Hyperbaric oxygen enhances neutrophil apoptosis and their clearance by monocyte-derived macrophages

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Hyperbaric oxygen enhances neutrophil apoptosis and their clearance by monocyte-derived macrophages

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Abstract

Neutrophil apoptosis and clearance by macrophages are essential for wound healing. Evidence suggests that hyperbaric oxygen (HBO) exposure may enhance neutrophil apoptosis, but HBO effects leading to neutrophil clearance by macrophages are still unclear. In the current study, bovine neutrophils and monocyte-derived macrophages (MDM Φ) were co-cultured under HBO (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), normoxia (air) and normobaric hypoxia (5% O₂, 5% CO₂). Phagocytosis of fresh and 22 h-aged neutrophils by MDM Φ was increased after HBO pre-treatment, assessed using flow cytometry and light microscopy. Enhanced clearance of neutrophils was accompanied by an increase in H₂O₂ levels following HBO pre-treatment with up regulation of IL-10 (anti-inflammatory cytokine) mRNA expression in LPS-stimulated MDM Φ that had ingested aged neutrophils. TNF- α (pro-inflammatory cytokine) gene expression did not change in LPS-stimulated MDM Φ that had ingested fresh or aged neutrophils after HBO, pressure and hyperoxia. These findings suggest that HBO-activated MDM Φ participate in the clearance of apoptotic cells. Uptake of neutrophils by MDM Φ exposed to HBO may contribute to resolution of inflammation, because HBO induced up-regulation of IL-10 mRNA expression.

Keywords: hyperbaric oxygen, neutrophils, macrophages, apoptosis

1. Introduction

Neutrophils are key players in wound healing; large numbers migrate to wound sites during the acute inflammatory phase of wound healing, their numbers being controlled by their rates of infiltration and apoptosis (Simon 2003). Upon activation, neutrophils release reactive oxygen species (ROS) and enzymes that are stored in granules (Henson and Johnston 1987). These are essential for their antimicrobial function, but can also cause damage if the inflammatory response is prolonged. Hence, apoptosis is important in preventing release of inflammatory mediators from neutrophils and allowing them to be cleared safely by macrophages (Savill *et al.* 1989). Apoptotic cells undergo specific surface changes, e.g. exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane, which lead to their recognition and ingestion by phagocytes (Fadok *et al.* 2001a). PS exposure is essential for the uptake of apoptotic cells by macrophages (Fadok *et al.* 2001b, Fadok *et al.* 1992).

Hypoxia is a key feature of chronic wounds, where destruction of vasculature in the wound and high oxygen consumption by inflammatory cells such as neutrophils, result in low oxygen levels (Tandara and Mustoe 2004). Under hypoxic conditions neutrophil apoptosis and subsequent clearance are markedly inhibited (Mecklenburgh *et al.* 2002, Walmsley *et al.* 2005). Prolonged hypoxia coupled with bacterial colonization leads to release of inflammatory cytokines from inflammatory cells at wound sites, prolonging the inflammation (Wolcott *et al.* 2008). Increased levels of the pro-inflammatory cytokines TNF- α and IL-6, but decreased levels of IL-10, are characteristic of non-healing wounds, where a major source of these cytokines is macrophages (Werner and Grose 2003). The successful phagocytosis of apoptotic cells results in suppression of pro-inflammatory cytokines and the progress of wound healing (Lee *et al.* 1993, Khanna *et al.* 2010).

Hyperbaric oxygen (HBO) therapy is the administration of 100% oxygen by inhalation at pressures greater than one atmosphere (1 ATA) for 60-90 min, repeated once or twice daily (Gill and Bell 2004). HBO has been found to increase apoptosis in HL-60 cells, Jurkat cells and lymphocytes (Weber *et al.* 2009a, Ganguly *et al.* 2002), as well as in neutrophil-like cells derived from HL-60 cells (Almzaiei *et al.* 2013). An immunosuppressive effect of HBO treatment on the inflammatory process in ischaemic wounds and trauma has been demonstrated (Al-Waili and Butler 2006, Zhang *et al.* 2008) together with decreased levels of TNF- α and IL-1 β secreted by monocytes and macrophages collected from rats or from human peripheral blood after stimulation with LPS (Lahat *et al.* 1995, Benson *et al.* 2003). Hence, the effects HBO on clearance of apoptotic cells by macrophages may be potentially important in the resolution of inflammation and hence in the progress of wound healing.

