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

Anwar J. Almzaiel, Richard Billington, Gary Smerdon, and A. John Moody

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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 (MDMΦ) were co-cultured under HBO (97.9% O_2, 2.1% CO_2 at 2.4 atm absolute (ATA)) (1 atm = 101.325 kPa), hyperbaric normoxia (8.8% O_2 at 2.4 ATA), normobaric hyperoxia (95% O_2, 5% CO_2), normoxia (air), and normobaric hypoxia (5% O_2, 5% CO_2). Phagocytosis of fresh and 22 h aged neutrophils by MDMΦ was increased after HBO pre-treatment, assessed using flow cytometry and light microscopy. Enhanced clearance of neutrophils was accompanied by an increase in H_2O_2 levels following HBO pre-treatment with upregulation of IL-10 (anti-inflammatory cytokine) mRNA expression in LPS-stimulated MDMΦ that had ingested aged neutrophils. TNF-α (pro-inflammatory cytokine) gene expression did not change in LPS-stimulated MDMΦ that had ingested fresh or aged neutrophils after HBO, pressure, and hyperoxia. These findings suggest that HBO-activated MDMΦ participate in the clearance of apoptotic cells. Uptake of neutrophils by MDMΦ exposed to HBO may contribute to resolution of inflammation, because HBO induced up-regulation of IL-10 mRNA expression.

Key words: hyperbaric oxygen, neutrophils, macrophages, apoptosis.

Résumé : L'apoptose des neutrophiles et leur nenlèvement par les macrophages sont essentiels à la cicatrisation. Des données suggèrent qu'une exposition à l'oxygène hyperbare (OHB) peut accroitre l'apoptose des neutrophiles, mais les effets de l'OHB menant à l'enlèvement des neutrophiles par les macrophages sont encore flous. Dans l'étude actuelle, des neutrophiles et des macrophages dérivés des monocytes (MDMΦ) bovins ont été co-cultivés sous OHB (97.9% O_2, 2.1% CO_2 à 24 ATA), normoxie hyperbare (8.8% O_2 à 2.4 ATA), hyperoxie normobare (95% O_2, 5% CO_2), normoxie (air) et hypoxie normobare (5% O_2, 5% CO_2). La phagocytose de neutrophiles frais et âgés de 22h par les MDMΦ, évaluée par cytométrie en flux et microscopie optique, était accrue après un traitement préalable à l'OHB. L'enlèvement accru des neutrophiles était accompagné d'une augmentation des niveaux de H_2O_2 à la suite du prétraitement à l'OHB et d'une régulation à la hausse de l'expression l'ARNm de l'IL-10 (une cytokine anti-inflammatoire) chez les MDMΦ stimulés au LPS qui avaient ingérés des neutrophiles âgés. L'expression génique du TNF-α (une cytokine pro-inflammatoire) ne changeait pas chez les MDMΦ stimulés au LPS qui avaient ingérés des neutrophiles frais ou des neutrophiles âgés après traitement à l'OHB, la pression et l'hyperoxie. Ces résultats suggèrent que les MDMΦ activés par l'OHB participent à l'enlèvement des cellules apoptotiques. L'ingestion des neutrophiles par les MDMΦ exposés à l'OHB peut contribuer à la résolution de l'inflammation parce que l'OHB a induit une régulation à la hausse de l'expression de l'ARNm de l'IL-10. [Traduit par la Rédaction]

Mots-clés : oxygène hyperbare, neutrophiles, macrophages, apoptose.

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. 2001*a*). PS exposure is essential for the uptake of apoptotic cells by macrophages (Fadok et al. 1992, 2001*b*).

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 atm absolute (ATA), 1 atm = 101.325 kPa) for 60–90 min, repeated

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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. 2009; Ganguly et al. 2002), as well as in neutrophil-like cells derived from HL-60 cells (Almzaiel 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-1β 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 MDMΦ in a non-inflammatory process. To elucidate the effects of HBO on resolution of inflammation and the potential involvement of ROS-mediated apoptosis in this process, monocyte-derived macrophages (MDMΦ) 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 Ca^{2+} and Mg^{2+} 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*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 resuspended 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 (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 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*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*g* for 25 min at room temperature. The neutrophils form a pellet, and any contaminating mononuclear cells remained at the sample/medium interface. The neutrophil pellet was washed by resuspending in 50 mL HBSS and centrifuging at 600*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 were obtained 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 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 cm × 21.5 cm × 11 cm). This achieved the correct concentration inside the 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 resuspension to either 1×10^6 or 2×10^6 cells·mL^{-1} in PBS, supplemented RPMI-1640 medium or 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 (MDMΦ)

Monocytes (4×10^6 cells·mL^{-1}) were cultured in DMEM (containing 20 mmol/L glutamine, 100 U·mL^{-1} penicillin, and 10 μg·mL^{-1} streptomycin) and 10% (*v*/*v*) heat-inactivated bovine autologous serum (derived from platelet-rich plasma by re-calcification with 20 mmol/L $CaCl_2$ 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.

