B through the degradation of the inhibitor I κ B α (De Moissac *et al.*, 1998; Fig. 8.2). This is consistent with the present results, which showed a decrease of expression of Bcl-2 after HBO treatment in TNF- α -treated neutrophil-like cells (see Chapter 5). Furthermore, a possible role for HIF-1 α may be in NF- κ B regulation. HIF-1 α is a direct target gene of NF- κ B and itself contributes to the activation of the NF- κ B pathway. This important interaction was confirmed in a previous study (Van Uden *et al.*, 2008) which suggested that several members of the NF- κ B family can bind to the HIF-1 α promoter under basal conditions as well as under exposure to TNF- α .

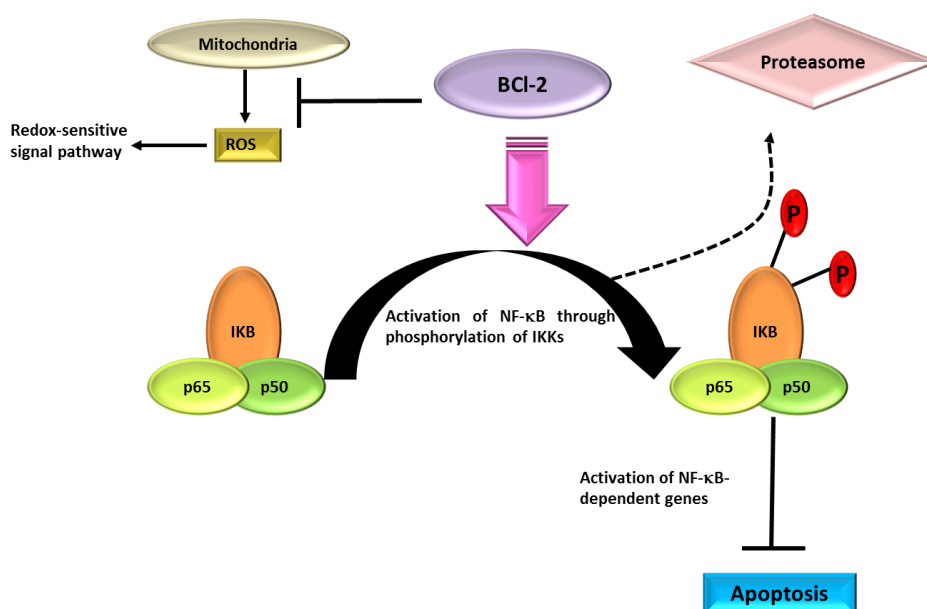


Fig. 8.2 Proposed molecular mechanisms underlying constitutive activation of NF- κ B and Bcl-2. Bcl-2 activates NF- κ B through a mechanism that involves the N-terminal phosphorylation and degradation of I κ B α (inhibitor of kappa B) by proteasomes. This permits NF- κ B to translocate to the nucleus and activate downstream expression necessary to suppress apoptosis, stimulate IKK activity and cause augmentation of NF- κ B activation. Bcl-2 expression protects against ROS-induced apoptosis and is associated with regulation of redox sensitive signal pathways such as changes in gene expression or the inflammatory process (Wang *et al.*, 1999, Zong *et al.*, 1999).

To confirm that the HBO-induced neutrophil apoptosis occurred in a non-inflammatory way, pro-inflammatory (TNF- α and IL-1 β) and anti-inflammatory (IL-10) cytokine levels were measured. HBO inhibited pro-inflammatory cytokine production and enhanced anti-inflammatory effects which could be due to HBO-induced apoptosis.

Given that the resolution of inflammation at the wound site requires removal and clearance of apoptotic neutrophils from the inflamed site, it is reasonable to suppose that failure to achieve this could result in chronic, persistent inflammation. In Chapter 5, the investigation of apoptotic neutrophil-like cells demonstrated an integral response to HBO-mediated ROS production, but the design of this particular experiment meant that it was not possible to find out whether these apoptotic cells would remain at the wound site or be removed in a safe manner. Consequently, experiments using co-culture of apoptotic neutrophils and macrophages with HBO treatment may be useful in following up the present work by investigating the clearance of apoptotic neutrophils at wound sites. Initially, isolated bovine neutrophils and monocytes were used, which may be more directly applicable to an *in vivo* situation rather than the cell line model. MDM Φ differentiated from bovine monocytes using autologous serum were used as an *in vitro* macrophage model. Initially, apart from morphological changes during apoptosis, changes on the apoptotic cell surface also occurred. These changes are important in stimulating the efficient removal of apoptotic cells by phagocytes. HBO and pressure treatments enhanced the apoptosis of fresh and 22 h-aged neutrophils and their clearance by MDM Φ . Oxidation of PS may stimulate its externalisation, the possible mechanism being that oxidised PS inhibits aminophospholipid translocase, the enzyme that is responsible for the maintenance of plasma membrane phospholipid asymmetry (Tyurina *et al.*, 2004). HBO and pressure treatments enhanced H₂O₂ production in both fresh and 22 h-aged neutrophils as well as in co-culture experiments. Hence, the activation of the NADPH oxidase burst of ROS after HBO treatment might be coupled with the externalisation of PS. Oxidants have been implicated previously in apoptosis associated with aging neutrophils (Oishi and Machida, 1997, Kasahara *et al.*, 1997). The present study suggests that HBO induced PS oxidation through activation of the

NADPH oxidase that generates ROS, and hence catalyses oxidation of different cellular constituents, including phospholipids, due to triggering of the apoptotic program.

