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A single exposure to hyperbaric oxygen does not cause oxidative stress in isolated platelets: No effect on superoxide dismutase, catalase, or cellular ATP

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Abstract

Objectives: The aim of the study was to investigate whether a single hyperbaric oxygen exposure causes oxidative stress in isolated platelets.

Design and methods: Isolated horse platelets were exposed to 100% oxygen at 2.2 atmospheres, or 100% oxygen under normobaric conditions, or air under normobaric conditions for 90 min.

Results: There were no differences in platelet SOD activity between conditions, but there was a rise in SOD in all cases after 24 h (in control platelets at 24 h, SOD was 11.9 ± 1.9 nmol/min/mg protein compared to initial background levels of 8.2 ± 1.9 nmol/min/mg protein) ($P < 0.05$). Neither platelet catalase activity nor platelet GSH concentration changed over time, nor between conditions (catalase activity remained at around 12 units/mg protein, and GSH at around 1.58 nmol/mg protein).

Conclusions: These data suggest that a single HBO exposure has no detrimental effect on platelet biochemistry, and does not cause overt oxidative stress in vitro.
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Keywords: Catalase; Hyperbaric oxygen; Oxidative stress; Platelets; Reduced glutathione; Superoxide dismutase

Introduction

Hyperbaric oxygen (HBO) therapy can be defined as the intermittent inhalation of 100% oxygen, at a pressure greater than 1 atmosphere absolute (ATA) [1]. The therapy has been used for a number of years to treat problem wounds such as diabetic ulcerations, osteomyelitis, and radionecrosis [2–4]. Effects of HBO therapy on platelet function have been investigated in both animals and humans. For example, Amin et al. [5] delivered 100% oxygen at 2.8 ATA to rats for 6 h, and showed both platelet count and fibrinogen concentration to be increased compared with control. A decrease in ADP- and collagen-induced platelet aggregation following exposure to 100% oxygen at 2.4 ATA has been observed in rabbits [6]. Labrouche et al. [7] found an increase in both haematocrit, and platelet count in humans, following 135 min of 100% oxygen at 1.8 ATA, although no change in haemostasis was observed. In addition, arachidonic acid-dependent, but not collagen-dependent, aggregation tended to increase in humans following 20 sessions of exposure to 100% oxygen at 2.2 ATA [8].

Hyperoxia and hyperbaric oxygen therapy have been shown to result in the increased production of reactive oxygen species (ROS) [9,10], and it is well established that such ROS are involved in the process of platelet activation [11–13]. The appearance of ROS is typically measured by

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assessing either oxidative damage (e.g., lipid peroxidation, DNA damage) or antioxidant status. It has been suggested that HBO therapy may cause harm to the recipient in the form of DNA damage [14]. Hyperbaric oxygen therapy has also been shown to affect the antioxidant status of human red blood cells (SOD, catalase, ascorbate radical) [10,15], although Dennog et al. [16] found no such changes in either plasma or red blood cells; therefore, this remains controversial. To our knowledge, the effect of HBO on platelet antioxidant status has not yet been investigated. Thus, the aim of the present study was to investigate whether the antioxidant status of isolated platelets is altered following a single HBO exposure.

Methods

Platelet isolation

Platelet-rich plasma (PRP) was obtained from whole horse blood collected in acid citrate dextrose (TCS Biosciences, U.K.) using a modification of the method of Holmsen and Robkin [17]. Briefly, blood was centrifuged for 15 min at $160g_{av.}$ at room temperature (Denley BR401, UK), and the PRP was gently aspirated and washed in an equal volume of ice-cold washing solution (20 mM sodium HEPES, pH 7.4, containing 120 mM NaCl, 5 mM glucose, and 3 mM EDTA). This solution was then centrifuged for 10 min at $2000g_{av.}$ at 4°C (Denley BR401 refrigerated centrifuge, UK). The platelet-free plasma (PFP) was removed, and the platelet pellet resuspended to the original volume of the PRP in modified Tyrode's buffer (platelet buffer) (134 mM NaCl, pH 7.4, containing 12 mM NaHCO₃, 2.9 mM KCl, 0.34 mM Na₂HPO₄, 1 mM MgCl₂, 10 mM sodium HEPES and 10 mM glucose). The mean platelet count was $5.07 \pm 2.31 \times 10^7$/ml (mean ± SD; $n = 6$).

Experimental design

Isolated platelets were exposed to HBO (100% oxygen at 2.2 atmospheres), or normobaric air, or normobaric 100% oxygen as controls, for 90 min. Samples were analysed for various biochemical parameters pre-treatment, immediately post-treatment, and at 3 h and 24 h post-treatment.

