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Response of blood vessels *in vitro* to hyperbaric oxygen (HBO): Modulation of VEGF and NO_x release by external lactate or arginine

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ABSTRACT

Hyperbaric oxygen therapy (HBO) is suggested to promote angiogenesis during wound healing, but the mechanisms involved are not understood. This study used a novel isolated blood vessel preparation to explore the effects of air, normobaric oxygen or hyperbaric oxygen (2.2 ATA for 90 min) on the angiogenesis factor, vascular endothelial growth factor (VEGF), nitrite and nitrate (NO_x), lactate dehydrogenase (LDH) and lactate release from the tissue in normal Krebs Ringer, and the Ringer supplemented with either L-arginine, or 15 mM lactate to mimic a wound environment, or both (L-arginine + lactate). The *in vitro* blood vessel preparation remained viable during all experiments. There were no effects of HBO treatment on any of the parameters measured in normal Krebs Ringer, but some treatment-dependent effects were observed in supplemented Krebs Ringer. In the lactate supplemented Krebs Ringer, medium LDH levels increased in response to either normobaric oxygen (NBO) or HBO, compared to air alone. There were also small, but statistically significant increases in total glutathione due to HBO treatment, compared to NBO or air in the lactate supplemented medium, and in the combined supplement. There were no effects of HBO on NO_x, changes in external medium lactate levels, or tissue VEGF in any of the Krebs Ringers tested. However, post treatment increases in VEGF were observed in the lactate supplemented medium, and for lactate release into the medium for the combined supplement. We conclude that HBO does not cause NO or VEGF production from the blood vessel in normal Krebs Ringer, but the data from supplemented medium show that the response of the tissue is subtly affected by the chemical environment around the blood vessel, and the tissue is more responsive to HBO when wound conditions are mimicked.

1. Introduction

Hyperbaric oxygen (HBO) therapy is the administration of 100% oxygen at more than one atmosphere. Clinical application and experimental trials of HBO treatment on chronic wounds have shown new blood vasculature formation and blood flow improvement in granulation tissue [1–4]. Although *in vivo* studies have provided evidence that HBO promotes angiogenesis in chronic wound, the mechanisms of how HBO therapy facilitates angiogenesis are far from understood.

Angiogenesis is a highly orchestrated event involving the endothelium of the blood vessel, the underlying smooth muscle cells, and a diverse array of angiogenesis factors. Of these, vascular endothelial growth factor (VEGF) is considered one of the most important promoters of angiogenesis [reviews, 5–9]. Nitric oxide release from smooth muscle cells in the vessel wall is implicated in the signalling cascade that results in VEGF production [6,8] and the promotion of angiogenesis. The wound environment is often characterised by reduced oxygen tensions, acidic pH and the presence of metabolites from glycolysis such as lactate [e.g., 10,11]. This chemical environment in the wound is therefore likely to affect the local production of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide [12]. Modulation of ROS generation has been implicated in the activation of VEGF and angiogenesis [13].

The moderate pressures (about 2 ATA) and durations (about 90 min) used in multiple HBO therapy for patients with chronic wounds do not cause acute oxidative stress or disturbances to haematology [14,15], but promote a beneficial level of angiogenesis and tissue repair [3,4]. One hypothesis is that the HBO therapy causes low or moderate ROS production, and that these ROS act as signalling molecules, perhaps via NO and VEGF, to promote angiogenesis [13,16,17].

However, direct effects of oxygen and/or pressure on NO and VEGF release, and thus angiogenesis, also cannot be excluded. Thus there may be at least two major modes of HBO-dependent initiation of angiogenesis. In patients this is further complicated by the fact that, prior to HBO therapy, the initial wound is likely to generate ROS via inflammation reactions. The presence of anaerobic metabolites and low pH in this initial wound might also influence NO chemistry, or the ability of the endothelium to release VEGF; and therefore the initiation

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of angiogenesis will also depend on the status of the wound. In this study, we attempt to unravel this problem by exploring NO, VEGF, and ROS effects during HBO treatment in two very different situations; (i) in normal physiological solutions, and (ii), in a solution with elevated lactate levels to mimic the wound environment [10,11]. Our experimental approach uses a novel isolated blood vessel preparation, so that the local effects of NO and VEGF production by the endothelium can be investigated, without the complication of other systemic responses found *in vivo*. In addition, we also ensure the ability of cells to produce NO in some experiments by adding L-arginine, which serves as a physiological precursor for NO formation in blood vessels [18,19].

2. Materials and methods

Male Sprague Dawley rats (350–400 g, $n=60$) were purchased from Harlan UK Ltd (Shaw's Farm Blackthorn, Bicester, UK). They were kept in a 12 h dark:12 h light cycle and had free access to food and water before tissue collection. Experiments were conducted in accordance with ethical approval. All chemicals except those otherwise specified were obtained from Sigma Aldrich (Poole, UK).

2.1. Experimental design

Rats were euthanized with an intraperitoneal injection of Sagatal (90 mg kg^{-1} body weight; Rhône Mérieux Limited, Harlow, Essex, UK). Once the aorta (8–10 cm) had been removed, it was washed in 250 ml of ice-cold phosphate-buffered saline (PBS) and then spread in a culture dish with 10 ml of modified Krebs Ringer solution (composition in mM: NaCl 118.6, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.1, HEPES 10, glucose 10, pH 7.4) and any remaining fat was carefully removed from the exterior of the blood vessel. The aorta was then sectioned into 1 cm segments, and these were randomly placed (one per well) in 6-well tissue culture plates (Nunclon Delta SI, Nunc, InterMed, Denmark) with each well containing 6 ml of either unsupplemented Krebs Ringer solution (normal control), or Krebs Ringer supplemented with 100 μM L-arginine ('+arg.'), or 15 mM sodium L-lactate ('+lac.'), or both ('+lac. +arg.') in order to mimic the wound environment [20–22]. The temperature was maintained at 37 °C throughout. Segments of aorta from one rat were used for each medium in each trial.

