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Different oxygen treatment pressures alter inflammatory gene expression in human endothelial cells

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ABSTRACT

Hyperbaric oxygen has proven to be a useful treatment for chronic wounds. However, therapeutic conditions vary between treatment centers, and we wished to investigate the effects of different treatment pressures on cells under inflammatory conditions. Endothelial cells were exposed to a chronic wound model comprising hypoxia (2% O_2 at 1 atmosphere absolute (atm abs); PO_2 ~ 2 kPa) in the presence of 0.5 μg/ml lipopolysaccharide and 1 ng/ml TNF-α for 24 hours, then treated with normobaric oxygen (NBO_2; 95%O_2/5%CO_2 at 1.0 atm abs; PO_2 ~ 96.3 kPa), hyperbaric oxygen (HBO_2) at 1.5 atm abs (1.5HBO_2; 96.7%O_2/3.3%CO_2 at 1.5 atm abs; PO_2 ~ 147 kPa), and HBO_2 at 2.4 atm abs (2.4HBO_2; 97.9%O_2/2.1%CO_2 at 2.4 atm abs; PO_2 ~ 238 kPa). The mRNA expression of 92 inflammatory genes was then analyzed, and we identified changes in genes involved in adhesion molecule expression, angiogenesis and tissue remodeling, intracellular signaling, and cellular oxygen responses and redox signaling. We noted differences in expression between different treatment pressures, highlighting the need for further research into the use of different therapeutic protocols in the treatment of inflammatory conditions such as chronic wounds.

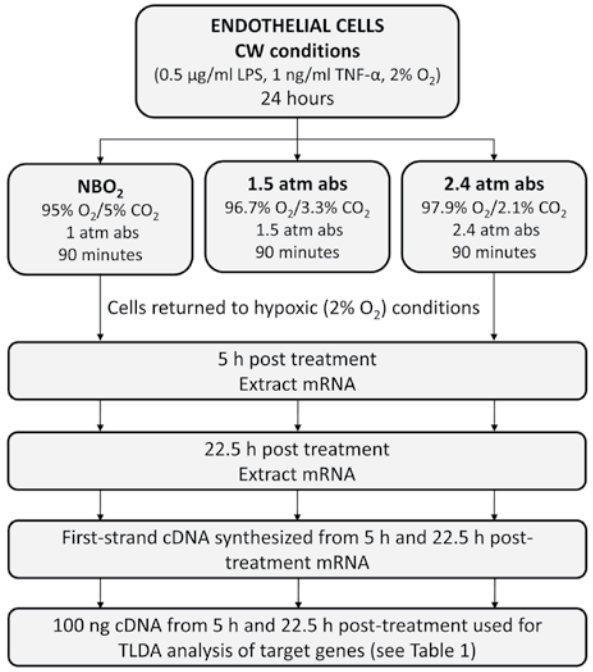
INTRODUCTION

Hyperbaric oxygen (HBO_2) has proven to be a useful and cost-effective adjunctive therapy for the treatment of chronic diabetic ulcers [1,2]. However, patients are treated according to protocols based on historical pressures and durations, and treatment protocols can vary considerably between different treatment centers [3]. It is important to review these treatment protocols to optimize patient care, safety and cost-effectiveness.

Chronic wounds are a highly inflammatory environment, often characterized by bacterial infection, hypoxia and a pro-inflammatory cytokine profile [4] leading to excessive recruitment of inflammatory cells, especially neutrophils, which further contribute to the inflammatory environment through the release of antibacterial enzymes [5]. These cause significant collateral damage to host tissue; degrading pro-healing cytokines and growth factors, prolonging the inflammation and preventing the wound from healing [6]. HBO_2 has been shown to aid the healing of chronic diabetic ulcers, promoting progression through the inflammatory phase of wound healing and into the granulation and remodeling phases [2].

One potential marker for monitoring the effect of HBO_2 treatment is a change in gene expression. Although gene changes following HBO_2 treatment have recently been examined, these have included only typical treatment pressures or pressures in excess of those safe to use for patients [7-9]. While effects of different treatment pressures within the range used for patients have been investigated at a tissue level [10], this is the first study we are aware of to examine effects at the gene level. We investigated the mRNA expression of 92 different inflammatory genes in human endothelial cells following treatment with selected chronic wound conditions and normobaric oxygen (NBO_2), hyperbaric oxygen at 1.5 atmospheres absolute (atm abs; 152 kPa; 1.5HBO_2) or HBO_2 at 2.4 atm abs (243 kPa; 2.4HBO_2).

