Oxidative metabolism in platelets, platelet aggregation, and hematology in patients undergoing multiple hyperbaric oxygen exposures.

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Handy RD, Bryson P, Moody AJ, Handy LM, Sneyd JR. Oxidative metabolism in platelets, platelet aggregation, and hematology in patients undergoing multiple hyperbaric oxygen exposures. Undersea Hyperb Med 2005; 32(5):327-340. Repeated hyperbaric oxygen (HBO_2) treatments at 2.2 ATA for 90 minutes each are used to treat chronically ill patients with problem wounds, but there are concerns about the cytotoxicity of oxygen to blood cells and platelet function during prolonged HBO_2 therapy. We recruited 31 consenting patients scheduled for multiple HBO_2 treatments to evaluate oxidative metabolism in platelets, platelet aggregation, and hematology (mean age ± standard error, 61 ± 2.6 years, 20 males, 11 females). Venous blood was collected before and after the 1st and 20th HBO_2 treatments. No effect of HBO_2 was observed on red cell counts, hematocrit, hemoglobin, mean red cell volume (MCV), platelet counts, basal levels of lactate production by platelets, ferric reducing ability of plasma (FRAP), or plasma protein. The capacity for oxidative metabolism (lactate ratio) in platelets was not affected by HBO_2, except in smokers where it increased by the 20th HBO_2 treatment. Mean lymphocyte count was increased by 38% after the 20th treatment. There was also a 23% increase in platelet protein content, and a 24% increase in arachidonic acid-dependent platelet activation. Collagen-dependent platelet aggregation was unaffected. Blood glucose showed HBO_2-dependent variability, but remained in the normal range. Plasma lactate levels decreased significantly from 3.2 to 2.5 mmol/l by the end of the study. Overall, we found no evidence that 20 HBO_2 sessions caused adverse effects on platelet aggregation or oxidative metabolism in platelets, red or white cell counts, or total antioxidant status of the plasma.

INTRODUCTION

Hyperbaric oxygen (HBO_2) therapy for chronic conditions typically consists of a period of 100% oxygen breathing at pressures of 2.0 and 2.4 ATA for one to two hours per session. Patients may receive multiple sessions of HBO_2 therapy over days or weeks depending on the indication (1-3). Multiple HBO_2 therapies have been used to treat many types of patients, including those with major soft tissue trauma (1), diabetic foot and peripheral ischaemia (4), antibiotic-resistant infections (5), and maxillo-facial radionecrosis (6). HBO_2 therapy has been reported to improve healing, and recovery from infection, in a variety of patients (3, 7). However, there are also some concerns that HBO_2 therapy may not improve healing (8), perhaps due to chronic oxygen toxicity and/or other adverse effects of oxidative stress during HBO_2 treatment (1, 9).

The suggested toxic effects of HBO_2 are controversial and include oxidative DNA damage to lymphocytes from healthy subjects following 100% oxygen at 2.5 ATA, after a total of 3 x 20 minute HBO_2 treatments with 5 minute air breaks (10). However, in the same patients antioxidants in the plasma such as

vitamin C, vitamin E, and reduced glutathione (GSH) were not affected (11). Furthermore GSH levels in lymphocytes were unchanged despite some DNA damage (11). Similarly, Benedetti et al. showed no depletion in plasma GSH or Vitamin E in 12 patients receiving 15 x daily treatments of 100% oxygen at 2.5 ATA for 30 min, even though there was some evidence of lipid peroxidation as measured by malondialdehyde concentration in the plasma (9). Enzymes involved in antioxidant defences in red cells such as superoxide dismutase (SOD) and catalase show either no change (11) or a decrease in activity (9) in these human studies. Sub-lethal studies in rodents at higher pressures and longer durations (2.8 ATA and 100% oxygen for periods up to 11 hours) demonstrate that antioxidant enzyme activities in the lung can eventually be compromised (12). However, reports on blood function in animal or human studies are varied. For example, HBO_2 therapy had no effect on collagen-induced platelet aggregation in rabbits (2.4 ATA HBO_2 for 90 min daily for 20 days, 14). Alternatively, acute exposure to HBO_2 (2.8 ATA for 6 hours) in rats decreased both platelet count and fibrinogen concentrations (13). HBO_2 has no effect on immunity in healthy subjects treated with daily 90 minute oxygen exposures at 2.4 ATA for 4 weeks (15), but only 8 days treatment of 100% oxygen at 2.4 ATA (twice a day) caused immunosupression in mice (16).

These varied observations suggest that there are several possible mechanisms for the effects of HBO_2 therapy on blood function, depending on the treatment regime. Oxidative stress and subsequent tissue injury is clearly implicated in animal studies where the treatment pressures are high or the HBO_2 exposure is long enough (12). However the human studies above, that have been performed at clinically relevant pressures (e.g. 2-2.4 ATA for 90 minutes or less, 1), do not find depletion of plasma antioxidants, and either small or no reduction in antioxidant enzyme defences in blood cells (9-11). This suggests that, although some oxidative stress may be present, the effects of clinically relevant HBO_2 treatments may be more subtle and could include other mechanisms such as changes in oxidative metabolism or the capacity of cells to utilize oxygen (22, 30). The latter may be particularly important in platelet function, where the capacity for oxidative metabolism is vital to initiate aggregation (22).