Segments of aorta were allowed to equilibrate for 30 min before oxygen treatment; they were then randomly assigned to exposure to air at 1 ATA ('air') or 100% oxygen at 1 ATA ('NBO') or 100% oxygen at 2.2 ATA ('HBO'). After 90 min exposure, the aorta segments were allowed to recover in normobaric air for 4 h. Tissue and medium samples were collected at the end of the 30 min equilibration ('Pre'), immediately post treatment ('Post') and 4 h post treatment ('4 h Post'). All tissue and medium samples were snap-frozen in liquid nitrogen, and stored at −80 °C until used for analysis.

2.2. Biochemical analysis

Several biochemical parameters were measured during the study. Lactate dehydrogenase (LDH) release to the medium was used to assess viability, whilst lactate accumulation in the medium was used to assess anaerobic metabolism. The possibility of oxidative stress during oxygen exposure was monitored by measuring total glutathione levels in the medium. Total content of nitrite and nitrate (NO_x) in the medium was measured as a surrogate for NO production by the tissue. Since NO and lactate are potential stimulatory factors for vascular endothelial growth factor (VEGF) production, the levels of VEGF in both the medium and the tissue were tested.

2.2.1. Lactate dehydrogenase

Lactate dehydrogenase (LDH) activity was measured essentially according to [23]. One hundred microlitres of 21 mM sodium pyruvate solution was rapidly added into a 4 ml cuvette which already contained a mixture of 0.3 ml medium sample or tissue homogenate, 2.5 ml phosphate buffer (0.1 M, pH 7.4) and 0.1 ml of 3.5 mM NADH. The latter mixture had been equilibrated at 37 °C within the thermostatically heated cell housing in the ultraviolet spectrophotometer (Perkin Elmer, UV/VIS, Bio 20). The change in absorbance at 340 nm was recorded for 3 min. One unit of activity is defined as the amount of enzyme that will reduce 1 μmol of pyruvate to L-lactate per minute at pH 7.5 and 37 °C.

2.2.2. Total glutathione

The total level of glutathione in culture medium was determined by the glutathione reductase enzymatic recycling method essentially as described by Adams et al. [24]. The reaction buffer was prepared by adding 16 U of glutathione reductase (GR) to 26 ml of assay buffer (100 mM potassium phosphate and 5 mM potassium EDTA, pH 7.5). Sample (20 μl) or GSH standard solution (up to 10 μM) was mixed with 20 μl of buffered DTNB (10 mM DTNB in assay buffer). After equilibration for 1 min the reaction was started by adding 20 μl of 3.6 mM NADPH, and the absorbance at 405 nm (A_{405}) was measured for 5 min using an OPTImax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.2.3. Nitrite plus nitrate

Nitrite plus nitrate were measured using a modified Griess assay essentially according to Moody and Shaw [25]. Assay mixture was prepared by mixing (per ml) 0.72 ml of 50 mM sodium phosphate buffer (pH 7.4), 0.08 ml (1.6 U ml^{-1} nitrate reductase from *Aspergillus niger* in buffer), and 0.2 ml of 1 mM NADPH. Nitrate standard (50 μl, up to 20 μM) or samples were mixed with 50 μl assay mixture to give a final concentration of 0.1 U ml^{-1} nitrate reductase and 0.2 mM NADPH in the wells of a 96-well plate. The plate was then incubated for 30 min at 20 °C to allow complete reduction of nitrate to nitrite. The diazotization reaction was started by adding 100 μl of sulphanilamide (1% [w/v] in 1 M HCl), followed by 100 μl of 0.1% NED 30 s later. The absorbance at 550 nm was measured a few minutes later in a Dynatech Laboratories MRX plate reader (Billingshurst, UK).

2.2.4. Lactate

The assay mixture contained 0.43 M glycine, 0.34 M hydrazine, 3.1 mM NAD^+ and 19 U ml^{-1} LDH. Sample (30 μl) or lactate standard solution (up to 2 mM) was mixed with 300 μl of assay mixture, and then incubated at 37 °C for 30 min to complete the reaction. The absorbance at 340 nm was measured in a Dynatech Laboratories MRX plate reader (Billingshurst, UK).

2.2.5. Vascular endothelial growth factor (VEGF)

Crude homogenates of each segment were made by homogenizing about 15 mg of rat aorta tissue in 0.6 ml hypotonic Tris buffer (pH 7.4, 20 mM Tris chloride, 1 mM EDTA) in the presence of protease inhibitors (Sigma P-2714, according to recommended usage). Tissues were then homogenized on ice using a TD20 rotor of a CAT-X5 20 D homogenizer at 16,000 rpm and using 3×10 second bursts to avoid frictional heating in the samples.

VEGF levels in tissue homogenate and medium were measured using a commercially available mouse VEGF immunoassay kit (R&D Systems, MMV00). Because the kit has not been used before for blood vessel homogenates, sample volume and first binding incubation time were optimised and validated at 50 μl and 2 h at 25 °C, respectively (data not shown). Assays were performed in duplicate and optical density was determined using the OPTImax microplate reader set to 450 nm. Absorbance at 540 nm (A_{540}) was also measured at the same time, and A_{540} values were subtracted from A_{450} values, as suggested in the manual to correct for optical imperfections in the plate.

