Hyperbaric oxygen treatment reduces neutrophil-endothelial adhesion in chronic wound conditions through S-nitrosation

Alexandra C. Kendall, PhD[1*†]; Jacqueline L. Whatmore, PhD[1*]; Paul G. Winyard, PhD[1]; Gary R. Smerdon, PhD[2]; Paul Eggleton, PhD[1,3]

1. Exeter University School of Medicine, Exeter,
2. Diving Diseases Research Centre, Plymouth, Devon, United Kingdom, and
3. Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

Reprint requests:
Dr. P. Eggleton, Exeter University School of Medicine, University of Exeter, Exeter EX1 2LU, UK.
Tel: +44 1392 722940;
Fax: +44 1392 722926;
Email: p.eggleton@exeter.ac.uk

*ACK and JLW made an equal contribution to this paper.
†Present address: Manchester Pharmacy School, University of Manchester, Manchester, United Kingdom.

Manuscript received: September 19, 2012
Accepted in final form: July 10, 2013

DOI:10.1111/wrr.12108

ABSTRACT

Hyperbaric oxygen (HBO) therapy is an effective treatment for diabetic chronic wounds. HBO reduces inflammation and accelerates wound healing, by mechanisms that remain unclear. Here we examined a mechanism by which HBO may reduce neutrophil recruitment, through changes in endothelial and neutrophil adhesion molecule expression and function. Human umbilical vein endothelial cells and neutrophils were exposed to selected chronic wound conditions, comprising hypoxia in the presence of lipopolysaccharide and tumor necrosis factor-alpha, and then treated with HBO. We observed neutrophil adhesion to endothelial cells following treatment with chronic wound conditions, which was reversed by HBO treatment. This was partly explained by reduced expression of endothelial intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 by HBO. No changes in neutrophil adhesion molecule expression (CD18, CD11b, CD62L, CD31) were observed following HBO treatment. However, HBO decreased hydrogen peroxide generation by neutrophils, and induced nitrous oxide–related protein modifications. The transnitrosating agent S-nitroso-L-cysteine ethyl ester (600 μM) also reduced neutrophil adhesion to human umbilical vein endothelial cell monolayers, and the iNOS inhibitor 1400W (10 μM) and $HgCl_2$, which promotes the decomposition of S-nitrosothiols (1 mM), reversed the effect of HBO, suggesting that S-nitrosation may inhibit neutrophil-endothelial cell adhesion. This study indicates that HBO could reduce inflammation in wounds through reduced neutrophil recruitment, mediated by S-nitrosation.

Chronic wounds result from the failure of normal wounds to heal in response to conventional treatments, greatly impair patients' quality of life, and represent a huge healthcare burden. The treatment of chronic wounds is complicated by the variation in pathological processes underlying the development of different types of wounds, such as arterial ulcers, venous ulcers, and diabetic ulcers.[1] However, there are some features that are common to several types of chronic wound. Hypoxia is a key feature of many chronic wounds, resulting from destruction of local vasculature, and high oxygen consumption by cells such as leukocytes and fibroblasts.[2] Indeed, the PO_2 in nonspecified chronic wounds has been found to lie in the range of 5–20 mmHg (compared with typical values of 30–50 mmHg in healthy tissue).[3] Additionally, chronic wounds are typically colonized by numerous species of bacteria, particularly opportunistic pathogens such as *Pseudomonas aeruginosa*.[4] The potential complications of hypoxia and bacterial infection to an already inflammatory environment in the wound site can propagate the release of inflammatory cytokines from host cells. Macrophages, which marginate to wound sites, release inflammatory cytokines, and are subjected to prolonged stimulation by the continuous hypoxia and bacterial contamination in the chronic wound, exacerbating inflammation. The inflammatory cytokine tumor necrosis factor-alpha (TNF-α), which is released by various cell types including macrophages and endothelial cells, is often found in much higher levels in chronic wounds, including diabetic and venous ulcers, compared with acute wounds.[5] While not all chronic wounds are influenced by each factor mentioned previously, the inflammatory environments produced by any combination of these factors impair wound healing through several mechanisms, including impaired angiogenesis and collagen synthesis and deposition, as well as prolonged inflammation through excessive recruitment of inflammatory cells. Neutrophils are particularly affected by exposure to hypoxia, bacteria, and the local pro-inflammatory cytokine profile present in chronic wounds, leading to neutrophil accumulation in the wound site.[6]

Hypoxia has been shown to affect the recruitment of neutrophils into the wound site by endothelial cells. Buras et al.[7] demonstrated a 1.5-fold increase in ICAM-1 expression on endothelial cells subjected to 4 hours of hypoxia, in the absence of any additional stimulants. Kalra et al.[8] showed that human umbilical vein endothelial cell (HUVEC) monolayers exposed to hypoxia (3% O_2) allowed a significantly higher transmigration of monocyte-like HL60 cells than HUVEC

exposed to normoxia. However, hypoxia is rarely the only physiological stimulus at a site of injury, especially in chronic wounds, and soluble factors present, including cytokines and bacterial endotoxins, also have an effect.[5,9] Lipopolysaccharide (LPS) and TNF-α are also known to induce up-regulation of adhesion molecule expression, including ICAM-1, VCAM-1, and E-selectin, and this has an important role in the enhanced neutrophil recruitment by endothelial cells necessary to fight infections at wound sites.[10] Crucially, hypoxia can enhance stimulus-dependent changes in adhesion molecule expression and neutrophil adhesion.[11]Enhanced adhesion molecule expression due to inflammatory stimulants and hypoxia could, in part, explain the increased numbers of neutrophils found in chronic wounds.

In the hypoxic environment of some chronic wounds, there is very little oxygen available for neutrophil antibacterial respiratory burst activity.[3,12] Hyperbaric oxygen (HBO) therapy is a treatment that involves the intermittent inhalation of 100% oxygen at greater than atmospheric pressure, usually in the range 2–3 atmosphere absolute (ATA).[13] HBO increases oxygen delivery to the wound, and has been shown to be an effective treatment for chronic diabetic wounds, through mechanisms such as increased angiogenesis and antibacterial activity.[14] During HBO therapy, the increased oxygen available may also lead to changes in reactive oxygen species (ROS) and nitrogen species production within the wound proximity. Our previous study showed that elevated oxygen exposure to endothelial cells in a chronic wound model transiently regulates the expression of a number of genes involved in nitric oxide production and oxidative stress, but not other genes regulating hypoxia or angiogenesis, such as HIF-1α or vascular endothelial growth factor (VEGF).[15] This highlights the potential limitations of conducting in vitro studies on isolated cells, given that there is some disagreement in the literature as to the effect of HBO on VEGF and HIF-1 expression, with some in vivo studies finding different HBO-induced effects.[16,17] Indeed, HBO treatment has been shown to lead to increased nitrous oxide (•NO) production within the proximity of the wound and to promote increased granulation tissue deposition, epidermal migration, and wound closure.[18] Although the mechanisms of these wound healing processes remain to be elucidated, a number of studies have shown that HBO therapy can lead to increased •NO production via activation of •NO synthases.[19,20] In addition, the same group has provided evidence that posttranslational modification of actin occurs following hyperoxia, resulting in the S-nitrosylation of β-actin, possibly via generation of •NO by iNOS under these conditions. The nitrosylation of the actin cysteine moieties impacts on the formation of actin-integrin complexes, and ultimately neutrophil-endothelial cell adhesion.[21]