The Hyperbaric Medical Centre in Plymouth receives patients with a variety of chronic non-healing wounds, who are treated with around 20-30 HBO_2 sessions at 2.2 ATA for 90 minutes each, over a period of 2-4 weeks, with an improved clinical outcome (17). The original cause of injury in these patients may vary, but these patients share the common problem of chronic illness and receive at least 20 HBO_2 sessions. To our knowledge only one study has explored the biochemical toxicology of oxygen during HBO_2 therapy in patients receiving a clinically realistic number of treatments (2 patients, 15 consecutive treatments at 2.5 ATA, 9). In this study we investigated patients who had been referred for HBO_2 therapy. Our primary objective was to determine whether multiple HBO_2 therapies cause any additional, clinically significant, changes in oxidative metabolism in platelets which could be associated with reduced platelet aggregation, or changes in red or white cell counts in the blood of patients who are already chronically ill prior to HBO_2 treatment. Secondly, we hoped to expand our knowledge of the potential effects of multiple HBO_2 therapies, by measuring the blood parameters above and overall antioxidant capacity of the blood after an extreme number of 20 repeated HBO_2 treatments.

MATERIALS AND METHODS

Experimental Design

Thirty one patients with non-healing wounds gave informed consent in writing to participate in the study. All these patients had been referred for HBO_2 therapy at the Hyperbaric Medical Centre in Plymouth as part of their clinical care, and were approached to participate in the study on admission to the center. The study was a pre-treatment/post-treatment design using the patients as their own controls (18). Venous blood was collected immediately before and after their 1st HBO_2, and again immediately before and after their 20th HBO_2. Each HBO_2 session consisted of 100% oxygen at 2.2 ATA for 90 minutes. Patients typically completed this course of treatment over 2-4 weeks. An anonymous questionnaire was used to record life style, general health, and clinical history, such as the use of substances with anticoagulant or anti-oxidant properties (e.g. aspirin, vitamin supplements). The questionnaire was completed during the recruitment stage when patients were referred for HBO_2 therapy, and was also used to identify patients to be excluded from the study. Patients who had taken aspirin on the day of the study, or in whose work exposure to oxidizing chemicals was likely, were excluded. In addition, patients taking any prescribed anti-coagulants (e.g. warfarin, heparin), diabetics with abnormal blood glucose or a history of poor glucose control, and patients with known cardiovascular disease or metabolic disorders related to platelet function (uremia, von Willebrand Syndrome, or Bernard-Soulier Syndrome) were excluded.

Blood Collection, Hematology and Platelet Aggregation

20 ml of venous blood was collected into Vacutainers (Becton-Dickinson) before and after the 1st and 20th HBO_2 sessions (4 x 20 ml blood in total/patient). The 20 ml venous sample was collected into 3 separate Vacutainers for appropriate analysis (2 ml into EDTA-tubes for hematology, 8 ml into sodium citrate tubes for platelet aggregation, and 10 ml into a sodium citrate tube for biochemistry). Routine hematology was performed immediately using an automated analyser (Bayer, H1). Platelet aggregation was performed promptly in a PAP-4 platelet aggregation profiler (Biodata Corporation) at 37°C in platelet rich plasma (PRP) adjusted to a known platelet concentration with autologous platelet-poor plasma. After recording baseline values for each sample, an aggregating agent (1.9 mg/ml collagen or 5 mg/ml arachidonic acid) was added to the PRP and the maximum sustainable rate of aggregation recorded (see ref. 19 for original manual method).

Biochemistry

Blood used for biochemistry was collected into a separate sodium citrate tube for chilling during sample preparation to preserve metabolites. This blood was not used for platelet aggregation assays or hematology. The general status of aerobic metabolism in blood from patients was assessed by measuring plasma glucose and lactate. The ferric reducing ability of plasma (FRAP assay) was used as a general measure of antioxidant capacity that would decline if significant oxidative stress occurred in the blood (21). Whole blood (10 ml) was centrifuged (10 min, 200 *g*, Denley BR 401, 4°C) and platelet-rich plasma (PRP) collected on ice. Aliquots of PRP were centrifuged (6 min, 2000 *g*, Denley BR 401, 4°C) and the plasma assayed for glucose (Sigma glucose kit No. 16-UV), lactate (Sigma kit No. 735-10), protein (20), and FRAP (21). The FRAP assay was modified for use with a plate reader. Briefly, 30 µl of deionized water, 10 µl of undiluted serum, and 300 µl of FRAP reagent were mixed in a 96-well microplate (in triplicate).

Microplates were incubated for 60 minutes at room temperature and absorbances read at 550 nm (Dynex MRX plate reader) against 0-1 mmol/l $FeSO_4$ standards.

Lactate production (22) and protein content (20) of freshly isolated platelets were also measured in aliquots of the cold PRP collected above. Platelet lactate production was measured because oxidative damage to mitochondrial oxidative phosphorylation may increase reliance on glycolytic energy production (22). The capacity for oxidative phosphorylation can be assessed by measuring lactate production in the presence/absence of antimycin A, a mitochondrial respiratory chain inhibitor (22). The lactate ratio (lactate production in the presence of antimycin/lactate production without added antimycin) therefore gives a measure of the ability of platelets to generate ATP during mitochondrial respiration. Platelets were washed twice in an equal volume of ice cold buffer (in mmol/l; 20 HEPES, 120 NaCl, 3 EDTA, 5 glucose, pH 7.4) to initially remove external lactate that was present in the blood samples. This step was performed in the cold to reduce metabolic rate so that platelets did not produce extra lactate during washing (22). Cells were then re-suspended (~6 x 10^8 cells/ml) in ice cold washing buffer (without glucose). The lactate ratio measurement was performed by transferring 100 µl of the resulting glucose-free platelet suspension to each of two Eppendorf tubes, and adding 2 µl of antimycin A (1 mg/ml in 96% ethanol) to one tube (aerobic metabolism-inhibited) or 2 µl of 96% ethanol to the other (control). Pairs of Eppendorf tubes containing the platelet suspension were incubated for 15 min at 37 ºC to stimulate lactate production. The reaction was stopped on ice and the platelet suspension was quickly centrifuged to remove platelets (6 min, 2000 *g*, Denley BR 401, 4 ºC). Lactate was then determined in the supernatants (23). Briefly, 30 µl of supernatant was mixed with 300 µl of lactate reagent (10 ml, freshly prepared containing 20.6 mg ß-NAD^+, 4.3 ml of 1 M glycine, 170 µl of 98% hydrazine, 19 U/ml of lactate dehydrogenase, LDH, and 5.4 ml of deionized water) in a microplate, incubated at 37°C for 30 mins, and absorbances read at 340 nm (Dynex MRX) against 0-2 mmol/l lactate standards.