Given this background, the current study aimed to determine whether HBO-induced apoptosis was able to enhance neutrophil clearance by MDM Φ in a non-inflammatory process. 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 neutrophils.

2 Materials and methods

2.1 Tissue culture reagents

Dulbecco's Modified Eagle Medium (DMEM) and Hank's Balanced Salt Solution (HBSS) without Ca²⁺ and Mg²⁺ were obtained from Gibco Liga Technologies (Paisley, UK). Fetal calf serum was purchased from Lonza (Wokingham, UK) and was heat inactivated (56 °C, 1 h) before addition to the media (10%).

2.2 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.

2.3 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) combined with density centrifugation in Histopaque[®] 1077/1119 to obtain relatively 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 density of 4×10^6 cells ml^{-1} . 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 with DMEM.

The remaining RBC and leucocytes from earlier (mostly neutrophils) were split between three 50 ml centrifuge tubes, diluted with 5 ml of cold distilled water and gently mixed for about 2 min in order to lyse the RBC. Immediately after this period 2.5 ml of 0.2% NaCl were 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 neutrophils form a pellet and any contaminating mononuclear cells remain at the sample/medium interface. The neutrophil pellet was washed by resuspending in 50 ml HBSS and centrifuging at $600 \times g$ for 10 min at room temperature. Purified neutrophils were resuspended in 5 ml of DMEM medium.

2.4 Hyperbaric oxygen and pressure control treatments

Hyperbaric chambers were supplied by DDRC Healthcare, Plymouth, UK) and connecting hoses from Pressure Lines Ltd (Plymouth, UK). Different gas mixes were kindly prepared by DDRC Healthcare (Plymouth, UK). To achieve hyperbaric oxygen conditions, the chambers were flushed for 4 min at 3 l min^{-1} with 97.9% O_2 , 2.1% CO_2 , and then pressurized to 2.4 atmosphere absolute (ATA) over 2 min; 2.1% CO_2 at 2.4 ATA was used to achieve a CO_2 concentration equivalent to 5% CO_2 at 1 ATA in a conventional incubator. To investigate the effect of pressure alone, cells were treated with 8.8% O_2 , 2.1% CO_2 , balance N_2 at 2.4 ATA; 8.8% O_2 was used such that the O_2 concentration would be equivalent to 21% at 1 ATA. For normobaric hyperoxic or hypoxic treatments, cells were flushed with gas mixtures containing 95% O_2 , 5% CO_2 or 5% O_2 , 5% CO_2 balance N_2 , respectively, for 2-4 min at a rate of 4 l min^{-1} in gas-tight plastic boxes ($21.5 \text{ cm} \times 21.5 \text{ cm} \times 11 \text{ cm}$). This achieved the correct concentration inside the chamber box, based on direct measurement with an oxygen meter (PCE-007, Dwyer Instruments Ltd, High Wycombe, UK). Control cell cultures for each experiment were placed in similar boxes under 21% O_2 , 5% CO_2 , balance N_2 , at 1 ATA. 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 1×10^6 or $2 \times 10^6 \text{ cells ml}^{-1}$ 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.

2.5 Generation of monocyte-derived macrophages (MDM Φ)

Monocytes (4×10^6 cells ml⁻¹) were cultured in DMEM (containing 20 mM glutamine, 100 U ml⁻¹ penicillin and 10 μ g ml⁻¹ streptomycin) and 10% (v/v) heat-inactivated bovine autologous serum (derived from platelet-rich plasma by re-calcification with 20 mM CaCl₂ 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% CO₂ (balance air). The medium was replaced every three days and the cells were used for experiments within seven days of plating.