HBO is an effective treatment for chronic diabetic wounds, reducing inflammation and promoting healing.[14] In the present study, we investigated whether neutrophil-endothelial cell interactions could, in part, be mediated by HBO. We also wished to further expand the mechanistic work of Thom et al.[21] and Khiabani et al.,[22] who have implicated S-nitrosation in the reduced adhesion of neutrophils following HBO in healthy or ischemia-reperfusion (I/R) animal models. In this study we show that treatment of neutrophils with HBO or the •NO donor and transnitrosating agent S-nitroso-L-cysteine ethyl ester (SNCEE) significantly reduce neutrophil adhesion to endothelial cells, in an iNOS- and S-nitrosation-dependent manner.

MATERIALS AND METHODS

Reagents

Ham's F-12 medium, RPMI medium, and fetal bovine serum (FBS), were purchased from Lonza (Slough, United Kingdom). Endothelial cell growth supplement (ECGS), collagenase, gentamicin, LPS from *P. aeruginosa*, bovine serum albumin (BSA), gelatin, streptavidin alkaline phosphatase, *p*-nitrophenyl phosphate, FITC-conjugated mouse anti-human PECAM-1 antibody, Sigmacote (Sigma, Poole, United Kingdom) 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), L-glutamine, glucose, cytochrome *c*, phenol red, phenol red-free Hank's balanced salt solution (HBSS), N-formyl-L-methionyl-L-leucyl-phenylalanine (FMLP), superoxide dismutase (SOD), horseradish peroxidase (HRP), cytochalasin B, 3,3',5,5'-tetramethylbenzidine, and the HRP-conjugated rabbit anti-mouse antibody used in the Western blots were purchased from Sigma-Aldrich (Poole, United Kingdom). TNF-α was purchased from the NIBSC (Potters Bar, United Kingdom). 1400W was purchased from Merck Biosciences (Nottingham, UK). Mercury (II) chloride was purchased from Fisher Scientific (Loughborough, United Kingdom). Gas mixes were purchased from BOC (Manchester, United Kingdom). The ICAM-1 and VCAM-1 mouse anti-human primary antibodies and the biotinylated goat anti-mouse secondary antibody used for the enzyme-linked immunosorbent assay (ELISA) were purchased from AutogenBioclear (Calne, United Kingdom). The ICAM-1, and VCAM-1 mouse anti-human primary antibodies used for the Western blots, the E-selectin ELISA kit, the CD18, CD11b and L-selectin mouse anti-human primary antibodies, and the FITC-conjugated chicken anti-mouse secondary antibody used for flow cytometry, were purchased from Abcam (Cambridge, UK). Optilyse C was purchased from Beckman Coulter (High Wycombe, United Kingdom). Flow slides were made by Ibidi and purchased from Thistle Scientific (Glasgow, United Kingdom).

Cell isolation and culture

Human umbilical cords were obtained within 24 hours of birth from normal pregnancies. The anonymous collection of umbilical cords for this project was approved by the North and East Devon Medical Research Ethics Committee. HUVECs were isolated from the vein by collagenase (0.3 mg/mL) digestion[23]and cultured in Ham's F-12 medium containing 20% (v/v) FBS, 50 μg/mL gentamicin, and 20 μg/mL ECGS (complete Ham's + ECGS) at 37°C in 5% CO_2/air. The identity of endothelial cells in culture was confirmed by the presence of the typical "cobblestone" morphology and by the expression of von Willebrand factor and PECAM-1, as determined by immunocytochemical staining.

Neutrophils were isolated from whole blood, taken from healthy volunteers (with full approval from the North and East Devon Medical Research Ethics Committee), using Polymorphprep™ as previously described.[24]

Cell viability of endothelial cells and neutrophils was determined using the annexin V/7-AAD assay, as previously described.[25]

In vitro chronic wound conditions and HBO/pressure control treatment

Endothelial cells or neutrophils were left unstimulated, or exposed to selected in vitro chronic wound conditions (CW) by the addition of LPS from *P. aeruginosa* (0.5 μg/mL) and TNF-α (1 ng/mL) in hypoxia (2% O_2 at 1 ATA; PO_2 ~2 kPa ≈ 15 mmHg) at 37°C. Cells were exposed to HBO (97.5% O_2 at 2.4 ATA; PO_2 ~237 kPa ≈ 1778 mmHg) or pressure control (PC) conditions (0.83% O_2 at 2.4 ATA; PO_2 ~2 kPa ≈ 15 mmHg) for 90 minutes in separate pressure vessels at 37°C. The PO_2 of the single dose of HBO used to treat cells in this study was higher than the PO_2 range that wounds in patients would normally be exposed to during multiple HBO treatments (PO_2 ~27–107 kPa ≈ 200–800 mmHg). Reactive nitrogen species (RNS) donors or inhibitors were added during CW/HBO treatment (600 μM SNCEE [prepared as previously described[25,26]], 10 μM 1400W and 1 mM $HgCl_2$). These concentrations of RNS donors and inhibitors were tested for cell toxicity, and were used at a concentration that was shown not to affect cell viability, as determined by Trypan blue exclusion.

Analysis of neutrophil adhesion to an endothelial monolayer under flow

HUVECs were cultured and treated in collagen IV-coated gas-permeable flow slides. Before neutrophil flow, the HUVECs were rinsed with PBS containing 0.15% BSA + 5 mM glucose for 1 minute at 37°C. Treated neutrophils (1×10^6 cells/mL in RPMI containing 10% [v/v] FBS, 25 mM HEPES, and 2 mM L-glutamine) were then flowed over the HUVEC at 1 dyn/cm^2 for 4 minutes at 37°C (see Figure 1A). To remove any nonadherent neutrophils, the slide was again rinsed with PBS containing 0.15% BSA + 5 mM glucose for 1 minute at 37 °C. The flow slide was then examined using a Nikon Eclipse TS100 microscope, and the numbers of adherent neutrophils in 10 random fields of view were counted.

