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HYPERBARIC OXYGEN THERAPY INCREASES FREE RADICAL LEVELS IN THE BLOOD OF HUMANS

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It has been postulated that exposure to high concentrations of oxygen results in increased oxygen radical production which may account for the toxic effects of excessive exposure to oxygen. Examination of blood from persons undergoing hyperbaric oxygen (HBO) exposure, by low temperature electron spin resonance (ESR) spectroscopy, demonstrated a marked increase in the magnitude of a signal with properties consistent with a free radical ($g = 2.006$). The signal diminished to baseline levels within 10 minutes of cessation of HBO exposure. Further *in vitro* studies of blood revealed an ESR signal generated in red blood cells by oxygen, and dependent on oxyhaemoglobin, which had characteristics indistinguishable from those of the ESR signal of ascorbate radical and the signal in blood from persons undergoing HBO exposure. It is postulated that HBO exposure increases ascorbate radical levels in blood, which is likely to reflect increased ascorbate turnover in human red blood cells.

KEY WORDS: hyperbaric oxygen, ascorbate, semidehydroascorbate radical, ESR, blood

INTRODUCTION

Exposure to oxygen at partial pressures of >0.5 ATA (atmospheres absolute) is known to be damaging to humans and experimental animals, with prolonged exposure to high partial pressures of oxygen often proving fatal to the latter. The effects of high concentrations of oxygen on lungs have been well described[1].

In spite of oxygen's toxicity, controlled HBO therapy has been used successfully for the treatment of a variety of conditions. Commonly accepted clinical indications for its use include decompression sickness, air embolism, carbon monoxide poisoning and gas gangrene[2]. In other situations, such as multiple sclerosis, no significant clinical benefit has been shown. It may be that some of the potential benefits of HBO are limited by the adverse effects of oxygen exposure. For example, any benefit of HBO therapy following stroke may be limited to that produced by the early HBO sessions and then only at minor differential pressures (1.2–1.3 ATA)[3].

It has been suggested that the detrimental effects of exposure to high concentrations of oxygen are due to the increased production of oxygen radicals[4,5] or other

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reactive metabolites derived from oxygen. There is an increasingly large body of evidence, mostly indirect, to support this view[6].

ESR allows the direct measurement of free radical levels. Most oxy-radicals have a short half-life[7] and therefore are unlikely to be detected by direct ESR measurement, even with very rapid freezing of samples. However other oxy-radical products may be more stable. In this study we applied direct ESR spectroscopy, at low temperature, to the investigation of free radical levels in the blood from persons undergoing a regimen of HBO exposure, as used therapeutically.

MATERIALS AND METHODS

Human Study

The study was approved by the Royal Hobart Hospital Ethics Committee and the subjects gave informed consent. The subjects in this study were ten healthy male Tasmania Police divers, aged 21 to 48 years, mean age 31 years. They were exposed to 95% oxygen at a pressure of 2.7 ATA in an hyperbaric chamber for a total of 3×20 minute periods, interspersed with 5 minute periods of air breathing; this being a usual HBO treatment protocol at this hospital.

Venous blood samples were taken -via an indwelling catheter in the forearm- before HBO exposure, after each 20 minute period of HBO breathing, and upon decompression to ambient pressure breathing air. The blood samples were immediately transferred to 4 mm i.d. quartz ESR tubes (Wilmad Glass Company, inc.) and frozen in liquid nitrogen (-196°C).

Samples to control for the effect of pressure were taken outside of the chamber, transferred to the chamber at 2.7 ATA, and similarly frozen.

ESR analysis of the samples was performed using a JEOL FE-3X spectrometer at X-band (9.122 GHz) with: modulation frequency 100 KHz; modulation width 6.3 Gauss; temperature -150°C; scan rate 31.25 Gauss/min.; sensitivity 2×1000; response 1 s.

In Vitro Studies

Preparation of Red Blood Cells

Human blood was obtained by venepuncture, collected in lithium heparin coated tubes, and centrifuged. After removal of plasma and buffy layer, red blood cells were washed three times with phosphate buffered saline, containing 5 mM glucose, and resuspended in an equal volume of the same buffer. Studies were performed using such red blood cell suspensions incubated at 37°C in a water bath, with portions transferred to quartz ESR tubes and frozen in liquid nitrogen at appropriate times for subsequent analysis by ESR.

