NTNU – Trondheim
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Heliox Saturation Diving to 500kPa. Effects of Saturation Diving on Oxidative Stress in a Rat Model

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ABSTRACT

Formation of vascular gas bubbles following decompression has for a long time been considered to be the main initiator of decompression related illness. However, the presence of "silent bubbles" may suggest another underlying cause to the problem. Oxygen has toxic properties that are particularly prominent under hyperbaric conditions, producing reactive oxygen species (ROS) that alter physiological functions. **Aim and hypotheses:** The aim of this study was to examine the effects of hyperbaric conditions on oxidative stress in a rat model. It was hypothesized that lipid peroxidation would increase, and antioxidant concentrations of SOD and catalase would decrease in response to simulated saturation diving. **Methods:** Forty rats were divided into four groups; two diving groups and two respective control groups. Heart and pulmonary artery of the rats were scanned for vascular gas bubbles, *in vivo*, for one or four hours after surfacing to indicate decompression stress. Blood was drawn and serum measurements of TBARS, SOD and catalase were performed. **Results:** No vascular gas bubbles were detected, except for one rat with maximum bubble grade 2-3. There were no significant differences in TBARS or SOD concentrations between the four groups. Catalase concentrations did however differ, with significantly lower concentrations in diving groups compared to the respective control groups. **Conclusion:** Bubble formation, lipid peroxidation and SOD were not significantly affected by hyperbaric exposure, thus the applied dive profile does not seem to cause excessive oxidative stress to the rats.

ACKNOWLEDGEMENTS

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Trondheim, July 2014

Ingunn Lie

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INTRODUCTION

Many underwater tasks are too complex to be carried out by machines, and humans must undergo challenging deep dives to complete different work assignments. Saturation diving involves being in a closed environment with a high ambient pressure and elevated oxygen pressure for several days, maybe weeks. Even though there is a strict focus on safety in today's diving improvement of procedures are necessary to reduce the risk of adverse health effects. Health problems related to diving are often connected to changing behavior of gases under pressure and knowledge about these mechanisms is crucial to practice safe diving.

Diving and the pathophysiological effects of decompression and hyperoxia

Several physiological functions are challenged by altered breathing gas-composition and by the demand for rapid gas exchange in body tissues in response to variation in ambient pressure during a dive (Brubakk et al., 2005, Nossum et al., 2002). When diving using compressed gas, the gas is taken up into tissues in proportion to the depth and duration of the dive until tissue saturation is reached. While returning to the surface a reduction of pressure (decompression) occurs, and dissolved gas can diffuse across membranes and be eliminated from the body through expired air. If the pressure is reduced faster than the gas can be eliminated a state of supersaturation occurs, and gas will come out of solution in form of bubbles. Supersaturation and bubble formation are in some cases associated with pathological consequences (Philp, 1974, Brubakk et al., 2005, Francis and Mitchell, 2003), and studies have revealed a correlation between increasing amount of bubbles and likelihood of adverse effects of decompression (Eftedal et al., 2007). Such observations have been done in both venous and arterial vasculature where endothelial function was reduced (Brubakk et al., 2005, Nossum et al., 2002). The amount of vascular bubbles can be measured with ultrasonic devices and graded on a scale from 0 to 5 accordingly: 0: no bubbles, 1: an occasional bubble, 2: at least one bubble every fourth heart cycle, 3: at least one bubble every heart cycle, 4: continuous bubbling, and 5: massive bubbling (Eftedal and Brubakk, 1997). It has long been thought that vascular gas bubbles are the main cause of adverse effects by decompression, but it seems that the bubble grade is not a reliable measure for which divers will develop certain types of decompression related illnesses (Neuman,

2002). Decompression from any depth cause some degree of bubbles, but in the majority of cases the diver seems not to be harmed (Lippmann and Mitchell, 1991). This indicates that bubbles alone are not enough to cause the different adverse effects seen following decompression, but that their presence could aggravate an already vulnerable situation that originates during a dive (Madden and Laden, 2009). A lack of clear conclusions concerning bubble induced injury have led to the assumption that adverse effects originate during the compression phase of a dive, and that bubbles caused by decompression will worsen the situation. A diver undergoes hyperoxia, which is a higher than normal partial pressure of oxygen, that can lead to oxygen toxicity (Clark and Thom, 2003). When breathing oxygen at elevated partial pressure the oxygen can have numerous harmful effects. The present study elucidates the occurrence of oxidative stress as a possible consequence of hyperoxia, and how this can be related to adverse health effects.

Oxidative stress and the generation of reactive oxygen species (ROS)

The vital oxygen we breathe can under certain conditions become toxic (Fridovich, 1998, Clark and Thom, 2003). During the reduction of oxygen to water, O_2 gives rise to reactive intermediates called reactive oxygen species (ROS). These molecules are partly responsible for the toxicity of oxygen. Oxidants are formed as natural products of aerobe metabolism, but can under pathophysiological conditions be produced at elevated rates. The term “oxidative stress” is used to describe the situation where oxidants overexert the cumulative activity of oxidant defense systems in the body (Sies, 1997, Turrens, 2003). Overexertion of this antioxidant capacity can potentially lead to damage.

ROS are so-called free radicals that involve oxygen, and a free radical is an atom or a molecule that have one or more unpaired valence electron (Valko et al., 2007). The unpaired electrons give the free radicals an extremely unstable configuration, which makes them highly chemically reactive to other radicals and biological molecules as they seek to achieve a stable configuration. Radicals derived from oxygen are the most important class of radicals within living systems. The oxygen molecule, O_2, is by itself a stable molecule and is not very reactive with other compounds. However, during metabolic processes of reduction of oxygen to water, the mitochondrial electron transport chain can leak electrons to oxygen prematurely and form superoxide anion radicals ($O_2^{\bullet -}$):

$$O_2 + e^- \rightarrow O_2^{-}\bullet \quad (1)$$

Superoxide is a ROS, but also acts as a mediator in oxidative chain reactions to produce even stronger oxidants (Turrens, 2003). Especially the reaction between H_2O_2, a natural by-product of oxidative metabolism, and $O_2^{-}\bullet$ in the presence of certain metals yields the highly reactive hydroxyl radical (•OH), the most reactive radical known in chemistry:

$$O_2^{-}\bullet + H_2O_2 \rightarrow \bullet OH + OH^- + O_2 \quad (2)$$

OH• reacts as soon as it comes in contact with other molecules (Halliwell, 1991). These radicals are able to attack and damage almost all molecules found in living cells, and they do so in less than a microsecond after being generated.