2.6 Induction of apoptosis

Following isolation from peripheral blood, neutrophils were cultured at 37 °C under 5% CO₂ (balance air) at a density of 5×10^6 cells ml⁻¹ in DMEM. After treatment with different oxygen or pressure conditions (Section 2.4), 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*, 1989a). Cells were harvested at the indicated times and resuspended in DMEM (serum free) and analysed immediately.

2.7 Assay for cell viability

Samples of each suspension of neutrophils (50 μ l of 10^6 cells ml⁻¹ in supplemented RPMI-1640) were mixed with 50 μ 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.

2.8 MTT assay

Neutrophils 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) was added to each well and incubated for 2-3 h at 37 °C. After incubation, 100 μl DMSO was added to each well and incubated for 20-30 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 (Vistica *et al.* 1991).

2.9 Morphological assessment of cell apoptosis

Neutrophils were first washed in supplemented RPMI-1640 medium after which cytocentrifuge preparations of 10^6 cells, made up to 100 μl in medium, were prepared using a Shandon Cytospin centrifuge (Cheshire, UK, 400 g 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. Morphological changes characteristic of apoptosis were assessed microscopically using a $\times 100$ oil-immersion objective (Olympus BH, Japan). Triplicate slides were prepared for each condition, and at least 500 cells per slide were counted. This method has been previously demonstrated to correlate closely with other parameters of apoptosis (Savill *et al.* 1989). Apoptotic cells were characterized by nuclear condensation and fragmentation, while membrane integrity was maintained.

2.10 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 (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). Neutrophils were pelleted by centrifugation at $400 \times g$ for 5 min. This was performed either immediately after isolation or after aging the neutrophils. 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, and then washed and resuspended in 100 μ l of annexin V binding buffer, after which 5 μ l of PI ($1 \mu\text{g ml}^{-1}$) was added. 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 10000 events were 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.

2.11 Assay of DNA fragmentation in apoptotic neutrophils

Determination of apoptotic cell death was confirmed by detection of DNA laddering using gel electrophoresis (Counis and Torriglia 2000, Filipski *et al.* 1990). Briefly, apoptotic neutrophils (2×10^6 cells ml^{-1}) 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. DNA samples were stored at -80°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 TAE for 1 to 2 h at 90 V. The gel was visualized under UV a using a

transilluminator (UVIttec Limited, England). Gel images were evaluated for typical of low molecular weight DNA fragments separated in size by 180-200 base pairs, a hallmark of apoptosis (Wyllie *et al.* 1980). DNA fragment sizes were determined by using DNA size markers in the range 0.5 to 12 kb (New England Biolabs, Hitchin, UK).

2.12 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, Invitrogen, Paisley, UK) at a final concentration of 2 $\mu\text{g ml}^{-1}$ (1 μl of 1 mg ml^{-1} stock solution per 1×10^6 neutrophils) 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) after which the cells were exposed to different oxygen and pressure conditions (Section 2.4). Finally they were co-incubated 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. MDM Φ with ingested neutrophils, were detached from the plates using 300 μl of 0.25% trypsin/1 mM EDTA for 15 min at 37 °C followed by 15 min at 4 °C, washed and resuspended in PBS and transferred to FACS tubes. In order to ensure that all the MDM Φ were removed from wells, plates were examined by light microscopy. Cells were analysed using an ArialTM II FACS (Becton-Dickinson, San Jose, USA; excitation 492 nm/emission 517 nm) using FACS Diva software[®]. MDM Φ were initially gated by their characteristic forward scatter (FSC) and side scatter

(SSC) profiles, after which quadrants were set up based on matched controls including unstained MDM Φ alone and neutrophils stained with TrackerTM Green alone.