Experimental protocol

Platelets were aliquoted (6 ml/dish) into twenty 60 × 15 mm Petri dishes (Becton Dickinson Ltd., UK), and left to rest at 4°C overnight to recover from the isolation procedure. After 20 h, the platelet buffer was poured off, and replaced with 6 ml of fresh buffer. Samples of platelet buffer were immediately collected (pre-treatment control) from two plates by careful pipetting to avoid disturbing the layer of cells. The samples were combined, aliquoted into

1.5-ml tubes, snap frozen in liquid nitrogen, and stored at −80°C for the later analysis of lactate dehydrogenase (LDH), lactate, and glucose. The remainder of the platelet buffer was used for the measurement of pH.

Platelets were then harvested from the plate by resuspending in 3.5 ml of fresh platelet buffer with the aid of a plate scraper. The contents of the two plates were pooled and frozen as above for the later analysis of superoxide dismutase (SOD), catalase, reduced glutathione (GSH), protein, and ATP (see below). The remaining 18 plates were then placed in three separate pressure vessels, and exposed for 90 min to (a) 100% oxygen at 2.2 atmospheres, (b) 100% oxygen under normobaric conditions, and (c) air under normobaric conditions. The flow of medical grade oxygen into the vessels was delivered by a direct feed from a low-pressure port on a diving regulator attached to a high-pressure diving cylinder (5 l × 232 bar; all oxygen cleaned). Immediately post-treatment, and at 3 h and 24 h post-treatment, samples of buffer and platelets were collected for biochemical analysis as described above.

Assays

All assays were performed in triplicate, except the GSH assay. Platelet protein [18], lactate release [19], and glucose consumption (kit 16-UV, Sigma Aldrich, U.K.) were all measured using a MRX microplate reader (Dynatech Laboratories, U.K.). Platelet SOD [20] and catalase [21] activities, and LDH activity [22] were all measured using a Perkin Elmer Lambda Bio 20 UV/Vis spectrophotometer (Perkin Elmer, U.K.). Additional experiments using Triton X-100 (0.05%) to lyse untreated cell suspensions were performed to determine the maximum LDH activity in cells. Platelet reduced glutathione concentration [23] was quantified using a Perkin Elmer LS50B fluorometer (Perkin Elmer, U.K.). Platelet ATP was measured with a commercially available kit (Biothema, Sweden) using bioluminescence (Microbeta Trilux, EG and G Wallac, Finland).

Statistics

Data were normalised by assigning the pre-treatment data a value of 1, and analysed using two-way ANOVA followed by Tukey's HSD test as a post hoc test. A probability level of $P < 0.05$ was chosen as the threshold for acceptance of statistical significance. All data are expressed as the mean value ± SD, and are for $n = 6$ replicates.

Results

There was no evidence of metabolic disturbance in isolated platelets, and cells remained viable throughout the experiment. Platelet ATP was stable between post-treatment and 3 h, but eventually declined by 24 h in isolation (Fig. 1). For example, the post-treatment ATP concentration in the
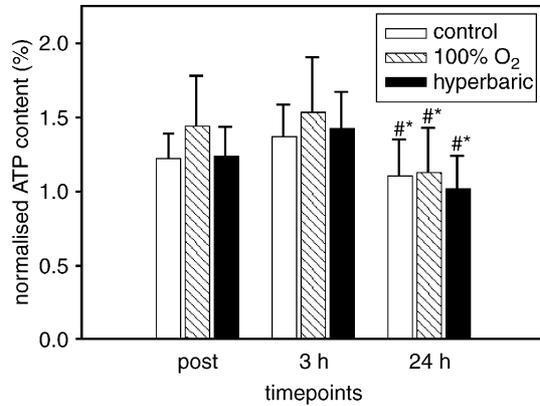
Fig. 1. The effect of oxygen on ATP content with the data (mean ± SD; $n = 6$) normalised by assigning the pre-treatment data a value of 1. ATP concentration decreased significantly in all conditions at 24 h compared with post-treatment (*$P < 0.05$) and 3 h ($^{\#}P < 0.05$), but there was no significant difference between conditions at any time point. ATP pre-treatment value was 8.53 ± 1.44 nmol/mg protein.

Fig. 2. The effect of oxygen on LDH activity, with the data (mean ± SD; $n = 6$) normalised by assigning the pre-treatment data a value of 1. LDH activity increased significantly at 24 h compared with post-treatment (*$P < 0.05$) and 3 h in all conditions ($^{\#}P < 0.05$), but there was no significant difference between conditions at any timepoint. The absolute LDH pre-treatment value was 0.4 ± 0.1 pmol/cell or, expressed as a % of the maximum determined using Triton X-100, 1.32 ± 0.48%.

