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HYPERBARIC HYPEROXIA AND THE BRAIN *IN VIVO*: THE BALANCE BETWEEN THERAPY AND TOXICITY

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Hyperbaric oxygenation (HBO) treatment protocols utilize low pressures up to 3 ATA. Higher pressures may induce side effects such as convulsions due to brain toxicity. The optimal HBO pressure allowing for maximal therapy and minimal toxicity is under controversy. However, it can be evaluated by monitoring oxygen delivery, saturation, and consumption. In this study, the monitoring system fixed on the rats' brain cortex included a time-sharing fluorometer-reflectometer for monitoring mitochondrial NADH and hemoglobin oxygenation (HbO_2) combined with Laser Doppler Flowmetry (LDF) for blood-flow monitoring. Rats were located in a hyperbaric chamber and exposed to different pressures. The HBO pressure caused an increase in HbO_2 and a decrease in NADH in proportion to the increase in hyperbaric pressure, up to a nearly maximum effect at 2.5 ATA. At 6 ATA, 15 minutes before convulsions started, blood volume and NADH started to increase, while tissue O_2 supply by hemoglobin remained stable. Oxygen pool includes oxygen dissolved in the plasma and also bounded to hemoglobin. Above 2.5 ATA, hemoglobin is fully saturated and the oxygen pool nourishment derives only from the oxygen dissolved in the plasma, exceeding the physiological ability for autoregulation; hence, homeostasis is disturbed and convulsions appear. This information is vital because pressures around 2.5 ATA–3 ATA are standard clinically applied pressures used to treat most of the pathophysiological problems considering the potential benefit which must be balanced against the potential toxicity. This study enables, for the first time, to evaluate the oxygenation level of hemoglobin in the microcirculation. Furthermore, our study showed that additional oxygen pressure (above 2.5 ATA) caused brain oxygen toxicity within a short variable period of time after the pressure elevation.

Keywords: NADH redox state; brain tissue hemoglobin oxygenation; HBO therapy; HBO toxicity.

1. Introduction

The systemic circulation involves O_2 diffusion from the capillary bed to the mitochondria that entirely depends on the oxygen gradient between the microcirculation and the mitochondria.[1] Thus, there is a direct connection between blood and tissue

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hemoglobin saturation, which enables metabolic mitochondrial activity and affects the oxidation-reduction state of the respiratory chain.[2] Under normal conditions, most of the oxygen carried in the blood is bound to hemoglobin, which is 98% saturated in the blood, leaving the heart at sea-level pressure. However, some dissolved oxygen is carried in the plasma and, according to Henry's Law, its level is proportional to the increase in O_2 partial pressure, maximizing tissue oxygenation.[3] In our previous article,[4] we hypothesized that brain hemoglobin oxygenation (HbO_2) at the microcirculation level, is in the range of 50%–60% and in order to achieve 100% Hb oxygenation in the microcirculation, the tissue has to be exposed to hyperbaric oxygenation (HBO). Then, the O_2 consumed by the cells will be supplied by high levels of dissolved O_2. Araki *et al.*[5] found that at 2 ATA O_2 hemoglobin in the brain tissue is only 80% saturated. We showed in a previous study[4] that tissue HbO_2 reaches a maximal level at 2.5 ATA. Higher pressures did not increase significantly the tissue hemoglobin saturation and all O_2 supply to the cells was provided by the O_2 dissolved in the plasma. At higher pressures, such as 4.5 ATA and 6 ATA, tissue HbO_2 remained unchanged even when convulsion periods were noted. These pressures are known to cause oxygen toxicity manifested by convulsion periods and even death.[6–9]

The aims of this study were to verify the effects of high HBO pressures on oxygen supply (CBF and HbO_2) and on tissue oxygen balance (mitochondrial NADH redox state) and to examine the interrelation between these parameters during the development of oxygen toxicity in relation to HBO therapy.