2.6. Induction of apoptosis

Following isolation from peripheral blood, neutrophils were cultured at 37 °C under 5% CO_2 (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% CO_2 (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 (serum free) and analysed immediately.

2.7. Assay for cell viability

Samples of each suspension of neutrophils (50 μL of 10^6 cells·mL^{-1} in supplemented RPMI-1640) were mixed with 50 μL 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, California, USA) at 540 nm (Vistica et al. 1991).

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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, 400g for 5 min). Slides were stained using 20% modified Wright-Giemsa stain (pH 7.0, Sigma-Aldrich) 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 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 400g 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 × 10^6 cells in annexin V binding buffer. The cells in 100 μL of annexin V binding buffer were pelleted, before addition of FITC-conjugated annexin V (5 μL; eBioscience, Hatfield, UK). The tubes were incubated on ice in the dark for 15 min, and then washed and resuspended in 100 μL of annexin V binding buffer, after which 5 μL of PI (1 μg·mL^{-1}) was added. Flow cytometric analysis of cell suspensions was performed by using an Arial™ II FACS (excitation 520 nm and emission 620 nm, Becton Dickinson, San Jose, California, USA) within 1 h of staining. Data for 10 000 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, Wisconsin, 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×) and TAE for 1 to 2 h at 90 V. The gel was visualized under UV a using a transilluminator (UVItec 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 MDMΦ phagocytosis by flow cytometry. Neutrophils were labelled with the fluorescent dye Tracker™ Green (5-chloromethyl fluorescein diacetate, CMFDA, Invitrogen, Paisley, UK) at a final concentration of 2 μg·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% CO_2, 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 MDMΦ-containing well (which had been washed with HBSS without Ca^{2+} and Mg^2 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% CO_2 (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 mmol/L EDTA for 15 min at 37 °C, followed by 15 min at 4 °C, washed and resuspended in PBS, and transferred to FACS tubes. 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, California, USA; excitation 492 nm/emission 517 nm) using FACS Diva software©. MDMΦ 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 MDMΦ alone 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. Uningested neutrophils were identified by the combination of their green fluorescence due to Tracker™ green labelling and their relatively smaller size (FSC) when compared with the MDMΦ. MDMΦ populations were gated based on FSC and SSC characteristics, MDMΦ 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

To assess phagocytosis by microscopy, MDMΦ 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*) H_2O_2 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 MDMΦ 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). MDMΦ that had ingested neutrophils were MPO positive, while MDMΦ which had not were MPO negative. Five hundred cells per each of three replicate wells were examined. The percentage of MDMΦ 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 μmol/L 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 μmol/L H_2O_2) was used to determine the H_2O_2 concentration in each well (Baggiolini et al. 1986).