Measurement of HUVEC adhesion molecule expression by ELISA

For cell-surface ELISAs, HUVECs were cultured and treated in 0.1% (w/v) gelatin-coated 96-well plates. Following treatment, the cells were washed three times with complete Ham's + ECGS per well. Mouse anti-human antibodies against either ICAM-1 or VCAM-1 (5 μg/mL in PBS-BSA) were then added to the wells (50 μL per well) and the plates were incubated for 1 hour at 4°C. Following three washes with PBS-BSA to remove unbound antibody, a biotinylated goat anti-mouse secondary antibody (0.64 μg/mL in PBS-BSA) was applied to the cells (50 μL per well) and incubated for 1 hour at 4 °C. Unbound secondary antibody was removed with three PBS-BSA washes, and a solution of streptavidin alkaline phosphatase (2 μg/mL in Tris buffered saline) was added to the wells (100 μL per well) and incubated for 30 minutes at 4 °C. Plates were washed three times with PBS-BSA, before *p*-NPP substrate solution was added (200 μL per well) and incubated for 30 minutes at 4 °C in the dark. The enzyme reaction was stopped by the addition of 50 μL 1 N NaOH per well, before the absorbances were read on a plate reader (BMG LABTECH FLUOstar OPTIMA, BMG LABTECH Ltd. Aylesbury, United Kingdom) at 405 nm.

Shed E-selectin in conditioned medium collected from HUVEC following treatment with the various conditions was analyzed by commercial sandwich ELISA.

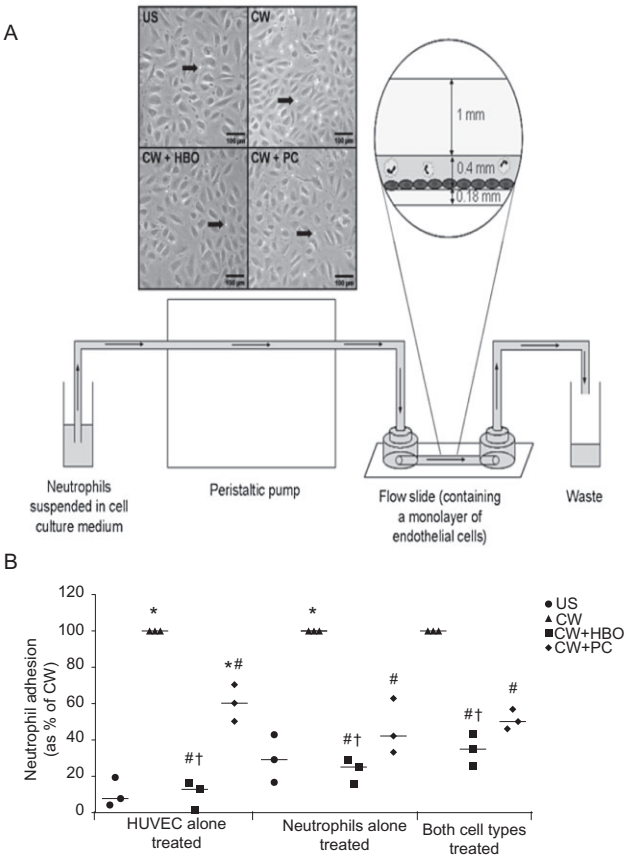

Figure 1. HBO and pressure changes reduce neutrophil adhesion to endothelial cells under chronic wound conditions. (A) Schematic showing neutrophil-endothelial cell adhesion chamber, photomicrograph inset shows neutrophils binding to endothelial cells (depicted by arrows) under unstimulated (US), chronic wound (CW), with hyperbaric oxygen (CW + HBO), or pressure control (CW + PC). Bar depicts 100 μm. Neutrophil adhesion to HUVECs under flow was assessed when HUVECs alone, neutrophils alone, and both HUVEC and neutrophils were treated with chronic wound (CW), HBO, or pressure control (PC) conditions (B). HUVECs were cultured on flow slides and treated with CW conditions and HBO or the PC. Following a 1 minute wash, neutrophils were flowed over the endothelial cells, at a shear stress of 1 dyn/cm^2, for 4 minutes. Following a further 1 minute wash to remove nonadherent neutrophils, adherent neutrophils in 10 random fields of view were counted using phase-contrast microscopy. Data are expressed as a percentage of the number of neutrophils adhering under CW conditions, $n = 3$. $*p < 0.05$ vs. US, $\#p < 0.05$ vs. CW, $\dagger p < 0.05$ vs. CW + PC. HBO, hyperbaric oxygen; HUVEC, human umbilical vein endothelial cell.

Measurement of HUVEC ICAM-1 and VCAM-1 total protein by Western blot

HUVECs were treated as above and collected 22.5 hours posthyperbaric treatment (corresponding to the time at which patients would receive their next HBO treatment). Total protein samples were prepared by cell lysis in sample buffer containing 62.5 mM Tris HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 25% (v/v) glycerol, and 0.01% bromophenol blue (with the addition of 50 mM DTT for analysis of VCAM-1). Samples were heated at 100°C for 5 minutes and 25 μg protein per sample was separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane at 60 V for 1 hour. Blots were blocked for 1 hour with 5% (w/v) milk powder in PBS containing 0.02% Tween-20 (PBS-T). Blots were then incubated with mouse anti-human ICAM-1 or VCAM-1 primary antibody diluted 1:1000 in PBS-T, overnight at 4°C with gentle agitation. Blots were washed three times for 5 minutes in PBS-T, and then incubated with an HRP-conjugated rabbit anti-mouse secondary antibody, diluted 1:1000 in blocking buffer for 1 hour at room temperature (RT). Blots were washed six times for 5 minutes in PBS-T before bands were detected and their densities quantified using enhanced chemiluminescence detection on a Chemidoc XRS system with Quantity One software (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Blots were stripped and reprobed for actin to assess protein loading.

Analysis of neutrophil adhesion molecule expression

Whole blood, taken from healthy volunteers (with full approval from the North and East Devon Medical Research Ethics Committee and with written informed consent), was mixed with dipotassium ethylenediaminetetraacetic acid (EDTA) (1.5 mg/mL) and divided into 100 μL aliquots in Sigmacote-treated 1.7 mL tubes. Following treatment, FITC-conjugated anti-CD18, -CD11b, -L-selectin, or -PECAM-1 mouse anti-human primary antibodies were added (10 μL per sample) and incubated at RT in the dark for 30 minutes. Optilyse C (500 μL) was then added to each tube and vortexed immediately. The tubes were incubated at RT for 10 minutes (in the dark). PBS (500 μL) was added to each tube and vortexed, before being incubated in the dark at RT for 5 minutes. The samples were then analyzed on a Beckman Coulter Cell Lab Quanta SC flow cytometer, and data for 10,000 events were collected. The neutrophil subpopulation was then identified and gated, and the mean fluorescence intensity of the cells was recorded.