Alterations in the cytoskeleton (polymerisation, depolymerisation) interacting with cell apoptosis signals might enhance the activation of caspases or disrupt mitochondria and cause the release of cytochrome *c* (Thomas *et al.*, 2006). During phagocytosis of apoptotic cells, major changes in the cytoskeleton of the cell occurs, leading to the formation of filopodia surrounding an apoptotic cell that is to be engulfed. Alteration in actin depolymerisation plays an important role in this process and the signalling mediators in pathways resulting in cytoskeleton rearrangement all involve Rac-1. Engulfment is stimulated by activation of Rac-1 and Cdc42, both members of the Rho family of GTPases, and they affect cytoskeletal organization (Somersan and Bhardwaj, 2001). Cytoskeletal rearrangement enables the phagocyte to enclose the apoptotic cell into a phagosome where degradation occurs. It has been suggested that HBO has effects on the cytoskeleton and actin polymerisation. HBO exposure causes a marked increase in Rac activity (Thom *et al.*, 2008). The results support the hypothesis that actin depolymerisation in apoptotic and phagocytic cells acts as a target of a signalling cascade during exposure to HBO (Fig. 8.3). This is an important topic for further research.

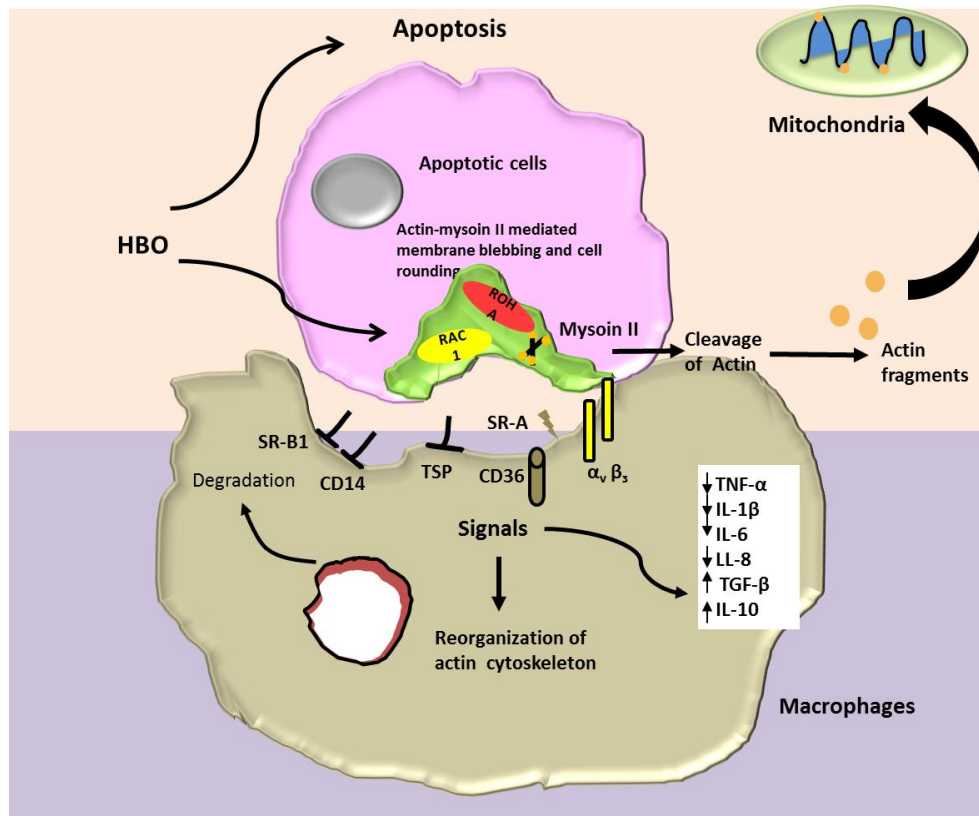


Fig. 8.3 Hypothesised mechanisms of HBO effects on the actin cytoskeleton during neutrophil apoptosis and their clearance by macrophages. Neutrophils undergoing apoptosis following HBO treatment show pronounced membrane blebbing, and alterations in phospholipid asymmetry leading to phosphatidylserine (PS) exposure. Binding and engulfment of apoptotic cells are achieved through one of a number of membrane receptors, which may include SR- B, CD14, SR-A and CD36/VnR (vitronectin receptor $\alpha_v \beta_3$), together with thrombospondin (TSP) in macrophages (Fadok *et al.*, 1992, Zwaal and Schroit, 1997). HBO-induced apoptosis results in changes in the actin cytoskeleton in apoptotic cells leading to the following biochemical changes: actin-myosin II contraction mediates morphological hallmarks of apoptosis including membrane blebbing and cell rounding. Activation of the caspase cascade pathway enhances actin cleavages and triggers their N-myristoylation with actin being translocated to the mitochondria. Internalization of the dying cell involves reorganization of elements of the cytoskeleton through adaptor/signalling molecules in macrophages (Swanson and Baer, 1995). These signalling events might also change the profile of cytokine and/or chemokine secretion by the macrophages (Hughes *et al.*, 1997). Internalized apoptotic material might be extensively degraded and directed to a salvage pathway (Brazil *et al.*, 1997).

Phagocytosis by macrophages actively suppresses inflammation; this is often referred to as a ‘silent event’, reducing pro-inflammatory TNF- α production and increasing anti-inflammatory TGF- β 1 production, but only following the ingestion of apoptotic, not secondary necrotic, cells (Savill and Fadok, 2000). Therefore it was of interest to study whether HBO-induced neutrophil apoptosis and clearance coincided with the

suppression of the inflammatory response. HBO appeared to elicit an anti-inflammatory effect and decreased the expression of TNF- α , and significantly increased IL-10.