2. Materials and Methods

The hemodynamic and metabolic functions of the cerebral cortex, were monitored by the tissue vitality monitoring system (TVMS) that includes two devices: a time-sharing fluorometer-reflectometer (TSFR) for mitochondrial NADH redox state and microcirculatory hemoglobin oxygen saturation (HbO_2) measurements combined with a Laser Doppler Flowmeter for cerebral blood flow (CBF) monitoring. Details of this monitoring system and methods have been presented in our previous articles.[2,4,10]

2.1. *Animal preparation*

All experiments were performed in accordance to the guidelines of the Animal Care Committee of Bar-Ilan University. Male Wistar rats ($n = 28$) weighing 220 g–320 g, were used. The animals were anesthetized by intraperitoneal injection of Equithesin, 0.3 mg/100 g body weight. A black delrin light-guide holder combined with silver wires, for measuring bipolar electrocorticogram (ECoG), was placed epidurally on the left parietal brain and cemented to the skull by dental acrylic cement. More details can be found in our previous study.[15] Body rectal temperature was monitored continuously using a thermistor (Yellow Springs Instruments Co. Inc., type 402), and was kept constant at 36°C–37°C. The animal was placed in

a Plexiglas cage, fitted to the rat's size, and was allowed to recover for about 30 minutes. The normal response to anoxia was checked by the NADH increase and HbO_2 decrease. Thereafter, the rat (in the Plexiglas cage) was placed in a 150-L hyperbaric chamber, containing a special cage heated by water (Bethlehem Corp. FM-21-A).

2.2. *Experimental procedure*

Four hours after anesthesia, in a fully-awake state, the experiment started. The chamber was flushed with 100% oxygen, to establish normobaric hyperoxia (NH) for 15 minutes at atmospheric pressure (cleaning phase) in order to prevent acid–base disturbances and physiological CO_2 elevation in blood. Then, the oxygen pressure was elevated at a rate of 1 atmosphere per minute to a maximum pressure of the hyperbaric hyperoxia (HH). The respective maximum pressures were applied to 4 groups of animals. Group 1 ($n = 7$) was exposed to 2.5 ATA for 140 minutes. Ten minutes after decompression, the animals were exposed to pure N_2 inhalation until death. Group 2 ($n = 7$) was subjected to 4.5 ATA until the appearance of the first tonic-clonic convulsions, or until a maximum of 140 minutes, and then decompression was performed. Ten minutes later, the animals were exposed to pure N_2 inhalation until death. Group 3 ($n = 7$) was pressurized to a maximum of 6 ATA. The animals were left in the chamber until their death which occurred approximately 0.5 hour after the compression induction. An additional group of rats ($n = 7$), the control group of normobaric normoxia (NN), underwent the same preparation and procedures. The rats were monitored at 0.2 ATA air in the chamber for 2.5 hours without pressure and were exposed to pure N_2 inhalation at the end of the experiment.

2.3. *Data collection, processing and statistical analysis*

Since neither of the optical signals is calibrated in absolute units, we set all signals to read 100% after connecting the brain to the monitoring system in the normoxic state, and measured the percentage change of the signal. Data were collected and stored in computer files, using LabView A/D hardware and software (National Instruments Inc., USA). Simultaneously, electrocorticogram (ECoG) and electrocardiogram (ECG) were recorded on a polygraph.

All statistical analyses and calculations were done by Matlab software (Ver. 2006b, The MathWorks, Inc.). The latency time for the first convulsion was measured from the experiment initiation until the appearance of the first convulsion, or until a maximum of 166 minutes (a maximum of 140 minutes under pressure). The distribution of the time until the first convulsion was found to be abnormal. Therefore, we used the Wilcoxon signed-rank test to calculate the changes in the appearance of the first convulsion between the pressure groups. The median amplitudes at 5, 20, and 30 minutes after experiment initiation (in the 2.5 ATA and 6 ATA groups) and 5, 10, and 15 minutes before the first convulsion (in the 6 ATA

group), were calculated. In the 2.5 ATA group, the times until convulsions (for comparative calculations) were set at 61 minutes from the experiments' initiation, since this is the median time to the first convulsion in the 6 ATA group. Additionally, the differences between the control and 2.5 ATA groups as well as between 6 ATA and 2.5 ATA groups at specific time points were tested by the Student t-test. A value of $p < 0.05$ was considered significant.