Measurement of neutrophil ROS release

To measure H_2O_2 production, 50 μL aliquots of neutrophil suspension, containing 5×10^5 neutrophils in phenol red-free HBSS, were added to the wells of a 96-well plate. One hundred μL 10 mM potassium phosphate buffer, pH 7.0, containing 140 mM NaCl, 5.5 mM D-glucose, 280 μM phenol red, and 1.14 μM HRP, with or without TNF-α and LPS (1 ng/mL and 0.5 μg/mL, respectively), was then added to each well. For blank wells, 10 μL 1 N NaOH was added to kill the neutrophils and set the pH to 12.5. H_2O_2 standards ranging from 0 to 100 μM were set up in cell-free wells. Plates were then treated with (normal air containing 21% O_2 at atmospheric pressure, representing the air that patients not undergoing HBO therapy would be exposed to), hypoxia, HBO, or the pressure control, all at 37°C, for 90 minutes, before 10 μL 1 N NaOH was added to all nonblank wells. Sham control 96-well plates were also set up in parallel containing the appropriate medium, exposure to the same gaseous conditions and pressures. A blood gas analyzer was then used to confirm the oxygen levels were correct. After brief shaking, plates were centrifuged at 200×*g* for 10 minutes to remove cells, and the supernatant from each well was transferred to a new plate. The absorbance of the supernatant was then read at 595 nm. A standard curve was constructed from the H_2O_2 standard wells, and used to calculate the H_2O_2 concentration in treatment wells, having first subtracted the blanks.

Superoxide production was measured by placing 50 μL aliquots of neutrophil suspension, containing 5×10^5 neutrophils in phenol red-free HBSS with 8% BSA, into wells of a 96-well plate. Phenol red-free HBSS (100 μL) containing 8% BSA and 160 μM cytochrome *c*, with or without TNF-α and LPS (1 ng/ml and 0.5 μg/mL, respectively), was then added to each well. Some wells for each treatment were also treated with 300 U/mL SOD. The cell suspensions were then exposed to21% O_2/5% CO_2balanced air, hypoxia, HBO, or the pressure control treatment, all at 37°C, for 90 minutes. Plates were centrifuged at 129 × g for 10 minutes to remove cells, and the supernatant from each well was transferred to a new plate. The absorbance of the supernatant was then read on a plate reader at 550 nm. SOD-inhibitable O_2^- production was calculated by subtracting the mean optical density (OD) of the SOD-containing wells from the mean OD of the non-SOD-containing wells for each treatment, and using the extinction coefficient of cytochrome *c* (21 mM/cm) while allowing for a path length of 4.5 mm (150 μL total volume per well).

Data analysis

The data were produced from repeated experiments (minimum $n = 3$, using cells isolated from three or more different donors, and with all experiments employing multiple replicates) of cell viability, neutrophil adhesion under flow, measurement of adhesion molecule expression by ELISA; Western blot and flow cytometry were analyzed using Mann–Whitney *U*-tests or one-way analyses of variance (ANOVAs) with Bonferroni posttests. The data from repeated experiments ($n = 3$) of H_2O_2 and O_2^- release were analyzed using two-way ANOVAs.

RESULTS

HBO reduces neutrophil adhesion to endothelial cells under flow

We investigated the effects of HBO on neutrophil adhesion to endothelial cells under physiological flow conditions. To ensure any changes in adhesion were not a consequence of altered viability caused by any of the treatments, endothelial cell and neutrophil viability were assessed by annexin V/7-AAD at various times post-HBO treatment. There was no significant loss in the percentage of live endothelial cells following all treatment conditions. However, there was some

loss in neutrophil viability following treatment with HBO or the PC. To assess cell adhesion, neutrophils at a density of 1×10^6 cells/mL were pumped over a monolayer of HUVEC, generating a shear stress of 1 dyn/cm^2, similar to that found in postcapillary venules. Treatment of HUVEC with CW conditions led to an increase in the number of adherent neutrophils, which was partially reversed by treatment with HBO or PC conditions as shown in Figure 1. HBO treatment of HUVEC alone (Figure 1B, left panel) neutrophils alone (Figure 1B, middle panel), and both HUVEC and neutrophils (Figure 1B, right panel) led to a significant decrease ($p = 0.037$) in the number of neutrophils adhering to the endothelial cell monolayer (by 89.9 ± 7.8, 76.5 ± 6.7, and $65.2 \pm 8.8\%$, respectively). Treatment of HUVEC alone, neutrophils alone, or both HUVEC and neutrophils, with the PC conditions, also led to a significant decrease ($p = 0.037$) in neutrophil adhesion (by 39.7 ± 10.1, 53.9 ± 15.2, and $48.9 \pm 5.4\%$, respectively), compared with cells that just received the CW treatment. However, in all three cases, neutrophil adhesion following HBO was significantly lower than neutrophil adhesion following treatment with the PC conditions.

HBO reduces endothelial, but not neutrophil, adhesion molecule expression

To investigate whether the reduced neutrophil adhesion observed when HUVECs alone were treated with HBO was dependent on changes in endothelial adhesion molecule expression, ICAM-1 and VCAM-1 expressions were measured by ELISA and Western blot. ELISAs revealed that HBO reduced surface expression of both ICAM-1 and VCAM-1 (Figure 2A, left and right panels, respectively). At 22.5 hours posttreatment, cells receiving a 90-minute HBO treatment expressed significantly less ICAM-1 and VCAM-1 than cells treated with CW conditions alone or the PC. This result was confirmed by analysis of total cell content of both endothelial adhesion molecules, as determined by Western blot 22.5 hours posttreatment (Figure 2B). ICAM-1 expression decreased by $10.1 \pm 8.2\%$ following treatment with HBO, compared with cells that received treatment with CW conditions alone, while VCAM-1 total cell content decreased by $27.8 \pm 6.1\%$ following HBO treatment. Figure 2C shows that there was significant shedding of E-selectin from the endothelial cell surface only in HBO-treated cells.