In spite of the fact that HBO exposure is applied for chronic wound treatment in a repeated manner, it also leads to oxidative stress. Other studies have shown that HBO treatment leads to increased ROS formation that causes cellular damage to lipids, proteins and DNA (Narkowicz *et al.*, 1993, Jamieson *et al.*, 1986). The present study was based on *in vitro* experiments to investigate the effects of HBO; therefore knowledge of biochemical indexes and anti-inflammatory effects of HBO treatment for patients with chronic wounds could lead to developments in treatment protocols. Thirty *in vivo* HBO exposures led to a reduction in the antioxidant enzymes SOD and CAT in the plasma, with changes in the levels of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 and the anti-inflammatory cytokine IL-10, and subsequent changes in the levels of the anti-apoptotic factors NF- κ B and Bcl-2. Several experiments in the present study were limited in their power to detect statistically significant differences between treatments because only four patients were available and there are potentially large variations between different patients. Increasing the scope of some of these studies by performing further replicates might allow detection of statistically significant differences, thereby increasing the robustness of the results.

In the present study, neutrophils were treated with 8.8% O₂ at a pressure of 2.4 ATA, to give a final PO₂ equal to that observed under normoxic conditions, but at a pressure more than double atmospheric pressure. Neutrophils treated with the pressure control (pressure alone) demonstrated increases in H₂O₂ and O₂^{•-} production, and apoptosis compared to normoxic conditions. The mechanical effects of pressure could be responsible for most of the changes seen after this treatment. Mechanical deformation of PMNs results in an increase in cytoplasmic free Ca²⁺ and up regulation of cell surface

adhesion molecules CD11b/CD18 (Anderson *et al.*, 2001). This could explain the observed pressure effects, and suggests that priming of neutrophils with a chemoattractant such as FMLP would make the cells more sensitive to activation by mechanical stress (Doerschuk *et al.*, 1993). Neutrophil functions such as H₂O₂, OH[•] and HOCl production are directly related to the level of PO₂ (Davis *et al.*, 1988). Additionally, the formation of microbubbles during decompression may have an effect on the cell surface molecules (Madden and Laden, 2007). However, in the present study a sealed chamber was used. This did not allow the measurement of microbubble formation in the cell culture medium during decompression. Therefore the measurement of microbubbles, for example by ultrasound (Bollinger *et al.*, 2009) using a multiplace HBO chamber would be a possible way of investigating whether or not microbubble formation is plays a role in this situation.

The response to fluid shear involves specific mediators on the cell membrane. In neutrophils, G protein-coupled receptors (GPCRs) mediate the response to fluid shear stress. Formyl peptide receptor (FPR) is the most predominant mechanoreceptor in neutrophils (Makino *et al.*, 2006). This receptor is also involved in the initiation of apoptosis in fibroblasts; stimulation of FPR triggers a signalling cascade leading to cytochrome *c* release from mitochondria and the initiation of apoptosis. This could explain pressure-mediated neutrophil apoptosis (Revankar *et al.*, 2004). However, *in vivo*, neutrophil function involves processes that could alter the pressure on the cell membrane, which in turn initiates changes in cell status such as L-selectin binding or G-protein ligation leading to activation of the leukocyte and initiating actin polymerization, respiratory burst and granulation (Zarbock and Ley, 2008).

8.2 Limitations of the present work

Although the various experiments have been conducted as part of this research, there are limitations, some of which may be addressed in the future.

Most chronic wounds are mainly characterised by hypoxia, infection and the presence of inflammatory cytokines. Due to fact that it is difficult to control the experimental conditions, the study was unable to proceed with other independent experiments to investigate the effects of HBO on neutrophils and macrophages under pathological conditions.

The *in vivo* results in the present study were limited in their power to detect statistically significant differences between treatments because only four patients were available for the study. Increasing the sample size of these studies by involving more patients with chronic wounds, would allow for the detection of statistically significant differences.

As an appropriate incubator was not available, a small HBO chamber and custom-prepared sealed boxes were used to perform the experiments at room temperature, and the present study was unable to demonstrate the exact effects of oxygen or pressure on the cells at actual body temperature (37 °C) under optimum conditions as would be found *in vivo*.

8.3 Future work

Hyperbaric oxygen (HBO) works to aid the healing of chronic wounds via various mechanisms including the killing of anaerobic bacteria, promotion of neovascularisation and the relief of relative tissue hypoxia in affected tissues. However, there are thought to be many other mechanisms by which HBO aids the healing of chronic wounds. Taking into account the limitations of current work and possible further research, the following selected experiments could be undertaken in future.

In the present study, the apoptotic effects of HBO were partly dependent on ROS production that suggested a contribution by the mitochondrial pathway. Therefore measurement of apoptotic substances released from mitochondria (for example, cytochrome *c*) by Western blotting could be helpful in the analysis of the mechanisms of apoptosis induction by HBO.

The mediators of the effects of HBO on neutrophils should be examined. These potential mediators are HIF-1 and NF- κ B. HIF-1 is a DNA-binding complex which consists of a HIF-1 α and a HIF-1 β subunit, and is responsible for the regulation of cellular responses to hypoxia. Whilst oxygen has no effect on HIF-1 β expression, HIF-1 α demonstrates high translational regulation by oxygen. In cells exposed to oxygen, HIF-1 α is quickly ubiquitinated and degraded by the proteasome system, whereas hypoxia induces HIF-1 α by decreasing its ubiquitin-proteasome-mediated degradation (Huang *et al.*, 1998). Whilst a few studies have examined the effect of HBO on HIF-1 α expression (Li *et al.*, 2005, Peng *et al.*, 2008), none have examined its effect on HIF-1 α transcription. From these studies, HBO may have different effects on HIF-1 α in different cell types, or the effect may depend on the state of the cells before HBO treatment and also the HBO exposure protocol. NF- κ B has been investigated in this study. Therefore it is important to examine the effect of HBO on HIF-1 α using Western

blotting and electromobility shift assays (EMSA), combining these with measurements of NF- κ B which show the effect of HBO on transcription signals and its importance in regulation of pathways.