Antioxidant defense systems

Cells have a variety of defenses against the harmful effects of ROS, and among them are antioxidants, which are molecules that inhibit oxidation of other molecules (Santos et al., 2005, Benzie, 2000). They are needed in biological systems because of the constant production of reactive radicals originated from aerobe metabolism. There are both non-enzymatic (vitamin E and C, glutathione) and enzymatic (superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px)) antioxidant defenses. Detoxification of radicals by different defense mechanisms is essential because oxidation of lipids, DNA and proteins change both structure and functions of cellular components and result in mutation, cell damage and cell death. Different lines of defense systems have evolved to counter this problem (Clark and Thom, 2003). These include, among others, the enzymatic avoidance of production of reactive intermediates during reduction of molecular oxygen to water, termination of chain reactions of fatty acid radicals by formation of stable radicals, and repair processes like reactivation of oxidized enzymes and reduction of oxidized tissue components.

The present study emphasizes on the enzymatic antioxidant defense systems with a focus on SOD and catalase. SODs are a group of enzymatic antioxidants that protect against intracellularly

produced reactive oxygen species as they remove $O_2^{-}\bullet$ by catalyzing a dismutation reaction (Halliwell, 1991):

$$O_2^{-}\bullet + O_2^{-}\bullet + 2H^{+} \rightarrow H_2O_2 + O_2 \qquad (3)$$

The reaction involves oxidation of one $O_2^{-}\bullet$ to oxygen, and reduction of another $O_2^{-}\bullet$ to hydrogen peroxide. As mentioned earlier, and visualized in equation (2), H_2O_2 itself is a radical able to produce the highly reactive •OH, and thus removal of both $O_2^{-}\bullet$ and H_2O_2 is biologically advantageous. The reduction of H_2O_2 is accomplished by catalase and peroxidase. Catalase catalyze the conversion of H_2O_2 into oxygen and water (H_2O):

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad (4)$$

Peroxidase on the other hand uses different reductants to reduce H_2O_2 to H_2O (Fridovich, 1998). Figure 1 gives a schematic overview of the described enzymatic antioxidant defense systems.

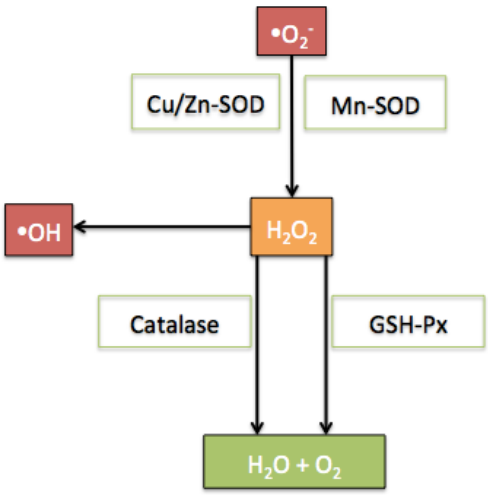

Figure 1. Schematic presentation of the main enzymatic antioxidants defence systems that deals with oxidative stress. Cu/Zn-SOD is within the cytosol, while Mn-SOD is in mitochondria. Both catalase and GSH-Px (glutathione peroxidase) convert hydrogen peroxide into water and oxygen.

Both hyperoxia and inert gas bubbles formed during decompression are likely to trigger oxidative stress (Obad et al., 2010, Obad et al., 2007, Ikeda et al., 2004). Pre-dive administration of non-

enzymatic antioxidants has been shown to counteract this, supporting the involvement of oxidative stress in diving-induced reduction of different biological functions. Hyperoxia increases the production of free radicals (Jain, 1990), and it has been shown that as the oxygen concentration increases the rate of mitochondrial O_2^{-}• -production increases linearly (Turrens, 2003). The oxidant damage seen in different cellular components and membranes due to increased oxygen pressure are however primarily caused by secondary generation of more reactive intermediates, and not directly by O_2^{-}• and H_2O_2 (Clark and Thom, 2003). Human studies have shown alterations in antioxidant enzyme activity following saturation dives, where an increase in catalase activity indicates occurrence of increased oxidative stress (Zwart et al., 2012).

Oxidative damage: lipid peroxidation

ROS are capable of damaging all macromolecules including lipids, proteins and DNA (Koeppen and Stanton, 2009). A well-known effect of oxygen radicals is damage to cellular membranes through a process known as lipid peroxidation. All cells are surrounded by a plasma membrane that separates it from the extracellular environment. The membrane creates a barrier that allows the cell to keep an interior composition different from the composition of the extracellular fluid. Free radicals can produce a variety of functional consequences by interaction with the plasma membrane (Clark and Thom, 2003). Peroxidation of membrane lipids, oxidation of proteins, and inactivation of membrane-bound enzymes can increase the permeability of the membrane and result in loss of important membrane functions. In addition, more toxic radicals can be produced during destruction of the membrane. •OH is best known for stimulating the free radical chain reaction that trigger lipid peroxidation, which disrupts biological membranes and damage their function and structure (Halliwell, 1991). This happens when the hydroxyl radical is generated close to membranes and attacks the membrane phospholipids. The removal of a hydrogen atom from a biological molecule leaves behind an unpaired electron on the atom where the hydrogen was attached (Halliwell and Chirico, 1993). In the case of lipid peroxidation the abstraction of hydrogen from lipids results in the formation of a lipid radical (L•) that is converted to a lipid peroxyl radical (LOO•) by oxygen fixation (Klaassen and Casarett, 2008). Further follows a cascade of reactions producing several reactive free radicals and endogenous toxicants (Figure 2). These substances can react with adjacent molecules or diffuse to more distant sites such as DNA.