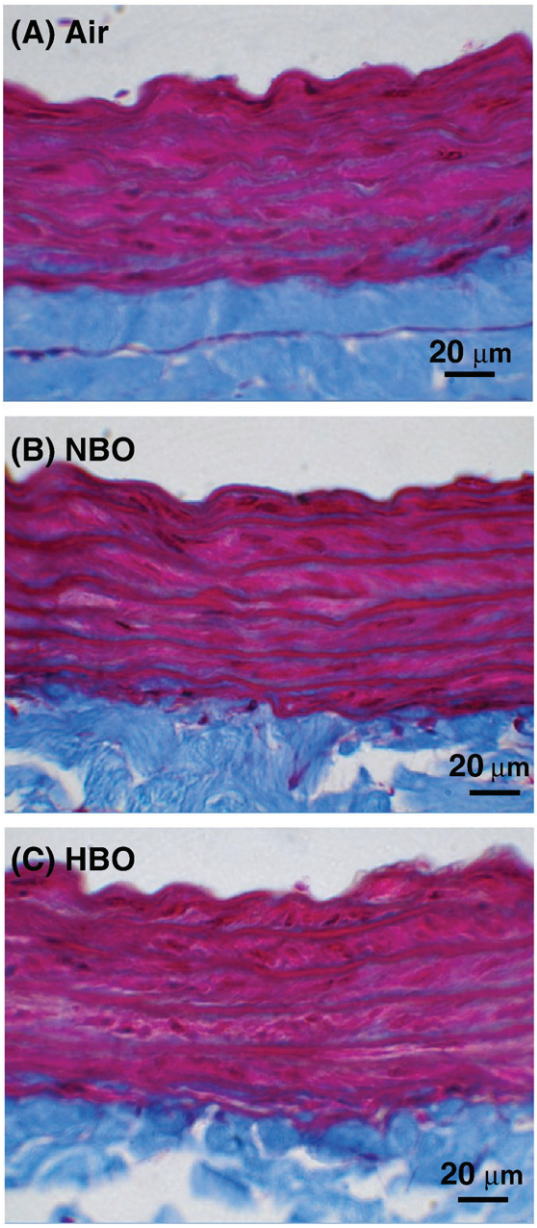

Fig. 1. Histological integrity of the *in vitro* blood vessel preparation in Krebs Ringer. Sections (8 μm, stained with Mallory's trichrome) show normal histology of the vessel wall at the end of experiments (A) air, (B) normobaric oxygen, NBO and (C) hyperbaric oxygen, HBO treatments.

2.3. Histology

Histological assessment was performed as part of the viability assessment of the *in vitro* preparation to ensure the structure of the endothelium and underlying layers of the blood vessel were intact. At the end of the experiments, segments of aorta were fixed in 15 ml of Bouin's solution (75 ml of 1% picric acid, 25 ml of 10% formalin, 5 ml of glacial acetic acid) for 24 h, and then processed for routine wax histology. Sections were cut to 8 μm and stained with Mallory's trichrome. Sections were prepared in batches from all treatments to eliminate staining artefact between treatments, and photographed (Nikon E990 digital camera and Leica DM1RB light microscope) to collect at least 3 representative images from each specimen. In addition to general light microscopy observations on the integrity of each specimen, some quantitative histology was performed to measure the dimensions of the endothelium. The thickness of the tunica intima and tunica media was determined for each specimen (in triplicate using Image J software). In order to correct the for the absolute size of each blood vessel in the analysis of tunica thickness, the data for the innermost layer (tunica intima) were expressed as a percentage fraction of the total tunica thickness and we have called this the "endothelium index":

Endothelium index = thickness of tunica intima / (thickness of tunica intima + tunica media) × 100%.

2.4. Statistics

Data are shown as means ± S.E.M. Statistical analysis was carried out using Statgraphics Plus 5.1 (Statistical Graphics Corp.). Multiple range tests (Tukey) and *t* tests, as appropriate, were carried out after ANOVA in order to determine significant differences. Throughout a *P* value of <0.05 is considered significant.

3. Results

In this study sections of rat aorta were incubated for 30 min in either normal Krebs Ringer medium or Krebs Ringer supplemented with lactate and/or arginine, and then subjected to 90 min of one of three treatments: normobaric air (air at 1 ATA, 'air'); normobaric oxygen (100% O_2 at 1 ATA, 'NBO'); and hyperbaric oxygen (100% O_2 at 2.2 ATA, 'HBO'). After treatment the aorta sections were returned to normobaric air for a further 4 h. The *in vitro* blood vessel preparation remained viable during all experiments, as judged by the histological integrity of the vessels at the end of the experiments (Fig. 1) and the absence of large changes in LDH in the Krebs Ringer over 4 h (Fig. 2). The wall of rat aorta showed a normal tunica intima (innermost, the endothelial cell lining), tunica media (middle layer containing smooth muscle cells), and tunica adventitia (outer layer, mainly connective tissue). There was no evidence of tissue damage such as oedema,

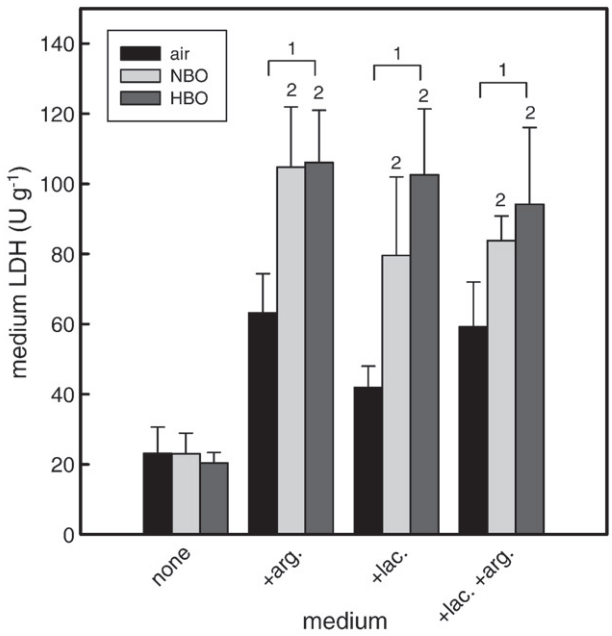

Fig. 2. Effects of medium and oxygen treatment on LDH activity in the medium surrounding sections of rat aorta. The data are the pooled values for samples of media taken immediately after the oxygen treatment and 4 h after. Means ± S.E.M. ($n = 18$) are shown. 'None' is unsupplemented Krebs Ringer solution, whereas '+arg.', '+lac.' and '+lac. +arg.' are Krebs Ringer supplemented with 100 μM arginine and/or 15 mM lactate, as indicated. Oxygen treatment was for 90 min with air at 1 ATA (air) or 100% O_2 at 1 ATA (NBO) or 100% O_2 at 2.2 ATA. '1', statistically significant difference compared to values seen with unsupplemented Krebs Ringer as the medium. '2', statistically significant difference compared to values seen in the same medium with air treatment.