Statistics

Data were analysed using Statgraphics version 4.0. All data were initially tested for skewness, kurtosis and a Bartlett's variance check prior to application of one-way analysis of variance (ANOVA) to test the pre and post-treatment effect of HBO_2 over all time intervals on the data for all patients. The least squares difference (LSD) multiple range test was used to locate statistical differences within each ANOVA. The Kruskal-Wallis test was used instead of ANOVA when data were non-parametric, or failed Bartlett's test. These statistical tests were used primarily to compare: (i) pre-treatment with post-treatment at each time interval (effect of each HBO_2 treatment), (ii) pre-treatment values before the 1st and 20th HBO_2 treatment (change in background level of response over time). Since the ANOVA compared all time points, it also incidentally provided a comparison of data before the 1st treatment with after the last treatment. This comparison is not strictly valid for identifying the specific effects of HBO_2 (and is not intended as such), but does reflect the overall effect of all aspects of patient care from admission to discharge, and is therefore of clinical interest to the Hyperbaric Medical Centre. Data were also analysed to compare differences in HBO_2 response over time in both male and female subjects, and in other sub-groups such as smokers versus non-smokers, and diabetics versus non-diabetic patients. This was done using the ANOVA or Kruskal-Wallis test as above. In some cases, to improve statistical

resolution data at individual time points were also analysed using a two-tailed paired Students *t*-test for patients that completed all HBO_2 sessions. All statistical analysis used a rejection level of P = 0.05.

RESULTS

The Patient Group

Thirty one patients were recruited in the study (Table 1), most of whom were elderly non-smokers with relatively normal BMI and moderate alcohol intakes (0 patients>21 units/ week, 22 patients 0-14 units/week, 9 patients 14-21 units/week). Although 32% of patients smoked, only 2 smoked more than 20 cigarettes per day. Incidental ingestion of dietary supplements that were also anti-oxidants was small and only 3 patients used multi-vitamins or vitamin C, routinely. One patient did not eat fruit, and also did not take vitamin supplements. The remaining 9 patients who took supplements used cod liver oil, garlic capsules or iron (or combinations of these). Only 4 patients used Sensodyne toothpaste (which contains aspirin), and no patients were taking antioxidant herbal remedies such as ginseng. About one third of patients periodically used aspirin (but not on the day of blood sampling), and one third of patients routinely used paracetamol for pain relief. Five patients reported current use of antibiotics at the start of the trial. The types of patients referred with non-healing wounds were primarily associated with radiation tissue damage (23 patients), chronic osteomyelitis (5 patients), and diabetic leg ulcers (3 patients). None of the radionecrosis patients were receiving radiation therapy or chemotherapy at the time of the study, and the last treatments were months to years prior to HBO_2 .

Plasma glucose, lactate and FRAP

Plasma glucose and lactate concentrations remained within the normal clinical ranges before and after the 1st and the 20th HBO_2 session, indicating there was no clinically important adverse effect of HBO_2 on aerobic metabolism (Fig. 1), although some subtle changes of statistical significance did occur within the normal clinical range. Plasma glucose concentrations were not affected by HBO_2 when all patient data were

Table 1. Patients with non-healing wounds recruited to the HBO_2 study.

Parameter	Males	Females	All patients
Number	20	11	31
Age (years)	62 ± 3.1 (45-81)	58 ± 4.5 (37-81)	61 ± 2.6
Weight (kg)	71.9 ± 6.0 (50-133)	59.5 ± 4.7 (42-88)	67.0 ± 4.2
Body Mass Index (BMI)	24.1 ± 1.5 (17-41)	23.5 ± 2.2 (15-36)	23.9 ± 1.2
Smokers (%)	40	20	32
Aspirin (%)	40	20	32
Paracetamol (%)	26	40	32
Alcohol (%)	73	40	70
Vitamin/Mineral supplement (%)	15	15	38

Data are means ± S.E. with ranges indicated in parenthesis. No differences in means were served between males and females. However, the BMI of smokers was lower than non-smokers for all patients (P = 0.04, ANOVA). Values as % indicate the proportion of patients who smoke, take aspirin, paracetamol, drink alcohol, or take any form of vitamin or mineral supplement.

analysed together (Kruskal-Wallis, P = 0.65). This included only one diabetic patient who completed all treatments, and since this patient had normal blood glucose throughout (in mmol/l, Pre 1, 4.43; Post 1, 5.09; Pre 20, 4.71; Post 20, 4.77) and was not a statistical outlier, was therefore included in the analysis. However, all female patients showed a statistically significant increase in plasma glucose of 21% over the experiment (paired *t*-test, P <0.05) compared to a statistically significant 17% decrease in males (paired *t*-test, P <0.05). These changes in blood glucose over the entire experiment were not caused by a gradual change in pre-treatment (baseline) values in blood glucose over time (Pre 1 compared Pre 20, paired *t*-test, P>0.05 in both males and females, Fig. 1a, b, c).