FIGURE 1



Schematic representation of endothelial cell treatment, RNA isolation and cDNA preparation for TLDA analysis of gene expression. HUVEC were treated with selected chronic wound (CW) conditions for 24 hours, before treatment with oxygen at 1.0 atm abs (NBO_2), 1.5 atm abs ($1.5HBO_2$) or 2.4 atm abs ($2.4HBO_2$) for 90 minute. Cells were returned to hypoxia and RNA was collected for analysis 5 hours and 22.5 hours later. Abbreviations: NBO_2 – normobaric oxygen; TLDA – TaqMan low-density array; atm abs – atmospheres absolute.

METHODS AND MATERIALS

Reagents

Ham's F-12 medium and fetal bovine serum (FBS) were purchased from Lonza. Endothelial cell growth supplement (ECGS), collagenase, gentamicin and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich UK. TNF-α (Tumor Necrosis Factor α) was purchased from the National Institute for Biological Standards and Control, UK (NIBSC). Gas mixes were purchased from BOC or provided by the Diving Diseases Research Centre, Plymouth (DDRC). RNeasy® Mini Kit, RNA stabilization reagent, TURBO DNA-free™ kit, TaqMan® Reverse Transcription Reagents kit, custom TaqMan® Low Density Array (TLDA) plates, RNase-free water and TaqMan® Universal Polymerase Chain Reaction (PCR) Master Mix were purchased from Applied Biosystems.

Cell 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. Human umbilical vein endothelial cells (HUVEC) were isolated from the vein by collagenase (0.3 mg/ml) digestion [11] and cultured in Ham's F-12 medium containing 20% (v/v) Fetal Bovine Serum (FBS), 50 µg/ml gentamicin, and 20 µg/ml Endothelial Cell Growth Supplement (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 platelet/endothelial cell adhesion molecule 1 (PECAM-1), as determined by immunocytochemical staining.

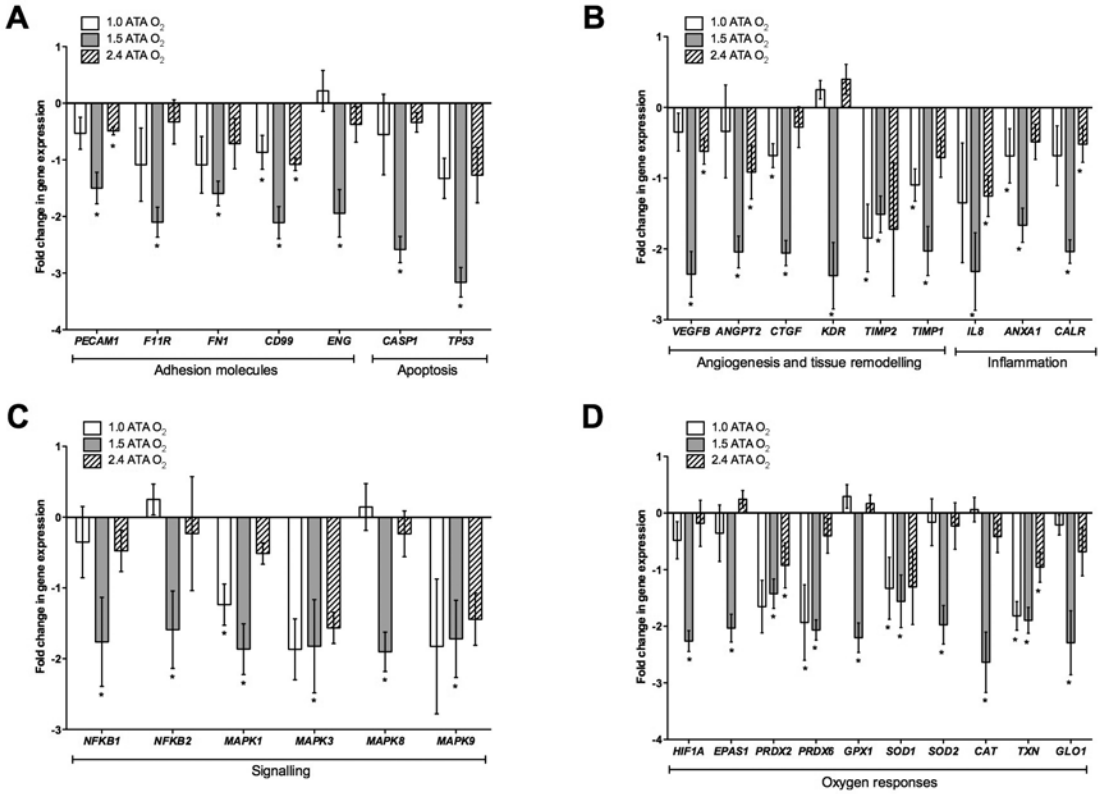
In vitro chronic wound conditions and HBO_2/pressure control treatment