Table 1
Biochemical parameters after incubation of segments of rat aorta in normal Krebs Ringer for 30 min.

	Supplementation to normal Krebs Ringer medium			
	None	Arginine	Lactate	Lactate + arginine
LDH (U g^{-1})[a]	10.3 ± 2.3	15.0 ± 3.3	12.3 ± 2.0	14.5 ± 3.2
Total glutathione (nmol g^{-1})	79.8 ± 7.8	105.0 ± 12.0	106.8 ± 7.2	117.0 ± 10.2[b]
NO_x (μmol g^{-1})	0.96 ± 0.06	2.82 ± 0.48[b,c]	1.62 ± 0.18[d]	1.92 ± 0.18
Tissue VEGF (ng mg^{-1} protein)	0.27 ± 0.08[d]	0.25 ± 0.05	0.30 ± 0.04	0.28 ± 0.04

[a] All values are per g wet weight of tissue except for VEGF, which is per mg protein.
[b] Significantly different to that in medium without supplementation.
[c] Significantly different to that in medium with only lactate supplementation.
[d] $n = 8$.

epithelial lifting or necrosis in any of the specimens. This was supported by measurement of the endothelium index which did not change during the experiments (ANOVA, $P = 0.31$). For example, the endothelium index was (mean ± S.E.M.; $n = 5$ rats/treatment): 2.97 ± 0.32, 2.59 ± 0.27 and 2.45 ± 0.31% for air, NBO and HBO immediately post exposure when Krebs Ringer was used (the pre-treatment control, 2.83 ± 0.24%). The index did not change by 4 h post exposure (mean ± S.E.M.; $n = 5$ rats/treatment): 2.86 ± 0.19, 2.36 ± 0.22 and 2.46 ± 0.37% for air, NBO and HBO. The *in vitro* preparations therefore appeared viable with normal structural integrity.

In each experiment a range of biochemical parameters was measured after the initial 30 min incubation (pre-exposure; 'Pre'); after the oxygen treatment (post exposure; 'Post'); and again 4 h after the treatment (4 h post exposure; '4 h Post') in either the medium or, in the case of VEGF, in tissue homogenate. Table 1 shows the 'Pre' values for these biochemical parameters. There were no significant differences in LDH activities in the four media at this point, or in tissue VEGF levels. VEGF in the media was below the detection limit of the assay method used. At this point ('Pre' values) total glutathione levels were higher in the supplemented media compared to the unsupplemented medium, but the difference was only significant ($P < 0.05$) for the lactate + arginine supplemented medium. NO_x levels were higher in media supplemented with arginine; where NO_x levels were significantly higher than in the two media without arginine supplementation ($P < 0.05$).

For each biochemical parameter shown in Table 1, and also medium lactate levels, the 'Post' and '4 h Post' data were analysed using multifactorial ANOVA with medium (no supplementation, 'none'; plus arginine, '+arg.'; plus lactate, '+lac.'; and plus lactate and arginine, '+lac. +arg.'), oxygen treatment ('air', 'NBO' and 'HBO') and time ('Post' and '4 h Post') as factors, and initially including the three second order interactions between these factors. In each case, if after the initial analysis one or more of these interactions was found to be insignificant, they were omitted and the data were re-analysed.

There was no significant effect of time on medium lactate dehydrogenase (LDH) levels, but the effects of type of medium and treatment were both significant ($P < 0.00005$) as was the interaction between type of medium and treatment ($P = 0.0219$). Since there was no effect of time on medium LDH the 'Post' and '4 h Post' data were pooled. Fig. 2 shows a plot of the pooled LDH data for each medium and each oxygen treatment. The pattern of LDH release for each medium is broadly the same as that seen in the 'Pre' data (Table 1), with medium LDH levels being significantly higher in all the supplemented media compared to the unsupplemented Krebs Ringer. Exposure to 100% O_2, either normobaric ('NBO') or hyperbaric ('HBO'), caused significant elevation of medium LDH in the supplemented Krebs Ringer, but not in the unsupplemented medium.

There were significant effects of all factors on total glutathione levels in the medium ($P < 0.00005$ for medium; $P < 0.00005$ for treatment; and $P = 0.0239$ for time), and there was an interaction between medium and treatment ($P = 0.0135$). Fig. 3 shows plots of total glutathione for each medium and each oxygen treatment immediately after the oxygen treatment ('Post') and again, 4 h after ('4 h Post'). Glutathione levels were higher in the media supplemented with lactate and/or arginine, and were also slightly higher 4 h post treatment compared to immediately post treatment. Exposure to 100%