Expression of the neutrophil adhesion molecules CD18, CD11b, CD62L, or CD31 was measured by flow cytometry immediately following a 90-minute treatment with CW conditions and after HBO or PC treatment. Cell surface expression of the adhesion molecules tested was not significantly affected by treatment (Figure 3), demonstrating that HBO-mediated impairment of neutrophil adhesion when neutrophils alone are treated with HBO is dependent on impaired adhesion molecule function, rather than reduced expression.

HBO-mediated impairment of neutrophil adhesion is mediated by S-nitrosation

To investigate the possible role of S-nitrosation in the HBO-mediated impairment of neutrophil adhesion to endothelial cells, we treated neutrophils with the cell-permeable

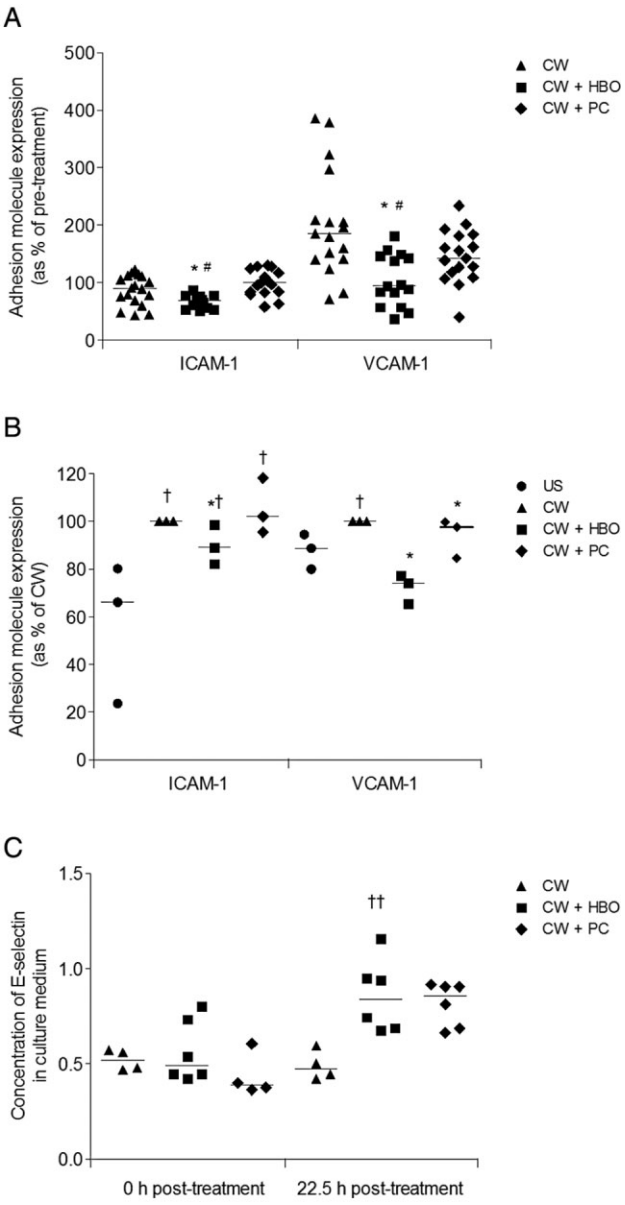

Figure 2. Effects of HBO and chronic wound conditions on endothelial cell adhesion molecules expression and release. HUVECs were treated with chronic wound (CW) conditions and HBO or the pressure control (PC), then cell surface ICAM-1 and VCAM-1 expression was measured at 0 hour and 22.5 hours posttreatment by ELISA (A) and total cell content was measured at 22.5 hours posttreatment by Western blot (B). E-selectin shed into the cell culture medium was measured by sandwich ELISA (C). Cell surface ELISA data are $n = 18$, Western blot data are $n = 3$, E-selectin ELISA data are $n = 6$. *$p < 0.05$ vs. CW, #$p < 0.05$ vs. CW + PC, †$p < 0.05$ vs. US. ††$p < 0.05$ vs. 0 hour post-treatment. ELISA, enzyme-linked immunosorbent assay; HBO, hyperbaric oxygen; HUVEC, human umbilical vein endothelial cell.

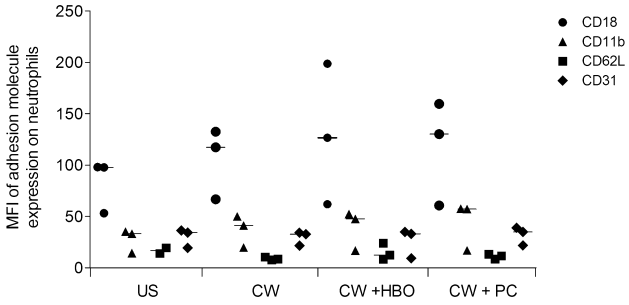

Figure 3. Effects of HBO and chronic wound conditions on cell surface neutrophil adhesion molecule expression. Neutrophils were treated with chronic wound (CW) conditions and HBO or the pressure control (PC) for 90 minutes, then adhesion molecule expression was immediately assessed by flow cytometry. Data are expressed as the mean fluorescence intensity (MFI) of the neutrophils, $n = 3$. HBO, hyperbaric oxygen; US, unstimulated.

S-nitrosating agent SNCEE, to determine if S-nitrosation could replicate the effects of HBO. Selective inhibitors were also employed to test the role of $NO^{\bullet}$ in this process—the selective iNOS inhibitor 1400W, used to impair iNOS-derived $NO^{\bullet}$ production, and $HgCl_2$, which promotes the decomposition of S-nitrosothiols. The addition of SNCEE (600 μM) to CW-treated neutrophils reduced neutrophil adhesion to an endothelial monolayer by 77.6 ± 4.14%, a similar reduction as that caused by HBO treatment (Figure 4). Additionally, the HBO-mediated impairment of neutrophil adhesion was inhibited by the addition of 1400W (10 μM) or $HgCl_2$ (1 mM), so that neutrophil adhesion was not significantly different from that in CW-treated cells (83.3 ± 13.9 and 94.1 ± 33.2%, respectively).

HBO decreases ROS release from neutrophils

We also investigated the effect of HBO on other specific aspects of neutrophil function. The release of ROS was monitored following exposure of cells to CW and HBO conditions (Table 1). Superoxide release, measured by cytochrome *c* reduction, was not significantly affected following HBO treatment. However, H_2O_2 release, measured by the H_2O_2-dependent oxidation of phenol red, was significantly decreased by treatment with both HBO and the PC conditions.