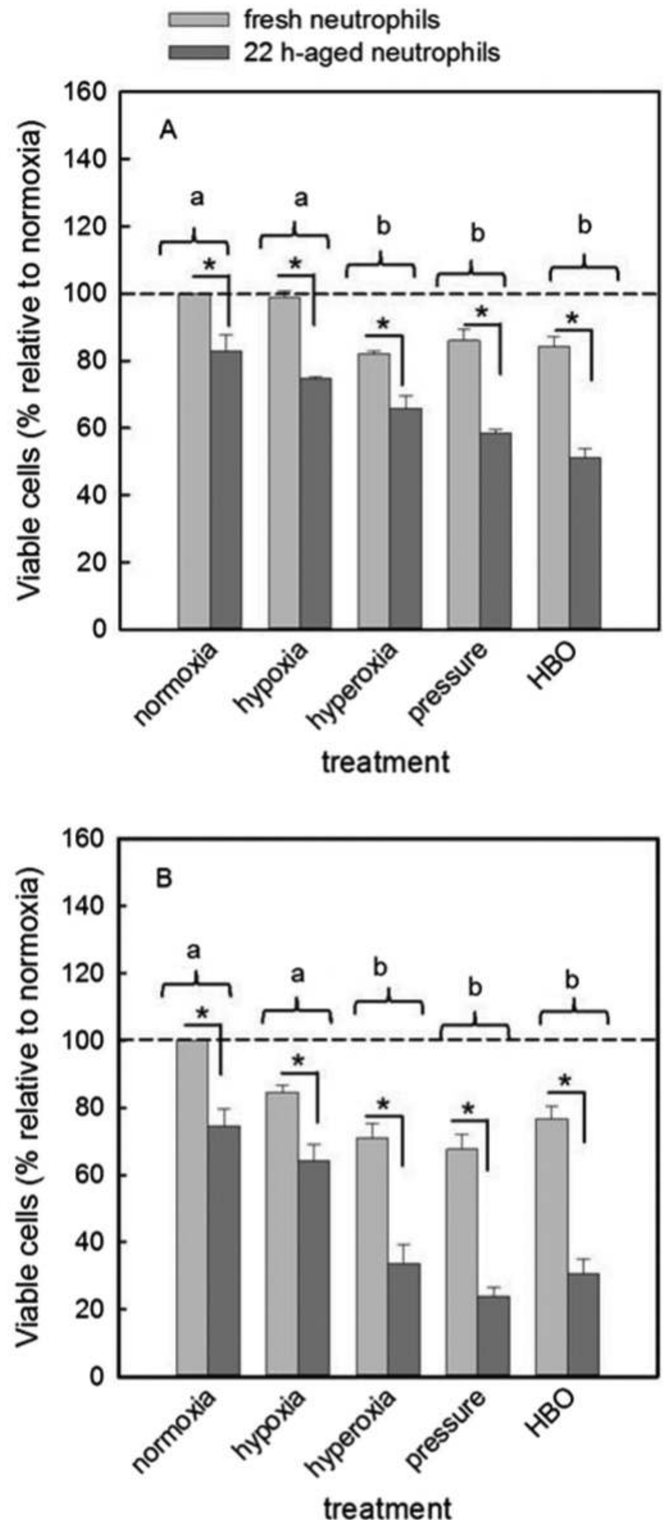

Fig. 1. Neutrophil cell viability (percentage relative to normoxia) after pre-treatment with hypoxia, hyperoxia, pressure and HBO. Neutrophils were isolated from bovine blood and exposed to a range of oxygen and pressure conditions for 90 min (Section 2.4). After exposure, cells were washed then immediately 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 ± SEM for 3 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. * indicates 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, $P < 0.05$).

2.15. Measurement of TNF-α and IL-10 expression using qPCR

MDMΦ 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 GeneElute™ 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: AAGCCGGCCTTGCACAT) 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 ± 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 1B). 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

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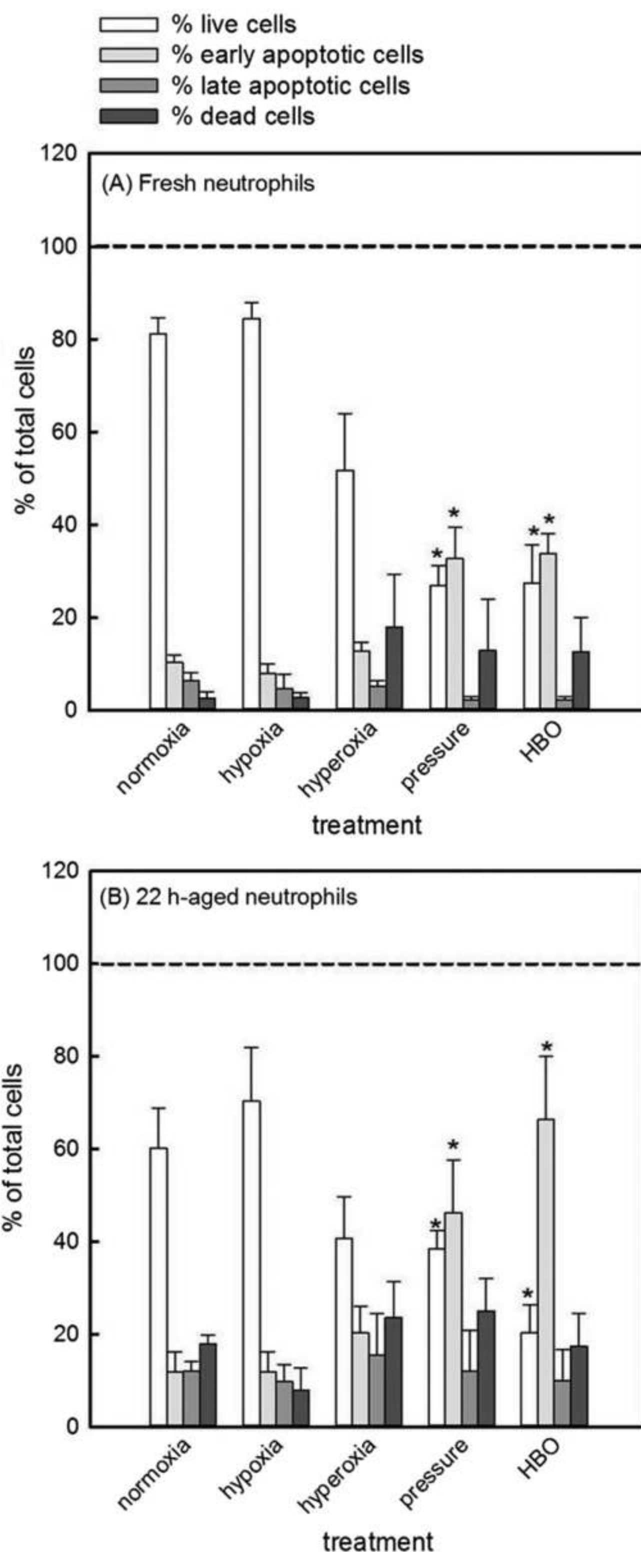

Fig. 2. Neutrophil populations after pre-treatment with normoxia, hypoxia, hyperoxia, pressure and HBO. Neutrophils were isolated from bovine blood and exposed to a range of oxygen and pressure conditions for 90 min (see Section 2.4). After exposure, cells were washed and then assayed immediately (fresh 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 ± SEM for 3 separate experiments (n = 3) with triplicate measurements. * significant difference versus treatment with normoxia (one-way ANOVA or Kruskal–Wallis, P < 0.05).