Plasma lactate in all patients consistently decreased by about 0.5 mmol/l after each HBO_2 session (Fig. 1d). These differences between pre- and post treatment lactate were statistically significant in all patients (paired *t*-test, P <0.02) immediately after the 1st HBO_2, suggesting a reduction in anaerobic lactate production even during a single HBO_2 session. However by the 20th treatment, this HBO_2 effect on lactate was lost with no statistically significant difference by paired *t*-test, (P = 0.25). However, the post 20th treatment lactate was lower than at the start of the study (paired *t*-test, P = 0.021), suggesting an overall improvement in lactate after the final treatment compared to on admission, which was not attributed to drift in baseline lactate (pre 1 and pre 20, paired t-test, P = 0.28). No sex differences were apparent in plasma lactate after either the 1st (ANOVA, P = 0.446) or the 20th HBO_2 (ANOVA, P = 0.15). No changes in the ferric reducing ability of plasma (FRAP, ANOVA, P = 0.97) were observed (Fig. 1e). Re-analysis of the FRAP data for HBO_2 effect for sub-groups of smokers (ANOVA, P = 0.932) and non-smokers (ANOVA, P = 0.949) also showed no statistical differences. There were no differences in FRAP in smokers compared to non-smokers on admission (Pre 1, *t*-test, P = 0.474; smokers, 0.78 ± 0.18 mmol/l; non-smokers 0.64 ± 0.10 mmol/l, mean ± S.E., n = 8 and 16 respectively) or at the end of the study (Post 20, *t*-test, P = 0.923; smokers, 0.67 ± 0.15 mmol/l; non-smokers 0.68 ± 0.10 mmol/l, mean ± S.E., n = 8 and 14 respectively). No effects of HBO_2 therapy on plasma protein (ANOVA, P = 0.82) were observed (Fig. 1f).

Platelet Aggregation

Collagen-dependent platelet aggregation was in the normal clinical range during HBO_2 treatment, although arachidonic-acid dependent aggregation was not and there were subtle changes in platelet activation (Table 2**)**. Collagen-dependent aggregation of platelets was unaffected by HBO_2 (ANOVA, P = 0.71) and no sex differences were evident (P>0.4, Table 2). Re-analysis of collagen-dependent aggregation for smokers versus non-smokers also showed no effect of HBO_2 (ANOVAs, P = 0.561 and 0.648 for smokers and non-smokers respectively). The maximum rate of collagen-dependent aggregation (% aggregation/minute) was also unaffected by HBO_2 (e.g. mean ± S.E., pre 1, 34.3 ± 2.7 %/min; pre 20 33.8 ± 2.2 %/min, n = 29 & 22, ANOVA over time for all patients, P = 0.680), and re-analysis of collagen-dependent aggregation rates by patient sub groups (smokers, non-smokers, males, females, aspirin and non-aspirin users) revealed no HBO_2 effect (ANOVAs, P>0.05). Similarly, the maximum rate of arachidonic acid-dependent aggregation was unaffected by HBO_2 (e.g. mean ± S.E., pre 1, 21.4 ± 2.6 %/min; pre 20 22.0 ± 2.5 %/min, n = 29 & 21, ANOVA over time for all patients, P = 0.326), and re-analysis of arachidonic acid-dependent aggregation rate by patient sub groups (as above) revealed no HBO_2 effects (ANOVAs, P>0.05). However, arachidonic acid-dependent platelet activation tended to increase over the entire experiment compared to the pre-