Excessive neutrophil recruitment is associated with impaired wound healing. Limited research to date has focussed on neutrophil influx to the wound site. Therefore development of an inflammatory animal model to assess the dynamics of neutrophil influx into the wound site and its association with neutrophil viability in circulation is important to identify the mechanisms that aid normal wound healing. A histological study using immunostaining with specific antibodies or biochemical detection of MPO activity as well as fluorescence-based studies using a flow cytometry or fluorescence microscopy protocols could be applied.

Adhesion molecules and their importance in inflammatory conditions have been widely investigated, but the effect of HBO on their expression and their contribution to chronic wound healing is not fully understood. Therefore an ELISA-based examination of the effects of HBO on some adhesion molecules such as ICAM-1, VCAM-1 and selectin, which play a key role in neutrophil recruitment to the wound site via the endothelium, could be of help in understanding the effects of HBO on neutrophil infiltration into the wound site.

IL-8 is potent in attracting and activating neutrophils. Production of IL-8 induces migration of neutrophils to the affected site where they inactivate pathogens by phagocytosis and enhance production of $O_2^{\cdot-}$ and H_2O_2 via respiratory burst activity. Investigation of the effects of HBO treatment on IL-8 release from neutrophils using ELISA protocols will provide further information about HBO effects on neutrophil infiltration into wound sites.

There is mounting evidence to suggest that the actin cytoskeleton acts as a sensor and mediator of HBO effects. The remodelling of the actin cytoskeleton appears consistent

with apoptosis signalling. Therefore it would be desirable to conduct experiments involving analysis of actin deformation following HBO treatment by using techniques such as flow cytometry (Yap and Kamm, 2005), fluorescence microscopy or Western blotting to identify the changes in the distribution of the actin cytoskeleton.

The *in vitro* experiments used in the study involved only the examination of the short-term effects of HBO due to the limited life span of neutrophil-like cells, and there was a limited sample size for the *in vivo* studies. In order to study the effects of a course of HBO treatments, cells would need to be studied daily over the course of 20-30 treatments, which represents an actual patient treatment protocol. Isolated neutrophils from the wound site, obtained via lavage, may be a better alternative to determine the functions of neutrophils at the wound site, although the number of neutrophils obtained by this procedure is limited.

The coordinated activity of phagocytic cells in the inflammatory phase, as well as endothelial cells and fibroblasts is essential for wound healing. These cells are known to contribute substantially to the wound healing environment, including the resistance to infection and the sustaining of the inflammatory phase and also release of various growth factors. Interactions between the different cell types could be crucial for the effects of HBO. For example, platelets are known to interact with neutrophils and endothelial cells during the process of neutrophil recruitment. Therefore, it is important to investigate the effect of HBO on these cells alone or in co-culture *in vivo*.

HBO has been demonstrated to increase intracellular Ca^{2+} concentration. A further study to investigate the effects of HBO treatments on Ca^{2+} levels will aid understanding of Ca^{2+} signalling in cells and whether it is associated with ROS production.

8.4 Conclusions

The present study demonstrated several mechanisms by which HBO could resolve inflammation in chronic wounds, through alteration of neutrophil functions. The results and analyses have indicated that a single exposure to HBO *in vitro* enhances antimicrobial functions and apoptosis of the neutrophil-like cells, thus providing an important contribution to knowledge concerning the effects of HBO in chronic wounds. A single HBO treatment exhibited immunosuppressive effects through decreased pro-inflammatory cytokine production in neutrophil-like cells. In addition, a single therapeutic HBO treatment increased neutrophil clearance by monocyte-derived macrophages. More importantly, HBO is able to enhance clearance of apoptotic neutrophils in a non-inflammatory process suggesting the possibility for treatment of other inflammatory conditions with HBO. The contributions of hyperoxia and pressure conditions to HBO effects were identified, and further investigations to confirm the *in vitro* and *in vivo* findings are required. The work presented included research on some aspects of the *in vivo* effect of prolonged, repeated HBO treatment in patients with chronic wounds, and further work with a suitable sample size could provide evidence to support the more effective use of hyperoxia and pressure during HBO treatments.

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Appendix

Poster presentations



Effects of oxygen on neutrophil-like derivatives of HL-60 cells

Anwar J. Almazai, Richard Billington and A. John Moody
School of Biomedical and Biological Sciences, University of Plymouth, PL4 8AA, UK
Anwar almazai@plymouth.ac.uk



Background

Oxygen is an essential requirement for both cell survival and apoptosis (Hannah et al. 1995). Exposure to high levels of oxygen affects cell proliferation and viability, and can induce apoptosis in a variety of cell types both *in vitro* and *in vivo* (Muschel et al. 1995; Israel and Gougerot-Pocidaló 1997). Oxygen undergoes electron transfer (redox) reactions to produce highly reactive species (ROS), including hydrogen peroxide (H₂O₂), superoxide (O₂⁻), and hydroxyl radical (OH[•]). Through these intermediates oxygen seems to affect apoptosis (Stefaneli et al. 1995; Fiers, Beyaert et al. 1999).

