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## The acute antinociceptive effect of hyperbaric oxygen is not accompanied by an increase in markers of oxidative stress

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### Abstract

**Aims**—Exposure to hyperbaric oxygen (HBO<sub>2</sub>) causes an antinociceptive response in mice. However, breathing oxygen (O<sub>2</sub>) at an elevated pressure can potentially cause oxygen toxicity. The aim of this study was to identify the determinants of HBO<sub>2</sub> antinociception and the toxicity profile of HBO<sub>2</sub>.

**Main methods**—Male NIH Swiss mice were assessed for acute antinociceptive responsiveness under room air or 100% O<sub>2</sub> at 1.0 or 3.5 atmospheres absolute (ATA), using the acetic acid-induced abdominal constriction test. For the oxygen toxicity test, mice were exposed to 3.5 ATA oxygen for 11 min, 60 min, 60 min daily for 2 days (120 min) or 60 min daily for 4 days (240 min), then assessed by analyzing the levels of two oxidative stress markers, MDA (malondialdehyde) and protein carbonyl in brain, spinal cord and lung.

**Key Findings**—Only the combination of 100% O<sub>2</sub> and 3.5 ATA caused significant antinociception. The antinociceptive effect of 100% O<sub>2</sub> was pressure-dependent up to 3.5 ATA. In the oxygen toxicity test, mice exposed to HBO<sub>2</sub> for different time intervals had levels of brain, spinal cord and lung MDA and protein carbonyl that were comparable to that of control animals exposed to room air.

**Significance**—Treatment with 100% O<sub>2</sub> evokes a pressure-dependent antinociceptive effect. Since there was no significant increase in levels of the oxidative stress markers in the tested tissues, it is concluded HBO<sub>2</sub> at 3.5 ATA produces antinociception in the absence of oxidative stress in mice.

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## Keywords

hyperbaric oxygen; antinociception; oxidative stress; malondialdehyde; protein carbonyl

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## Introduction

Hyperbaric oxygen (HBO<sub>2</sub>) therapy has been approved by the FDA for a limited set of clinical indications [Gesell, 2008]. However, there are a number of clinical reports whereby HBO<sub>2</sub> therapy appears to be effective in a broader range of conditions, including several examples of chronic pain [Peach, 1995; Kiralp et al., 2004; Yildiz et al., 2004; Handschel et al., 2007]. There is clinical evidence for an analgesic effect of HBO<sub>2</sub>; however, the mechanism of this analgesic action is poorly understood.

There have been few animal studies exploring the efficacy of HBO<sub>2</sub> in the relief of pain, and these have focused largely on the anti-inflammatory rather than antinociceptive effects of HBO<sub>2</sub> [Warren et al., 1979; Sümen et al., 2001]. Other studies measured both antinociception and anti-inflammatory effects [Wilson et al., 2006]. However, the antinociception effect in this animal model did not manifest itself until nearly two hours after the HBO<sub>2</sub> treatment.

We have reported that treatment of mice with HBO<sub>2</sub> @ 3.5 atmospheres absolute (ATA) resulted in a very rapid-onset antinociceptive effect (i.e., within 5 min). However, whether this biological response is the effect of the 100% oxygen (O<sub>2</sub>) or both is unclear. It is equally uncertain whether the antinociceptive effect is associated with an oxidative stress induced by reactive species of oxygen and nitrogen (N<sub>2</sub>) as a result of HBO<sub>2</sub> treatment [Thom, 2009].

The present study was conducted to determine the optimal conditions for expression of the antinociceptive response to acute exposure to HBO<sub>2</sub>, ascertain whether the antinociceptive effect was pressure-dependent, and assess the possible oxidative injury caused by our hyperbaric oxygen exposure.

## Materials and Methods

### Animals

Male NIH Swiss mice, weighing 18–22 g, were purchased from Harlan Laboratories (Indianapolis, IN) and used in these experiments, which were approved by an institutional animal care and use committee and carried out in accordance with The Guide for the Care and Use of Laboratory Animals, 8th Edition (National Academies Press, Washington, DC, 2010).