Fig. I - a through f

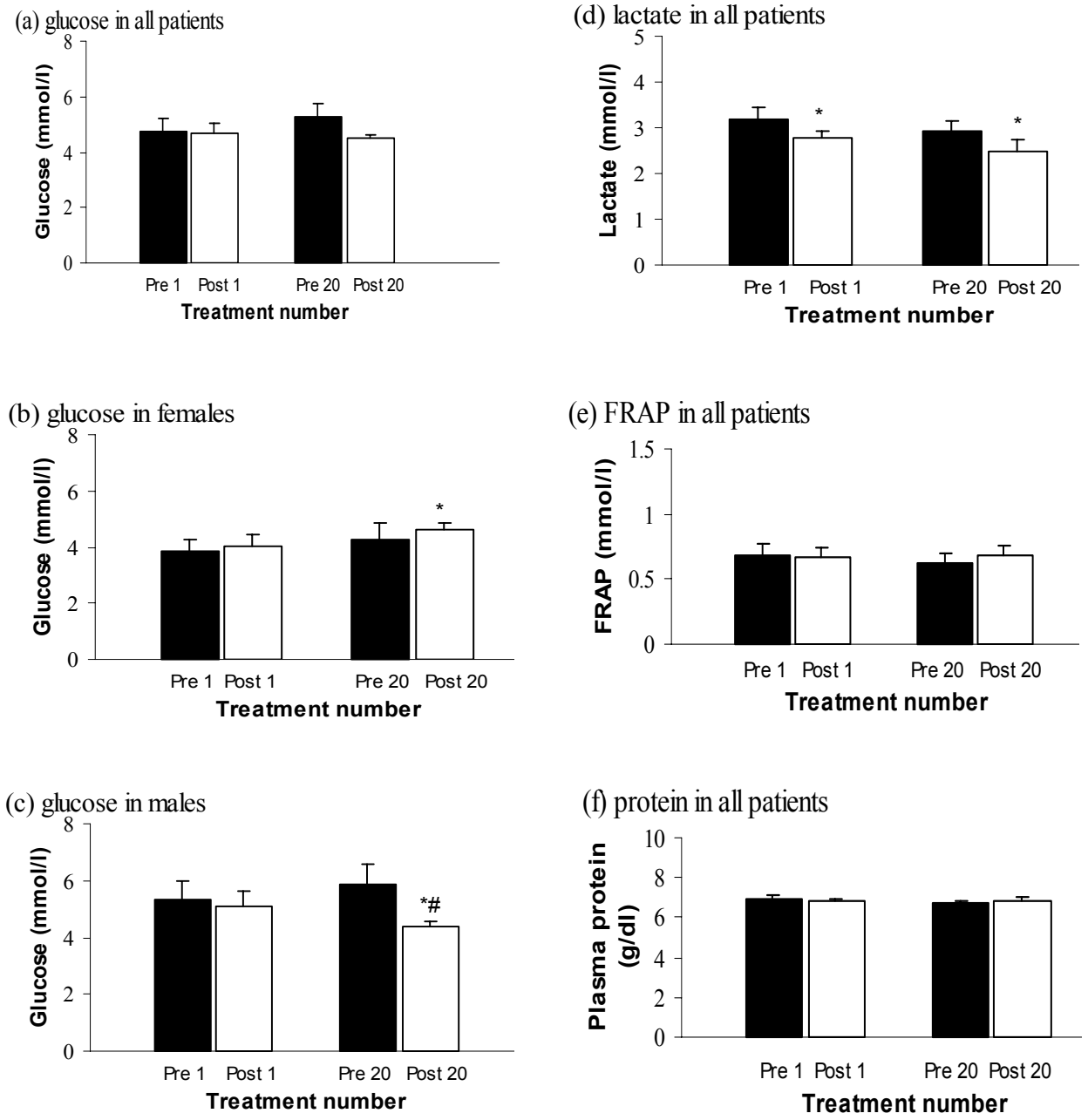

Fig 1. The effect of multiple hyperbaric oxygen therapy (HBO_2) sessions on (a) plasma glucose in all patients, (b) plasma glucose in female and (c) male patients, (d) plasma lactate in all patients, (e) ferric reducing ability of plasma (FRAP) in all patients and (f) total plasma protein in all patients. Data are means ± S.E. (n = 31-24 blood samples). Black bars indicate values before HBO_2, white bars indicate values immediately after HBO_2. Pre 1 and Post 1 are before and after the 1st HBO_2 respectively. Pre 20 and Post 20 indicate before and after the 20th HBO_2 respectively. * significantly different from Pre 1 by paired *t*-test ($P < 0.05$). # significantly different from Pre 20 by paired *t*-test ($P < 0.05$). Unlabelled bars indicate no statistical difference by ANOVA or Kruskal Wallis test ($P > 0.05$) for all patients, or by paired *t*-tests within male or female data (see text for details). Note, no statistical differences were observed between Pre 1 and Pre 20 in any variable.

treatment value before the 1st HBO_2, mainly due to an increasing trend in aggregation in males. Females showed a statistically significant decrease in arachidonic acid-dependent platelet aggregation immediately after the 20th HBO_2 (pre 20 compared to post 20, paired *t*-test, P = 0.042). Over the entire experiment arachidonic acid-dependent aggregation increased by 24% in females (Table 2), but this was not statistically significant because of patient variability (see aspirin usage below).

All patients who had a history of taking an aspirin daily (but not on the day of treatment) had much lower arachidonic acid-dependent aggregation rates at the start of the experiment compared to non-aspirin users (pre 1 comparison, *t*-test, P = 0.013), with values of (mean ± S.E., n = 22 and 7) 18.7 ± 6.7 and 53.4 ± 9.5 % respectively. HBO_2 improved arachidonic acid-dependent aggregation more in non-aspirin than aspirin users by the end of the experiment (overall median increases of 18% and 8% aggregation, respectively). However, this apparent improvement simply reflected the lower aggregation in aspirin users at the start of the study. No effect of HBO_2 over time was found in aspirin users (Kruskal Wallis, P = 0.594) or non-users who did not take aspirin (ANOVA, P = 0.597) when these sub-groups were analysed independently.

No effects of either paracetamol or smoking were observed on platelet aggregation, either at individual time points (*t*-tests smokers versus non smokers, or paracetamol users versus non-users, P>0.05), or within these sub-groups with respect to HBO_2 effect over time (ANOVAs, P>0.05). For example, on admission (sample pre 1) there was no difference (*t*-test, P

Table 2. Platelet aggregation ability in whole blood from patients receiving multiple hyperbaric oxygen therapy (HBO_2) sessions.