Neutrophils and MDM Φ that had ingested apoptotic neutrophils were separated (gated) by FSC and SSC profile, and at least 10000 events were counted in each sample. Uningested neutrophils were identified by the combination of their green fluorescence due to TrackerTM green labelling and their relatively smaller size (FSC) when compared with the MDM Φ . MDM Φ populations were gated based on FSC and SSC characteristics, MDM Φ that had ingested neutrophils and hence showed green fluorescence were deemed to have ingested apoptotic neutrophils. All results are reported as the percentage of total cells stained.

2.13 Detection of phagocytosis of apoptotic cells by light microscopy

In order to assess phagocytosis by microscopy, MDM Φ were incubated with neutrophils as described in Section 2.12. Following phagocytosis, the medium was removed from each well and the wells 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 min 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). MPO activity leads to formation of a soluble yellow-brown product detectable by light microscopy (Hart *et al.*, 1997). MDM Φ that had ingested neutrophils were MPO positive, while MDM Φ which had not were MPO negative. Five hundred cells per each of three replicate wells were examined. The percentage of MDM Φ that had ingested neutrophils was calculated.

2.14 Assay of extracellular hydrogen peroxide

A horseradish peroxidase (HRP)-linked assay using homovanillic acid was used to measure production of H_2O_2 by neutrophils. Briefly, 100 μl of cell suspension in HBSS, containing 10^6 cells ml^{-1} was added to each well of a 96-well plate. Then 100 μl of HBSS containing 200 μM homovanillic acid, 10 U ml^{-1} HRP (P8375, Sigma, Poole, 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; excitation 320 nm/emission 420 nm). A standard curve (0-100 μM H_2O_2) was used to determine the H_2O_2 concentration in each well (Baggiolini *et al.* 1986).

2.15 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-culture for 1 h at 37 °C. They were then washed in PBS and pelleted. Total RNA was extracted using a GeneEluteTM mammalian total RNA miniprep kit (Sigma, Poole, UK), cDNA synthesised and qPCR for TNF- α (primers: forward GGTGGCCCCTCCATCAACAGC, reverse GGCCACGAGGGCATTGGCATA) and IL-10 (primers: forward CCTGGAAGAGGTGATGCCACAGG, reverse CCTTCTCCACCGCCTTGCTCT) genes was carried out. For the housekeeping gene, β -actin (primers: forward, CGCCATGGATGATGATATTGC, reverse: AAGCCGGCCTTGACACAT) was used. Neutrophils cultured alone (fresh or 22 h-aged) were also stimulated with LPS (100 ng ml^{-1}) for 2 h before total RNA was isolated and processed for qPCR as described above.

2.16 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 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.

3 Results

3.1 Effect of oxygen treatments on neutrophil viability

Cell 'viability' using the trypan blue and MTT assays was assessed for fresh neutrophils and 22 h-aged neutrophils (Figs 1A 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 found for both fresh and 22 h-aged 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 (trypan blue and MTT) compared to normoxia (Figs 1A and B).

3.2 Effect of oxygen treatments on apoptosis

The effects of different oxygen treatments on neutrophil apoptosis were assessed using several assays: FITC-annexin V/PI staining via flow cytometry, morphology using Giemsa staining and DNA fragmentation. Dual staining with FITC-labelled Annexin V and PI showed a significant decrease in the percentage of live cells and an increase in early apoptotic cells in fresh neutrophils assayed immediately after HBO or pressure treatments (one-way ANOVA, $P = 0.045$ and 0.020 , respectively) compared to normoxia (Fig. 2A). Both hyperoxia and hypoxia pre-treatments showed no significant effect on the levels of live cells

and early apoptotic cells. Also, there was no significant difference between treatments in the percentage of the cell population that was late apoptotic or dead (Fig. 2A). The same pattern was seen with neutrophils aged for 22 h post oxygen treatments. There was a significantly higher proportion of early apoptotic cells (Kruskal-Wallis, $P = 0.030$) and a corresponding decrease in live cells (one-way ANOVA, $P = 0.0002$) post-HBO and pressure treatments compared to normoxia (Fig. 2B), but not following hyperoxia and hypoxia treatments. 22 h aged neutrophils also demonstrated no significant difference in the percentage of late apoptotic and dead cells (one-way ANOVA, $P > 0.05$).