Mice were housed five per cage in the AAALAC-accredited Wegner Hall Vivarium with access to food and water ad libitum. The facility was maintained on a 12-h light:dark cycle (lights on 0700–1900 h) under standard conditions (22°C room temperature, 33% humidity). Mice were kept in the holding room for at least four days following arrival in the facility and prior to experimentation. All measures to minimize pain or discomfort were taken by the investigators.

### Exposure to Hyperbaric Oxygen

Five groups of eight mice each were treated with 0.6% glacial acetic acid, placed in the hyperbaric chamber, exposed to 100% O<sub>2</sub> at 1.0, 2.0, 2.5, 3.0 or 3.5 ATA for 5 min, and assessed for antinociceptive responsiveness for 6 min for a total of 11 min of HBO<sub>2</sub> treatment. Mice were placed in a B-11 research hyperbaric chamber (Reimers Systems, Inc.,

Lorton, VA) as previously described [Zelinski et al., 2009]. The chamber was ventilated with 100% O<sub>2</sub>, U.S.P. (A-L Compressed Gases Inc., Spokane, WA) at a flow rate of 20 L/min to minimize nitrogen and carbon dioxide accumulation. The pressure within the cylindrical clear acrylic chamber (27.9 cm diameter × 55.9 cm L) was increased at a rate of 1.0 ATA/min to the desired pressure and maintained for 11 min (5 min for onset of nociception plus 6 min antinociceptive testing). The mice were allowed to breathe spontaneously during HBO<sub>2</sub> treatment. After completion of the HBO<sub>2</sub> exposure, mice were then decompressed at a rate of 1.0 ATA/min. Control groups of mice were exposed to room air, and experimental groups of mice were exposed to either compressed air (A-L Compressed Gases) or 100% O<sub>2</sub> circulated through the chamber at either 1.0 or 3.5 ATA and maintained for 11 min. Decompression occurred as described above.

In determining whether the antinociceptive effect of 100% O<sub>2</sub> was pressure-related, the pressure of the HBO<sub>2</sub> was varied at 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 ATA. In groups of control animals, the pressure of compressed air was set at 1.0, 2.0 or 3.5 ATA.

### Antinociceptive Testing

Antinociceptive responsiveness was assessed using the abdominal constriction test as previously described [Zelinski et al., 2009]. Mice were treated i.p. with 0.1 ml per 10 g body weight of 0.6% glacial acetic acid and placed into the hyperbaric chamber. Exactly 5 min later, the number of abdominal constrictions—lengthwise stretches of the torso with concave arching of the back—in each animal was counted for 6 min while under HBO<sub>2</sub>. Multiple raters were used for some but not all experiments; at least one of the raters was blinded to the drug treatment. All experiments were consistently conducted between 1300 and 1700 h. The control reference group was exposed to room air. The degree of antinociception (inhibition of abdominal constrictions) produced in various treatment groups of mice was calculated as:

$$\% \text{ antinociception} = 100 \times \frac{\# \text{ constrictions in control mice} - \# \text{ constrictions in treated mice}}{\# \text{ constrictions in control mice}}$$

### Measurement of Lipid Peroxidation and Protein Carbonyl

Mice were exposed to HBO<sub>2</sub> @ 3.5 ATA for 11 min, 60 min, 120 min (60 min daily for 2 days), 240 min (60 min daily for 4 days) or room air (eight mice were used for each treatment), then ketamine/xylazine anesthetized immediately after the final HBO<sub>2</sub> exposure. Then the mice were perfused with phosphate buffered saline (PBS) containing heparin to remove the blood. The tissue was collected and resuspended with PBS containing 1 mM EDTA and homogenized on ice at 50 mg/mL. After spinning at 10,000 × g for 5 min at 4°C, the supernatant was collected for assay. Levels of malondialdehyde (MDA) were determined, using a commercially available colorimetric assay for lipid peroxidation (TBARS Assay Kit, Cayman Chemical Company, Ann Arbor, MI). The absorbance at 530–540 nm was measured to obtain the concentration of MDA. Protein carbonyls were measured using a commercially available quantitative protein C carbonyl assay kit (Cayman Chemical, Ann Arbor, MI). The sample to be assayed was homogenized in 50 mM phosphate buffer containing 1 mM EDTA, pH 6.7, and then centrifuged at 10,000 × g for 5 min under 4°C. The supernatant was collected for assay. The protein concentration was determined using a BCA protein assay kit. The MDA content and protein carbonyl content were normalized to protein concentration for each sample. The purpose of the EDTA was to inhibit further production of these oxidative markers during the measurement process. Therefore, the oxidative marker levels in Tables 1 and 2 are tissues levels at the time of death.