Origin of the Free Radical Signal

Blood was fractionated into plasma, red cells and white cells by centrifugation. Plasma and freeze/thaw lysed red cells were extracted with twice their volume of ethyl acetate. Red cell membranes were isolated from the lysed cells by repeated centrifugation and washing. Plasma and red cell proteins were denatured, using acetonitrile, and isolated. Each fraction was analysed at low temperature by ESR spectroscopy.

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Requirement for Oxyhaemoglobin

Red blood cell suspensions were saturated first with oxygen for 10 minutes followed by carbon monoxide for 10 minutes and finally by a 1:1 mixture of oxygen and carbon monoxide.

Involvement of Glutathione Reductase

Red blood cell suspensions were pre-incubated with either 0 or 1 mM 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Bristol Laboratories), for 20 minutes, followed by saturation with oxygen for between 20 and 40 minutes.

Effect of Glutathione Depletion

Red blood cell suspensions were preincubated with N-ethyl maleimide (NEM) (Sigma), 0.3 mg/ml red blood cells, for 20 minutes, followed by saturation with oxygen for 20 to 40 minutes.

Ascorbate Radical

Ascorbate radical was generated in aqueous solution by autoxidation of 0.1 M ascorbate solution at pH7.5 as described by Lagercrantz[8]. Ascorbate radical was generated in blood by addition of ascorbic acid to well oxygenated blood at concentrations of up to 0.1 M. The presence of ascorbate radical was verified by room temperature ESR, using a flat sample cell, which confirmed the presence of the characteristic ascorbate radical spectrum doublet.

ESR

Spin concentrations were determined by reference to standard potassium peroxylamine disulphonate solutions. The *g*-values were measured by reference to a Mn^{2+} reference standard. To carry out temperature annealing frozen samples were warmed to around $-15°C$ in a ice/salt bath for ten minute periods. Treated samples were subsequently re-analysed by ESR at $-150°C$.

Statistics

Statistical correlations were evaluated using Student's t-Test. Differences were considered significant for $p < 0.05$. Results are expressed as mean ± standard error.

RESULTS

HBO Exposure of Subjects

Control whole blood produced an ESR signal with a *g*-value of 2.004 and a magnitude corresponding to a spin concentration of 90 ± 10 nM.

Blood obtained from subjects exposed to HBO (HBO treated blood) gave a significantly increased signal magnitude after each 20 minute period of exposure ($p < 0.05$, $p < 0.01$, $p < 0.05$) with values returning to baseline levels after the decompression period (Figure 1).

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Blood samples taken at ambient pressure and frozen within the hyperbaric chamber at 2.7 ATA gave ESR spectra indistinguishable from equivalent blood samples frozen at ambient pressure.

Analysis of HBO treated blood giving the most intense ESR signals gave a g-value of 2.006 and a spin concentration greater than 500 nM (Figure 2).

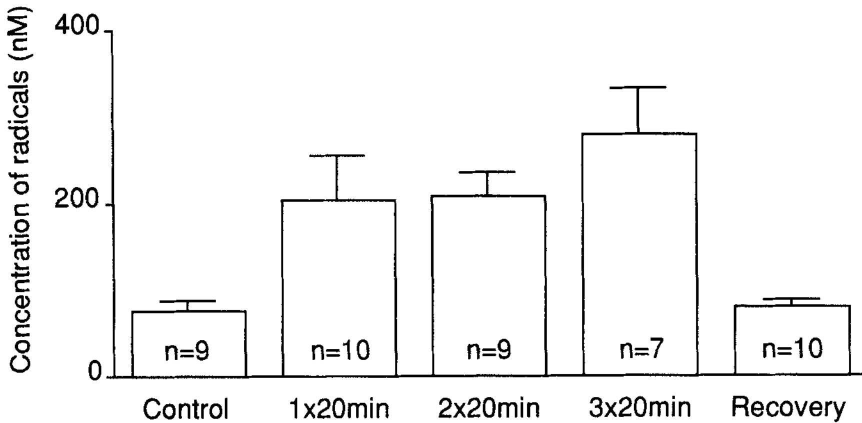

FIGURE 1 The effect of consecutive 20 minute periods of breathing 95% oxygen at 2.7 ATA in an hyperbaric chamber on the concentration of radicals in blood, as measured by ESR spectroscopy.