Endothelial cells were exposed to *in vitro* chronic wound (CW) conditions as previously described [9] by culturing them for 24 hours with LPS from *Pseudomonas aeruginosa* (0.5 µg/ml) and TNF-α (1 ng/ml) in hypoxia (2% O_2 at 1 atmosphere absolute (atm abs); PO_2 ~ 2 kPa) at 37ºC. Cells were exposed to normobaric oxygen (NBO_2; 95% O_2/CO_2 at 1.0 atm abs; PO_2 ~ 96.3 kPa), HBO_2 at 1.5 atm abs ($1.5HBO_2$; 96.7 % O_2/CO_2 at 1.5 atm abs; PO_2 ~ 147 kPa), and HBO_2 at 2.4 atm abs ($2.4HBO_2$; 97.92% O_2/CO_2 at 2.4 atm abs; PO_2 ~ 238 kPa) for 90 min at 37ºC (summarized in Figure 1). All of the gas mixes contained CO_2 at a level to give a final PCO_2 of 5 kPa, representing respiration-derived CO_2 at the cellular level.

RNA isolation and cDNA preparation for inflammatory gene QRT-PCR analysis by TaqMan® Low Density Array (TLDA)

RNA expression was analyzed as previously described [9,12,13]. Briefly, RNA was isolated from 2 x 106 HUVEC per treatment condition using an RNeasy® Mini Kit, and supplemented with 10 µl RNA stabilization reagent. Contaminating DNA was removed using a TURBO DNA-free™ kit. First stand complementary DNA (cDNA) was synthesised from 2.5 µg RNA per sample using a TaqMan® Reverse Transcription Reagents kit in preparation for gene expression analysis *(Figure 1)*.

FIGURE 2



Changes in inflammatory gene expression in HBO_2-treated endothelial cells. HUVEC were treated with chronic wound (CW) conditions for 24 hours followed by a 90-minute treatment with oxygen at 1.0 atm abs, 1.5 atm abs or 2.4 atm abs. mRNA was harvested five hours post-treatment and analyzed for changes in gene expression.

(A) In CW-treated HUVEC, seven genes involved in adhesion molecule expression or apoptosis demonstrated statistically significant changes in mRNA expression five hours post-treatment with HBO_2.

(B) In CW-treated HUVEC, nine genes involved in angiogenesis and tissue remodelling or the expression of inflammatory mediators demonstrated significant changes in mRNA expression five hours post-treatment with HBO_2.

(C) In CW-treated HUVEC, six genes involved in intracellular signaling demonstrated significant changes in mRNA expression five hours post-treatment with HBO_2.

(D) In CW-treated HUVEC, 10 genes involved in cellular oxygen responses or redox signalling demonstrated significant changes in mRNA expression five hours post-treatment with HBO_2. Data for mRNA expression in HBO_2-treated cells are expressed as a fold-change in expression from CW-treated cells (mean ± SD).

* $P<0.05$ *vs.* CW-treated.

QRT-PCR of inflammatory genes in endothelial cells under different oxygen conditions

For each treatment condition, 92 genes of interest *(Table 1)* were screened using custom-made TLDA plates (Applied Biosystems, Warrington, UK). Briefly, reaction mixtures were prepared by mixing 2 µl of each cDNA sample (containing 100 ng cDNA) with 48 µl RNase-free water and 50 µl TaqMan® Universal PCR Master Mix, before 100 µl reaction mixture was added to each loading port on the array. The card was centrifuged twice to ensure equal loading of cDNA in each sample well, and then sealed. Four samples were analyzed per plate. Samples were PCR amplified using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK) with the following amplification conditions: two minutes at 50ºC (to activate uracil N-glycosylase), 10 minutes at 94.5ºC (for activation), and 50 cycles of 30 seconds at 97ºC (denaturation) and one