Parameter	Pre 1	Post 1	Pre 20	Post 20
Collagen-dependent aggregation (%)				
All patients	72.4 ± 2.6	71.3 ± 2.7	67.9 ± 2.3	70.9 ± 3.3
Males	73.6 ± 3.7	74.6 ± 2.6	68.1 ± 12.5	70.8 ± 4.3
Females	70.4 ± 3.2	65.3 ± 5.9	67.8 ± 3.1	71.3 ± 4.4
Arachidonic acid-dependent aggregation (%)				
All patients	45.0 ± 6.2	45.8 ± 5.8	52.4 ± 6.0	56.5 ± 6.7
Males	46.0 ± 7.9	44.6 ± 7.2	50.7 ± 7.6	53.9 ± 7.9
Females	43.2 ± 10.4	48.2 ± 10.4	55.3 ± 10.2	#48.2 ± 13.3
Platelet protein (g/dl)	0.77 ± 0.028	*0.87 ± 0.033	0.76 ± 0.043	*#0.95 ± 0.053
Platelet Count (10^9/l)	274 ± 15.2	273 ± 16.3	274 ± 23.5	290 ± 22.6

All patients include both males and females, males = only male patients, females = only female patients. Platelet protein = protein concentration in washed platelets from all patients. Platelet count = count in whole blood from all patients. Data are means ± S.E. (n = 31-24 blood samples). Pre 1 and Post 1 are before and after the 1st HBO_2 respectively. Pre 20 and Post 20 indicate before and after the 20th HBO_2 respectively. * Significantly different from Pre 1 by paired *t*-test (P <0.05). # significantly different from Pre 20 by paired *t*-test (P<0.05). Unlabelled data indicate no statistical difference by ANOVA or Kruskal Wallis test (P >0.05) for all patients, or by paired *t*-tests within male or female data (see text for details). Note, no statistical differences were observed between Pre 1 and Pre 20 in any variable. Collagen-dependent platelet aggregation remained in the normal range (70-94%, ref. 28), the suggested normal range for arachidonic acid-dependent platelet aggregation is 70-90% for the assay used (see 19).

= 0.70) in arachidonic acid-dependent platelet aggregation between smokers (41.7 ± 11.2%) and non-smokers (46.8 ± 7.6%) respectively (mean ± S.E., n = 10 and 19). Similarly, at the end of the study (Post 20) there were no statistical differences in platelet aggregation between smokers and non-smokers (*t*-test, P = 0.44, smokers, 49.6 ± 11.8%; non-smokers, 60.5 ± 8.3%). Similarly for patients taking paracetamol, on admission (sample pre 1) there was no difference (*t*-test, P = 0.78) in arachidonic acid-dependent platelet aggregation between paracetamol users (48.5 ± 18.4%) and non-users (44.2 ± 6.4%) respectively (mean ± S.E., n = 5 and 17). At the end of the study (Post 20) there were no statistical differences in platelet aggregation between patients using or not using paracetamol (*t*-test, P = 0.69, using paracetamol, 61.6 ± 12.4%; without paracetamol, 55.1 ± 8.0%).

Capacity for oxidative metabolism in platelets

The capacity for oxidative metabolism (lactate ratio) in platelets was not affected by HBO_2 (analysis of all patients, Kruskal-Wallis, P = 0.86), and there were no statistical differences (ANOVA, P >0.05) in the response to HBO_2 over time in males or females (Table 3**)**. Re-analysis of lactate ratio data in non-smokers only showed no statistically significant effect of HBO_2 over time (ANOVA, P = 0.264). However, in smokers there was a statistically significant (1.3 fold, Kruskal Wallis, P = 0.082, differences in post-hoc box-whisker plots) increase in the Pre 20 lactate ratio compared to the Pre 1 lactate ratio (Table 3).

Basal levels of lactate production by platelets (ethanol controls in the lactate ratio measurements) were unaffected by HBO_2 with pre-HBO_2 treatment values of 0.35 ± 0.08 and 0. 35 ± 0.06 µmol/l (mean ± S.E., n = 31-24) prior to 1st and 20th treatments respectively. These basal levels of lactate increased slightly to 0.55 ± 0.14 and 0.43 ± 0.12 µmol/l at the end of the 1st and 20th HBO_2 sessions respectively, but were not statistically different from pre-treatment controls at any time (Kruskal-Wallis, P = 0.77). The protein content of washed platelet suspensions increased by 23% over the entire experiment (ANOVA, P = 0.007), and this increase was evident immediately following

Table 3. Capacity for oxidative metabolism (lactate ratio) in platelets from patients receiving multiple hyperbaric oxygen therapy (HBO_2) sessions.

Patient Group	Pre 1	Post 1	Pre 20	Post 20
All patients	1.72 ± 0.23	1.53 ± 0.31	1.62 ± 0.13	1.48 ± 0.13
Males	1.50 ± 0.16	1.03 ± 0.49	1.42 ± 0.17	1.47 ± 0.18
Females	1.85 ± 0.54	2.07 ± 1.22	1.85 ± 0.54	1.89 ± 0.24
Smokers	1.23 ± 0.12	1.66 ± 0.30	#1.70 ± 0.13	1.74 ± 0.20
Non-smokers	1.96 ± 0.32	1.47 ± 0.43	1.58 ± 0.20	1.30 ± 0.15

Data are means ± S.E. n = 31-24 patients at each time point for all patients, n = 12 or 8-9 at each time point for males and females respectively, and n = 17 non-smokers and 8-9 smokers respectively. Lactate ratio is calculated from lactate production by platelets in the presence or absence of antimycin. Lactate ratio = [lactate] in the presence of antimycin/[lactate] without antimycin (see text for details). Pre 1 and Post 1 are before and after the 1st HBO_2 respectively. Pre 20 and Post 20 indicate before and after the 20th HBO_2 respectively. No statistical differences within rows were observed except, # significantly different from Pre 1 within Kruskal Wallis (P = 0.082 for all comparisons within row, difference identified by post-hoc box-whisker plot). No differences were observed between males and females within each time point (column) by *t*-tests (P>0.05). No differences were observed between smokers and non-smokers within each time point (column) by *t*-tests (P>0.05).

the 1st HBO_2 session (Table 2). This effect of HBO_2 on platelet protein was not explained by a change in platelet counts, which remained constant (ANOVA, P = 0.88) (Table 2).