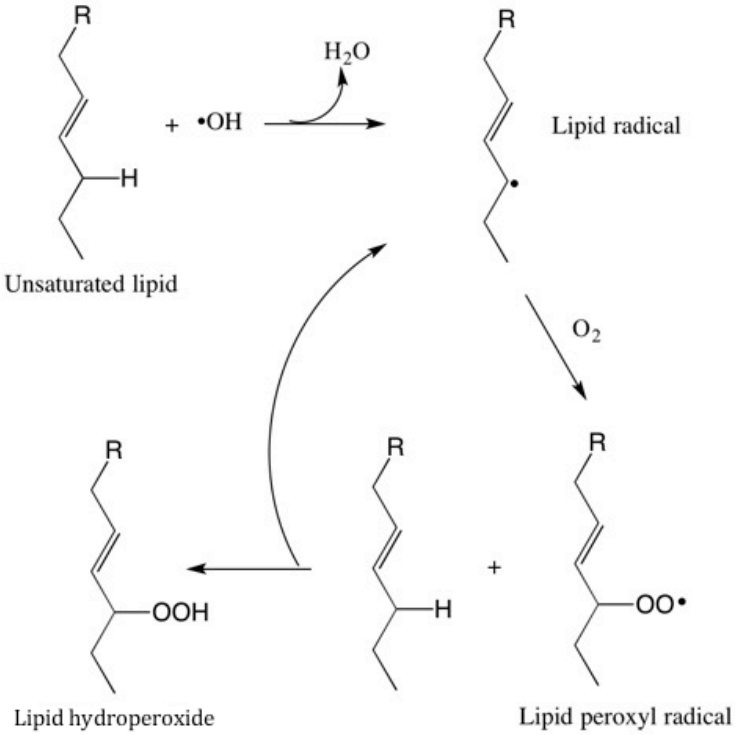

Figure 2. Molecular mechanism of lipid peroxidation; a hydroxyl radical removes a hydrogen atom from an unsaturated lipid, producing a lipid radical. This lipid radical is transformed into a lipid peroxyl radical by oxygen fixation. Lipid peroxyl radicals can react with adjacent molecules and produce several reactive radicals, propagating lipid peroxidation.

One single •OH can cause the conversion of hundreds of fatty acid side chains into lipid peroxides and result in collapse of the membrane (Halliwell, 1991).

Under normobaric conditions, i.e. when breathing at sea level, inspired air has an O_2 partial pressure of approximately 21 kPa (160 mmHg). In rats, exposure to hyperbaric oxygen has been shown to cause increased levels of lipid by-products in blood and other tissues (Oter et al., 2005). Also in humans, exposure to 250 kPa of oxygen revealed indications of oxidative stress by increased values of lipid peroxidation products and ROS in blood plasma (Benedetti et al., 2004). Direct detection of ROS is difficult as they are short-lived and highly reactive, but oxidative damage as a consequence of ROS can be analyzed through measurement of secondary products (Devasagayam et al., 2003, Nyska and Kohen, 2002). A variety of assays can measure different by-products from lipid peroxidation, and one common method is estimating aldehydic products from decomposition of lipid hydroperoxides, which reacts with thiobarbituric acid (TBA) and

form thiobarbituric acid reactive substances (TBARS). Thus, TBARS are markers of lipid peroxidation, which can further be measured with spectrophotometry.

The role of ROS in human health and disease

ROS are recognized to play a dual role as both deleterious and beneficial species. Free radicals are known to be a part of the pathophysiology of several human diseases as well as ageing (Devasagayam et al., 2004). But it is important to emphasize that ROS are important to help maintain cellular homeostasis in normal healthy tissues, and function as important signaling molecules. Most cell types seem to generate low concentrations of ROS when they are stimulated by growth factors and hormones, which give us the assumption that ROS are important signal molecules in different signals transduction cascades (Valko et al., 2007). Oxidative burst also occurs in inflammatory environments where ROS play a key role in the defense against environmental pathogens. Under such circumstances the enzyme NADPH oxidase produce superoxide radicals to help kill bacteria. Programmed cell death, known as apoptosis, is important for destruction of cells that appear harmful to an organism. One mechanism is increased levels of oxidants that give internal signals that trigger apoptosis.

In addition to ROS, it is worth mentioning the reactive nitrogen species (RNS) as a part of human health and disease. Nitric oxide (NO•) is a free radical produced as a part of normal physiology (Clark and Thom, 2003). It serves as an intercellular messenger that is responsible for vasodilation and relaxation of smooth muscle cells, and is therefore among other things an important radical in blood pressure regulation. In spite of its key role in cellular health, NO• is capable of causing damage by production of peroxynitrite (ONOO-) due to reaction with O_2^{-}• (Szabó, 2003). Peroxinitrite is an even stronger oxidant than both NO• and ONOO- and seems to be an important contributor to various forms of inflammatory diseases.

ROS is also a challenge in situations with altered ambient pressure, such as in diving. Hyperoxia is known to increase ROS production, and higher levels of several biological markers of oxidative stress are observed after deep dives (Obad et al., 2010). Oxygen radicals can reduce the bioavailability of NO by reacting with it or impairing the function of NO synthase through

interaction with cofactors important for its function. These are effects that can compromise vascular function. The exact mechanism of endothelial dysfunction following diving is unknown, but hypoxia-induced oxidative stress is a likely candidate for contribution to the problem. Also liver dysfunction has been observed as a consequence of oxidative stress during saturation diving (Ikeda et al., 2004). Studies with oral supplement of antioxidants seem to counteract the detrimental effects of ROS on liver- and endothelial function, an observation that strengthen the assertion of ROS being responsible for these destructive effects (Ikeda et al., 2004, Obad et al., 2007). Impairment of endothelial functions seems to become more prominent after repetitive dives (Obad et al., 2010). Despite normally being asymptomatic these effects can pose a potential risk for acute cardiovascular incidents.