For the positive control, each sample from room air group were homogenized (with buffer without EDTA), centrifuged, and then incubated with H<sub>2</sub>O<sub>2</sub> (5 mM), similar to Sewerynek et al. [1995]. Briefly, the homogenates were incubated with H<sub>2</sub>O<sub>2</sub> for 60 min at 37°C, then placed into ice for 10 min to stop the reaction. Then the homogenates were centrifuged at 13,200 × g for 5 min at 4°C. The supernatants were collected for assay.

### Statistical Analysis of Data

One-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to compare the antinociception data and levels of MDA and protein carbonyl in groups exposed to different HBO<sub>2</sub> regimens. Unpaired t-test was used to determine the difference between antinociceptive effects of HBO<sub>2</sub> and compressed air at the same pressure.

## Results

### Basic Characterization of HBO<sub>2</sub>-Induced Acute Antinociception

Mice were randomly assigned to four different treatment groups: normobaric air (NBA; room air or compressed air @ 1.0 ATA), normobaric oxygen (NBO<sub>2</sub>; room air replaced by 100% O<sub>2</sub> @ 1.0 ATA), hyperbaric air (HBA; compressed air @ 3.5 ATA) and hyperbaric oxygen (HBO<sub>2</sub>; 100% O<sub>2</sub> @ 3.5 ATA). Fig. 1 shows that NBA, NBO<sub>2</sub> and HBA treatment groups had no demonstrable antinociceptive response. While in the HBO<sub>2</sub> treatment group, increasing the pressure to 3.5 ATA resulted in a 93.8 ± 2.2% antinociceptive effect. This was significantly different from all three control groups.

### HBO<sub>2</sub>-Induced Antinociception Is Pressure-Dependent

Four groups of eight mice each were treated with 0.6% glacial acetic acid, placed in the hyperbaric chamber, exposed to 100% O<sub>2</sub> at 1.0, 2.0, 2.5, 3.0 or 3.5 ATA for 5 min, and assessed for antinociceptive responsiveness for 6 min for a total of 11 min of HBO<sub>2</sub> treatment. Fig. 2 shows a linear relationship between the pressure of the O<sub>2</sub> exposure and the percent antinociceptive effect.

### Effect of HBO<sub>2</sub> on Levels of Oxidative Stress Markers in Brain, Spinal Cord and Lung

To determine whether the designed hyperbaric oxygen exposure caused oxidative injury in mice, the oxidative injury was assessed by analyzing the levels of two oxidative stress markers, MDA (malondialdehyde) and protein carbonyl in the brain, spinal cord and lung after HBO<sub>2</sub> treatment @ 3.5 ATA for 11, 60, 120 or 240 min. There were no significant differences in MDA and protein carbonyl levels between mice exposed to HBO<sub>2</sub> and NBA mice in brain, lung and spinal cord (Table 1). The positive controls, on the other hand, showed a significant increase in levels of MDA and protein carbonyl.

## Discussion

HBO<sub>2</sub> therapy has been approved by the American Medical Association (AMA) and the Food and Drug Administration (FDA) for the treatment of the following fourteen conditions: air or gas embolism; carbon monoxide poisoning and smoke inhalation; carbon monoxide poisoning complicated by cyanide poisoning; clostridial myonecrosis (gas gangrene); crush injury, compartment syndrome and other acute traumatic ischemias; decompression sickness (the “bends”); enhancement of healing in selected problem wounds; exceptional blood loss (anemia); necrotizing soft tissue infections; refractory osteomyelitis; radiation tissue damage (osteoradionecrosis); compromised skin grafts and flaps; thermal burns; and idiopathic sudden sensorineural hearing loss [Gesell, 2008; Murphy-Lavoie et al., 2012]. But there are also clinical reports and anecdotal evidence of HBO<sub>2</sub> efficacy in a much broader range of clinical conditions [Helms et al., 2011].