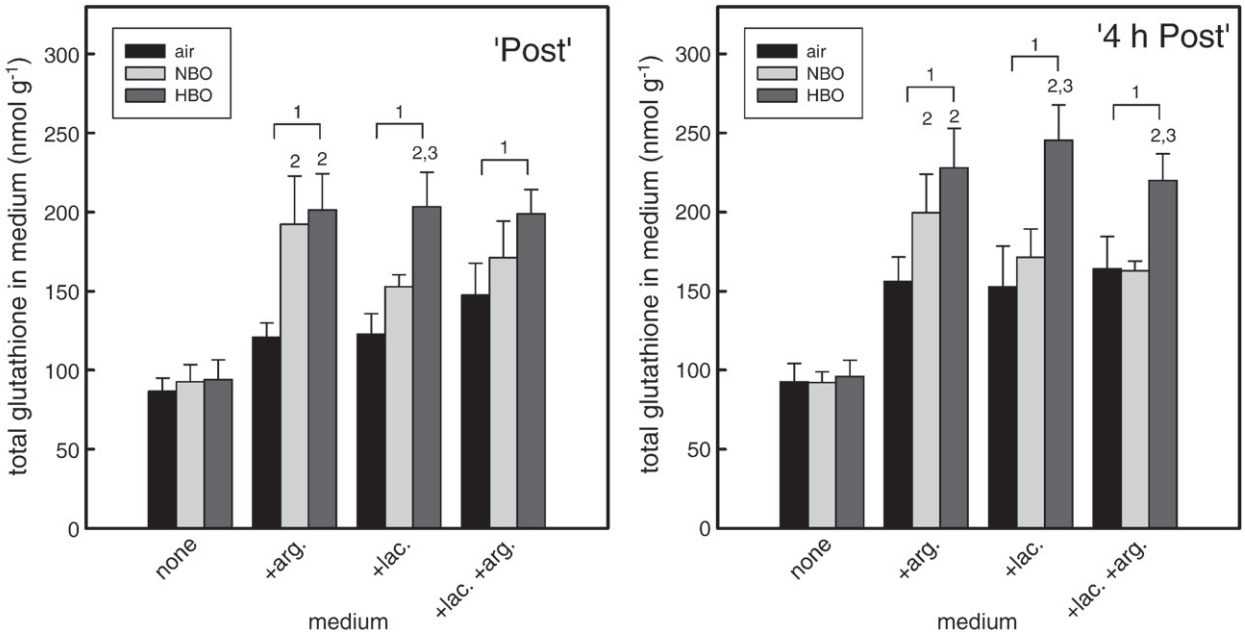

Fig. 3. Effects of medium and oxygen treatment on total glutathione in the medium surrounding sections of rat aorta. Left panel, immediately post treatment, and right panel, 4 h post treatment. Means ± S.E.M. ($n = 9$) are shown. 'None' is unsupplemented Krebs Ringer solution, whereas '+arg.', '+lac.' and '+lac. +arg.' are Krebs Ringer supplemented with 100 μM arginine and/or 15 mM lactate, as indicated. Oxygen treatment was for 90 min with air at 1 ATA (air) or 100% O_2 at 1 ATA (NBO) or 100% O_2 at 2.2 ATA. '1', statistically significant difference compared to values seen with unsupplemented Krebs Ringer as the medium. '2', statistically significant difference compared to values seen in the same medium with air treatment. '3' statistically significant difference compared to values seen in the same medium with normobaric oxygen treatment.

O_2, either normobaric ('NBO') or hyperbaric ('HBO'), caused significant elevation of medium glutathione in the supplemented Krebs Ringer, but not in the unsupplemented medium.

There was a significant effect of medium supplementation on NO_x (nitrate, NO_3^- plus nitrite, NO_2^-) levels ($P=0.0007$; Fig. 4) but not of oxygen treatment or time. NO_x levels were higher in the media supplemented with lactate and/or arginine than in the unsupplemented Krebs Ringer.

Lactate levels were measured in the media at all three time points ('Pre', 'Post' and '4 h Post'). However, they are not reported in Table 1 ('Pre' data) because of the high levels of lactate with which two of the media were supplemented. It was therefore difficult to judge whether or not there had been any further accumulation of lactate in these media at that point. Nevertheless, for the later time points it was possible to include the change in lactate in the media compared to the levels immediately before the oxygen treatment. There were significant effects of medium and time on lactate accumulation ($P=0.0007$ and $P<0.00005$, respectively) (Fig. 5). Immediately post treatment ('Post'–'Pre') there had been accumulation of lactate in all the media except for the medium supplemented with lactate alone, where there was a slight decrease. Accumulation of lactate was greatest in the media supplemented with arginine. However, by 4 h post treatment ('4 h Post'–'Pre') there had been accumulation of lactate in all the media, but more so in the media supplemented with arginine, and in particular the medium supplemented with both lactate and arginine.

There was no significant effect of oxygen treatment on tissue VEGF levels. However, there were significant effects of both medium and time ($P=0.0001$ and $P<0.00005$, respectively). Since there was no effect of treatment on tissue VEGF the data for the different treatments were pooled. Fig. 6 shows a plot of the pooled VEGF data for each medium and each time. Tissue VEGF levels were significantly higher 4 h after oxygen treatment ('4 h Post') than immediately after treatment ('Post'), but they were significantly lower at both time points in the media supplemented with lactate.

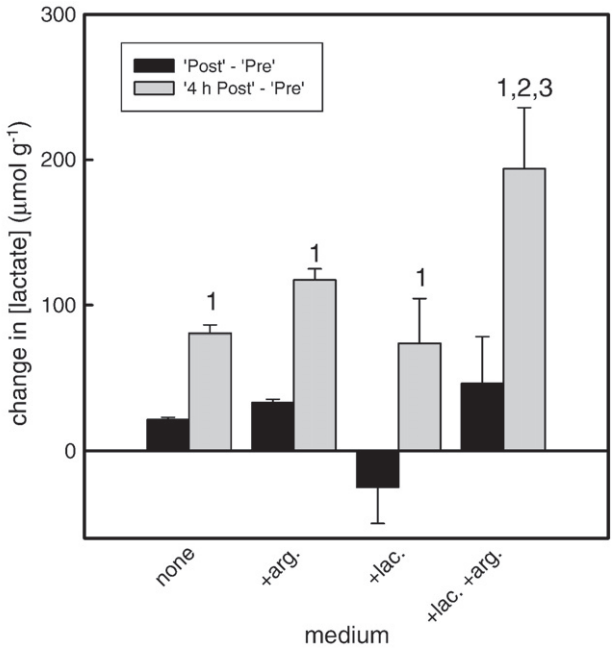

Fig. 5. Effects of medium composition on lactate in the medium surrounding sections of rat aorta immediately ('Post') and 4 h post ('4 h Post') oxygen treatment compared to lactate levels immediately before ('Pre') oxygen treatment. The data from all of the oxygen treatments are pooled. Means ± S.E.M. ($n=27$) are shown. 'None' is unsupplemented Krebs Ringer solution, whereas '+arg.', '+lac.' and '+lac. +arg.' are Krebs Ringer supplemented with 100 µM arginine and/or 15 mM lactate, as indicated. '1', statistically significant difference compared to values seen immediately post treatment ('Post'–'Pre'). '2', statistically significant difference compared to values seen in unsupplemented Krebs Ringer. '3', statistically significant difference compared to values seen in Krebs Ringer supplemented with lactate.