To further assess the effects of different treatments on neutrophil apoptosis, morphological changes were analysed by light microscopy using modified Wright-Giemsa staining (Figs 3A 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 post different treatment (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. 3B), compared with normoxia. As expected, the hypoxia treatment significantly decreased apoptosis of both fresh and 22 h-aged neutrophils compared with normoxia (Fig. 3B).

The cleavage of chromatin into multi-nucleosome sized fragments also indicates that cell death has occurred via apoptosis. Figs 4A 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. 4A). The degree of DNA fragmentation was more marked in 22 h-

aged neutrophils after HBO, with less genomic DNA visible (Fig. 4B). However, lower but detectable DNA fragmentation was observed in 22 h-aged neutrophils after pressure, hyperoxia and hypoxia treatments compared to normoxia (Fig. 4B).

3.3 Effects of oxygen treatments on phagocytosis of neutrophils

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. 5. 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 (Fig. 5). No differences in MDM Φ phagocytosis of fresh and 22 h-aged neutrophils were observed after hyperoxia and hypoxia pre-treatments compared with normoxia (Fig. 5).

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. 6A). 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. Exposure to hypoxia did not produce a significant change in percentage phagocytosis of neutrophils compared with normoxia (Figs 6 A and B).

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

The H_2O_2 concentrations in the supernatants from PMA-stimulated fresh or 22 h-aged neutrophils cultured alone or after co-culture with MDM Φ are shown in Fig. 7A. H_2O_2 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. In contrast, hypoxia pre-treatment had no effect on H_2O_2 levels in fresh and 22 h-aged neutrophils (Fig. 7A) compared with normoxia.

HBO, pressure and hyperoxia pre-treatments gave a significant increase in H_2O_2 levels in MDM Φ that had ingested fresh or 22 h-aged neutrophils (two-way ANOVA, $P < 0.00005$) compared with normoxia. No treatment effects were observed on H_2O_2 levels in co-cultures of MDM Φ with fresh or 22 h-aged neutrophils after pre-treatment to hypoxia (Fig. 7B).

3.5 Effects of HBO on TNF- α and IL-10 gene expression on co-culture of neutrophils with MDM Φ

It has been proposed that clearance of apoptotic neutrophils results in 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 co-cultured with neutrophils following different oxygen treatments and stimulation with 100 ng ml^{-1} LPS. As already demonstrated (Section 3.3), it is presumed that some ingestion of apoptotic neutrophils by MDM Φ will have occurred during the co-culture. For fresh and 22 h-aged neutrophils cultured alone, the data are normalised against fresh neutrophils under normoxia (Figs 8A and B) while co-culture data are normalised against MDM Φ cultured alone (Figs 8C and D). Note that for the co-culture experiments mRNA was extracted from the mixture of cell types and so the different contributions of the MDM Φ and neutrophils to the observed mRNA levels cannot be determined.

Although hyperoxia, pressure and HBO caused a significant increase in TNF- α expression in fresh and 22 h-aged neutrophils incubated alone (Fig. 8A), there were no increases when these neutrophils were co-cultured with MDM Φ except in the case of fresh neutrophils and hyperoxia (Fig. 8C) (Kruskal-Wallis, $P < 0.05$). For IL-10, HBO caused a small increase in gene expression for both fresh and aged neutrophils incubated alone, whereas hyperoxia and pressure caused a slight decrease in expression in fresh neutrophils (Fig. 8B). However, when aged neutrophils were co-cultured with MDM Φ , hyperoxia and HBO caused a marked increase in IL-10 expression, whereas there was no effect when fresh neutrophils were co-cultured with MDM Φ (Fig. 8D).