Although HBO<sub>2</sub> treatment is not currently indicated in treatment of pain per se, HBO<sub>2</sub> therapy is reported to induce an analgesic effect in relief of various types of pain in human subjects, including complex regional pain syndrome [Peach, 1995; Kiralp et al., 2004], fibromyalgia [Yildiz et al., 2004], headache [Wilson et al., 1998; Di Sabato et al., 1997], rheumatoid arthritis [Rui-Chang, 1994], chronic osteomyelitis [Handsichel et al., 2007] and radiotherapy of cancer [Jones et al., 2006; Dall'Era et al., 2006]. The mechanism of this clinical analgesic effect of HBO<sub>2</sub> treatment is not known, although some have attributed this effect of HBO<sub>2</sub> to its anti-inflammatory properties [Weisz et al., 1997; Yang et al., 2006].

Animal studies related to HBO<sub>2</sub> treatment of pain have been limited. Earlier studies focused largely on the anti-inflammatory rather than the analgesic effect of HBO<sub>2</sub> [Warren et al., 1979; Sümen et al., 2001]. More recently, inflammatory pain induced by carrageenan in rats was evaluated after HBO<sub>2</sub> exposure at 2.4 ATA for 90 min [Wilson et al., 2006, 2007]. HBO<sub>2</sub> treatment was found to reduce inflammation (as determined by reduced paw swelling) as well as mechanical hypersensitivity (as determined by increased threshold for paw withdrawal) [Wilson et al., 2006]. The ability of HBO<sub>2</sub> treatment to reduce inflammation and pain was comparable to that of acetylsalicylic acid treatment [Wilson et al., 2007].

In humans breathing air normally at sea level the alveolar pO<sub>2</sub> (pAO<sub>2</sub>) is approximately 102 mm Hg and breathing 100% oxygen it increases to 673 mm Hg. During HBO<sub>2</sub> therapy the patient breathes 100% O<sub>2</sub> and the alveolar pO<sub>2</sub> increases rapidly as the pressure in the hyperbaric chamber increases, so that at 2.0 ATA and at 2.5 ATA the pAO<sub>2</sub> increases to 1433 mmHg and to about 1813 mmHg respectively, the latter a 17-fold increase as compared to breathing air at 1.0 ATA [Jain, 1999]. These increases are attained within minutes, thus, the tissue O<sub>2</sub> concentrations correspondingly increase in a rapid manner.

The onset of the antinociception in this study occurred within 5 minutes of the exposure of the mice to HBO<sub>2</sub> and continued for another 6 minutes during the abdominal constriction test. It is possible that full antinociception may not have occurred within the 5 minutes of HBO<sub>2</sub> exposure prior to the testing period, especially during the lower pressures applied during the pressure-response experiments. However, there did not appear to be any noticeable time difference between periods of abdominal constrictions in the animals, and the pressure-response curve was linear.

Oxygen toxicity is the main physiologic limitation on the use of HBO<sub>2</sub>-enriched breathing mixtures for medical applications and scientific, technical and military diving [Hampson and Atik, 2003]. It has been argued that oxidative stress is fundamental to HBO<sub>2</sub> therapy [Thom, 2009]. Metabolic activation of molecular oxygen, as well as the chemical and physical interaction of cells with their environment, frequently give rise to reactive oxygen species (ROS), such as superoxide, hydrogen peroxide or singlet oxygen [Halliwell and Gutteridge, 1984]. Low levels of ROS result in an adaptation response by increasing cellular antioxidant activity. Higher levels that overwhelm the antioxidative capacity of the cells cause oxidative damage manifesting as protein oxidation, lipid peroxidation, DNA injury and membrane damage, eventually culminating in disease and death.

As our previous studies have routinely treated mice with HBO<sub>2</sub> @ 3.5 ATA [Zelinski et al., 2009; Ohgami et al., 2009; Chung et al., 2010; Quock et al., 2011], we have to ascertain whether the HBO<sub>2</sub> treatment induced oxidative stress. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, such as MDA [Esterbauer, 1985]. MDA is a naturally occurring product of lipid peroxidation. Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals and is used as an

indicator of oxidative stress in cells and tissues and the measurement of thiobarbituric acid reactive substances (TBARS) is a well-established method for screening and monitoring lipid peroxidation [Yagi, 1998; Armstrong and Browne, 1994]. The most general indicator and, by far, the most commonly used marker of protein oxidation is protein carbonyl content [Stadtman and Oliver, 1991]. The most convenient procedure is the reaction between DNPH and protein carbonyl. DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically.