DISCUSSION

HBO is a therapy used extensively worldwide for the treatment of chronic diabetic wounds.[14,27] Of the various cell types involved in the wound healing process, neutrophils are of particular interest as they play a crucial role in normal wound healing, but may also contribute to the prolonged inflammation observed in chronic wounds.[28] We investigated whether HBO could affect neutrophil recruitment by endothelial cells under conditions often found in diabetic chronic wounds (hypoxia, high levels of TNF-α and LPS[29,30]), offering a partial explanation for the reduced inflammation and improved healing observed in some chronic diabetic wounds following HBO treatment.

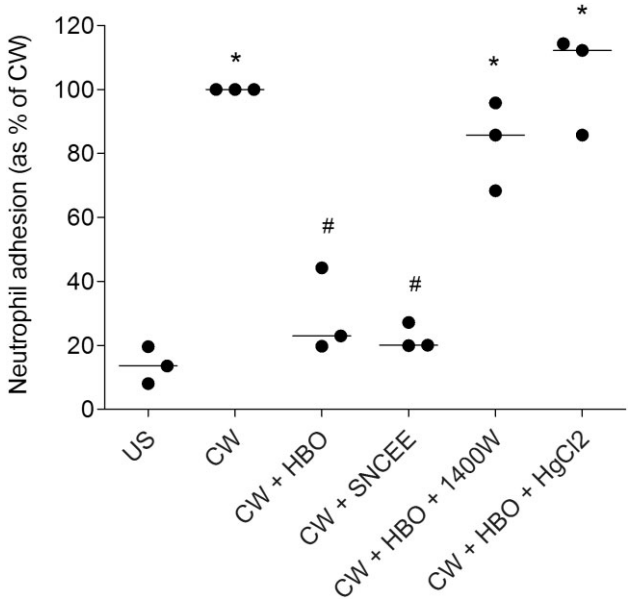

Figure 4. Effects of RNS and RNS inhibitors on neutrophil adhesion to endothelial cells under flow conditions. Neutrophils were treated with chronic wound (CW) conditions and SNCEE (600 μM), or HBO alone or in combination with 1400W (10 μM) or $HgCl_2$ (1 mM) for 90 minutes. Adhesion to an endothelial cell monolayer under flow was measured and data are expressed as a percentage of the number of neutrophils adhering under CW conditions, $n = 3$. $^*p < 0.05$ vs. US, $\#p < 0.05$ vs. CW. RNS, reactive nitrogen species; HBO, hyperbaric oxygen; SNCEE, S-nitroso-L-cysteine ethyl ester; US, unstimulated.

We observed HBO-mediated impairment of neutrophil adhesion not only when both endothelial cells and neutrophils were treated with HBO, but also when only one cell type was treated. This allowed dissection of some of the mechanisms involved. Endothelial-mediated impairment of neutrophil adhesion was partly due to altered endothelial adhesion molecule expression. Both surface and total ICAM-1 and VCAM-1 expression were reduced by HBO treatment, and E-selectin was shed significantly from the endothelial cell surface, an effect supported by previous research that showed

Table 1. Effect of HBO on ROS release from neutrophils under chronic wound conditions

	ROS release (nM per million cells per min)		
	CW	CW + HBO	CW + PC
H_2O_2	916.4 ± 5.3	792.6 ± 2.3***	747.2 ± 8.7***
$O_2^{\bullet-}$	2382.8 ± 312.1	2143.6 ± 210.0	1183.5 ± 220.1

Data are expressed as mean ± SE; $n = 9$.
***$p < 0.001$ vs. CW treatment.
CW, chronic wound; HBO, hyperbaric oxygen; PC, pressure control, ROS, reactive oxygen species.

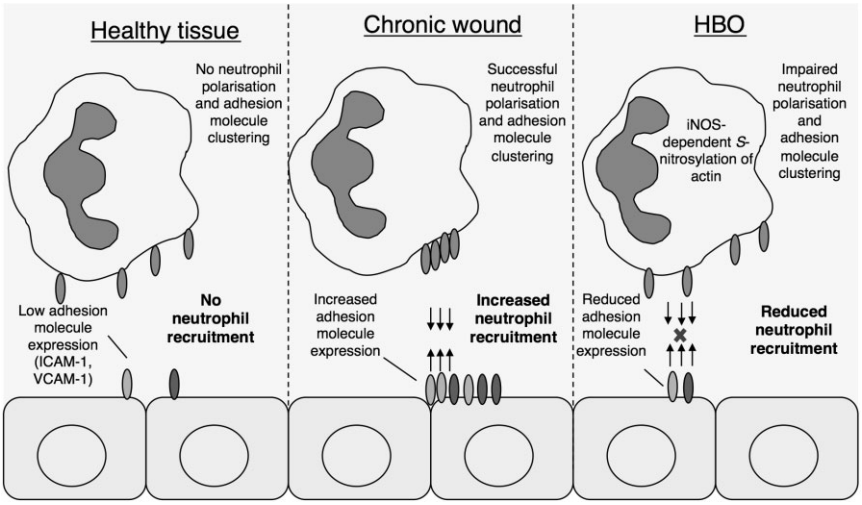

Figure 5. S-nitrosation is involved in HBO-mediated impairment of neutrophil adhesion to an endothelial cell monolayer. Chronic wound conditions increase endothelial adhesion molecule expression and cause neutrophil polarization and adhesion molecule clustering. HBO reduces endothelial adhesion molecule expression, and impairs neutrophil adhesion molecule clustering, reducing the number of neutrophils adhering to endothelial cells following treatment with HBO. HBO, hyperbaric oxygen.

reduced ICAM-1 expression in endothelial cells following HBO in an I/R model.[7] The relatively small changes observed in the surface expression of ICAM-1, VCAM-1, and E-selectin suggest that other endothelial adhesion molecules involved in the recruitment process might also have been affected by HBO in our work, and possible candidates include CD31 and CD99, which are known to be involved in neutrophil recruitment in wound healing.[31]

In a previous study we showed that a single exposure of HBO has no effect of surface expression of adhesion molecules CD31 and CD62P on platelets.[32] In this current study, treatment of neutrophils alone with HBO led to reduced neutrophil adhesion to endothelial cells, despite no effect of HBO on the total surface expression of a number of neutrophil adhesion molecules (CD18, CD11b, CD62L, or CD31). While our study is the first to our knowledge to investigate the effect of HBO on neutrophil CD62L or CD31 expression, Thom et al. [33]have also observed an impairment of CD18/CD11 b-dependent adhesion following HBO treatment (2.8 ATA or 3.0 ATA for 45 minutes), with no change in actual CD18 expression.