Aim and hypothesis

The aim of the present study was to examine the effect of simulated saturation diving on oxidative stress markers and antioxidant enzymes in the circulatory system in rats. Three biological oxidative stress markers (catalase, mitochondrial SOD, TBARS) were analyzed in rat serum. It was hypothesized that lipid peroxidation would increase in blood from rats exposed to a single bout of simulated saturation diving, and that protein levels of SOD and catalase would show an acute decrease as these proteins were consumed in response to increased oxidative stress. Vascular gas bubbles were measured to indicate decompression stress following saturation diving.

MATERIALS AND METHODS

Ethical approval

The local Animal Research Ethics Authority at the Norwegian University of Science and Technology gave ethical approval for the experiment. Prior to upstart of the work the FELASA category C animal researcher certificate was obtained.

Experimental animals

A total of 40 female Sprague Dawley rats divided into 4 groups were used for this experiment. This is an animal-model much used in decompression experiments, and previously established methods for ultrasound measuring, bubble grading and blood sample collection in rats are available (Wisløff et al., 2003, Eftedal et al., 2012). The species is suitable for studies of circulation changes because the similarity of its circular system to that of humans (Suckow et al., 2005).

Table 1. Mean body weight of all four groups of rats presented with standard deviation (SD). HeO-1: dive group with one hour observation time, Ctrl-1: control group with one hour observation time, HeO-4: dive group with four hours observation time, Ctrl-4: control group with four hours observation time.

Group	Mean (g)	SD
HeO-1	272.2	26.5
Ctrl-1	269.8	15.3
HeO-4	273.8	15.1
Ctrl-4	271.1	15.1

The rats delivered by Taconic (Lille Skensved, Denmark) were transported to the lab four weeks prior to the experiment for acclimatization. They were kept in cages with a volume of 30 liters in groups of five rats per cage. The room where the animals were held had a circadian rhythm of 12 hours light- and darkness, with a relative humidity of ~62 % and an ambient temperature of 21 ± 1°C. The rats had free access to food and water, and their bedding was changed every 14th day. From arrival to the end of the experiment the rats had daily supervision of people that were responsible for the welfare of the animals.

Simulated diving protocol

The rats were randomly assigned to 4 groups of 10 individuals each on the day of experiment. The rats had a body weight (BW) of 236-327 g. (mean BW of 271.7 g., Table 1, Appendix A). Two of the groups were exposed to hyperbaric heliox (500 kPa, 90% He and 10% O_2) in a 22 L hyperbaric chamber (Figure 3) to simulate conditions of human saturation diving. These groups were called HeO-1 and HeO-4 according to their exposure to hyperbaric heliox and observation time of either 1 or 4 hours. The two remaining groups were assigned to be the controls and were named Ctrl-1 and Ctrl-4 according to their observation time; they were treated simultaneously and identically to those exposed to simulated diving, but only breathing normobaric air (normal air at 100 kPa).

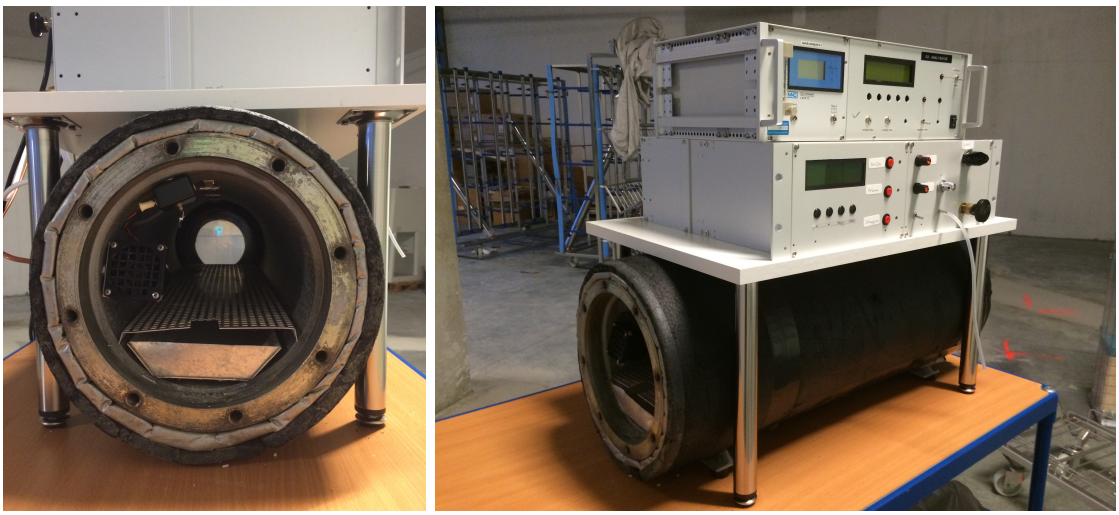

Figure 3. Pressure chamber used for rat dives.

The experiment took place over a period of two weeks where group HeO-1 and Ctrl-1 were run in parallel the first week, while group HeO-4 and Ctrl-4 were run in parallel the following week. Four rats went through the procedures each day; two controls rats and two dive rats from each parallel group. The dive profile (Table 2, Figure 4) was designed for the rats to gain full gas saturation, and held a decompression phase that was not expected to cause death or acute injury after ended dive (Skogland et al., 2002). Compression rates were 30 kPa/min until the maximum pressure of 500 kPa was reached, and the oxygen pressure was maintained at 50kPa until decompression. The bottom time was 180 minutes, calculated from start of compression to start of decompression. Decompression rates were 300 kPa/min with a stop at 160 kPa to rapidly flush the chamber with 100% oxygen before further decompression to the surface. This was done in order to keep sufficient breathing gas oxygen pressure, as the decompression was very rapid. The temperature in the chamber was 22 ± 1°C for all simulated dives.

Table 2. Dive profile with compression rate, pressure, dive time and decompression rate presented together with observation period for all four groups. The gas mixture at maximum pressure was 90% helium (450kPa) and 10% oxygen (50kPa).