Our results showed that levels of both lipid peroxidation and protein carbonyl were not significantly increased after the HBO<sub>2</sub> exposures, even following HBO<sub>2</sub> @ 3.5 ATA for 60 min daily for 4 days. It is known that higher pressures and longer exposure times of HBO<sub>2</sub> treatment can lead to oxygen toxicity to various organs, such as the brain [Liu et al., 2012] and the lung [Sun et al., 2011], but whether our exposure procedure produced oxygen toxicity was not previously known. HBO<sub>2</sub> treatment can activate antioxidant as well as oxidant systems. Whether oxygen toxicity is induced depends on the balance of these antioxidant/oxidant systems. It would appear that our 11-min and 60-min HBO<sub>2</sub> exposures didn't disrupt this balance. It is more interesting that even the daily 60-min HBO<sub>2</sub> treatments for 2 days or 4 days also failed to produce oxygen toxicity. Recently the preconditioning effect of HBO<sub>2</sub> has been widely explored. It was found that HBO<sub>2</sub> pretreatment could prevent oxidative injuries to organs [Yan et al., 2013; Liu et al., 2011]. Speit and Bonzheim [2003] implicated heme oxygenase-1 in HBO<sub>2</sub>-induced protection of A549 cells against oxygen toxicity induced by a second HBO<sub>2</sub> treatment 24 h later. Recently it was reported that daily exposure to 90 min HBO<sub>2</sub> @ 2.8 ATA for as many as 40 days failed to significantly elevated levels of MDA, protein carbonyl and glutathione peroxidase in the cerebral cortex, inner white matter and cerebellum [Simsek et al., 2012]. These findings, albeit at a lower atmospheric pressure than that applied in the present study, as well as our results are consistent in demonstrating a lack of oxygen toxicity. It is possible that in the present study, the 24-h interval between each HBO<sub>2</sub> exposure prevent the accumulation of oxygen toxicity, but the adaptive and preconditioning actions induced by earlier HBO<sub>2</sub> exposure(s) may contribute to these results.

We acknowledge that DNA damage is also an important marker of oxidative stress, and DNA strand breaks could be a site of oxidative damage from HBO<sub>2</sub> exposure. Endogenous antioxidant levels are other important indicators of oxidative injury. These indicators should be measured when we further explore the oxidative/antioxidative status and the measurement of NO and other ROS would also accumulate our knowledge on this aspect. Also, this study was conducted in rodents and the results need to be reassessed when applied to humans.

## Conclusions

The antinociceptive effect of 100% O<sub>2</sub> was pressure-dependent up to 3.5 ATA. Mice exposed to HBO<sub>2</sub> @ 3.5 ATA for 11 min, 60 min, 60 min daily for 2 days or 60 min daily for 4 days failed to show a significant increase in either protein carbonyl or MDA in the brain, spinal cord or lung. It is concluded that HBO<sub>2</sub> at 3.5 ATA is able to produce significant antinociception without causing oxygen toxicity in mice. This also suggests that the HBO<sub>2</sub>-induced antinociception is not associated with oxidative stress.