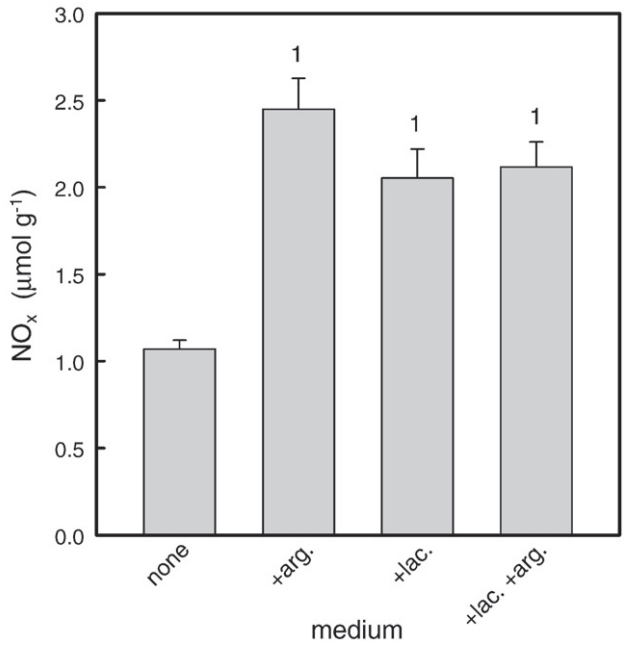

Fig. 4. Effect of medium on NO_x ($NO_2^- + NO_3^-$) in the medium surrounding sections of rat aorta. The data are the pooled values for samples of media taken immediately after the oxygen treatment and 4 h after, irrespective of oxygen treatment. Means ± S.E.M. ($n=54$) are shown. 'None' is unsupplemented Krebs Ringer solution, whereas '+arg.', '+lac.' and '+lac. +arg.' are Krebs Ringer supplemented with 100 µM arginine and/or 15 mM lactate, as indicated. '1', statistically significant difference compared to values seen with unsupplemented Krebs Ringer as the medium.

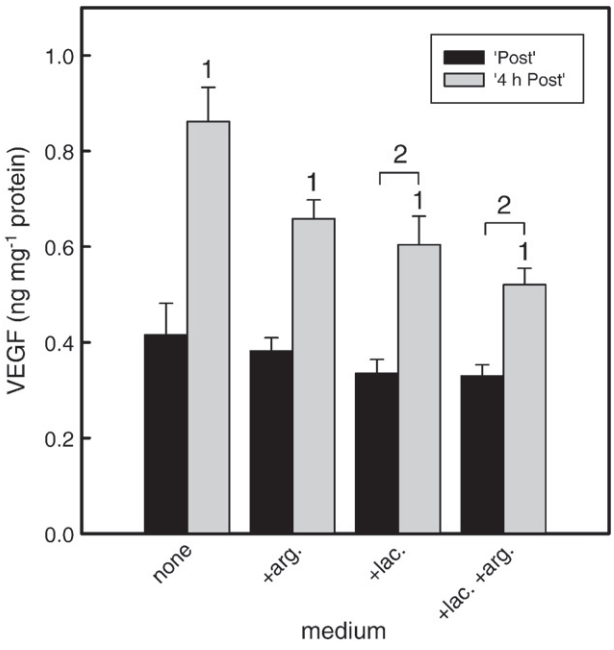

Fig. 6. Effects of medium composition on VEGF levels in sections of rat aorta immediately ('Post') and 4 h post ('4 h Post') oxygen treatment. The data from all of the oxygen treatments are pooled. Means ± S.E.M. ($n=27$) are shown. 'None' is unsupplemented Krebs Ringer solution, whereas '+arg.', '+lac.' and '+lac. +arg.' are Krebs Ringer supplemented with 100 µM arginine and/or 15 mM lactate, as indicated. '1', statistically significant difference compared to values seen immediately post treatment ('Post'). '2', statistically significant difference compared to values seen in unsupplemented Krebs Ringer.

4. Discussion

In this study, we used an *in vitro* aortic vessel preparation to investigate the effects of HBO on NO_x and VEGF production by the tissue, and to assess tissue damage via LDH release into the medium. For the first time, we also explore these effects in a Krebs Ringer that mimics the chemical conditions in a chronic wound situation. Overall, we have shown that a single 90 min therapeutic HBO treatment at 2.2 ATA in normal Krebs Ringer does not induce significant oxidative stress or damage the tissue, as evidenced by very low levels of LDH leak, and modest depletion of total glutathione pools (Table 1, Figs. 2 and 3). However, there is also no evidence of HBO-dependent NO or VEGF production in normal Krebs Ringer (no supplement, Table 1). Nonetheless, when the Krebs Ringers were supplemented with lactate to mimic wound conditions, or arginine to promote NO synthesis, statistically significant increases in LDH release, and glutathione depletion were observed in HBO groups (Figs. 2 and 3); along with some decrease in tissue VEGF mainly in the +lac, lac + arg media (Fig. 6), and increased NO_x production (Fig. 4). Thus we show that the response of the tissue is subtly affected by the chemical environment around the blood vessel, and the tissue is more responsive to HBO when wound conditions are mimicked.

This experiment, to our knowledge, is the first time an isolated *in vitro* blood vessel preparation has been used to investigate the effects of HBO therapy. One concern is that ROS production during HBO therapy will damage any *in vitro* preparation, but this does not seem to be the case in our experiment. In normal Krebs Ringer, the LDH leak into the medium was low (Table 1), and histological examination of vessels at the end of the experiment also confirmed normal structure of the tissue (Fig. 1). At the same time, total glutathione measurements in the medium suggested only small changes in tissue glutathione (Table 1) and therefore minimal loss of this antioxidant. In addition, there were no effects of oxygen or HBO on any of these parameters (Table 1, Figs. 2 and 3), suggesting that a single 90 min HBO treatment at 2.2 ATA does not induce oxidative stress or have adverse effects on this *in vitro* preparation. This finding is consistent with reports on blood from our patients [14] and biochemical investigations on blood platelets *in vitro* [15]. Together, this suggests that the *in vitro* blood vessel preparation was viable, and is a good tool for investigating the local effects of HBO on blood vessels.