TABLE 1. Target genes investigated in endothelial cells following treatment with selected chronic wound conditions and NBO_2, $1.5HBO_2$ and $2.4HBO_2$

Category	Protein (gene) name	Function
Adhesion molecules	ICAM-1 (*ICAM1*); VCAM-1 (*VCAM1*); E-selectin (*SELE*); P-selectin (*SELP*); PECAM-1 (*PECAM1*); Mucosal addressin cell adhesion molecule-1 (*MADCAM1*); Junctional adhesion molecule 1 (*F11R*); JAM2 (*JAM2*); JAM3 (*JAM3*); Fibronectin 1 (*FN1*); Lymphocyte-specific protein 1 (*LSP1*); CD99 (*CD99*); Endoglin (*ENG*)	Endothelial adhesion molecules involved in adhesion and transmigration of inflammatory leukocytes
Apoptosis	Caspase-1 (*CASP1*); Caspase-2 (*CASP2*); Caspase-3 (*CASP3*); Caspase-6 (*CASP6*); Caspase-7 (*CASP7*); Caspase-8 (*CASP8*); Caspase-9 (*CASP9*); Caspase-10 (*CASP10*); p53 (*TP53*)	Regulate both intrinsic and extrinsic apoptotic pathways
Angiogenesis and tissue remodelling	VEGF A (*VEGFA*); VEGF B (*VEGFB*); VEGF C (*VEGFC*); Angiogenin (*ANG*); Angiopoietin 1 (*ANGPT1*); Angiopoietin 2 (*ANGPT2*); Angiopoietin 4 (*ANGPT4*); Connective tissue growth factor (*CTGF*); EGF-like repeat and discoidin I-like domain-containing protein 3 (*EDIL3*); Thymidine phosphorylase - endothelial cell growth factor 1 (*TYMP*)	Angiogenic growth factors that promote endothelial cell proliferation and growth of blood vessels
	Fms-related tyrosine kinase 1 - VEGF receptor 1 (*FLT1*); Kinase insert domain receptor - VEGF receptor 2 (*KDR*); TEK tyrosine kinase, endothelial (*TEK*); Tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (*TIE1*)	Angiogenesis targets, including receptors for angiogenic growth factors
	A disintegrin and metalloproteinase with thrombospondin motifs 1 (*ADAMTS1*); TNF (ligand) superfamily - member 15 (*TNFSF15*); Vasohibin 1 (*VASH1*); Tissue inhibitor of metalloproteinases 2 (*TIMP2*)	Can inhibit the actions of angiogenic growth factors
	MMP-2 (*MMP2*); MMP-9 (*MMP9*); TIMP1 (*TIMP1*); TIMP3 (*TIMP3*); TIMP4 (*TIMP4*)	Enzymes involved in tissue remodeling, which is required for the growth of new blood vessels into ischemic tissue
Inflammation	TNF-α (*TNF*); TNF receptor 1 (*TNFRSF1A*); TNF receptor 2 (*TNFRSF1B*); IL-8 (*IL8*)	Cytokines and receptors involved in inflammation
	Phospholipase A2, group IIA (*PLA2G2A*); Phospholipase A2, group IID (*PLA2G2D*); Annexin A1 (*ANXA1*); Bradykinin receptor B1 (*BDKRB1*); Calreticulin (*CALR*); Calreticulin 3 (*CALR3*)	Inflammatory mediators involved in regulation of pro- and anti-inflammatory processes
	Prostaglandin endoperoxide synthase 1 (*PTGS1*); Prostaglandin endoperoxide synthase 2 (*PTGS2*)	Regulators of prostaglandin biosynthesis
Intracellular signaling	MAPK1 (*MAPK1*); MAPK3 (*MAPK3*); MAPK8 (*MAPK8*); MAPK9 (*MAPK9*); MAPK11 (*MAPK11*); MAPK12 (*MAPK12*); MAPK13 (*MAPK13*); MAPK14 (*MAPK14*)	MAP kinases, which regulate phosphorylation-dependent events
	NF-κB, subunit 1 (*NFKB1*); NF-κB, subunit 2 (*NFKB2*); c-Jun (*JUN*); c-Fos (*FOS*)	Regulators of gene expression
Oxygen responses and redox signaling	HIF-1α (*HIF1A*); HIF-1β (*ARNT*); Endothelial PAS domain protein 1 - HIF-2α (*EPAS1*); HIF-3α (*HIF3A*); Haem-oxygenase-1 (*HMOX1*); Peroxiredoxin 1 (*PRDX1*); Peroxiredoxin 2 (PRDX2); Peroxiredoxin 6 (*PRDX6*); Glutathione peroxidase 1 (*GPX1*); CuZnSOD (*SOD1*); MnSOD (*SOD2*); Catalase (*CAT*); Xanthine dehydrogenase (*XDH*); Glutathione S-transferase alpha 1 (*GSTA1*); Sulfiredoxin 1 homolog (*SRXN1*); Thioredoxin (*TXN*); NADPH oxidase 4 (*NOX4*); Glyoxalase 1 (*GLO1*)	Regulators of cellular responses to hypoxic or oxidative stress