Objectives

The aim of this study was to investigate the effect of oxygen concentration (normoxia, hyperoxia and hypoxia) on apoptosis in a human granulocytic cell line, and also to investigate whether the various oxygen concentrations stimulate or inhibit the activity of neutrophil-like cells and their inflammatory response.

Methods

- HL-60 cells were differentiated using all-trans retinoic acid.
- Cells were exposed to different oxygen concentrations for 90 minutes.
- Apoptosis measurements
 - Caspase 3/7 activity assayed by fluorescence using a fluorogenic substrate.
 - Quantitative microscopic measurements by using Sybr Green and Giemsa staining.
- Respiratory burst activity (superoxide production)
 - Spectrophotometric using cytochrome c reduction
 - Microscopic, using nitroblue tetrazolium (NBT) reduction
- Phagocytosis of *Staphylococcus aureus* NCIMB 6571 by all trans RA differentiated cells was also measured.
- TNF- α levels were measured by ELISA.

Results

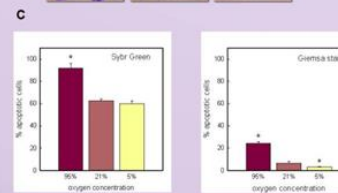
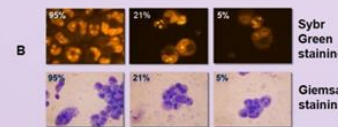
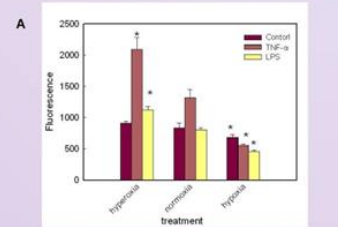


Figure 1 Effect of oxygen on apoptosis. HL-60 cells (1×10^6 /ml) differentiated with all-trans retinoic acid were incubated in an atmosphere containing 95%, 21%, or 5% O₂ for 90 min at 20 °C. They were then (A) incubated with either TNF- α (10 ng/ml) or LPS (100 ng/ml) for 3 h at 37 °C. The caspase 3/7 activity was then measured using a fluorogenic assay using a Promega Apo-ONE kit. Experiments were performed in triplicate, with duplicate assays in each case. Means \pm SE are shown. * P < 0.05 vs normoxic (21%).

(B) The slides show the effects of oxygen on apoptosis by neutrophil-like cells using Sybr Green and Giemsa staining. All-trans retinoic acid differentiated cells (1×10^6 /ml) were incubated in an atmosphere containing 95%, 21% or 5% O₂ for 90 min. The percentage of apoptotic cells was assessed morphologically by Sybr Green or Giemsa staining (C). Data are shown as means \pm SE of the percentage of the total number of cells counted (> 200) from five randomly selected fields on triplicate slides.

Results

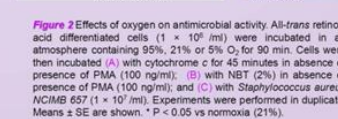
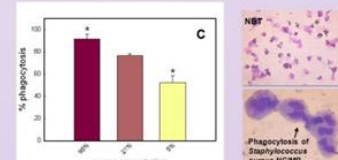
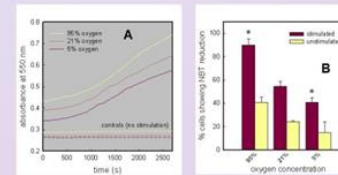


Figure 2 Effects of oxygen on antimicrobial activity. All-trans retinoic acid differentiated cells (1×10^6 /ml) were incubated in an atmosphere containing 95%, 21% or 5% O₂ for 90 min. Cells were then incubated (A) with cytochrome c for 45 minutes in absence or presence of PMA (100 ng/ml), (B) with NBT (2%) in absence or presence of PMA (100 ng/ml), and (C) with *Staphylococcus aureus* NCIMB 6571 (1×10^7 /ml). Experiments were performed in duplicate. Means \pm SE are shown. * P < 0.05 vs normoxia (21%).

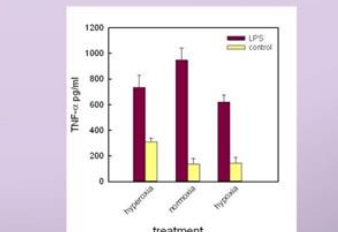


Figure 3 Effect of oxygen on TNF- α production by differentiated HL-60 cells (1×10^6 /ml) cultured in the presence and absence of LPS (100ng/ml) followed by exposure to different oxygen concentrations for 90 min. TNF- α was measured by ELISA. Experiments were performed in triplicate and the mean \pm SE are shown. * P < 0.05 vs normoxia (21%).

Results

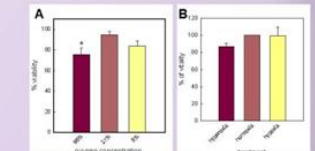


Figure 4 Effect of oxygen on cell viability. All-trans retinoic acid differentiated cells (1×10^6 /ml) were incubated in different oxygen concentrations for 90 min. Cells were then incubated (A) with Trypan blue (4%) for 5 minutes and then cells counted or (B) with MTT dye for 2-3 h after which the formazan was extracted over 24 h and its absorbance measured. Experiments were performed in triplicate and data shown are means \pm SE. * P < 0.05 vs normoxia.

Conclusions

- Exposure to hyperoxia for 90 min enhances apoptosis in differentiated HL-60 cells as shown by caspase 3/7 activity and changes in cell nuclear morphology.
- Prior exposure to hyperoxia enhances the respiratory burst activity of differentiated HL-60 cells and also their phagocytosis activity.
- Exposure to hyperoxia decreases stimulus-induced production of the proinflammatory cytokine TNF- α .