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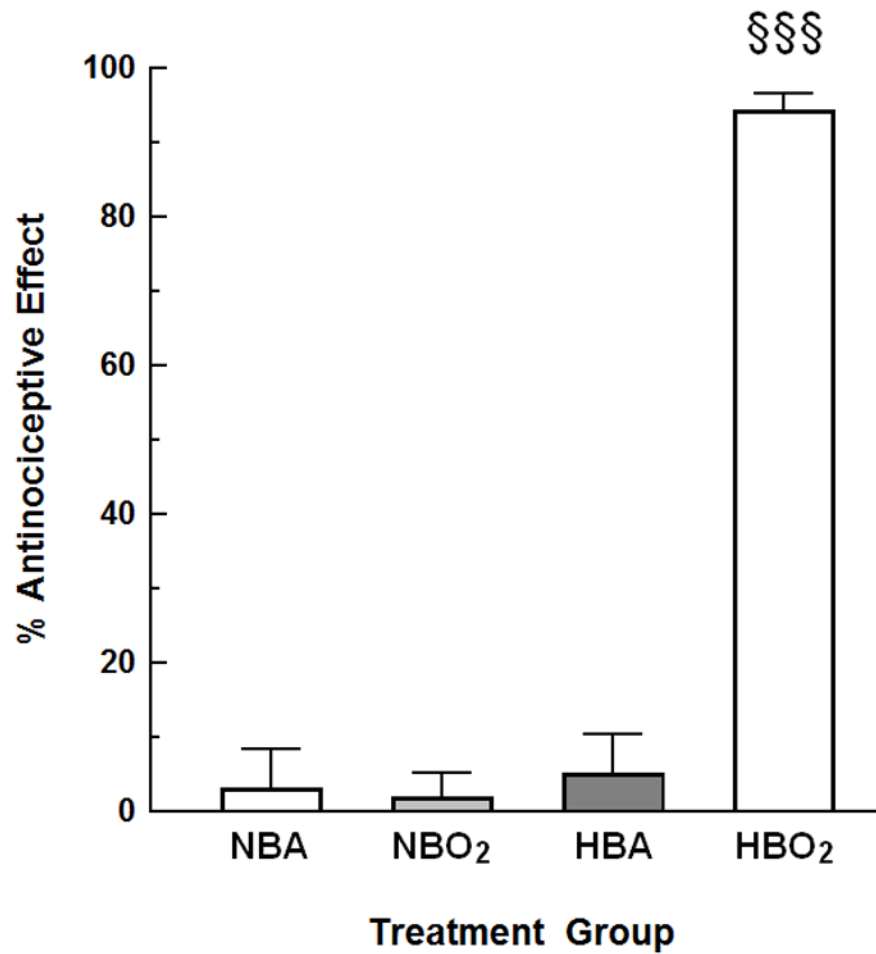
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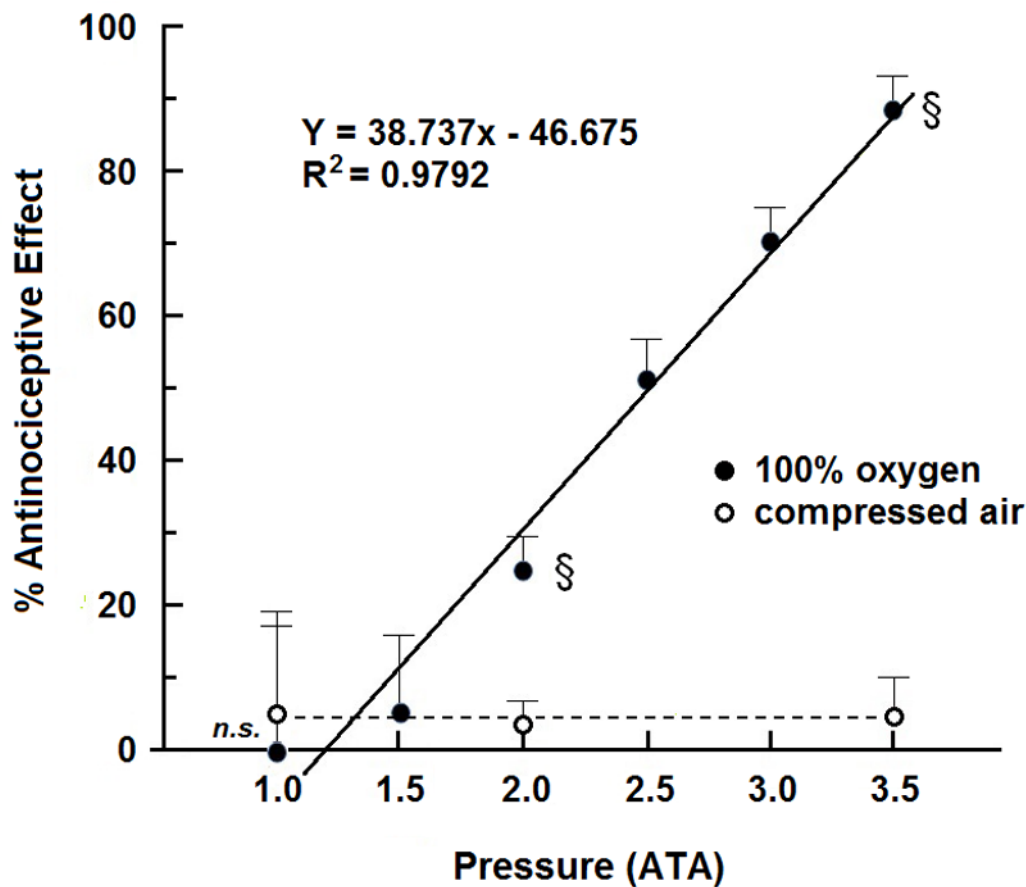
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**Fig. 1.**