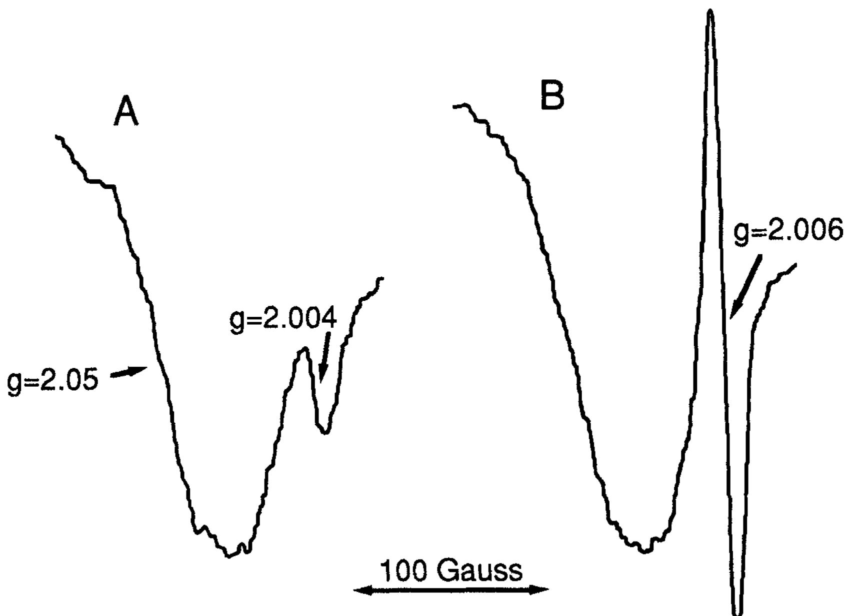

FIGURE 2 The ESR spectra of control blood (A) and blood following *in vivo* exposure to HBO (B). Control blood gave an absorption at $g = 2.05$ due to copper in caeruloplasmin, and an absorption at $g = 2.004$. HBO treatment resulted in a strong absorption at $g = 2.006$.

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In Vitro Studies of Red Blood Cells

Investigation of the various blood fractions revealed that the predominant contribution to the whole blood ESR signal at g = 2.004 was made by the red blood cells, and, more specifically, the red blood cell proteins, which gave an intense signal at $g = 2.004$.

It was further found that saturation of red blood cell suspensions with oxygen resulted in an ESR signal, having a g-value of 2.006, similar to that produced in blood following HBO treatment. Treatment with carbon monoxide was found to prevent the oxygen associated ESR signal increase (Figure 3).

The results of the incubations of red blood cell suspensions with BCNU and NEM are summarised in Figure 4. It was found that BCNU, at a concentration reported to be sufficient to inhibit erythrocyte glutathione reductase by >95%[9,10], did not result in significant changes to the red blood cell free radical signals, either before or after oxygen treatment. Treatment with sufficient NEM to bind all intracellular glutathione[11] resulted in significant ($p < 0.01$) increases in the red blood cell ESR signal levels.

The ascorbate radical generated by the autoxidation of ascorbate gave a signal in the low temperature ESR spectrum with a g-value of 2.006.

Comparative ESR Spectral Characteristics

The signal widths (at half signal height) of the upper and lower peaks of the various signals analysed are presented in Figure 5.

The changes in ESR signal intensity with changing power of μ-wave irradiation during ESR analysis (power saturation curves) for various sample signals are presented in Figure 6.

Temperature annealing of HBO treated blood, oxygen treated red blood cell suspensions and ascorbate radical solutions resulted in decreased ESR signal intensity. Temperature annealing of control blood had no effect on the ESR signal intensity (Figure 7).