HBO treatment was 10.6 ± 2.7 nmol/mg protein, and this decreased to 8.8 ± 2.4 nmol/mg protein at 24 h. However, the ATP content did not decrease below the pre-treatment value of 8.5 ± 1.4 nmol/mg protein in any of the conditions. The (approximately 20%) decrease in platelet ATP at 24 h was significantly different compared with post-treatment and 3 h within all three treatments ($P < 0.05$), but there was no condition-dependent effect ($P > 0.05$; Fig. 1). Also, this decrease was not caused by depletion of glucose from the medium, which remained close to 10 mM in all cases ($P > 0.05$). There was an expected accumulation of lactate in the medium at 24 h compared with post-treatment and 3 h ($P < 0.05$; data not shown). For example, the lactate concentration measured pre-treatment was 3.6 ± 4.8 pmol/cell, and this increased to a maximum of 44.6 ± 16.2 pmol/cell at 24 h in the HBO condition.

LDH activity was expressed as a percentage of the maximum LDH activity in the cell suspension (as determined by lysis of cells with Triton X-100) (Fig. 2). There was an expected accumulation of LDH in the medium at 24 h compared with post-treatment and 3 h ($P < 0.05$). For example, the pre-treatment LDH activity in the medium was 0.4 ± 0.1 pmol/cell (1.3%), and this increased to a maximum of 3.1 ± 1.1 pmol/cell (10.8%) at 24 h in the HBO condition. Thus, LDH release was less than 4% of the total available in the cells for most of the experiment, and although this increased by 24 h compared with post-treatment and 3 h, the LDH released remained less than 11% of the total. There were no significant differences between conditions ($P > 0.05$).

Table 1 shows that both platelet catalase activity and platelet GSH concentration did not change over time, nor was there any difference between conditions. For example, catalase activity was 13.4 ± 7.2 U/mg protein pre-treatment, and 9.2 ± 1.7 U/mg protein at 24 h in the HBO condition (Table 1). Similarly, the concentration of GSH was 1.6 ± 0.7 nmol/mg protein pre-treatment, and 1.8 ± 0.4 nmol/mg

protein at 3 h in the HBO condition (Table 1). Platelet SOD activity increased at 24 h compared with post-treatment in all three conditions ($P < 0.05$; Table 1). For example, platelet SOD activity post-treatment was 9.9 ± 3.1 nmol/min/mg protein, and this increased to 13.4 ± 5.1 nmol/min/mg protein at 24 h in the HBO condition (Table 1). There was no significant difference between conditions.

Discussion

This study shows that a single exposure of platelets to hyperbaric oxygen at 2.2 atmospheres does not cause metabolic disturbances as indicated by a stable ATP content, and low lactate production. The failure to affect either SOD or catalase activities, or the GSH content of isolated platelets implies that it is unlikely that they suffer significant oxidative stress in vitro, either as a consequence of loss of antioxidant enzyme activity, or because of depletion of

Table 1
Absolute catalase and SOD activities, and absolute GSH concentrations (mean ± SD; $n = 6$)

	Catalase (U/mg protein)	SOD (nmol/min/mg protein)	GSH (nmol/mg protein)
Pre-treatment	13.4 ± 7.2	8.2 ± 1.9	1.6 ± 0.7
HBO, post	11.4 ± 2.8	9.9 ± 3.1	1.6 ± 0.6
100% O₂, post	11.4 ± 2.9	9.1 ± 1.5	1.5 ± 0.7
Control, post	12.4 ± 4.5	9.0 ± 1.5	1.6 ± 0.4
HBO, 3 h	12.6 ± 4.7	10.8 ± 3.7	1.8 ± 0.4
100% O₂, 3 h	13.0 ± 7.0	9.3 ± 2.3	1.6 ± 0.6
Control, 3 h	11.1 ± 3.6	10.5 ± 2.0	1.4 ± 0.6
HBO, 24 h	9.2 ± 1.7	13.4 ± 5.1*	–
100% O₂, 24 h	9.4 ± 1.7	12.6 ± 1.7*	–
Control, 24 h	9.6 ± 2.3	11.9 ± 1.9*	–

SOD activity increased at 24 h compared with post-treatment in all three conditions (*$P < 0.05$).

antioxidant. To our knowledge, this is the first study to assess platelet GSH and antioxidant enzyme defences following exposure to hyperbaric oxygen.