Future Directions

- Effect of oxygen on markers of oxidative stress and levels of antioxidants.
- Effect of oxygen on gene expression.

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MECHANISMS BY WHICH HYPERBARIC OXYGEN THERAPY MAY RESOLVE INFLAMMATION

ALMZAIEL, A., SMERDON, G., BILLINGTON, R. AND MOODY, A.J.

School of Biomedical Sciences, University of Plymouth, PL4 8AA, UK
Anwar almzaiel@plymouth.ac.uk

Introduction

Hyperbaric oxygen (HBO) therapy involves the intermittent inhalation of 100% oxygen, whilst in a chamber above atmospheric pressure (Fig. 1). It is an effective treatment for chronic wounds, which are characterised by hypoxia, infection and prolonged inflammation. These factors attract excessive numbers of neutrophils, which are recruited from the blood into the wound site. These initiate and prolong the inflammation, and prevent wound healing. Although some of the mechanisms by which HBO aids wound healing have been investigated, the effects of HBO on the resolution of inflammation are not well understood.¹

Aim

The aim of this study was to examine the effect of HBO on neutrophil function (antimicrobial defences) and apoptosis in all-trans retinoic acid differentiated HL-60 cells as a model for neutrophils.



Figure 1: A multiplace chamber (left) and a monoplace chamber (right) at the Diving Diseases Research Centre (DDRC) in Plymouth.

Methods

To produce neutrophil-like cells, HL-60 cells were differentiated for 5 days using 1 μM all-trans retinoic acid.² These cells (at $1 \times 10^6/\text{ml}$) were then exposed to normoxia (21% O_2 ; 5% CO_2), elevated pressure alone (8.8% O_2 ; 2.1% CO_2 ; 2.4 ATA, i.e. $2.4 \times$ atmospheric pressure; Fig. 2) or hyperbaric oxygen (97.9% O_2 ; 2.1% CO_2 ; 2.4 ATA) for 90 min, after which a range of end-points were measured.



Figure 2: chamber used for HBO experiments.

Results

1. Apoptosis

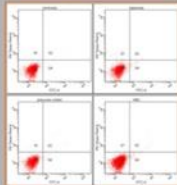


Figure 3: treatment of differentiated HL-60 cells with HBO or pressure alone did not significantly affect apoptosis, as assessed by flow cytometry using Annexin V and PI.

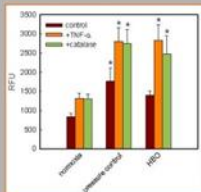


Figure 4: pressure and HBO significantly increased the caspase 3/7 activity in differentiated HL-60 cells. After O_2 treatment cells were either incubated with TNF- α (10 ng ml^{-1}) or catalase (1 $\mu\text{g ml}^{-1}$) for 3 h at 37 $^\circ\text{C}$. Caspase 3/7 activity was measured by using an Apo-ONE[®] assay kit (Promega). Data are means \pm SEM ($n = 9$). * $P < 0.05$.

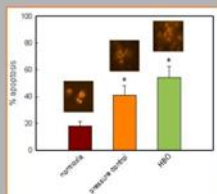


Figure 5: HBO and pressure significantly increase the morphological changes associated with apoptosis. Cells ($1 \times 10^6/\text{ml}$) were exposed to HBO or increased pressure alone for 90 min. The % apoptotic cells from >200 in 5 randomly selected fields was assessed using SYBR Green staining. Data are means \pm SEM ($n = 9$). * $P < 0.05$.

2. Antimicrobial activity

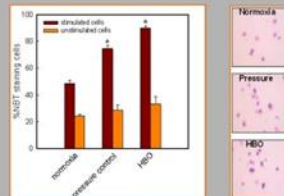


Figure 6: pressure and HBO increase levels of NBT positive cells. After O_2 treatment cells nitroblue tetrazolium (NBT; 5 mg ml^{-1}) was added in the absence (unstimulated) or presence (stimulated) of PMA (100 ng ml^{-1}). Data are means \pm SEM ($n = 9$). * $P < 0.05$.

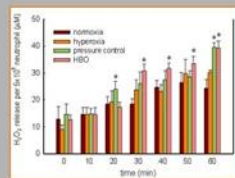


Figure 7: HBO and pressure both increased H_2O_2 production by neutrophil-like cells. H_2O_2 was detected using the HRP-catalysed H_2O_2 -dependent oxidation of homovanillic acid to give a fluorescent product. Data are means \pm SEM ($n = 9$). * $P < 0.05$.

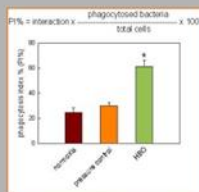


Figure 8: HBO increases phagocytosis of *Staphylococcus aureus* NCIMB 6571. After O_2 treatment cells were incubated for 30 min with *S. aureus* NCIMB 6571 ($1 \times 10^7/\text{ml}$). Data are means \pm SEM ($n = 9$). * $P < 0.05$.

3. Viability

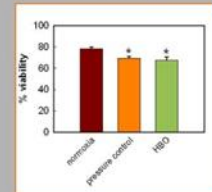


Figure 9: Cell viability was affected by HBO and pressure alone. After O_2 treatment cells were assayed for viability using trypan blue. Data are mean \pm SEM ($n = 9$). * $P < 0.05$.