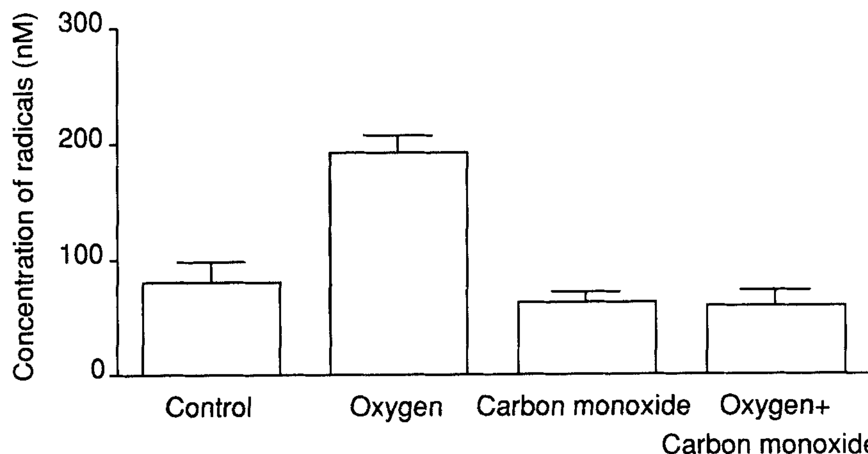

FIGURE 3 The effect of carboxyhaemoglobin formation on the ESR-measured concentration of radicals generated in red blood cell suspensions by oxygen saturation ($n = 4$).

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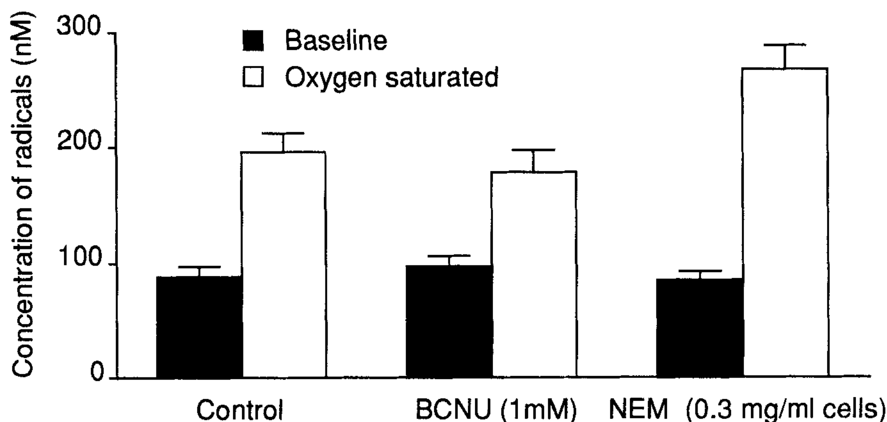

FIGURE 4 The effects of BCNU (n = 5) and NEM (n = 5) on the ESR-measured concentration of radicals in red blood cell suspensions, before and after saturation with oxygen. NEM treatment significantly increased the concentration of radicals -after oxygen saturation- compared with controls ($p < 0.01$).