3. Results

Four levels of pressure were selected: 1.75 ATA and 2.5 ATA (utilized for clinical treatments) and two high pressures, 4.5 ATA and 6 ATA, known for their toxic effects.[4,9,11] Figure 1 shows the effects of 2.5 ATA compared to the control group at 0.2 ATA, on CBF, reflectance, mitochondrial NADH fluorescence, and HbO_2. The experiments started from normal pressure (0.2 ATA), then, pressure was elevated gradually starting from NH at 100% O_2 and continued to hyperbaric pressure (2.5 ATA). Our results showed that 2.5 ATA had no toxic effects during the experiments' period. Starting with 100% O_2, HbO_2 and reflectance increased significantly ($p < 0.01$, $p < 0.001$, respectively), whereas NADH oxidized (decreased) significantly ($p < 0.001$). Under elevated pressure (2.5 ATA), a further augmentation in HbO_2 and reflectance was observed ($p < 0.001$). Also, HbO_2 reached a steady-state level about 5 minutes after the compression started. Parallel to these

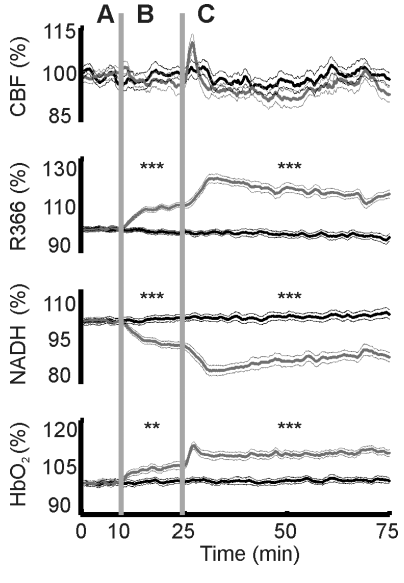

Fig. 1. The effect of 100% O_2 and 2.5 ATA O_2 (gray line) as compared to normobaric normoxia (black line). A–normobaric normoxia; B–100% O_2 and C–2.5 ATA. Data are presented as Median ± MAD. CBF–cerebral blood flow; R366–reflectance at 366 nm and NADH–mitochondrial NADH redox state; HbO_2–brain tissue hemoglobin saturation.
**= $p < 0.01$, ***= $p < 0.001$.

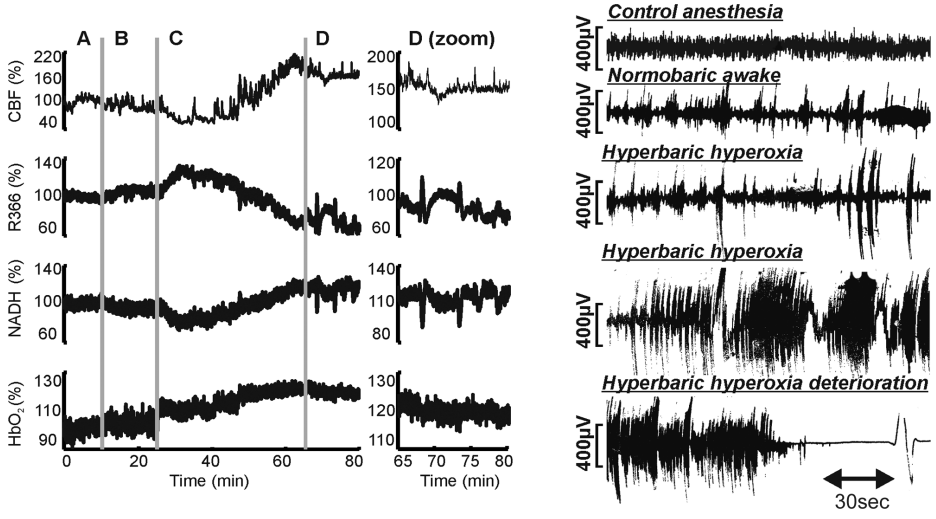

Fig. 2. Illustrative traces showing the effect of 6 ATA on: left side–the measured parameters; right side–the ECoG during the different phases of the experiment. Abbreviations are as described in Fig. 1 including A–Control; B–100% O_2; and C–high pressure (6 ATA); D–the first convulsion period during 6 ATA and D (zoom)–enlargement of the convulsion periods.