Hematology

HBO_2 had no effect on red blood cell count, mean red cell volume, blood hemoglobin concentration or hematocrit (Table 4), regardless of whether the data analysis was on all patients, males or females (all ANOVAs P>0.05). Differential white cell counts mostly showed an increasing trend with HBO_2 sessions. This was most notable in lymphocyte counts which showed a statistically significant (ANOVA, P = 0.05) 34% increase over the entire experiment, perhaps reflected partly by a 14% increase (NS, Kruskal Wallis, P = 0.29) in total white cell counts. Neutrophils also increased by 9.7% but were not significantly different over the experiment Kruskal Wallis, P = 0.38). No sex differences in the HBO_2 response of white cells were observed.

DISCUSSION

To our knowledge, this is the largest (31 patients) and longest (20 HBO_2 treatments) biochemical study of oxidative metabolism and platelet aggregation in patients receiving multiple HBO_2 sessions for non-healing wounds (larger than 9, or 11). Overall, we found no evidence of adverse effects on platelet aggregation, capacity for oxidative metabolism in platelets, or red and white cell counts. The absence of changes in FRAP also suggests a lack of oxidative stress of clinical significance.

The Patient Group

The study group was typical of patients with non-healing wounds, being mainly elderly people with a history of chronic wounds. Patients with a life style or drug history that might alter oxidative metabolism or induce oxidative stress in the blood were excluded, but one third of patients used over-the-counter nutritional supplements. The types of supplements used were too varied for statistical analysis of effects of individual supplements, but the absence of outliers in each sub group suggests the use of

Table 4. Hematology in patients receiving multiple hyperbaric oxygen therapy (HBO_2) sessions.

Parameter	Pre 1	Post 1	Pre 20	Post 20
RBC (10^{12}/l)	4.33 ± 0.07	4.28 ± 0.08	4.28 ± 0.09	4.17 ± 0.10
MCV (fl)	96.0 ± 1.4	97.2 ± 1.4	96.5 ± 1.7	97.3 ± 1.7
[Hb] (g/dl)	13.3 ± 0.2	13.1 ± 0.3	13.1 ± 0.2	13.1 ± 0.3
HCT (%)	41.4 ± 0.01	41.6 ± 0.01	40.9 ± 0.01	40.3 ± 0.01
Total white cells	7.93 ± 0.68	9.00 ± 0.70	7.65 ± 0.56	9.02 ± 0.84
Lymphocytes	1.39 ± 0.10	1.62 ± 0.13	1.42 ± 0.13	*#1.86 ± 0.16
Monocytes	0.48 ± 0.03	0.48 ± 0.03	0.47 ± 0.04	0.51 ± 0.05
Neutrophils	5.56 ± 0.58	6.41 ± 0.63	5.24 ± 0.50	6.11 ± 0.79
Basophils	0.059 ± 0.006	0.060 ± 0.007	0.064 ± 0.009	0.060 ± 0.005
Eosinophils	0.22 ± 0.03	0.19 ± 0.03	0.26 ± 0.04	0.23 ± 0.04

Data are means ± S.E. n = 31-24 patients at each time point. All counts of white blood cells are expressed as 10^9 cells/l. Pre 1 and Post 1 are before and after the 1st HBO_2 respectively. Pre 20 and Post 20 indicate before and after the 20th HBO_2 respectively. * significantly different from Pre 1 by ANOVA (P <0.05). # significantly different from Pre 20 by ANOVA (P<0.05). RBC, red blood cell count; MCV, mean red cell volume; Hb, hemoglobin; HCT, hematocrit.

supplements did not influence the findings. No effects of smoking or alcohol were observed.

Plasma glucose, lactate and FRAP

Multiple HBO_2 treatments did not effect the overall antioxidant capacity of the plasma (FRAP) from patients (Fig. 1). FRAP values were around 0.7 mmol/l and were consistent with normal subjects (21). Dennog *et al*. (11) also found no change in FRAP in four healthy subjects after 3 x 20 minutes of HBO_2 (2.5 ATA). Benedetti *et al*. (9) also found that thiobarbituric acid reactive substances (TBARS, a measure of lipid peroxidation products) were not different after the first and 15th HBO_2 treatments, although TBARS were 37% higher than controls before the 15th HBO_2 therapy, this effect resolved after the final 15th HBO_2 session.

A potential adverse effect of HBO_2 on blood could involve changes in oxygen utilization by blood cells during aerobic metabolism, and the latter can be assessed by monitoring changes in blood glucose or lactate levels (24). Plasma lactic acid levels are normally between 0.6-1.8 mmol/l (25), and with pre-treatment lactate levels around 2-3 mmol/l, it is clear that our patients started treatment with some reliance on glycolytic lactate production. HBO_2 reduced this lactate burden, even after a single treatment (Fig. 1). Together this suggests that the beneficial effects of multiple HBO_2 treatments on reducing lactate out weigh the risk of lactate production arising from oxidative stress or inefficient aerobic metabolism arising from oxygen toxicity in the blood cells. The latter is also supported by the absence of effects of HBO_2 on the capacity for oxidative metabolism in platelets (see below, and Table 2).