ANOVA, P = 0.0002) post-HBO and pressure treatments compared to normoxia (Fig. 2B), but not following hyperoxia and hypoxia treatments. Aged neutrophils (22 h) 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 3B). 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. Figures 4A and 4B 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 MDMΦ that had ingested neutrophils labelled with Tracker™ green was assessed by flow cytometric analysis, and the results are presented in Fig. 5. Phagocytosis of 22 h aged neutrophils by MDMΦ was significantly increased (two-way ANOVA, P < 0.005), compared with fresh neutrophils. Immediately after HBO and pressure treatments, MDMΦ 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 MDMΦ 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 MDMΦ was further confirmed by light microscopic examination of cytocentrifuged preparations of MDMΦ after the phagocytosis assay had been performed. This analysis clearly showed the presence of MPO-positive cells inside MDMΦ (Fig. 6A). Quantification of percentage phagocytosis confirmed the FACS data, in that MDMΦ 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, MDMΦ 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. 6A and 6B).

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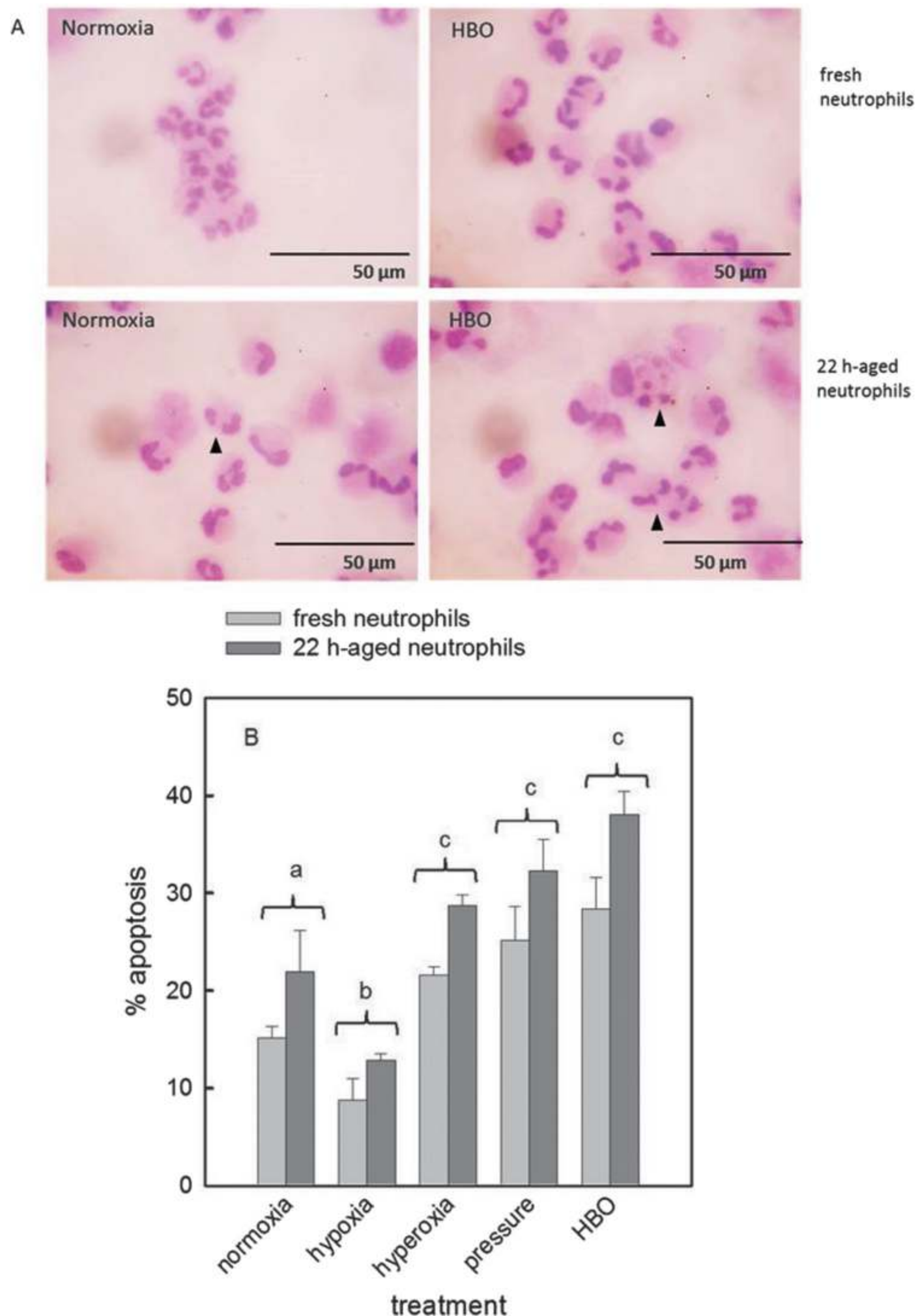
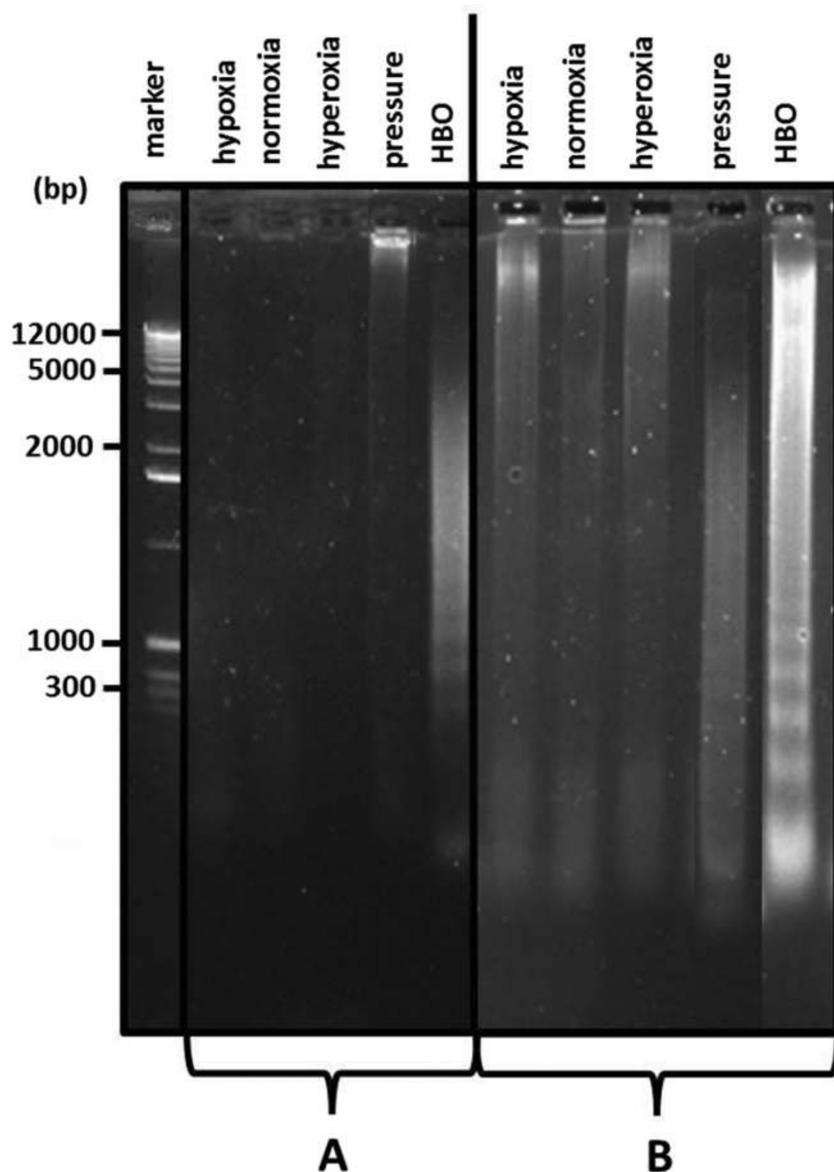
Fig. 3. Morphological analysis of apoptosis by light microscopy in neutrophils pre-treated with normoxia, hypoxia, hyperoxia, pressure, and HBO. Neutrophils were isolated from bovine blood and exposed to a range of oxygen conditions for 90 min (Section 2.4). After exposure, cells were washed and then 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 ± SEM for 3 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, $P < 0.05$).



3.4. Oxidants mediate neutrophil clearance by MDMΦ: 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 MDMΦ 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-

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×), 20 ng per lane, and visualized under UV.



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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 MDMΦ 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 MDMΦ 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 MDMΦ

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 MDMΦ were investigated by measuring the cytokine gene expression (TNF-α and IL-10) of fresh neutrophils, 22 h aged neutrophils and MDMΦ 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 MDMΦ 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 8B), while co-culture data are normalised against MDMΦ cultured alone (Figs. 8C and 8D). Note that for the co-culture experiments, mRNA was extracted from the mixture of cell types and so the different contributions of the MDMΦ 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 MDMΦ except in the case of fresh neutrophils and hyperoxia (Fig. 8C) (Kruskal–Wallis, $P < 0.05$).