changes, mitochondrial NADH oxidized and reached a steady-state. No significant changes were observed in CBF. Figure 2 presents an illustrative experiment, showing the effect of high pressures (6 ATA) on the measured parameters (left side) and on ECoG (right side). Trace D is zoomed to illustrate the epileptic convulsions and their effects on the measured parameters. High pressure (6 ATA) initially yielded changes identical to 2.5 ATA (Fig. 1) until the toxic effects started. At the 50th minute under 6 ATA, HbO_2 reached its maximal amplitude and remained stable until the end of the experiment (even during the epileptic convulsions). Fifteen minutes after 6 ATA induction, CBF and NADH started to increase whereas reflectance decreased and reached the maximum changes few minutes before the first convulsion was observed. The increase in pressure was followed by pre-convulsion ECoG spikes that became amplified and synchronized over time (Fig. 2, right). Figure 2 (right) shows 5 ECoG traces of one rat exposed to 6 ATA, from the beginning of the experiment (control anesthesia), during HH (6 ATA), the convulsions period and until death. As seen, ECoG showed clear synchronized tonic-clonic convulsions (the last 2 right-hand traces), synchronization of high amplitude activation that terminated in a silent trace (the last trace).

In the 2.5 ATA group, no convulsions occurred; at 4.5 ATA, 3 of the 7 animals developed convulsions and 2 of the 3 died. In contrast, in the 6 ATA group, all the animals developed convulsions and died during 166 minutes of measurement. Thus, the gradual decrease in latency is parallel to the pressure increase. The differences in the latency time to the first convulsion between 4.5 ATA and 6 ATA pressure groups were assessed by the Wilcoxon signed-rank test and were found to be significant

($p < 0.016$). For the statistical calculations, animals in the 4.5 ATA group that did not develop convulsions during the 166 minutes period, were considered to develop the first convulsion at 166 minutes. Not only the convulsion latency (time) decreased with increasing pressure, but the time until oxygen toxicity death also diminished. In the 6ATA group, all the animals died during the recorded time (between 78 and 148 minutes of experiment), whereas in the 4.5 ATA group, only two animals died very near to the end of the experiment (at 158th and 165th minutes within the 166 minutes of recording).

Substantial points, after the experiments' initiation, were chosen over the 2.5 ATA and 6 ATA time course, in order to find the pre-convulsion significant changes in the measured parameters (Table 1). Since during 2.5 ATA application, no convulsions were observed. To find the parallel time for comparing the changes in the measured parameters between the two pressures, we used the median time to the first convulsion in the 6 ATA group, which was 61 minutes into the experiment. The effects of 2.5 ATA and 6 ATA pressures on the measured parameters were similar from the beginning of the experiment and lasted until 10 minutes before the first convulsion. Ten and five minutes before the convulsions started, CBF and NADH increased significantly ($p < 0.05$) while the reflectance significantly decreased ($p < 0.05$). The HbO_2 levels did not change at both 2.5 ATA and 6 ATA despite the convulsions at 6 ATA (Table 1).

4. Discussion

This study examines the relationship between microcirculatory blood flow, tissue reflectance, HbO_2, and intracellular mitochondrial NADH redox state under normoxia (21% O_2), normobaric hyperoxia (100% O_2), and variable levels of hyperbaric hyperoxia (HH). The combination of these parameters may significantly contribute to the understanding of brain energy metabolism *in vivo* at various levels of oxygen supply above normoxia. We tested the hypothesis that the mitochondrial

Table 1. Median amplitude values (%) ± MAD of the measured parameters, at selected time points before the first convulsion started. Abbreviations as described in Fig. 1. min = minutes and * = $p < 0.05$.

		Median % Values ± MAD at time before the first convulsion		
Parameter	Pressure (ATA)	15 (minutes)	10 (minutes)	5 (minutes)
CBF	2.5	90 ± 6	91 ± 6	96 ± 4
(%)	6	106 ± 14	113 ± 27*	101 ± 45
Ref-366	2.5	118 ± 4	118 ± 5	115 ± 4
(%)	6	113 ± 20	108 ± 19	94 ± 14*
NADH	2.5	87 ± 3	87 ± 3	88 ± 3
(%)	6	87 ± 17	93 ± 15	107 ± 13*
HbO_2	2.5	107 ± 2	108 ± 2	109 ± 2
(%)	6	111 ± 19	115 ± 23	116 ± 37

NADH is not fully oxidized in the normoxic brain *in vivo* and can be oxidized even further under HH conditions. The majority of studies investigating the effect of HBO focused on monitoring intracranial pressure (ICP), CBF, EEG, and blood pressure.[12] Consequently, important information was to be gained on the relationship between brain tissue O_2 delivery, O_2 consumption and utilization (mitochondrial NADH redox state).