The present results implicate an impairment of neutrophil adhesion molecule function, but not overall cell surface adhesion molecule expression, following HBO under chronic wound conditions. A question raised by our observations and the work of others is the potential involvement of posttranslational modification of cellular proteins by HBO, specifically S-nitrosation of cellular proteins involved in adhesion.[21,34] This is of particular relevance given the evidence that HBO therapy increases iNOS and •NO production.[18,35] The inhibitory effect of SNCEE on CW-induced neutrophil adhesion indicates the involvement of S-nitrosation in the HBO-mediated impairment of neutrophil adhesion. Indeed, the addition of $HgCl_2$ to neutrophils, which selectively displaces the •NO moiety from S-nitrosothiols, with the formation of nitrite, reversed the effects of HBO, so that neutrophil adhesion to endothelial cells was at the same level as that in CW-treated cells. Additionally, the specific iNOS inhibitor, 1400W, reversed the HBO-mediated reduction in neutrophil adhesion, highlighting the potential involvement of iNOS-derived •NO. A potential mechanism of HBO-mediated impairment of neutrophil adhesion molecule function could be S-nitrosation of actin.[21] Actin-dependent redistribution of cell membrane components, leading to adhesion molecule clustering, is a crucial component of neutrophil polarization, which is necessary for firm adhesion to an endothelial monolayer. S-nitrosation of cytoskeletal actin impairs actin polymerization, and redistribution of the adhesion molecules on the cell membrane, which could explain the observed impairment of neutrophil adhesion in the absence of any changes in total adhesion molecule expression (Figure 5). This effect of HBO has previously been observed in studies using healthy mice or rats with I/R injury,[22,36] but this is the first study to observe a potential S-nitrosation-mediated effect in human cells under chronic wound conditions.

The impaired release of ROS from activated neutrophils observed in our work could also be explained by impaired actin polymerization due to S-nitrosation. Actin is crucial for assembly of the enzyme complex nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and impairment of actin function has been shown to decrease ROS release in vitro.[37] Additionally, the $p47^{phox}$ component of NADPH oxidase is susceptible to S-nitrosation, leading to decreased ROS release.[38] Therefore, S-nitrosation of both $p47^{phox}$ and actin could explain the decreased H_2O_2 release as observed in the present study.

While this study has shown exciting results, the limitations of using an in vitro setup have to be acknowledged, and the findings warrant further investigation in vivo. This study was limited in sample size due to the availability of HUVEC and neutrophil donors, and studies on larger numbers of patients might provide crucial information about why some patients respond better to HBO therapy than others.[39] Additionally, the complicated milieus of chronic wounds include more variables than we were able to include in our study, and so caution must be taken in extrapolating our in vitro data to humans.[40]Moreover, in this study we cultured cells under gaseous conditions of 21% O_2/5% CO_2 balanced air as a standard "normoxia" control. However, this will not reflect the true "normoxia" conditions seen in various tissues, which can differ greatly, depending on the vascular density of the

tissue. In future experiments, a "normoxic" control of 7–8% O_2 may be more appropriate as representative as the conditions at the cellular level.

We have demonstrated a potential mechanism by which an effective, yet inadequately understood (and therefore underused) therapy for chronic diabetic wounds works to reduce inflammation. HBO reduces neutrophil adhesion to endothelial cells through reduced endothelial adhesion molecule expression and impaired neutrophil adhesion molecule function, which is mediated by S-nitrosation resulting from HBO-induced iNOS-derived $^{\bullet}$NO production. This impaired neutrophil adhesion would reduce the inflammatory burden on the chronic wound and could explain, in part, how HBO treatment reduces inflammation in chronic diabetic wounds.

ACKNOWLEDGMENTS

Source of Funding: This work was supported by a grant from the Diving Diseases Research Centre (DDRC), Plymouth, UK [RR100098]. ACK was supported by a Peninsula College of Medicine and Dentistry Studentship awarded to PE, JLW, GRS, and PGW. We thank the clinical staff of the DDRC for help and assistance in the preparation of this manuscript.

Conflict of Interests: We declare that no author cited in this manuscript has actual or potential conflict of interest including any financial, personal, or other relationships with other people or organizations within 3 years of beginning the submitted work that could inappropriately influence, or be perceived to influence, this work.