TABLE 1

Category	Protein (gene) name	Function
Nitric oxide synthesis	eNOS (*NOS3*); iNOS (*NOS2*); nNOS (*NOS1*); Nitric oxide synthase trafficker (*NOSTRIN*); Nitric oxide synthase interacting protein (*NOSIP*)	Regulators of nitric oxide production
Endogenous controls	Eukaryotic 18S RNA (*18S*); Beta actin (*ACTB*); Beta-2-microglobulin (*B2M*); Glyceraldehye-3-phosphate dehydrogenase (*GAPDH*)	Essential housekeeping genes

minute at 59.7ºC (annealing and extension). Gene expression data were analyzed using relative quantification software, SDS 2.3 (Applied Biosystems, Warrington, UK) and the comparative threshold cycle method (ΔΔCt). Data were normalized against the average expression of four endogenous control genes (eukaryotic 18S RNA – 18S, beta actin – ACTB, beta-2-microglobulin – B2M and glyceraldehye-3-phosphate dehydrogenase – GAPDH). Quality control was achieved by comparison of the quadruplicate crossing points for each gene per sample. At each time-point, gene expression levels of the oxygen-treated samples were normalized against those of chronic wound-treated cells.

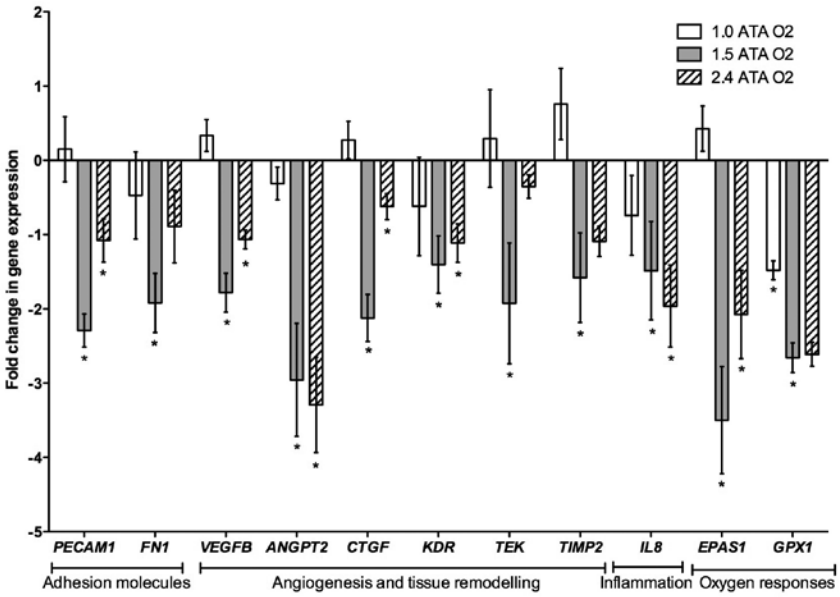
Data analysis

Differences in quadruplicate measurements of gene expression data between different treatment conditions were analyzed using the Mann-Whitney *U* test.

RESULTS

Changes in the mRNA expression of genes involved in several aspects of inflammation were observed five hours *(Figure 2)* and 22.5 hours *(Figure 3)* following treatment with NBO_2, $1.5HBO_2$ or $2.4HBO_2$ compared with cells maintained under chronic wound conditions.

FIGURE 3



Changes in inflammatory gene expression in HBO_2-treated endothelial cells. HUVEC were treated with chronic wound (CW) conditions for 24 hours, then given a 90-minute treatment with oxygen at 1.0 atm abs, 1.5 atm abs or 2.4 atm abs. mRNA was harvested 22.5 hours post-treatment and analyzed for changes in gene expression. In CW-treated HUVEC, 11 inflammatory genes demonstrated significant changes in mRNA expression five hours post-treatment with HBO_2. Data for mRNA expression in HBO_2-treated cells are expressed as a fold-change in expression from CW-treated cells (mean ± SD). * $P<0.05$ *vs.* CW-treated.