VEGF has been suggested to have a pivotal role in promoting angiogenesis [e.g., 7, 8]. However, the evidence for its direct activation during hyperbaric conditions is controversial, not least because experiments on rats show that there is a delay in VEGF production during repeated daily HBO treatments (40% rise in VEGF after 5 days during a twice daily regime of 90 min HBO at 2.1 ATA for 7 days), and any rise in VEGF is quickly lost (within 3 days) after HBO therapy [4]. *In vitro* studies on human umbilical vein endothelial cells show no effect of HBO on VEGF expression [26]. Similarly, in our experiments, there was no oxygen or HBO-dependent change in tissue VEGF when using normal Krebs Ringer, and VEGF levels in the medium remained below the detection limit (<3 pg ml^{-1}). Nitric oxide appears to be important for the induction of VEGF during wound healing [13], and it was theoretically possible that low NO production was limiting VEGF release from our *in vitro* preparation when normal Krebs Ringer was used. However, supplementation with a physiological level of L-arginine (100 μM for rat plasma, [20]) did not alter the findings for VEGF. L-arginine supplementation has been used in endothelial cell cultures to promote the synthesis of NO [27], and this was clearly successful in our experiments because NO_x levels increased with L-arginine supplementation (Fig. 4). Overall, in our experimental conditions, neither normobaric oxygen nor HBO treatment alters VEGF levels in the tissue.

Chronic wounds often suffer from insufficient oxygen supply, which can cause large concentrations of lactate (10–25 mM) to accumulate in the wound during anaerobic energy production [21,22]. It has been suggested that these abnormally high levels of lactate can initiate angiogenesis, and the release of regulatory factors including VEGF [28–30]. Lactate supplementation had no effect on tissue VEGF at the beginning (Table 1) or end of the experiment (Fig. 6), and no effects on NO_x production (Fig. 4). Our experiment therefore does not support the notion of a local effect of lactate on the blood vessel, but we cannot exclude other systemic effects of lactate (not local at the wound site).

Lactate supplementation had no effect on lactate production in the air, NBO or HBO treated groups. Sheikh et al. [4] also found that HBO did not affect lactate levels in wounds. However, supplementing the medium with lactate did slow down the rate of lactate release from the tissue (Fig. 5), regardless of air, oxygen or HBO treatment. This might be explained in part to a small loss of tissue LDH activity (increasing in the medium, Fig. 2), but is more likely due to the added external lactate slowing the net efflux of lactate from the endothelial cell (monocarboxylate transporters saturated for lactate uptake, K_m around 2–3 mM, [31]).

Lactate supplementation did reveal a HBO treatment-dependent effect on both medium LDH levels and total glutathione (Figs. 2 and 3). After, HBO treatment the glutathione and LDH release to the medium was statistically higher than the air controls, and for glutathione, also higher than the normobaric oxygen treatment. This suggests the *in vitro* preparation is more leaky to LDH and glutathione after HBO, but these are very small changes (e.g., <20 μmol g^{-1} tissue for total glutathione) and not likely to be of toxicological significance.

The wound environment can have both high levels of lactate and NO *in vivo*, and the role of NO has been explained in terms of its vasodilatory effects to enable access of the immune system and inflammatory factors to the wound site, and then later on, to promote angiogenesis [13]. It is therefore possible, that several beneficial effects of HBO are driven by the particular combination of metabolites present in the wound, and that any one chemical aspect alone (lactate, NO, wound hypoxia etc.) is not sufficient to trigger HBO-dependent angiogenesis. In this study, we also included a combined supplement of lactate plus L-arginine. This combined supplement had no effect on VEGF (Table 1. Fig. 6) or NO_x production (Fig. 4), and overall produced similar changes in LDH and glutathione levels in the medium to the lactate treatment alone (Figs. 2 and 3). There was no indication of an additive effective of these supplements.

In conclusion, we have demonstrated an *in vitro* blood vessel preparation for investigating the effects of HBO on blood vessels. The data suggests that HBO therapy does not cause VEGF release locally from the blood vessels used, or changes in NO_x. Although VEGF release was not stimulated by supplementing media with L-arginine, the effects of lactate, and/or arginine supplementation on changes in glutathione and LDH release suggest that using a medium that mimics the wound environment reveals effects of HBO therapy that are not seen in normal physiological solutions.

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References

[1] R.E. Marx, W.J. Ehler, P. Tayapongasak, L.W. Pierce, Relationship of oxygen dose to angiogenesis induction in irradiated tissue, Am. J. Surg. 160 (1990) 519–524.
[2] A. Mechine, S. Rohr, F. Toti, C. Aysoy, F. Schneider, C. Meyer, J.D. Tempe, J.P. Bellocq, Wound healing and hyperbaric oxygen. Experimental study of the angiogenesis phase in the rat, Ann. Chir. 53 (1999) 307–313.
[3] A. Muhonen, M. Haaparanta, T. Gronroos, J. Bergman, J. Knuuti, S. Hinkka, R.-P. Happonen, Osteoblastic activity and neoangiogenesis in distracted bone of irradiated rabbit mandible with or without hyperbaric oxygen treatment, Int. J. Oral Max. Surg. 33 (2004) 173–178.