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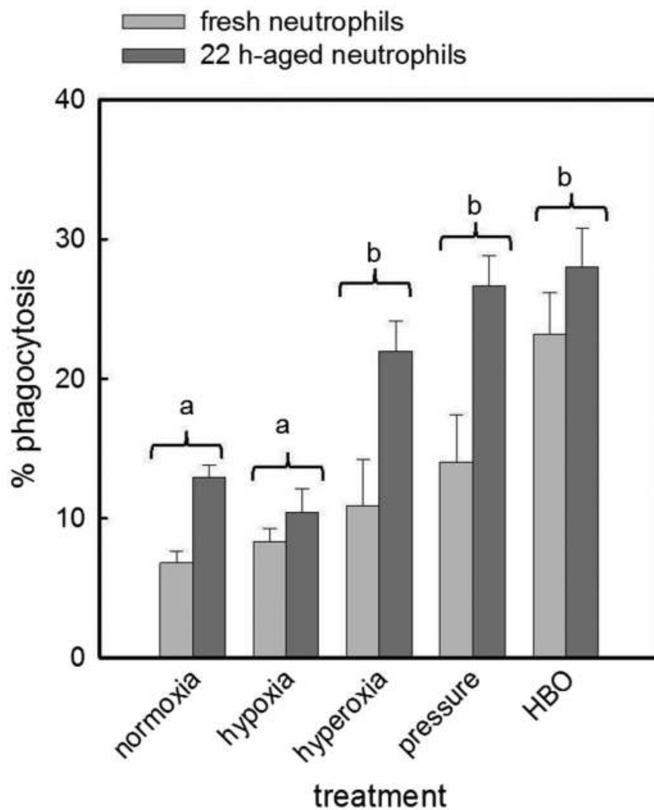
Fig. 5. Flow cytometry analysis of phagocytosis of neutrophils by MDMΦ after pre-treatment of the neutrophils with normoxia, hypoxia, hyperoxia, pressure, and HBO. Neutrophils (either fresh or 22 h aged) were labelled with Tracker™ Green and co-cultured with MDMΦ, after which they were exposed to a range of 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 MDMΦ that had ingested neutrophils. Data are expressed as means ± SEM for 3 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, $P < 0.05$).



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 MDMΦ, hyperoxia and HBO caused a marked increase in IL-10 expression, whereas there was no effect when fresh neutrophils were co-cultured with MDMΦ (Fig. 8D).

4. Discussion

The ability of MDMΦ 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.

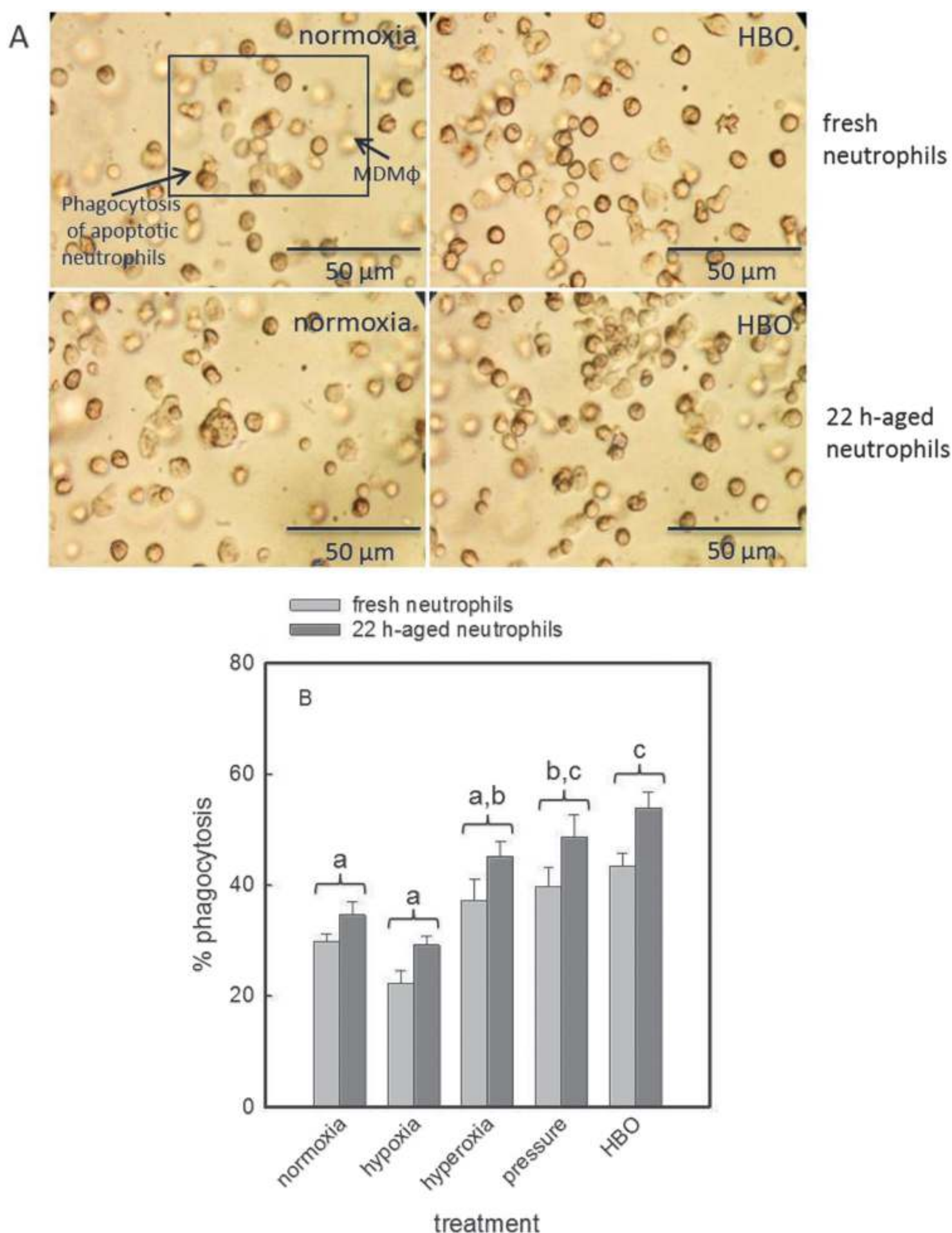
Having established that HBO induced apoptosis of neutrophils, the mechanisms involved were investigated. One possibility is that HBO-induced apoptosis is a function of oxygen partial pressure. Pre-treatment with HBO has been shown to up-regulate NADPH oxidase activity and caspase-3/7 activity, which 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. 2009). Exogenous H_2O_2 produced by the activity of xanthine oxidase or glucose oxidase induces apoptosis in neutrophils (Rollet-Labelle et al. 1998), and 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 that 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 (MDMΦ). MDMΦ 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 MDMΦ 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. 2001*a*), 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 MDMΦ 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