The stability of the platelet ATP content between post-treatment and 3 h indicates that the cells were not damaged by the isolation method. It is well established that platelet concentrates must be stored at room temperature (22°C) prior to transfusion: on transfusion, platelets stored at 4°C are rapidly cleared from the blood (see Andrews and Berndt [24]). However, for the purposes of in vitro experimentation, it has been shown to be advantageous to store platelets at 4°C to maintain both ATP stability and optimum pH conditions [25]. Thus, in the present study, platelets were left to rest overnight at 4°C. The decrease in platelet ATP by 24 h would seem to suggest that platelets kept in simple physiological buffers deteriorate after 24 h at room temperature. The absence of a condition effect on platelet ATP content demonstrates that the cells were not damaged by the HBO condition. Lactate dehydrogenase activity is a reliable measure for cell viability, because it demonstrates cell membrane integrity. Thus, the absence of a condition effect on LDH release also demonstrates that the cells were not damaged by the HBO condition.

Lactate release was measured because oxidative damage to mitochondrial oxidative phosphorylation may increase reliance on glycolytic energy production [17]. Although there was an expected cumulative increase in the lactate concentration of the medium, there was no significant difference between conditions, indicating that the HBO condition did not adversely affect oxidative phosphorylation. Indeed, the lactate concentrations were low (a mean of 0.05 mmol/L at 3 h across all three conditions), and were in keeping with published values for human platelets [26]. In addition, the glucose concentration of the medium did not change over time or between conditions, and with glucose remaining around 10 mM, glucose supply therefore was not a limiting factor in platelet metabolism.

The cellular formation of reactive oxygen species (ROS) has been shown to increase during HBO therapy [10], and it has been suggested that the therapy may cause oxidative stress in the recipient [10,15]. The measurement of steady state levels of ROS is difficult; therefore, the depletion of antioxidant enzymes can be used as an indirect measurement of oxidative stress. For example, singlet oxygen and peroxyl radicals have been shown to inactivate both SOD and catalase [27]. Thus, it would be expected that an increase in ROS would result in a decrease in enzyme activity. Such a decrease in enzyme activity would suggest that antioxidant defences are partly compromised. Benedetti et al. [15] found a significant decrease in the activity of the antioxidant enzymes SOD, and catalase in red blood cells following 15 exposures to 100% oxygen at 2.5 ATA. However, they also demonstrated that the reduced glutathione (GSH) concentration, a key component of the cellular defence system (see Schafer and Buettner [28]), remained stable; thus, the issue of oxidative stress following HBO therapy remains controversial.

There was no decrease in antioxidant enzyme activity in the present study. Indeed, platelet SOD activity was increased at 24 h compared with post-treatment in all three conditions. Although platelets are able to synthesise proteins [29], such synthesis is limited, and in the present study, there were no amino acids present in the platelet buffer to promote the synthesis of new proteins. Also, according to the LDH results, there was a loss of approximately 10% of LDH by 24 h post-treatment. Superoxide dismutase is a smaller protein than LDH; thus, it would be reasonable to assume that a similar percentage of SOD was lost due to leakage. However, it must be remembered that SOD activity was measured in the present study, and not SOD protein. The assay used to measure SOD activity was of the indirect type, and thus has limitations. For example, any agent that detoxifies superoxide would be detected as SOD activity. Platelet catalase activity did not change over time, nor was there any difference between conditions. There was a slight decrease in catalase activity at 24 h but this loss was not statistically significant.

Reduced glutathione is used as a hydrogen atom donor in the reduction of both hydrogen peroxide and lipid peroxides to nontoxic products. Although most cells can tolerate a decrease in GSH levels of up to 80% without adverse consequences, it has been suggested that depletion of the mitochondrial glutathione pool may be an important trigger in the apoptotic pathway (see Slater et al. [30]). Thus, GSH is an essential component of the platelet antioxidant defence system, and platelets are known to be abundant in GSH. However, although the intracellular level of GSH may fall temporarily on exposure to an increased production of ROS, many cells are able to rapidly restore their levels (see Hall [31]). Thus, any decrease in GSH concentration may be masked, and this could explain the results of studies which did not find a decrease in GSH concentration [15]. In the present study, platelet GSH concentration did not change over time, nor was there any difference between conditions.

The data from the present study suggest that a single hyperbaric exposure does not cause oxidative stress in isolated platelets. Previous work by this group has shown that there is no decline in the capacity of platelets for oxidative phosphorylation following HBO exposure in vivo [8]. This provides indirect evidence of a lack of oxidative damage to mitochondria under these conditions (see Lenaz et al. [32]). Further work is required to determine the effect of HBO on the antioxidant status of platelets isolated from subjects exposed to HBO therapy.

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