Group	Compression rate (kPa/min)	Maximum dive pressure (kPa)	Time at maximum pressure (min)	Decompression rate (kPa/min)	Observation period (h)
HeO-1	30	500	180	300	1
Ctrl-1	0	0	0	0	1
HeO-4	30	500	180	300	4
Ctrl-4	0	0	0	0	4

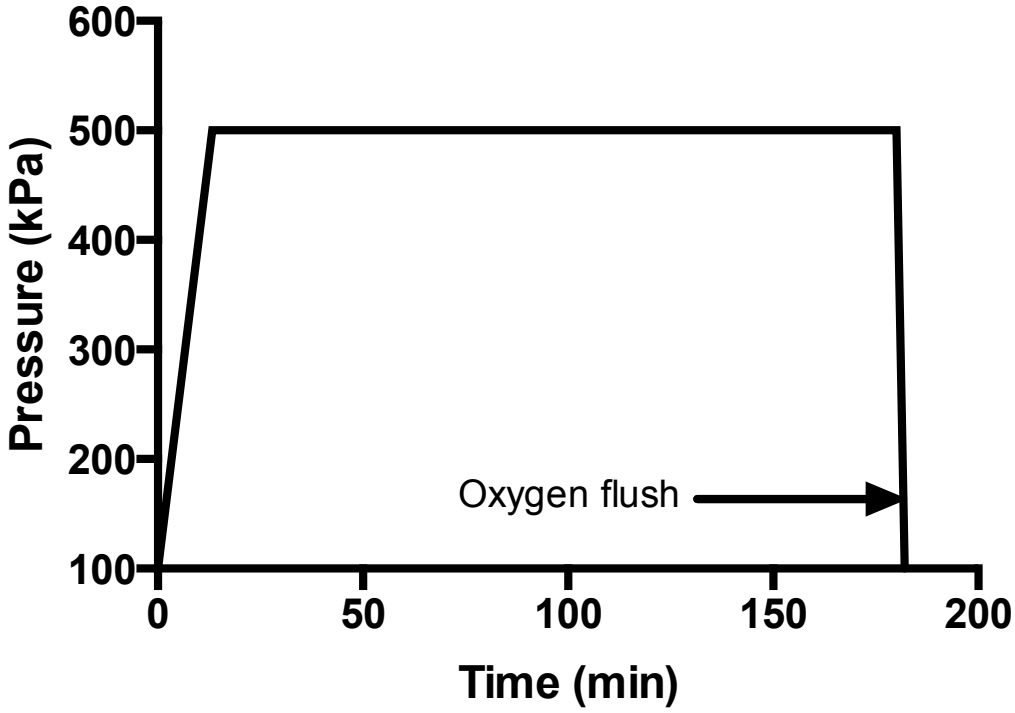

Figure 4. Schematic presentation of the dive profile for the two dive groups (HeO-1, HeO-4). At 160kPa the chamber was rapidly flushed with 100% oxygen in order to keep sufficient breathing gas oxygen pressure during the fast decompression.

Anesthesia and observation

Immediately after completion of the simulated dives both the diving rats and the unexposed control rats were anesthetized with a mixture of midazolam 0.5 mg· 100 g^{-1}, fentanyl 5 µg· 100 g^{-1} and haldol 0.33 mg· 100 g^{-1} given as a subcutaneous injection. Rats from group HeO-1 and Ctrl-1 were then observed for one hour, while rats from group HeO-4 and Ctrl-4 were observed for four hours. Throughout the entire observation period the animal's reflexes were regularly tested to see if they were properly anesthetized. If the animals showed any sign of reaction an addition of midazolam 0.3 mg· 100 g^{-1} ·$hour^{-1}$, fentanyl 3 µg· 100 g^{-1} ·$hour^{-1}$ and haldol 0.2 mg· 100 g^{-1} ·$hour^{-1}$ was given.

Bubble measurement

During observation each rat was scanned for vascular gas bubbles using ultrasonic measurement (Figure 5). The heart and pulmonary artery were monitored using a 13-24 MHz transducer (MS250) connected to a VisualSonics Vevo 2100 ultrasound scanner. Bubbles would appear on the monitor as white spots in the pulmonary artery, and the amount of bubbles were counted and graded on a scale from 0 to 5 accordingly: 0: no bubbles, 1: an occasional bubble, 2: at least one bubble every fourth heart cycle, 3: at least one bubble every heart cycle, 4: continuous bubbling, and 5: massive bubbling (Eftedal and Brubakk, 1997).