TABLE 2. Various HBO_2 conditions that have been used to treat chronic wounds over the past 35 years

Condition	HBO_2 conditions	Patient type	Reference
Chronic leg ulcers	3 atm abs for 1 h	Various chronic infections	[17]
Chronic varicose leg ulceration	2 atm abs for 2 h	Varicose vain	[18]
Chronic foot ulcer	2.5 atm abs for 1.5 h	Type II diabetic & chronic arterial disease	[19]
Chronic foot ulcer	3 atm abs for 45 min	Type II diabetic	[20]
Chronic leg ulcers	2.5 atm abs for 1.5 h	Non-diabetic patients	[21]
Foot/leg ulcers	2 atm abs fro 2 h	Type II diabetic	[22]
Chronic foot ulcer	2.5 atm abs for 1.5 h	Type II diabetic	[23]
Lower limb ulcers	2.4 atm abs for 1.5 h	Systemic sclerosis	[24]
Lower limb ulcers	2 atm abs for 1.5 h	Types I & II diabetes	[25]
Chronic foot ulcer	2.55 atm abs for 1.5 h	Type II diabetic	[26]
Non-healing lower limb ulcers	2.5 atm abs for 1.5 h	Not specified	[27]

Adhesion molecules

Several adhesion molecules involved in inflammatory cell recruitment and attachment to EC cells demonstrated reduced mRNA expression five hours post-HBO_2 treatment compared to cells treated with CW conditions alone *(Figure 2A)*. The platelet/endothelial cell adhesion molecule 1 (PECAM-1; CD31) expression was reduced significantly following treatment at either 1.5 atm abs or 2.4 atm abs, while fibronectin 1 (FN1) expression was reduced only by treatment at 1.5 atm abs. The effects on both of these adhesion molecules were maintained at 22.5 hours post-treatment *(Figure 3)*. Other adhesion molecules that showed decreased expression at five hours post-treatment were junctional adhesion molecule 1 (F11R), and endoglin (ENG), which were affected only at 1.5 atm abs, and MIC2 (CD99), which was affected by treatment at both 1.5 atm abs and 2.4 atm abs These effects were no longer evident 22.5 hours post-HBO_2, when expression was not significantly different than that of HUVEC that received only the CW treatment.

Apoptosis

Two genes involved in apoptosis (CASP1 and TP53) demonstrated an HBO_2-mediated decrease in mRNA expression *(Figure 2A)*, but this was evident only following treatment at 1.5 atm abs, not at 2.4 atm abs. This effect was short-lived, and was no longer evident when cells were analyzed 22.5 hours post-HBO_2 treatment.

Angiogenesis and tissue remodelling

The effects of HBO_2 on mRNA expression of genes involved in angiogenesis and tissue remodelling were generally longer lasting than those of other genes affected. VEGFB, ANGPT2, CTGF and TIMP2 all demonstrated reduced mRNA expression at both five hours *(Figure 2B)* and 22.5 hours post-treatment with $1.5HBO_2$ or $2.4HBO_2$ *(Figure 3)*. Additionally, KDR and TIMP1 both demonstrated reduced mRNA expression at five hours post $1.5HBO_2$, while TEK demonstrated reduced expression following $1.5HBO_2$, but only after 22.5 hours.

Inflammation

The genes of two important inflammatory mediators, IL8 and CALR, showed reduced mRNA expression following both 1.5HBO2 and $2.4HBO_2$ five hours post-treatment. Additionally, ANXA1 demonstrated reduced mRNA expression five hours post-treatment, but only at 1.5 atm abs. IL8 mRNA expression remained reduced 22.5 hours after treatment at either pressure.

Signaling

The genes of several proteins involved in intracellular signaling were affected by HBO_2 five hours post-treatment. NFKB1, NFKB2, MAPK1, MAPK3, MAPK8 and MAPK9 all demonstrated reduced mRNA expression, but only following treatment at 1.5 atm abs, not at 2.4 atm abs *(Figure 2C)*. The effects on all of these genes, however, were short-lived, and the reduced expression was no longer evident 22.5 hours post-treatment.