4. Glutathione

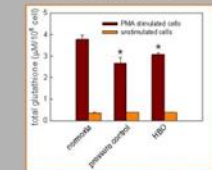


Figure 10: HBO and pressure treatments significantly decreased total glutathione in neutrophil-like cells. Total glutathione was measured using the enzymatic recycling method with DTNB. Means \pm SEM ($n = 9$). * $P < 0.05$.

Conclusions

- HBO for 90 min enhances apoptosis in differentiated HL-60 cells as shown by increased caspase 3/7 activity and changes in cell nuclear morphology (Figs 3 & 4), but this was not seen by flow cytometry (Fig. 5).
- Prior exposure to HBO enhances the respiratory burst activity of differentiated HL-60 cells (Figs 6 and 7) and also their phagocytosis activity (Fig. 8).
- Prior exposure to HBO significantly decreased total glutathione in PMA stimulated cells (Fig. 10).
- Prior exposure to HBO may affect apoptosis in differentiated HL-60 cells by increasing their production of H_2O_2 thereby increasing the level of oxidative stress.

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Effect of HBO on neutrophil clearance by monocyte-derived macrophages (MDM ϕ)



Anwar J. Almazaleh, Richard Billington and A. John Moody
School of Biomedical and Biological Sciences,
University of Plymouth, PL4 8AA, UK

Anwar.almazaleh@plymouth.ac.uk

Aim

The aim of this study was to examine the effect of HBO on apoptosis of neutrophils and their clearance by monocyte-derived macrophages from bovine blood.

RESULTS

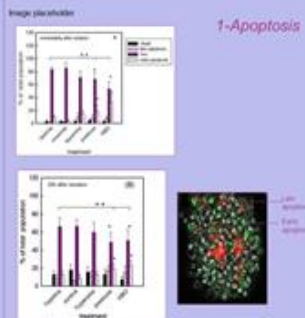


Fig. 3. Effect of HBO on neutrophil cell population by flow cytometric analysis using Annexin V-FITC binding and propidium iodide staining in neutrophils (A) isolated immediately or (B) aged for 20 h (C) fluorescence staining of apoptotic neutrophil using Annexin V-FITC binding and propidium iodide under normoxic conditions.

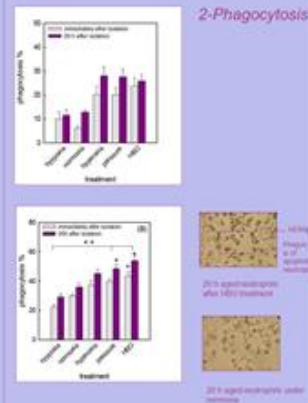


Fig. 4. Effects of HBO on phagocytosis of apoptotic neutrophils by MDM ϕ (A) by flow cytometry using Tracker green and (B) MPO. Data are means \pm SEM (n = 9). * P < 0.05 compared to normoxia, ** P < 0.05 compared to hypoxia.

Introduction

HBO therapy involves the intermittent inhalation of 100% oxygen, whilst in a chamber above atmospheric pressure (Fig. 1). It is an effective treatment for chronic wounds (1), which are characterised by hypoxia, infection and prolonged inflammation, that results in an excess influx of neutrophils. This may be related to dysregulation in mechanisms leading to resolution of inflammation, in particular neutrophil apoptosis and their clearance by macrophages (2,3). Although some of the mechanisms by which HBO aids wound healing have been investigated, the effects of HBO on the resolution of inflammation are not well understood.



Fig. 1. A multiplace chamber (left) and a monoplace chamber (right) at the Diving Diseases Research Centre (DDRC) in Plymouth.

Results

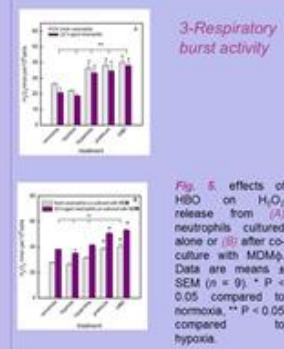


Fig. 5. effects of HBO on H₂O₂ release from (A) neutrophils cultured alone or (B) after co-culture with MDM ϕ . Data are means \pm SEM (n = 9). * P < 0.05 compared to normoxia, ** P < 0.05 compared to hypoxia.

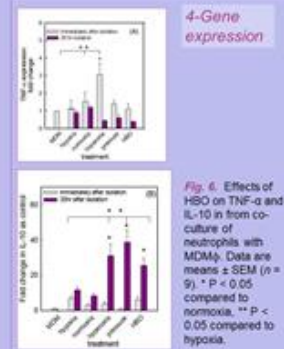


Fig. 6. Effects of HBO on TNF- α and IL-10 in co-culture of neutrophils with MDM ϕ . Data are means \pm SEM (n = 9). * P < 0.05 compared to normoxia, ** P < 0.05 compared to hypoxia.

MATERIALS & METHODS

Cell isolation and culture

Neutrophils and monocytes were isolated from bovine blood using hypotonic lysis of erythrocytes according to Hahn and Tolle (4) with some modification. These cells (at $1 \times 10^6/ml$) were then exposed to different oxygen conditions for 90 min.

- normoxia (21% O₂; 5% CO₂)
- hypoxia (95% 5% CO₂)
- hypoxia (5% O₂; 95% CO₂)
- elevated pressure alone (8.8% O₂; 2.1% CO₂) 2.4 ATA, i.e. 2.4 \times atmospheric pressure
- hyperbaric oxygen (97.9% O₂; 2.1% CO₂; 2.4 ATA)



Fig. 2. (A) chamber used for HBO experiments (B) Gas cylinders and boxes

Co-culture

Adherent monocytes were cultured for 5 days in DMEM with 10% (v/v) heat-inactivated bovine autologous serum to generate MDM ϕ . Neutrophils, immediately after isolation or aged for 20 h were added to MDM ϕ and co-cultured for 1 h at 37 $^{\circ}$ C, and different measurements were applied.