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4 Discussion

The ability of MDM Φ to phagocytose neutrophils is an important step in the resolution of inflammation and the progress of wound healing (Serhan and Savill 2005). The hypothesis behind the present study was that oxidative stress induced by hyperbaric oxygen (HBO) contributes to the apoptosis of neutrophils and that this augments clearance by macrophages through their recognition of the apoptotic neutrophils. Flow cytometric analysis using FITC-annexin V/PI (Fig. 2); assessment of morphological changes (Fig. 3); and DNA fragmentation (Fig. 4) all showed that pre-exposure of neutrophils to HBO or pressure for 90 min induced apoptosis in both fresh neutrophils and neutrophils that had been aged for 22 h after the exposure, with the greater response detected in aged neutrophils. This is the first demonstration that HBO induces apoptosis in bovine neutrophils, although previous studies have shown that HBO induces apoptosis in cultured cells, e.g. Jurkat-T cells (Ganguly *et al.* 2002, Chen *et al.* 2007), HL-60 cells (Ganguly *et al.* 2002), NCI-H929 cells (Chen *et al.* 2007) and murine thymocytes (Ganguly *et al.* 2002). It is worth bearing in mind that there may be significant functional differences between phagocytic cells from different mammalian species, so issues in extrapolation from the behaviour of bovine phagocytes to human phagocytes cannot be precluded.

Having established that HBO induced apoptosis of neutrophils, the mechanisms involved were investigated. One possibility is that HBO-induced apoptosis is a function of oxygen partial pressure. Pre-treatment with 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, so HBO-induced apoptosis could be via the mitochondrial pathway (Weber *et al.* 2009a).

Exogenous H_2O_2 produced by the activity of xanthine oxidase or glucose oxidase induces apoptosis in neutrophils (Rollet-Labelle *et al.* 1998) and also, endogenous production of ROS has been shown to be involved in the activation of apoptosis by Fas and TNF- α receptors (Kasahara *et al.* 1997, Scheel-Toellner *et al.* 2004, van den Berg *et al.* 2001). Consistent with this, after pre-exposure to HBO or pressure, H_2O_2 levels produced by both fresh and aged neutrophils were increased, which in turn could have enhanced neutrophil apoptosis. This is also in agreement with our previous study that indicated an increase in ROS production and apoptosis in neutrophil-like cells (derived from HL-60 cells) post 90 min HBO exposure (Almzaiel *et al.* 2013).

Other mechanisms could explain the FITC-annexin V/PI assay results. Annexin V binds to PS that has been externalised on surface of cells undergoing apoptosis. Pressure could have caused a deformation which led to conformational changes that might enhance PS translation to the outer leaflet of the cell membrane (van Engeland *et al.* 1997, Mateo *et al.* 2002). Consistent with this, we found an increase in apoptosis and a decrease in viability of fresh and aged neutrophils after pre-exposure to both pressure alone and HBO.

An important functional characteristic of macrophages is their ability to ingest apoptotic cells. The present data clearly indicate that HBO enhanced ingestion of apoptotic neutrophils by monocyte-derived macrophages (MDM Φ). MDM Φ showed a higher level of ingestion of fresh neutrophils after HBO exposure than after other treatments, which is consistent with the previously noted effect of HBO resulting in a high percentage of early apoptotic neutrophils. However, aged neutrophils were recognised and ingested to a greater extent than fresh neutrophils after HBO, pressure and hyperoxia treatments. There were discrepancies between the percentages of phagocytic MDM Φ observed via flow cytometry and via morphology, although the pattern was similar. This may in part have arisen because the data presented are

from separate experiments with cells derived from different animals, and the assays were not performed in parallel.

The clearance of apoptotic cells from an inflamed wound site takes place via a pathway that involves exposure of PS on the outer leaflet of the cell membrane (Fadok *et al.* 2001a), but the mechanisms of PS externalization are not fully characterized. In this study, PS expression on the outer membrane layer was detected by the measurement of FITC-conjugated annexin V using flow cytometry. HBO and pressure treatments showed an increase in exposure of PS in fresh and aged neutrophils which was perhaps recognized by MDM Φ via multiple phagocyte receptors including PS receptors, vitronectin receptors ($\alpha_v\beta_3$) and CD36. Shiratsuchi and Basson (2004) found that increasing extracellular pressure to 20 mm Hg above ambient pressure for 2 h 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 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 our study, the pressure utilized during HBO may have contributed to the increase in phagocytosis percentage as hyperoxia alone did not have the same effect.