Oxygen responses and redox signaling

The genes of several proteins involved in cellular oxygen responses and redox signaling were affected by HBO_2 treatment. Five hours post-HBO_2, HIF1A, EPAS1, PRDX2, PRDX6, GPX1, SOD1, SOD2, CAT, TXN, GLO1 all demonstrated reduced mRNA expression following treatment at 1.5 atm abs, with PRDX2, PRDX6 and TXN also demonstrating reduced expression following HBO_2 treatment at 2.4 atm abs *(Figure 2D)*. Only GPX1 expression remained reduced at 22.5 hours post-treatment with EPAS1 also demonstrating reduced mRNA expression at this time-point.

DISCUSSION

While the effects of HBO_2 on inflammatory gene mRNA expression under chronic wound conditions have previously been investigated [9], this is the first study to compare different treatment pressures. The treatment pressures used during HBO_2 therapy vary between treatment centers and depend on the condition being treated, yet there is a lack of comparative studies providing evidence for the benefits of certain treatment pressures over others.

The genes studied here were chosen for their known or potential involvement in the process of acute wound healing, and their dysregulation in chronic wound healing [9]. Their modification by HBO_2 treatment highlights some of the mechanisms by which the treatment could expedite the healing of chronic wounds. The downregulation of genes encoding several adhesion molecules was observed, and confirms our previous observations at the protein level that HBO_2 affects adhesion molecules expression in blood cells [14]. Down regulation of adhesion molecules, or their reduced cell surface expression would impair the recruitment of inflammatory immune cells, especially neutrophils, which are a key problem in the prolonged inflammation observed in chronic wounds [5]. Angiogenesis in response to oxygen at different pressures has been assessed *in vitro*, and been shown to peak at 2.0 atm abs, with less angiogenic activity at 1.0 atm abs, 2.5 atm abs and 3.0 atm abs [10]. Our study demonstrates a significant effect of PO_2 on mRNA expression of genes involved in angiogenesis (VEGFB, ANGPT2 and CTGF) with the greatest reduction being seen at 1.5 atm abs when compared to the chronic wound model conditions *(Figures 2B and 3)*. IL-8 is a potent neutrophil chemoattractant, and we have previously shown that HBO_2 at 2.4 atm abs reduces LPS/TNF-α/hypoxia-induced IL-8 mRNA and protein expression in HUVEC, which could lead to reduced neutrophil recruitment and improved healing in chronic wounds [9]. It is interesting to note that the reduction in expression is more pronounced following treatment at 1.5 atm abs than at 2.4 atm abs, and the effects at the protein level would be of interest in the context of chronic wound healing.

The downregulation of the genes for several antioxidants (PRDX2, PRDX6, GPX1, SOD1, SOD2 and CAT) observed at five hours post-HBO_2 could be a response to the decrease in cellular oxygen upon decompression, since cells were returned to hypoxic conditions for five hours before analysis. Having exposed endothelial cells to higher oxygen levels for 90 minutes, and then experiencing a relatively hypoxic environment when decompressed and returned to 2% O_2, the need for antioxidant enzymes would be reduced.

Most significant changes in mRNA expression were no longer evident at the second time-point, highlighting the involvement of mRNA half-lives and the short-lived effects of HBO_2 on mRNA expression. This study supports the requirement for frequent repetitive treatment sessions, which are currently employed in most treatment protocols.

Interestingly, oxygen at 1.5 atm abs affected many genes much more strongly than oxygen at 2.4. The reasons for this effect are unknown, but it does raise questions about the most appropriate treatment pressures for inflammatory conditions. As well as different facilities differing in their treatment protocols for the same conditions, different conditions are treated with different treatment pressures, and more research is needed to investigate the most appropriate treatment pressures for individual conditions. The benefit of reduced risks of oxygen toxicity when treating with oxygen at lower pressures have long been recognized [15], and rather than treating patients according to convention, there are important advantages to be gained by performing more studies to monitor wound healing under different treatment pressures.

This study is the first to compare the effects of different hyperbaric treatment pressures on inflammatory gene expression. We have identified numerous inflammatory genes that are affected by treatment with oxygen at above atmospheric pressure, and have highlighted differences in the response of genes to hyperbaric treatments used at different atmospheric pressures, namely 1.5 atm abs (used to treat brain trauma injury [16]) and 2.4 atm abs (2.4-2.5 atm abs commonly used for chronic wound healing – see Table 2).

Acknowledgments

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Conflict of interest: *None*

■

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