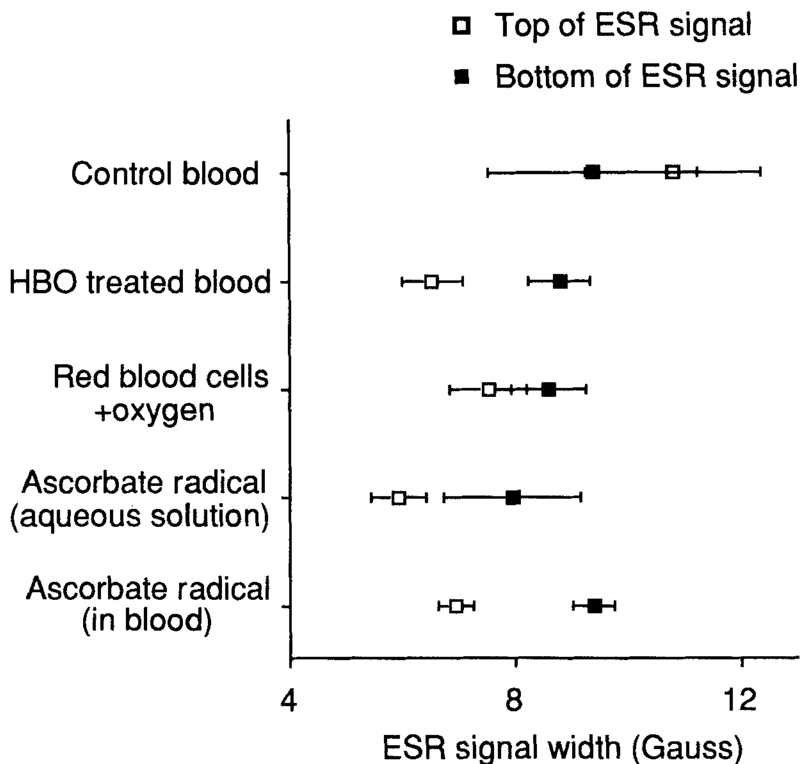

FIGURE 5 The half-height signal widths of the upper (top) and lower (bottom) components of the ESR absorption signals of the specified samples when measured at a scan width of ±500 G, modulation of 6.3 G and a scan rate of 62.5 G/min. Error bars designate 95% confidence intervals (t distribution).

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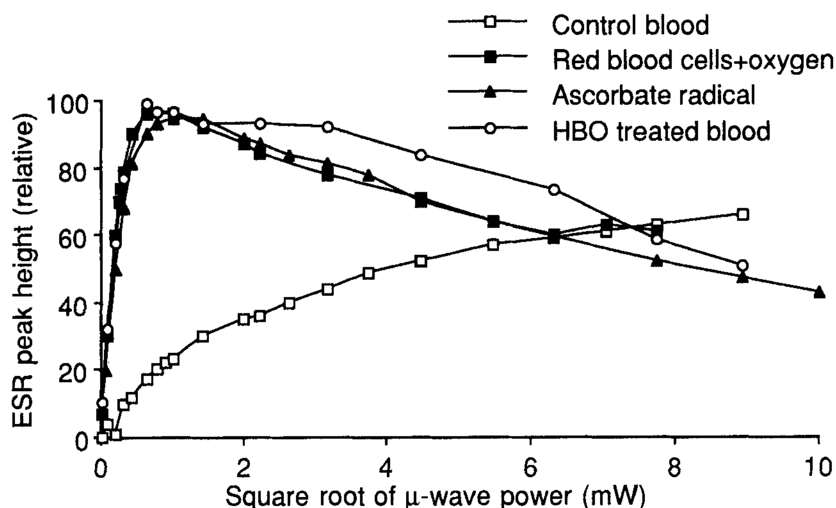

FIGURE 6 The ESR power saturation curves for various signals in the free radical region of the ESR spectrum.

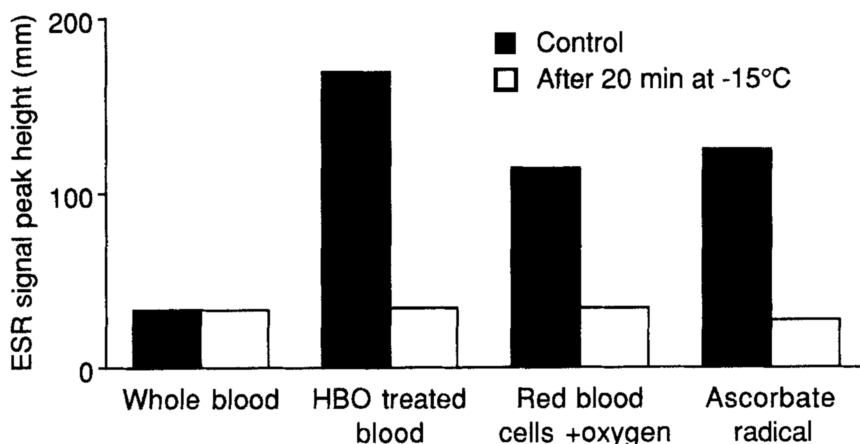

FIGURE 7 The effect of partial warming on the amplitude of various ESR signals in the free radical region of the ESR spectrum.

DISCUSSION

ESR analysis of untreated whole blood gave an absorption peak at a g-value of 2.004, consistent with that of a free radical but with power saturation occurring at high values of μ-wave power. This signal has been previously described[12,13] but not, to our knowledge, unequivocally assigned. Other major signals in whole blood at $g = 2.05$ and $g = 4$ originate in the plasma and have been attributed to Cu^{2+} in caeruloplasmin and Fe^{3+} in transferrin[14] respectively. Analysis of white cells resulted in a signal similar to that in whole blood but, given their minor volume

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in whole blood, their contribution to the whole blood signal was minimal. Red blood cells were found to be responsible for most of the signal at $g = 2.004$ in whole blood.