The effect of oxygen and/or hyperbaric pressure on nociception in mice. Groups: NBA, normobaric (room) air; NBO<sub>2</sub>, normobaric (100%) oxygen; HBA, hyperbaric (room) air (3.5 ATA); and HBO<sub>2</sub>, hyperbaric oxygen (100% oxygen at 3.5 ATA). The data are expressed as the mean ± S.E.M. of 8–13 mice per group. Significance of difference: §§§,  $P < 0.001$ , compared to NBA, NBO<sub>2</sub> and HBA treatment groups (one-way ANOVA and post-hoc Bonferroni's multiple comparison test). There were no significant differences among NBA, NBO<sub>2</sub> or HBA.



**Fig. 2.**

The influence of increasing pressure on oxygen-induced antinociceptive effect in mice. Each symbol (solid square for compressed air and solid diamond for 100% oxygen) represents the mean antinociceptive response of 8 mice at each pressure. The solid line shows the linear relationship between percent antinociceptive effect to 100% O<sub>2</sub> and pressure; the dotted line shows that pressure alone fails to evoke an antinociceptive effect in mice exposed to compressed air. Significance of difference: §,  $P < 0.05$ , *n.s.*, not significant, compared to compressed air at the same pressure (unpaired t-test).

Table 1

Influence of HBO<sub>2</sub> Treatment on Tissue Levels of Oxidative Stress Markers

<i>Influence of HBO<sub>2</sub> on Tissue MDA Levels (nmol/mg protein)</i>						
Tissue	NBA	11 min HBO <sub>2</sub>	60 min HBO <sub>2</sub>	120 min HBO <sub>2</sub>	240 min HBO <sub>2</sub>	Positive Control
Brain	9.16 ± 0.85	7.30 ± 1.06	7.81 ± 1.77	8.23 ± 2.19	8.62 ± 2.47	19.88 ± 3.88 <sup>a</sup>
Spinal Cord	7.01 ± 1.74	8.33 ± 1.12	7.15 ± 1.21	7.75 ± 1.24	7.18 ± 1.78	17.51 ± 5.21 <sup>a</sup>
Lung	6.23 ± 1.54	7.15 ± 2.89	6.73 ± 1.76	7.48 ± 2.38	7.00 ± 1.99	29.52 ± 8.89 <sup>a</sup>

<i>Influence of HBO<sub>2</sub> on Tissue Protein Carbonyl Levels (nmol/mg protein)</i>						
Tissue	NBA	11 min HBO <sub>2</sub>	60 min HBO <sub>2</sub>	120 min HBO <sub>2</sub>	240 min HBO <sub>2</sub>	Positive Control
Brain	8.68 ± 3.64	9.95 ± 3.24	8.68 ± 2.28	8.16 ± 1.25	8.34 ± 2.44	24.54 ± 3.21 <sup>a</sup>
Spinal Cord	8.42 ± 0.83	8.66 ± 3.48	9.44 ± 2.99	8.01 ± 2.20	8.13 ± 2.60	20.98 ± 2.63 <sup>a</sup>
Lung	6.64 ± 1.98	7.55 ± 2.07	8.40 ± 1.65	6.85 ± 3.10	9.35 ± 2.41	36.94 ± 18.13 <sup>a</sup>

Significance of difference:

<sup>a</sup> p < 0.01, compared to the Room Air control group (one-way ANOVA and post-hoc Bonferroni's multiple comparison test).