Fig. 6. 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 neutrophils were co-cultured with MDMΦ 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 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 (B). Data are expressed as means ± SEM for 3 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).



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 MDMΦ 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 MDMΦ. In the present study, a significant increase in H_2O_2 was observed immediately after HBO and pressure exposure in fresh and aged neutrophils. Furthermore, the increase was correlated with enhanced neutrophil

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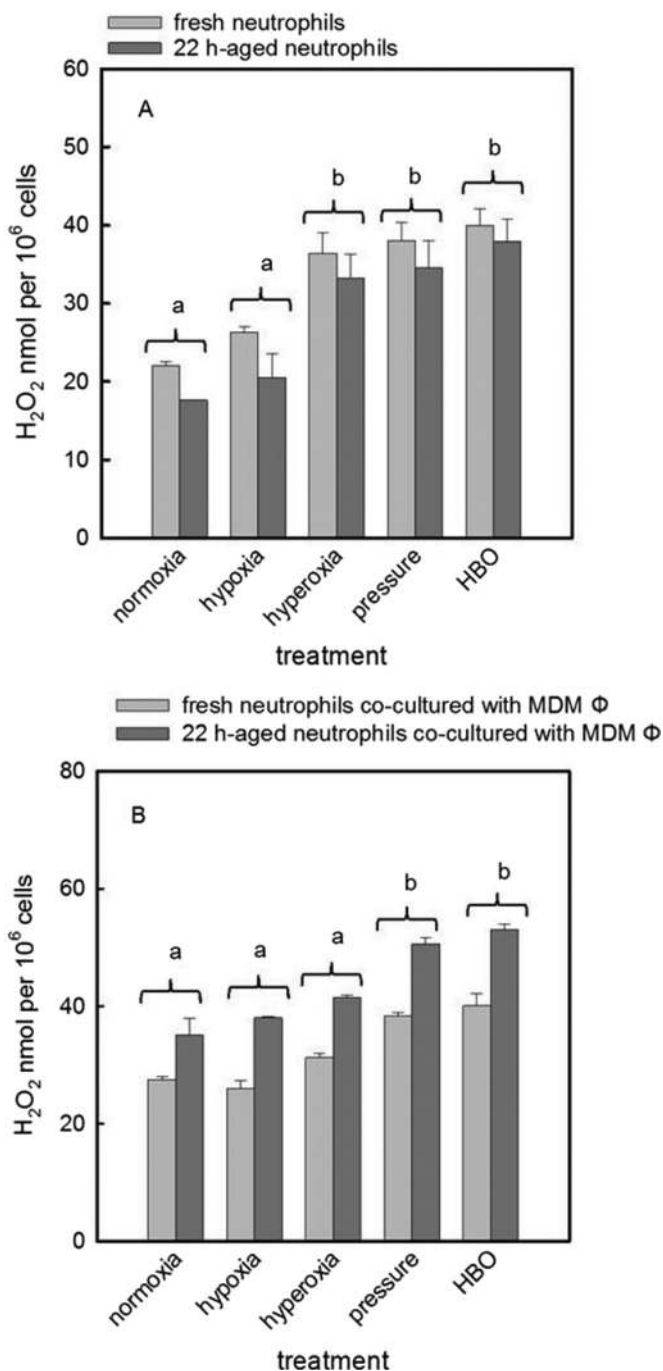
Fig. 7. H_2O_2 release by isolated neutrophils and MDMΦ that had ingested neutrophils after exposure to normoxia, hypoxia, hyperoxia, pressure, and HBO. Neutrophils were isolated from bovine blood exposed to a range of oxygen and pressure conditions for 90 min (Section 2.4). After exposure, fresh or 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 MDMΦ, and exposed to a range of conditions for 90 min. H_2O_2 levels in the medium were determined in both neutrophils and MDMΦ that had ingested neutrophils using HRP/homovanillic acid (Section 2.14). These are expressed as the amount of H_2O_2 produced per 10^6 cells. Data are means ± SEM for 3 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).