[4] A.Y. Sheikh, M.D. Rollins, H.W. Hopf, T.K. Hunt, Hyperoxia improves microvascular perfusion in a murine wound model, Wound Repair Regen. 13 (2005) 303–308.
[5] G. Neufeld, T. Cohen, S. Gengrinovitch, Z. Poltorak, Vascular endothelial growth factor (VEGF) and its receptors, FASEB J. 13 (1999) 9–22.
[6] I. Zachary, G. Gliki, Signaling transduction mechanism mediating biological actions of the vascular endothelial growth factor family, Cardiovasc. Res. 49 (2001) 568–581.
[7] E. Hormbrey, P. Gillespie, K. Turner, C. Han, A. Roberts, D. McGrouther, A.L. Harris, A critical review of vascular endothelial growth factor (VEGF) analysis in peripheral blood: is the current literature meaningful? Clin. Exp. Metastasis 19 (2002) 651–663.
[8] N. Ferrara, H.P. Gerber, J. LeCouter, The biology of VEGF and its receptors, Nat. Med. 9 (2003) 669–676.
[9] G.M. Gordillo, S. Roy, S. Khanna, R. Schlanger, S. Khandelwal, G. Phillips, C.K. Sen, Topical oxygen therapy induces vascular endothelial growth factor expression and improves closure of clinically presented chronic wounds, Clin. Exp. Pharm. Physiol. 35 (2008) 957–964.
[10] J. Niinikoski, Current concepts of the role of oxygen in wound healing, Ann. Chir. Gynaecol. Suppl 215 (2001) 9–11.
[11] W.A. Zamboni, L.K. Browder, J. Martinez, Hyperbaric oxygen and wound healing, Clin. Plastic Surg. 30 (2003) 67–75.
[12] J. Gutteridge, Biological origin of free radicals, and mechanisms of antioxidant protection, Chem. Biol. Interact. 91 (1994) 133–140.
[13] J.D. Luo, A.F. Chen, Nitric oxide: a newly discovered function on wound healing, Acta Pharmacol. Sin. 26 (2005) 259–264.
[14] R.D. Handy, P. Bryson, A.J. Moody, L.M. Handy, J.R. Sneyd, Oxidative metabolism in platelets, platelet aggregation, and hematology in patients undergoing multiple hyperbaric oxygen exposures, Undersea Hyperb. Med. 32 (2005) 327–340.
[15] F.L. Shaw, R.D. Handy, P. Bryson, J.R. Sneyd, A.J. Moody, A single exposure to hyperbaric oxygen does not cause oxidative stress in isolated platelets: no effect on superoxide dismutase, catalase, or cellular ATP, Clin. Biochem. 38 (2005) 722–726.
[16] C.K. Sen, S. Khanna, G. Gordillo, D. Bagchi, M. Bagchi, S. Roy, Oxygen, oxidants, and antioxidants in wound healing, An emerging paradigm, Ann. N.Y. Acad. Sci. 956 (2002) 239–249.
[17] C.K. Sen, S. Khanna, B.M. Babior, T.K. Hunt, E.C. Ellison, S. Roy, Oxidative-induced vascular endothelial growth factor expression in human keratinocytes and cutaneous wound healing, J. Biol. Chem. 277 (2002) 33284–33290.
[18] S.J. Kirk, M. Hurson, M.C. Regan, D.R. Holt, H.L. Wasserkrug, A. Barbul, Arginine stimulates wound healing and immune function in elderly human beings, Surgery 114 (1993) 155–160.
[19] R.E. Abbott, D. Schachter, Regional differentiation in rat aorta: L-arginine metabolism and cGMP content *in vitro*, Am. J. Physiol. 266 (1994) H2287–H2295.
[20] S. Osowska, C. Moinard, C. Loi, N. Neveux, L. Cynober, Citrulline increases arginine pools and restores nitrogen balance after massive intestinal resection, Gut 53 (2004) 1781–1786.
[21] M.J. Im, J.E. Hoopes, Enzyme activities in the repairing epithelium during wound healing, J. Surg. Res. 10 (1970) 173–179.
[22] T.K. Hunt, M.J. Banda, I.A. Silver, Cell interaction in post-traumatic fibrosis, Ciba Found. Symp. 114 (1985) 127–149.
[23] D.T. Plummer, An Introduction to Practical Biochemistry, McGraw Hill, London, 1971, pp. 238–240.
[24] J.D. Adams, B.H. Lauterburg, J.R. Mitchell, Plasma glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress, J. Pharmacol. Exp. Ther. 227 (1983) 749–754.
[25] A.J. Moody, F.L. Shaw, Re-evaluation of the Greiss reaction: how much of a problem is interference by nicotinamide nucleotides? Anal. Biochem. 356 (2006) 154–156.
[26] S. Lin, K.G. Shyu, C.C. Lee, B.W. Wang, C.C. Chang, Y.C. Liu, F.Y. Huang, H. Chang, Hyperbaric oxygen selectively induces angiopoietin-2 in human umbilical vein endothelial cells, Biochem. Biophys. Res. Commun. 296 (2002) 710–715.
[27] R.M.J. Palmer, D.D. Rees, D.S. Ashton, S. Moncada, L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation, Biochem. Biophys. Res. Commun. 153 (1988) 1251–1256.
[28] J.A. Jensen, T.K. Hunt, H. Scheuenstuhl, M.J. Banda, Effect of lactate, pyruvate, and pH on secretion of angiogenesis and mitogenesis factors by macrophages, Lab. Invest. 54 (1986) 574–578.
[29] D.D. Zabel, J.J. Feng, H. Scheuenstuhl, T.K. Hunt, M.Z. Hussain, Lactate stimulation of macrophage-derived angiogenic activity is associated with inhibition of Poly (ADP-ribose) synthesis, Lab. Invest. 74 (1996) 644–649.
[30] J.S. Constant, J.J. Feng, D.D. Zabel, H. Yuan, D.Y. Suh, H. Scheuenstuhl, T.K. Hunt, M.Z. Hussain, Lactate elicits vascular endothelial growth factor from macrophages: a possible alternative to hypoxia, Wound Repair Regen. 8 (2000) 353–360.
[31] R.C. Poole, A.P. Halestrap, S.J. Price, A.J. Levi, The kinetics of transport of lactate and pyruvate into isolated cardiac myocytes from Guinea-pig — kinetic evidence for the presence of a carrier distinct from that in erythrocytes and hepatocytes, Biochem. J. 264 (1989) 409–418.