REFERENCES

1. Kranke P, Bennett MH, Martyn-St James M, Schnabel A, Debus SE. Hyperbaric oxygen therapy for chronic wounds. *Cochrane Database Syst Rev* 2012; (4): CD004123.
2. Tandara AA, Mustoe TA. Oxygen in wound healing – more than a nutrient. *World J Surg* 2004; 28: 294–300.
3. Sheffield PJ. Measuring tissue oxygen tension: a review. *Undersea Hyperb Med* 1998; 25: 179–88.
4. Trøstrup H, Thomsen K, Christophersen LJ, Hougen HP, Bjarnsholt T, Jensen PØ, et al. Pseudomonas aeruginosa biofilm aggravates skin inflammatory response in BALB/c mice in a novel chronic wound model. *Wound Repair Regen* 2013; 21: 292–9.
5. Trengove NJ, Bielefeldt-Ohmann H, Stacey MC. Mitogenic activity and cytokine levels in non-healing and healing chronic leg ulcers. *Wound Repair Regen* 2000; 8: 13–25.
6. Yager DR, Nwomeh BC. The proteolytic environment of chronic wounds. *Wound Repair Regen* 1999; 7: 433–41.
7. Buras JA, Stahl GL, Svoboda KK, Reenstra WR. Hyperbaric oxygen downregulates ICAM-1 expression induced by hypoxia and hypoglycemia: the role of NOS. *Am J Physiol Cell Physiol* 2000; 278: C292–302.
8. Kalra VK, Shen Y, Sultana C, Rattan V. Hypoxia induces PECAM-1 phosphorylation and transendothelial migration of monocytes. *Am J Physiol* 1996; 271: H2025–34.
9. James GA, Swogger E, Wolcott R, Pulcini E, Secor P, Sestrich J, et al. Biofilms in chronic wounds. *Wound Repair Regen* 2008; 16: 37–44.
10. Magder S, Neculcea J, Neculcea V, Sladek R. Lipopolysaccharide and TNF-a produce very similar changes in gene expression in human endothelial cells. *J Vasc Res* 2006; 43: 447–61.
11. Zund G, Nelson DP, Neufeld EJ, Dzus AL, Bischoff J, Mayer JE, et al. Hypoxia enhances stimulus-dependent induction of E-selectin on aortic endothelial cells. *Proc Nal Acad Sci U S A* 1996; 93: 7075–80.
12. Hopf HW, Rollins MD. Wounds: an overview of the role of oxygen. *Antioxid Redox Signal* 2007; 9: 1183–92.
13. Kendall AC, Whatmore JL, Harries LW, Winyard PG, Eggleton P, Smerdon GR. Different oxygen treatment pressures alter inflammatory gene expression in human endothelial cells. *Undersea Hyperb Med* 2013; 40: 115–23.
14. Londahl M, Katzman P, Nilsson A, Hammarlund C. Hyperbaric oxygen therapy facilitates healing of chronic foot ulcers in patients with diabetes. *Diabetes Care* 2010; 33: 998–1003.
15. Kendall AC, Whatmore JL, Harries LW, Winyard PG, Smerdon GR, Eggleton P. Changes in inflammatory gene expression induced by hyperbaric oxygen treatment in human endothelial cells under chronic wound conditions. *Exp Cell Res* 2012; 318: 207–16.
16. Chen L, Gajendrareddy PK, Dipietro LA. Differential expression of HIF-1 alpha in skin and mucosal wounds. *J Dent Res* 2012; 91: 871–6.
17. Fok TCO, Jan A, Peel SAF, Evans AW, Clokie CML, Sándor GKB. Hyperbaric oxygen results in increased vascular endothelial growth factor (VEGF) protein expression in rabbit calvarial critical-sized defects. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2008; 105: 417–22.
18. Boykin JV, Jr, Baylis C. Hyperbaric oxygen therapy mediates increased nitric oxide production associated with wound healing: a preliminary study. *Adv Skin Wound Care* 2007; 20: 382–8.
19. Thom SR, Bhopale V, Fisher D, Manevich Y, Huang PL, Buerk DG. Stimulation of nitric oxide synthase in cerebral cortex due to elevated partial pressures of oxygen: an oxidative stress response. *J Neurobiol* 2002; 51: 85–100.
20. Thom SR, Fisher D, Zhang J, Bhopale VM, Ohnishi ST, Kotake Y, et al. Stimulation of perivascular nitric oxide synthesis by oxygen. *Am J Physiol Heart Circ Physiol* 2003; 284: H1230–9.
21. Thom SR, Bhopale VM, Mancini DJ, Milovanova TN. Actin S-nitrosylation inhibits neutrophil b2 integrin function. *J Biol Chem* 2008; 283: 10822–34.
22. Khiabani KT, Bellister SA, Skaggs SS, Stephenson LL, Nataraj C, Wang WZ, et al. Reperfusion-induced neutrophil CD18 polarization: effect of hyperbaric oxygen. *J Surg Res* 2008; 150: 11–6.
23. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 1973; 52: 2745–56.
24. Castell L, Vance C, Abbott R, Marquez J, Eggleton P. Granule localization of glutaminase in human neutrophils and the consequence of glutamine utilization for neutrophil activity. *J Biol Chem* 2004; 279: 13305–10.
25. Tarr JM, Winyard PG, Ryan B, Harries LW, Haigh R, Viner N, et al. Extracellular calreticulin is present in the joints of patients with rheumatoid arthritis and inhibits FasL (CD95L) – mediated apoptosis of T cells. *Arthritis Rheum* 2010; 62: 2919–29.
26. Clancy R, Cederbaum AI, Stoyanovsky DA. Preparation and properties of S-nitroso-l-cysteine ethyl ester, an intracellular nitrosating agent. *J Med Chem* 2001; 44: 2035–8.

27. Kaya A, Aydin F, Altay T, Karapinar L, Ozturk H, Karakuzu C. Can major amputation rates be decreased in diabetic foot ulcers with hyperbaric oxygen therapy? *Int Orthop* 2009; 33: 441–6.
28. Ai S, Cheng XW, Inoue A, Nakamura K, Okumura K, Iguchi A, et al. Angiogenic activity of bFGF and VEGF suppressed by proteolytic cleavage by neutrophil elastase. *Biochem Biophys Res Commun* 2007; 364: 395–401.
29. Kumari R, Willing LB, Krady JK, Vannucci SJ, Simpson IA. Impaired wound healing after cerebral hypoxia-ischemia in the diabetic mouse. *J Cereb Blood Flow Metab* 2007; 27: 710–8.
30. Xu F, Zhang C, Graves DT. Abnormal cell responses and role of TNF-alpha in impaired diabetic wound healing. *Biomed Res Int* 2013; 2013: 754802.
31. Ager A. Inflammation: border crossings. *Nature* 2003; 421: 703–5.
32. Shaw FL, Winyard PG, Smerdon GR, Bryson PJ, Moody AJ, Eggleton P. Hyperbaric oxygen treatment induces platelet aggregation and protein release, without altering expression of activation molecules. *Clin Biochem* 2009; 42: 467–76.
33. Thom SR, Mendiguren I, Hardy K, Bolotin T, Fisher D, Nebolon M, et al. Inhibition of human neutrophil b2-integrin-dependent adherence by hyperbaric O_2. *Am J Physiol* 1997; 272: C770–7.
34. Jones SR, Carpin KM, Woodward SM, Khiabani KT, Stephenson LL, Wang WZ et al. Hyperbaric oxygen inhibits ischemia-reperfusion-induced neutrophil CD18 polarization by a nitric oxide mechanism. *Plast Reconstr Surg* 2010; 126: 403–11.
35. Lin CD, Wei IH, Lai CH, Hsia TC, Kao MC, Tsai MH, et al. Hyperbaric oxygen upregulates cochlear constitutive nitric oxide synthase. *BMC Neurosci* 2011; 12: 21.
36. Thom SR, Bhopale VM, Milovanova TN, Yang M, Bogush M, Buerk DG. Nitric-oxide synthase-2 linkage to focal adhesion kinase in neutrophils influences enzyme activity and β2 integrin function. *J Biol Chem* 2013; 288: 4810–8.
37. Bengtsson T, Orselius K, Wetterö J. Role of the actin cytoskeleton during respiratory burst in chemoattractant-stimulated neutrophils. *Cell Biol Int* 2006; 30: 154–63.
38. Selemidis S, Dusting GJ, Peshavariya H, Kemp-Harper BK, Drummond GR. Nitric oxide suppresses NADPH oxidase-dependent superoxide production by S-nitrosylation in human endothelial cells. *Cardiovasc Res* 2007; 75: 349–58.
39. Kaur S, Pawar M, Banerjee N, Garg R. Evaluation of the efficacy of hyperbaric oxygen therapy in the management of chronic nonhealing ulcer and role of periwound transcutaneous oximetry as a predictor of wound healing response: a randomized prospective controlled trial. *J Anaesthesiol Clin Pharmacol* 2012; 28: 70–5.
40. Wong VW, Crawford JD. Vasculogenic cytokines in wound healing. *Biomed Res Int* 2013; 2013: 190486.