Blood from persons undergoing HBO exposure gave significantly increased ESR signal intensity in the free radical region of the ESR spectrum. The changes in g-value and power saturation characteristics of the ESR signal from blood following HBO treatment indicate the formation of relatively high concentrations of (a) radical species different from the radical(s) primarily responsible for the signal from control blood.

Hyperoxia is thought to result in increased oxygen radical production[6,15,16]. However, given their short half-lives and their efficient scavenging in blood, detection of oxygen free radicals by direct ESR spectroscopy, at the concentrations found in this study, would be unlikely.

Glutathione plays an important role in maintaining the reduced status of cells. It has been suggested that the free radical signal in red blood cells is due to semi-reduced flavine-adenine dinucleotide, a cofactor of glutathione reductase[13]. However, given that pre treatment with BCNU, an inhibitor of glutathione reductase, had no effect on the measured levels of free radicals, either before or after oxygen saturation, it appears that the contribution of semi-reduced flavine-adenine dinucleotide to the ESR signal in blood was not significant. Nor does glutathione radical itself appear to contribute significantly to the oxygen induced free radical signal in blood, given the failure of NEM treatment to reduce the signal intensity.

Ascorbate was investigated because it is present in red blood cells and is readily oxidised to give the moderately stable ascorbate radical (semidehydroascorbate). ESR analysis of ascorbate radical gave a signal with g-value, power saturation and temperature annealing characteristics indistinguishable from those obtained from HBO treated blood and oxygen saturated red blood cell suspensions. In aqueous solution the ascorbate radical gave ESR signals slightly narrower than the blood derived signals. However, ascorbate radical in blood gave an ESR signal with the same signal widths as the blood derived signals.

Given the above evidence, we propose that the increased free radical levels measured in blood by ESR following HBO treatment are primarily due to increased levels of ascorbate radical. It is not impossible that some other radical may have identical low temperature ESR spectral, power saturation and thermal stability characteristics to the ascorbate radical, but, given that ascorbate is among the most readily oxidised anti-oxidants[17], is present in relatively high concentrations in blood, and the ascorbate radical is moderately stable at physiological pH, ascorbate radical is the most likely candidate.

It has been reported that ascorbate scavenges superoxide, hydroxyl radical and singlet oxygen[18], and that ascorbate levels decrease dramatically in the brain[19] and the lungs[20] of rats following HBO exposure, presumably due to oxidant scavenging. So increased blood ascorbate radical levels caused by HBO treatment may be due to scavenging by ascorbate of increased levels of oxidants which, given the dependency of the free radical signal increase on oxyhaemoglobin, may arise due to increased rates of autoxidation of oxyhaemoglobin.

Oxyhaemoglobin is known to autoxidise *in vivo*, with around 3% of the body's haemoglobin oxidising per day[21]. By-products of haemoglobin oxidation include superoxide and hydrogen peroxide, with possible further production of hydroxyl radical and other radical species[22,23]. The rate of haemoglobin autoxidation, however, is reported to decrease with increasing haemoglobin saturation[24] so, if HBO

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treatment does increase autoxidation of haemoglobin, oxygen *per se* may not be the direct stimulus of this increase.

Paradoxically, however, ascorbate may act as a pro-oxidant in red blood cells[25], catalysing the redox cycling of haemoglobin, with net production of water and stimulation of the hexose monophosphate shunt. Under normal circumstances physiological concentrations of ascorbate would not be expected to have a pro-oxidant effect. However under high partial pressures of oxygen, which could promote autoxidation of ascorbate itself, circumstances may be conducive to increased redox-cycling of haemoglobin by ascorbate, with increased production of oxy-radicals, resulting in further increased levels of semidehydroascorbate.

In relation to the clinical use of HBO therapy, the elucidation of the role of ascorbate in red blood cells and the mechanism of its oxidation is of importance, given the common practice of ascorbic acid supplementation prior to HBO therapy. Measurement of ascorbate radicals in blood by ESR may also prove useful as a measure of oxidant stress in other clinical situations.

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Accepted by Professor B. Halliwell

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