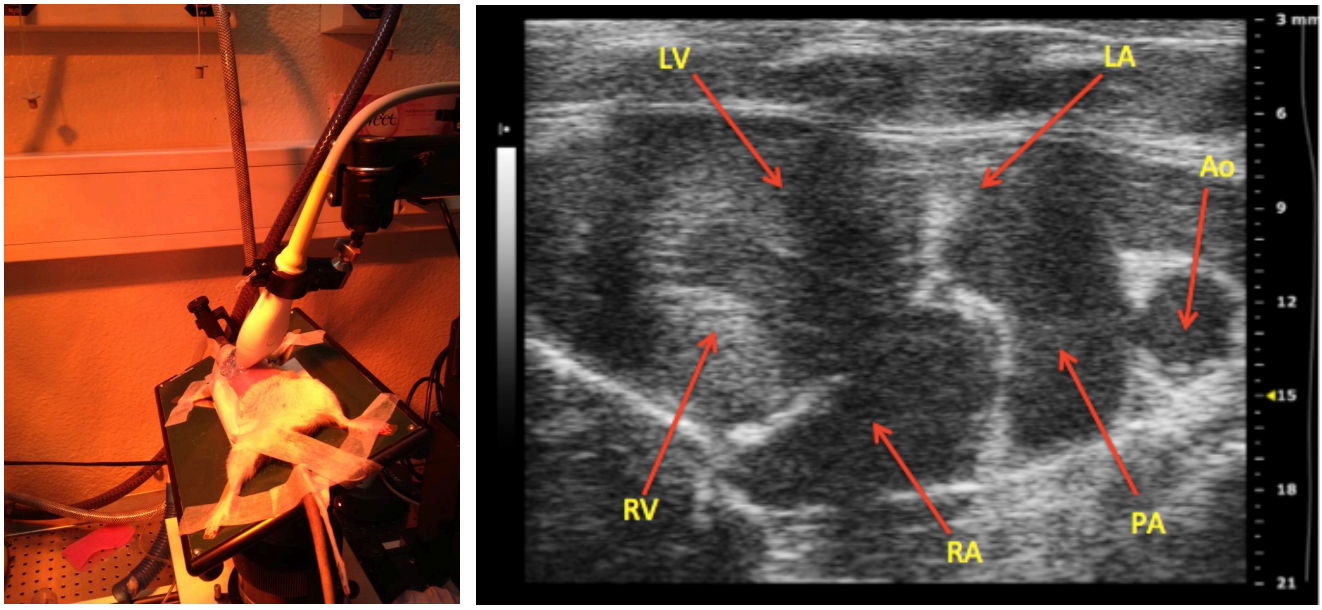

Figure 5. Left panel: ultrasonic measurement for detection of vascular gas bubbles in rats. Right panel: 2D ultrasound image of the rat heart. LV: left ventricle, RV: right ventricle, RA: right atrium, PA: pulmonary artery, Ao: aorta, LA: left atrium.

Blood sample collection

After ended observation the animals were euthanized by draining of blood directly from the vena cava via a cannula and into a syringe, still under total anesthesia. The blood was then transferred over to “serum sep clot activator”-tubes, and blood serum was prepared by centrifugation (3000 rpm x 10 minutes) 30 minutes after collection. Serum was allocated into cryotubes with 250 µl sample per tube, and frozen (-80 °C) until analysis.

Measurement of oxidative stress: TBARS

TBARS is a marker of lipid peroxidation and thus a marker of occurrence of oxidative stress. Many by-products formed in the process of lipid peroxidation and can be measured by different assays. One common method is the estimation of aldehydic products that react with thiobarbituric acid (TBA) and yield thiobarbituric acid reactive substances (TBARS), which further can be measured with spectrophotometry (Devasagayam et al., 2003). In the present study duplicate samples of rat serum were measured for malondialdehyde (MDA), a common product of lipid peroxidation. A TBARS Assay Kit was used for these measurements, where TBARS are expressed in terms of MDA equivalents that increase as a result of oxidative stress. MDA forms a 1:2 adduct with thiobarbituric acid and the MDA-TBA adduct produced can be measured by spectrophotometry. R&D Systems provides the TBARS Assay Kit that was used in this experiment, and detailed descriptions for execution of the assay are found in the referred protocol (R&DSystems, 2012).

Measurement of antioxidant enzymes

Measurement of protein levels of the two antioxidants superoxide dismutase (SOD) and catalase were carried out for further examination of oxidative stress responses. This was accomplished by using SOD- and catalase enzyme-linked immunosorbent assay (ELISA) kits, which measured the concentration of the respective antioxidants in duplicate samples of rat serum. Both assay kits used are designed by Cloud-Clone Corp. and assembled by Uscn Life Science Inc. Detailed descriptions for execution of the two ELISA's are found in referred instruction manuals (Corp., 2013b, Corp., 2013a).

Statistical analyses

A Shapiro-Wilk's test ($p > 0.05$) and a visual inspection of their histograms, normal Q-Q plots and box plots showed an approximately normally distribution for all data. The effect of saturation diving on oxidative stress was therefore analyzed using parametric statistical methods. One-way analysis of variance (ANOVA) was performed to assess significant intergroup differences, and post hoc analysis using a Bonferroni approach was carried out to further investigate group-to-group variance. The level of statistical relevance was set at $p < 0.05$. All statistical analyzes were performed using SPSS 21.0.

RESULTS

Bubble detection

Vascular gas bubbles were only seen in one out of the forty rats. This rat was from group HeO-4, and the bubbles were only seen during the first ultrasonic scanning immediately after surfacing. The rat had a maximum bubble grade of 2-3. No bubbles were observed at the second scan, which was performed about three hours after the dive. No bubbles were detected in any other rats.

Catalase measurement

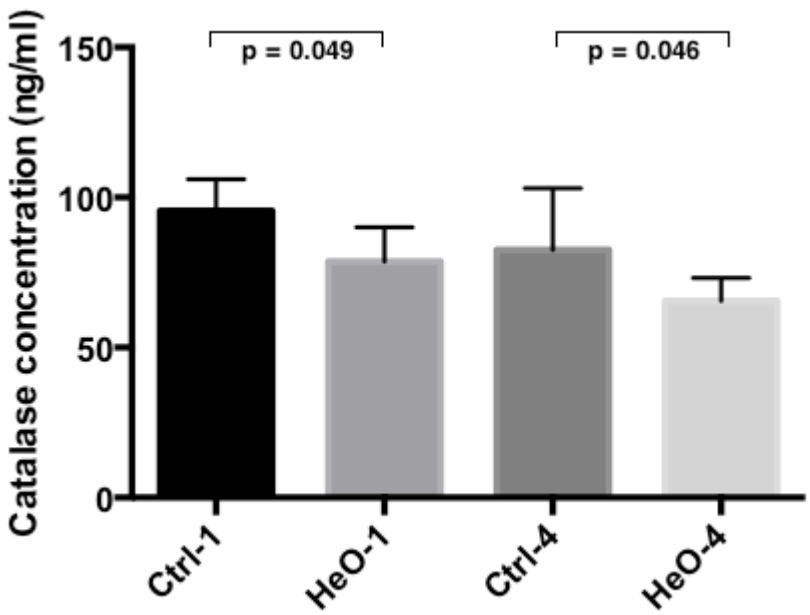

Figure 6. Catalase concentration (mean ± SD) presented for all four groups.