It is also possible that oxidants may be responsible for an increase in phagocytosis of apoptotic cells, due to ROS generation during HBO treatment. The present study suggests that functional NADPH oxidase may have a role in PS exposure in activated neutrophils as well as for their clearance by MDM Φ . In the present study, a significant increase in H₂O₂ was observed immediately after HBO and pressure exposure in fresh and aged neutrophils. Furthermore, the increase was correlated with enhanced neutrophil apoptosis, the process

necessary for clearance of neutrophils. PS exposure on apoptotic neutrophils can occur as a result of H_2O_2 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 H_2O_2 generated by both fresh and aged neutrophils following exposure to HBO and pressure may have 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 are supported by a marked increase in H_2O_2 levels in MDM Φ that had ingested fresh and aged neutrophils following HBO and pressure treatments. 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). These findings are consistent with the previous suggestion of Fadeel *et al.* (1998) that neutrophils have two pathways for PS externalisation: 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.

We hypothesized that HBO treatment enhances the clearance of apoptotic neutrophils and thereby elicits a non-inflammatory process. Consistent with this hypothesis it was found that co-culture of aged neutrophils with MDM Φ appeared to prevent an increase in the expression of TNF- α , a pro-inflammatory cytokine, which was induced in aged neutrophils alone by

HBO, whereas co-culture of aged neutrophils with MDM Φ appeared to induce a large increase in the expression of IL-10, an anti-inflammatory cytokine, above that induced in aged neutrophils alone by HBO. In general, almost the same pattern was seen with both hyperoxia and pressure treatments. IL-10 seems to have a regulatory role in phagocytosis of apoptotic cells (Savill 2000, Fadok *et al.* 1998, Voll *et al.* 1997), and it is also a powerful inhibitor of pro-inflammatory cytokine production (Moore *et al.* 1990, Denys *et al.* 2002). In addition, it has been shown that the phagocytosis of apoptotic cells by macrophages is associated with suppression of the inflammatory response via decreased production of the pro-inflammatory cytokines such as TNF- α and IL-6 (Voll *et al.* 1997, Fadok *et al.* 1998, Savill 2000). 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). Efficient clearance of apoptotic cells involves contact between the apoptotic cell and the phagocyte, specific recognition and phagocytosis of target. These processes are sufficient to induce profound inhibition of pro-inflammatory cytokine gene expression and secretion by MDM Φ (Cvetanovic and Ucker 2004).

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 with increased gene expression of IL-10. Overall, this study provides new insights into the role of HBO in regulation of the phagocytic removal of apoptotic cells, which may prove useful in developing a therapeutic approach to the resolution of inflammation.

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Figure Legends

Fig. 1 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 oxygen and pressure conditions for 90 min (Section 2.4). 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; for both fresh and aged neutrophils. * indicate significant differences between fresh and aged neutrophils after the same treatment. Treatments with the same lower case letter are not significantly different, whereas those with different lower case letters are significantly different (ANOVA, $P < 0.05$).

Fig. 2 Neutrophil populations after pre-treatment with normoxia, hypoxia, hyperoxia, pressure and HBO. Neutrophils were isolated from bovine blood and exposed to a range of oxygen and pressure conditions for 90 min (see Section 2.4). 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 (as described in Section 2.10). Data are expressed as means $\pm$ SEM for three separate experiments ($n = 3$) with triplicate measurements. * significant difference versus treatment with normoxia (one-way ANOVA or Kruskal-Wallis, $P < 0.05$).