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 MDMΦ that had ingested fresh and aged neutrophils following HBO and pressure treatments. Oxidation of PS may stimulate its externalisation, the possible mechanism being that oxidized 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 MDMΦ 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 MDMΦ 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 MDMΦ (Cvetanovic and Ucker 2004).

Conclusion

The findings presented here provide evidence that HBO enhances neutrophil engulfment by MDMΦ. 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 MDMΦ. Furthermore, the data demonstrate another aspect in that HBO treatment is able to enhance the removal of apoptotic cells by MDMΦ 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

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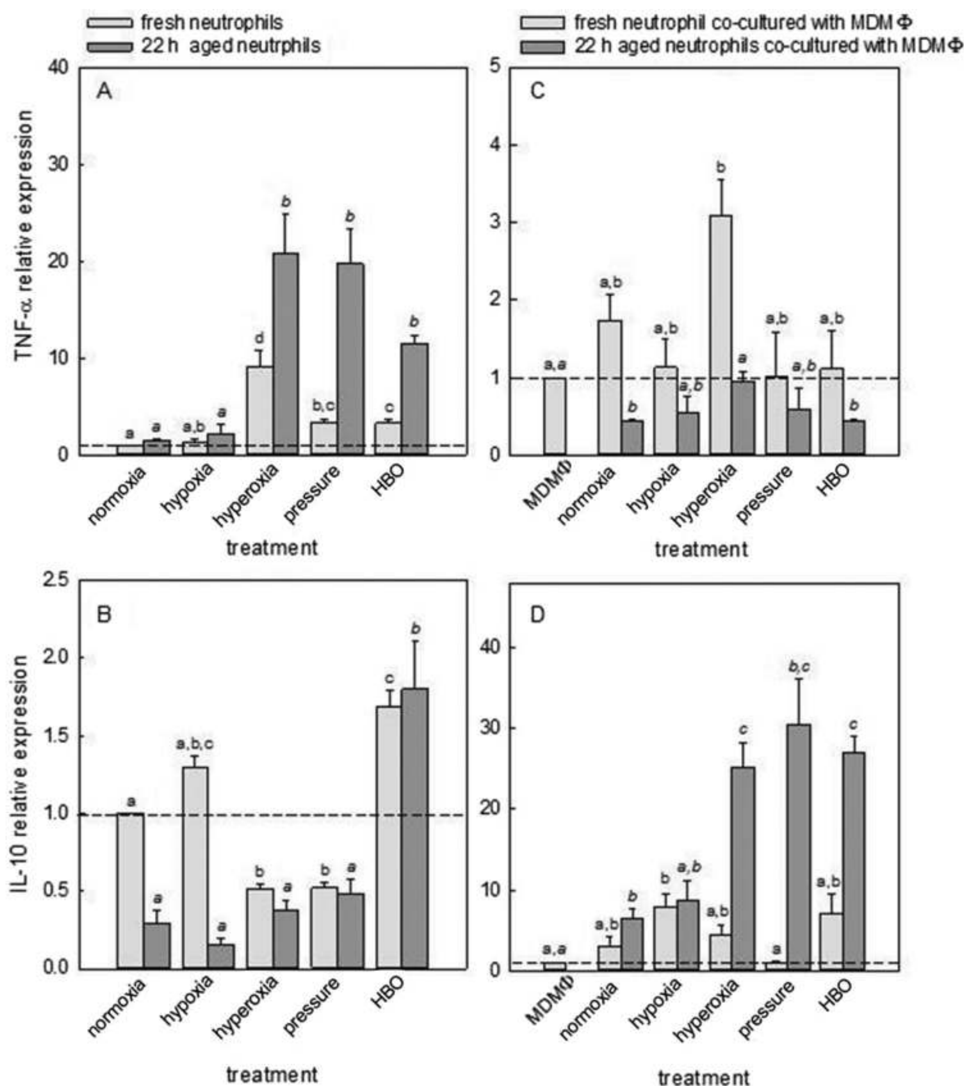
Fig. 8. TNF-α and IL-10 expression by neutrophils and MDMΦ that had ingested neutrophils after treatment with normoxia, hypoxia, hyperoxia, pressure, and HBO. Neutrophils isolated from bovine blood were exposed to a range of oxygen and pressure conditions for 90 min (Section 2.4). After exposure, fresh 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 MDMΦ 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 MDMΦ cultured alone (C and D). Data are means ± SEM for 3 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.



apoptotic cells, which may prove useful in developing a therapeutic approach to the resolution of inflammation.

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