Significant differences in catalase concentration were observed between the four groups (ANOVA $p < 0.001$), where serum catalase concentrations were lower in the diving groups than in their respective non-diving control groups (Figure 6).

As being the most conservative approach the Bonferroni correction was chosen to analyse the within-group variance and between-group variance. There was significant lower catalase concentration in blood serum one-hour post saturation dive to 500 kPa compared to one-hour controls (78.69 ± 11.31 ng/ml vs. 95.53 ± 10.59 ng/ml, respectively, p = 0.049, Figure 6). There was also statistically significant lower catalase concentration in blood serum from rats four-hour post saturation dive compared to the four-hour controls (65.56 ± 7.63 ng/ml vs. 82.52 ± 20.59 ng/ml, respectively, p = 0.046, Figure 6). No difference in catalase concentration was seen between four hours post dive rats and one-hour post dive rats (65.56 ± 7.63 ng/ml vs. 78.69 ± 11.31 ng/ml, respectively, p = 0.213, Figure 6).

TBARS measurement

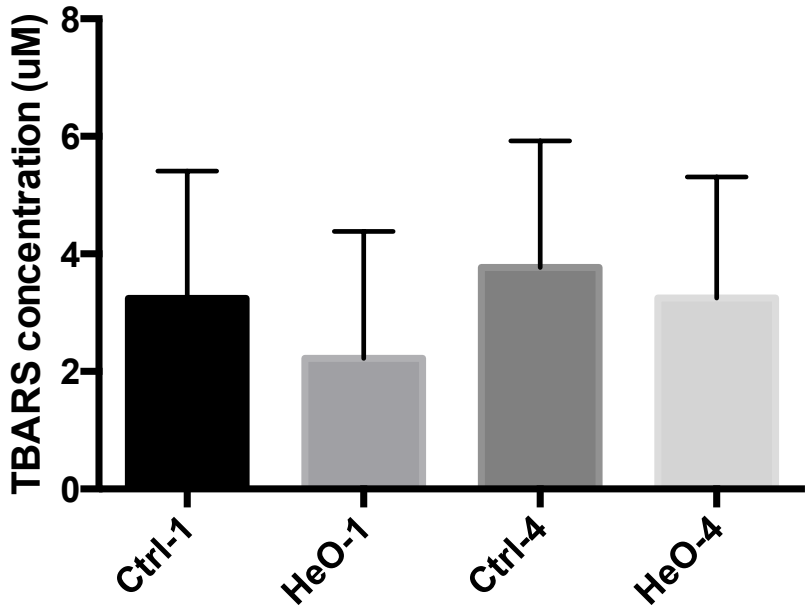

Figure 7. TBARS concentration (mean ± SD) presented for all four groups.

There was no significant difference in TBARS concentration between the groups (ANOVA p = 0.437).

SOD measurement

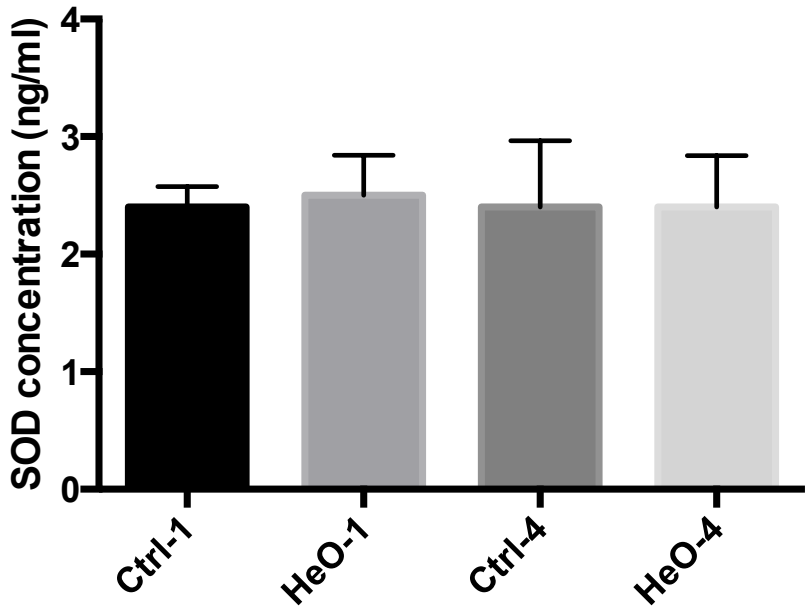

Figure 8. SOD concentrations (mean ± SD) presented for all four groups.

As shown in figure 8, there were no differences in SOD concentration between the groups (ANOVA p = 0.912).

DISCUSSION

The main findings in the present study were that saturation diving gave a significantly decrease in catalase concentrations. However, the hyperbaric exposure did not have any effect on lipid peroxidation as measured by TBARS, or on SOD concentration. Thus, the hypothesis that saturation diving would give an increase in lipid peroxidation and a decrease of measured antioxidants was not confirmed. Enzymatic activity of the two antioxidants was not measured.

Bubble detection

Decompression acts as a stressor and the amount of vascular gas bubbles can be an indicator of the magnitude of stress experienced by the diver (Brubakk and Mollerlokken, 2009). The lack of vascular gas bubbles in the present study may therefore suggest that the dive profile and gas mixture used exerts little decompression stress upon the animals.

In this study heliox was used as breathing gas under hyperbaric conditions (500 kPa) instead of air to best simulate the conditions of human saturation divers, and this gas mixture (10% oxygen, 90% Helium) may explain the lack of bubbles despite an otherwise provoking dive profile. When comparing shrinking rate of gas bubbles during breathing of either hyperbaric air or heliox, there is shown to be a significant faster shrinkage of bubbles while breathing heliox (Hyldegaard et al., 2001). Helium is more diffusible than nitrogen because of its lower atomic weight, and has lower tissue solubility (Moon and Gorman, 2003). This will prevent the inert gas from easily accumulating in tissues and probably contribute to a faster washout, thereby hindrance of bubbles to form during decompression. A fast shrinking rate of bubbles in the use of heliox as breathing gas may contribute to the absence of bubbles in the present study. Almost all saturation diving within the commercial diving industry is carried out with heliox mixtures with the main reason being avoidance of nitrogen narcosis and oxygen toxicity (Joiner, 2001).