Fig. 3 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 oxygen conditions for 90 min (Section 2.4). 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 Wright-Giemsa stain were then examined. 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. After pre-treatment with HBO there was a higher percentage of cells with typical features of apoptosis compared to those pre-treated with normoxia (B). Data are expressed as means $\pm$ SEM for three separate experiments with triplicate measurements ($n = 3$). Treatments with the same lower case letter are not significantly different, whereas those with different lower case letters are significantly different (two-way ANOVA, $P < 0.05$).

Fig. 4 Examples 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 oxygen and pressure conditions for 90 min (Section 2.4). 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 (as described in Section 2.11) run on 1% agarose gels containing SYBR[®] Safe (1 $\times$), 20 ng per lane, and visualized under UV.

Fig. 5 Flow cytometry analysis of phagocytosis of neutrophils by MDM Φ 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 MDM Φ , after which they were exposed to a range of oxygen and pressure conditions for 90 min (Section 2.4). Cells were washed and analysed using flow cytometry. The results are presented as the percentage of MDM Φ that had ingested neutrophils. Data are expressed as means $\pm$ SEM for three separate experiments ($n = 3$) with triplicate measurements. Treatments with the same

lower case letter are not significantly different, whereas those with different lower case letters are significantly different (two-way ANOVA, $P < 0.05$).

Fig. 6 Light microscopic detection of phagocytosis of neutrophils by MDM Φ after pre-treatment of the neutrophils with normoxia, hypoxia, hyperoxia, pressure and HBO. Fresh or 22 h-aged neutrophils were co-cultured with MDM Φ and exposed to a range of oxygen conditions for 90 min (Section 2.4). After exposure, cells were washed, fixed and stained for MPO, and then were analysed using light microscopy. Light microscopic analysis demonstrating MDM Φ that had engulfed apoptotic neutrophils for both fresh and 22 h-aged neutrophils after exposure to HBO or normoxia. MDM Φ that had ingested neutrophils contain a yellow-brown reaction product (MPO-positive cells). MDM Φ that had not ingested neutrophils were MPO-negative (A). Results are presented as the percentage of MDM Φ that had ingested neutrophils (B). Data are expressed as means $\pm$ SEM for three separate experiments ($n = 3$) with triplicate measurements. Treatments with the same lower case letter are not significantly different, whereas those with different lower case letters are significantly different (two-way ANOVA, $P < 0.05$).

Fig. 7 H₂O₂ release by isolated neutrophils and MDM Φ that had ingested neutrophils after exposure to normoxia, hypoxia, hyperoxia, pressure and HBO. Neutrophils were isolated from bovine blood exposed to a range of oxygen and pressure conditions for 90 min (Section 2.4). 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 MDM Φ and exposed to a range of conditions for 90 min. H₂O₂ levels in the medium were determined in both neutrophils and MDM Φ that had ingested neutrophils using HRP/homovanillic acid (Section 2.14). These are expressed as the amount of H₂O₂ produced per 10⁶ cells. Data are means $\pm$ SEM for three separate experiments ($n = 3$) measured in triplicate. Treatments with the same lower case letter are not

significantly different, whereas those with different lower case letters are significantly different (two-way ANOVA, $P < 0.05$).

Fig. 8 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 isolated from bovine blood were exposed to a range of oxygen and pressure conditions for 90 min (Section 2.4). After exposure, fresh or 22 h-aged neutrophils) were stimulated with 100 ng ml⁻¹ LPS and assayed (A and B). For co-culture experiments (C and D) fresh or 22 h-aged neutrophils were incubated with LPS-stimulated MDM Φ and exposed to a range of oxygen and pressure conditions for 90 min and assayed. 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 neutrophils under normoxia (A and B) or MDM Φ cultured alone (C and D). Data are means $\pm$ SEM for three separate experiments measured in triplicate ($n = 3$). For fresh neutrophils, treatments with the same lower case letter are not significantly different, whereas those with different lower case letters are significantly different (Kruskal-Wallis, $P < 0.05$). For aged neutrophils, the same is indicated using lower case italic letters.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

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Figure 8