Plasma glucose levels in all patients (Fig. 1) remained within the normal range (fasted range, 3.9-5.6 mmol/l, ref. 25). Pre-HBO_2 blood samples were typically collected 2 h or more after the last meal, and blood glucose was therefore close to baseline levels (< 6 mmol/l, Fig. 1). However, male patients consistently showed a small decrease in blood glucose after HBO_2 therapy, compared to females, which cannot be explained by fasting. A decreased plasma glucose in males is consistent with elevated tissue metabolic rate following HBO_2 (26), but the reason for increased plasma glucose in females is unclear (a stress response to HBO_2 treatment is unlikely, 27). Diabetic patients remained in the normal range, and reanalysis of the data excluding either diabetics or osteomyelitis patients did not change the trends.

Platelet Aggregation

Platelet aggregation at the wound site is an important step in the wound healing. Collagen-dependent platelet aggregation remained in the normal range (70-94%, ref. 28), and platelet counts were unaffected by HBO_2. Furthermore, HBO_2 may have had a beneficial effect on arachidonic acid-dependent platelet aggregation which was low on admission (<50%, suggested normal range 70-90% for the assay used, see 19), and recovery towards the normal range appeared to be enhanced by HBO_2 although not statistically significant over the experiment (Table 2). This possible benefit cannot be explained by oxidative stress, which would inhibit arachidonic acid-dependent platelet activation by interfering with arachidonic acid release from intracellular stores in the platelets and/or receptor activation (29). Even the small decrease in arachidonic acid-dependent platelet aggregation after the 20th HBO_2 treatment in females was probably not clinically important because of the overall trend of increasing platelet aggregation (Table 2).

Protein concentration in platelets also increased, both after the 1st and 20th HBO_2 (Table 2). This implies that either platelet

protein synthesis was enhanced by HBO_2 (also observed in skin explants, 26), or more likely, that the surface-connected canalicular system of the resting platelets were able to store more secretory material following HBO_2 therapy. High protein content could be associated with improved capacity for platelet aggregation. Elevation of apparent platelet protein content due to osmotic cell shrinkage is unlikely, as there was no evidence of MCV change in red cells with HBO_2 (Table 4). Further work is required to identify exogenous factors involved in platelet aggregation (e.g. epinephrine, ADP, arachidonic acid) that may be stimulated by HBO_2, but epinephrine does not increase (27), and the decrease in blood lactate (Fig. 1d.) argues against extracellular ADP accumulation.

Platelet aggregation was not affected by confounding factors such as type of patient, except for aspirin use in relation to arachidonic acid-dependent platelet activation. Patients had poor arachidonic acid-dependent platelet aggregation on admission and, as expected, this was worse in those using aspirin regularly (19). However, even in these patients, HBO_2 improved aggregation.

Capacity for oxidative metabolism in platelets

The lactate ratio is a measure of the relative production of lactate by platelets in the presence/absence of antimycin A. Antimycin inhibits oxidative phosphorylation in platelets and therefore increases the reliance on anaerobic metabolism and causes an increase in lactate production. The lactate ratio is therefore a measure of the capacity for aerobic metabolism, or more precisely, oxidative phosphorylation (22). A fall in the lactate ratio indicates a decline in the capacity for aerobic metabolism in the cell, and reflects disturbances to mitochondrial function (22, 30). A decline in lactate ratio in platelets is associated with the early stages of oxidative stress in the blood (30). Furthermore oxidative stress in platelets, and an associated decline in lactate ratio, precede the release of reactive oxygen species into the plasma that results in loss of plasma antioxidants (30). In this study the lactate ratio in platelets did not decline and was largely unaffected by HBO_2 (Table 3). This implies that there was no oxidative stress in the platelets, which is also consistent with the normal FRAP values obtained in this study (Fig. 1e). In smokers, HBO_2 may even benefit the oxidative metabolism in platelets, since the lactate ratio increased in this group by the 20^{th} treatment (Table 3).

Hematology

Red cells were not affected by HBO_2 and parameters remained in the normal range (Table 4), as previously observed (rats, 13; healthy humans, 11). This is also consistent with the normal erythrocyte glutathione levels found by Benedetti *et al*. (9) in patients receiving 15 HBO_2 treatments. However in this study lymphocyte counts increased (Table 4), suggesting a mild inflammatory response to multiple HBO_2 treatments in these chronically ill patients. This effect may possibly help stimulate host defences and wound repair, leading to improved healing (1). All the patients in our study showed improved wound healing during HBO_2 treatment. Most previous studies on HBO_2 and immunity have been in animals where stress-induced changes in immune organ function might alter circulating immune cells leading to immunosupression or have no benefit (16). This is clearly not the case in patients suffering from non-healing wounds (Table 4). Interestingly, a study of fit male pilots found that lymphocyte counts and antibody titres were not affected by 90 min HBO_2 at 2.4 ATA, repeated over 4 weeks (15). This suggests an absence of adverse effects of HBO_2 on immunity in humans, but that there may be benefit in chronically ill patients.

In conclusion, the end points we

employed in this study showed no evidence of loss of antioxidant capacity in the plasma, disturbances to aerobic metabolism in platelets, or adverse effects of HBO_2 therapy on platelet aggregation and haematology in chronically ill patients.

ACKNOWLEDGEMENT

The authors thank the many staff at DDRC, Triphasic Clinicals Trials Laboratory, and the University of Plymouth for skilled technical assistance, especially Jane Pimlott and Mark Neal for blood sampling, and Sharon Binney for aggregometry. The collection of blood samples was funded by DDRC, and subsequent analysis by the University of Plymouth.

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