In previous works,[8,9,13–15] we showed that the reflectance changes occurring immediately after increasing oxygen levels are due to changes in the hemodynamic responses tending towards vasoconstriction (decrease in blood volume). This vasoconstrictive effect was shown by a reduction in CBF and a significant increase in reflectance, indicating a significant decrease of blood volume in the awakened rat brain under various oxygen levels, immediately following an elevation in brain oxygenation (Fig. 1). A decrease in blood volume was also found in our previous studies.[4,14,15] In contrast, under high pressures, at the beginning of convulsions (at 4.5 ATA and 6 ATA), the CBF increased, while the reflectance decreased showing an increase in blood volume (clearly seen at 6 ATA in Fig. 2, left). Similar reductions in reflectance under high pressures were shown previously.[4,8,9,13–15] The effect of HBO on CBF was found to be highly dependent on the CBF monitoring method. A detailed discussion of this issue can be found in our previous article.[4] Studies using Laser Doppler Flowmetry[16–19] revealed no changes or only a small decrease or increase under exposure to 1 ATA–4 ATA. At pressures above 4 ATA, in the first few minutes, the CBF decreased and returned to the base line within a variable period. Several minutes before convolutions started, the CBF rose significantly (Fig. 2, left).

Van Hulst *et al.*[20] found that tissue pO_2 increases in a linear relation to the increase in the hyperbaric chamber O_2 pressure, due to the high plasma pO_2. We established that the tissue HbO_2 reached a maximal value at 2.5 ATA. Pressures above 2.5 ATA did not alter the tissue hemoglobin saturation.[4] In addition, mitochondrial NADH reaches a plateau concomitantly to tissue HbO_2. At 2.5 ATA, NADH oxygenation is maximal and in linear correlation to HbO_2. When increasing the hyperbaric pressure above 2.5 ATA, a reduced NADH molecule will be quickly transformed into an oxidized form[4] (see Figs. 1 and 2, left). A similar oxygenation effect was observed in our previous studies during various pressures ranging from 3 ATA to 11 ATA.[8,9,11,13–15] Pressures above 4 ATA caused oxygen toxicity within a variable period after the pressure induction. Similar results have been obtained by others.[8,9,11,13,14,16] The results of the present study show that during pressures higher than 4 ATA, the latency time to the first convulsion and death, decreases with increasing pressure. Thus, there is a gradual effect of oxygen pressure on the brain: the higher the pressure — the faster the emergence of convulsions and the shorter the time until oxygen toxicity and death.

In addition, under 6 ATA, 10 minutes before the onset of convulsions, mitochondrial NADH redox state increased (Table 1), even though CBF rose and reflectance decreased, which represent an increase in the blood flow and volume. Similar results

have been reported by other groups.[5,7,9,12,16,18,19] Furthermore, HbO_2 levels did not change during 6 ATA as compared to 2.5 ATA (Table 1) showing no decrease in oxygen supply. The increase in mitochondrial NADH may result from mitochondrial dysfunction due to oxygen toxicity triggered by the high pressures, since other works showed NADH oxidation during convulsion.[21–23] The convulsions period is very variable among animals, but in all the animals, we found spreading depression cycles.[8,9,11,13,14] The post-convulsive phase starts with the stops of the convulsions and flattening of the ECoG until death (Fig. 2, right).

In conclusion, the present work confirms the following: (1) The maximum saturation (100%) of hemoglobin in the tissue is achieved at 2.5 ATA and higher hyperbaric pressures produce no further hemoglobin oxygenation. (2) During pressures higher than 4 ATA, increasing pressure shortens the time to oxygen toxicity. (3) Multiparametric monitoring (CBF, mitochondrial NADH redox state, and tissue HbO_2) is important for detecting tissue oxygen toxicity during hyperbaric treatment, since monitoring only CBF and HbO_2 without NADH, impedes the identification of tissue oxygen toxicity.

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