Conclusion

Taken together, this study presents the first evidence demonstrating that HBO treatment increases clearance and phagocytosis of apoptotic neutrophils by MDM ϕ which in turn may lead to resolution of the inflammatory phase and enhanced progress of the healing process.

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Oxidative stress and inflammatory response in chronic wound patients undergoing multiple HBO exposures: an *in vivo* study

Anwar J. Almzaieel, Richard Billington, Gary Smerdon, A. John Moody

Abstract

Prolonged intermittent HBO treatment in patients with chronic wounds leads to a change in plasma cytokine profile and antioxidant capacity consistent with at least partial resolution of inflammation and progress towards healing.

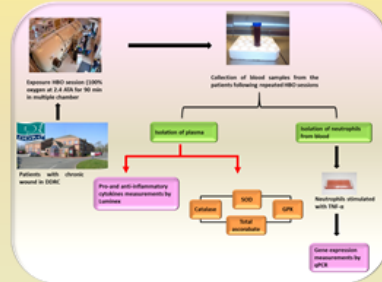
Introduction

Hyperbaric oxygen (HBO) therapy, the intermittent inhalation of 100% oxygen at pressure greater than atmospheric, seems to be an effective treatment for various inflammatory conditions, including chronic wounds¹. Although its clinical benefits are clear, HBO has also been reported to enhance ROS production and cause oxidative stress in several tissues². Recent studies have shown that HBO-induced oxidative stress is directly related to both the pressure used and the duration of exposure^{3,4}. However, these studies were performed with a single HBO treatment, whereas clinical use of HBO depends on long-term, repetitive exposure.

Aim

The purpose of this study was to determine the effects of long-term repetitive HBO on antioxidant capacity and inflammatory cytokines in patients with chronic wounds. In addition, the expression of NF- κ B (and downstream Bcl-2) genes, which control transcription of cytokine genes was examined.

Methods



Results

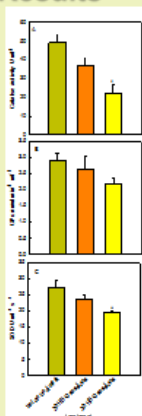


Fig. 1 Plasma antioxidant activities in patients with chronic wounds after exposure to repeated HBO sessions. Plasma samples were isolated from the blood of patients with chronic wounds before and after exposure to repeated HBO sessions. Antioxidant enzyme activities: (a) SOD, (b) GPx and (c) Catalase. Data are expressed as means \pm SEM, for four patients, with triplicate measurements. * Significant difference versus before HBO treatment (Student's *t*-test, $P < 0.05$).

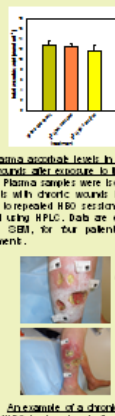


Fig. 2 Examples of a chronic wound, before the start of HBO treatment, and after 30 HBO sessions.

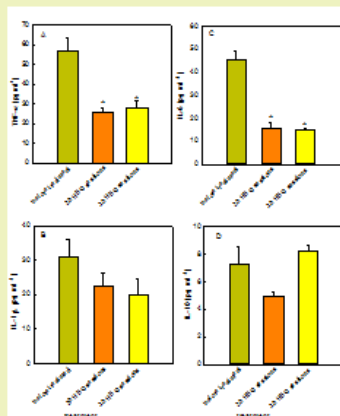


Fig. 4 Plasma pro- and anti-inflammatory cytokine levels in the patients with chronic wounds after exposure to the repeated HBO sessions. Plasma samples were isolated from blood from patients with chronic wounds before and after exposure to repeated HBO sessions. TNF- α , IL-10, IL-6, IL-17 levels were analyzed by Luminex and calculated against a standard curve. Data are expressed as the means \pm SEM, for four patients, with triplicate measurements. * Significant difference versus before HBO treatment (Student's *t*-test, $P < 0.05$).

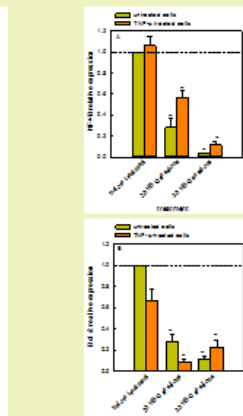


Fig. 5 NF- κ B and Bcl-2 gene expression in neutrophils from the patients with chronic wounds after exposure to the repeated HBO sessions. Neutrophils were isolated from blood from patients with chronic wounds before and after exposure to repeated HBO sessions, after which cells were washed and either treated with TNF- α (a) left untreated. cDNA was synthesized from extracted RNA. qPCR was used to determine expression of NF- κ B and Bcl-2. Data are expressed as the means \pm SEM, for four patients, with triplicate measurements. * Significant difference compared to untreated cells before HBO treatment (Student's *t*-test, $P < 0.05$).

Conclusion

A possible explanation for the decrease in antioxidant enzyme activities is that paradoxically HBO exposure may result in short-term oxidative stress that induces immunosuppressive effects which ultimately lead to a decrease in oxidative stress (reflected in the lower plasma antioxidant enzyme activities). However, the decrease in plasma antioxidant enzymes could also be explained as direct oxidative damage as a consequence of the HBO treatment, and so further studies should focus on whether the changes in enzyme activity result from chemical modifications caused by reactive oxygen species or from changes in expression of the proteins.

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