Catalase measurement

There were significant differences in catalase concentration in blood serum between the groups (ANOVA $p < 0.001$). The concentration of catalase decreased after hyperbaric exposure, as seen in both of the two dive groups (HeO-1, HeO-4) compared to their respective controls (Ctrl-1, Ctrl-4). These results can be interpreted as a consumption of catalase that is higher in diving animals compared to the controls and that it is not compensated with increased protein production within the time frame of this study, hence the decrease in catalase concentration. This could reflect a low stress level, and the system can maintain homeostasis without having to produce more catalase enzymes. Interestingly, studies have shown that the catalase gene is downregulated in response to oxidative stress (Quan et al., 2011, Min et al., 2010). ROS, in this case H_2O_2, seem to lower the expression of the transcriptional activator of the catalase gene, and thereby lowering the expression of catalase. Observations were made of cessation of transcriptional activity of the catalase promoter due to DNA methylation. When the protein is utilized in antioxidant defence and transcription of the gene is simultaneously downregulated, the level of catalase may decrease.

Over the past 10 years, NASA has conducted so-called NASA Extreme Environment Mission Operations (NEEMO) to simulate different aspects of spaceflight (Zwart et al., 2012, Zwart et al., 2009, Smith et al., 2004). This involved studies on hyperbaric environment by saturation diving to identify oxidative damage. Their results regarding catalase are in compliance with results from the present study. Notably, Zwart et al. (2012) measured the activity of different antioxidant enzymes in response to hyperbaic exposure (50kPa O_2), and not their concentration. They reported a significant increase in catalase activity during and after the dive compared to at baseline. It is reasonably to suggest that an increase in enzyme activity is a response to an increase in oxidative stress and amount of oxygen radicals present after a dive. When the activity is higher more of the enzyme is being used in the defence against radicals and depletes the total blood concentration of that enzyme if the loss is not compensated rapidly by increased protein production, giving a lower catalase concentration post-dive compared to pre-dive. It should however be taken into consideration that NASAs studies are not acute studies like the present one, a factor that may influence enzyme results despite similarities in PO_2 (50kPa).

TBARS measurement

No significant differences in TBARS concentration were observed between the groups ($p = 0.437$). These results indicate that the current dive profile did not give high levels of oxidative stress and thus no detectable lipid peroxidation. Higher oxygen pressures than what was employed in this study (50kPa) seems to be required in order to cause any significant lipid peroxidation. In a previous study a correlation between incrementing exposure pressures and increase of TBARS when treating rats with hyperbaric oxygen was found (Oter et al., 2005). In that study exposure to hyperbaric air was compared with exposure to 100% hyperbaric oxygen with a total maximum ambient pressure of 3 ATA (atmospheres absolute). No effects on TBARS were observed following air exposure, while there was a significant increase of lipid by-products after treatment with pure pressurized oxygen. Also the duration of hyperbaric exposure seem to have an influence on lipid peroxidation. No increase in TBARS were detected in mice exposed to hyperoxia for 30 minutes despite high pressure (585kPa), an observation that is in agreement with longer exposure time being necessary for significant lipid peroxidation to happen (Jamieson, 1991). A study of hyperbaric oxygen (HBO) treatment in humans showed an accumulation of TBARS when treating humans with HBO with an oxygen pressure of 250kPa (Benedetti et al., 2004). This involves hyperbaric exposure to 100% oxygen (2 x 30 minutes) with small intervals (3 minutes) in which the patients breathe air. They found no significant increase in MDA after only one treatment, but significantly higher TBARS concentrations after repeated exposures. These results indicate that consecutive diving may cause lipid peroxidation.

The lack of lipid peroxidation in the present study may indicate a low level of oxidative stress, but other types of oxidative stress could still occur. The decrease in catalase concentration suggests an increase in oxygen radicals as a consequence of higher oxidative stress levels after simulated dives. Even though this is not evident in the form of MDA products, other indices of oxidative stress may give different results. Therefore, further investigation of different types of oxidative stress is necessary to reveal the overall stress status after dives.

SOD measurement

Unlike catalase that showed a decrease in concentration following hyperbaric exposure, no significant difference was found in SOD concentration between the four groups. In a study of asymptomatic scuba diving Eftedal and colleagues (2013) analysed the expression of all genes in human white blood cells, including genes coding for different antioxidants (Eftedal et al., 2013). They found an upregulation of genes encoding mitochondrial SOD immediately after diving, which seemed to be caused by increased oxidative stress after air dives to a depth of 18 meters seawater (msw). These gene expression results may help interpret the SOD results from the present study. As there was no significant change in SOD concentration after diving, it is possible that a depletion of SOD is compensated by an upregulation of the mitochondrial SOD gene and no differences in SOD concentration between exposed and unexposed groups would be detected. Eftedal et al. (2013) only examined gene expression and not proteins concentrations, but a higher protein synthesis due to upregulation of the mitochondrial SOD genes would be expected.

The constant SOD concentration in the present study may have several explanations based on what is known about the behaviour for SOD enzymes. A stable enzyme concentration could be due to a depletion caused by increased activity, compensated with upregulation of gene expression and SOD protein synthesis. Antioxidants have different responses to the same stimuli, e.g. low level of oxidative stress may activate one enzyme and deactivate another, while a high level of stress do the opposite (Dotan et al., 2004). If a low level of oxidative stress, as indicated in the present study, activates SOD enzymes, then a depletion of SOD could be compensated by upregulation of the genes coding for SOD. In that way the SOD concentration could remain unchanged after hyperbaric exposure. The previously mentioned NASA studies found a decrease in SOD activity in all of the three conducted studies (Smith et al., 2004, Zwart et al., 2009, Zwart et al., 2012) Oxygen partial pressure for all NASA studies and the present study was the same (50kPa), but bottom time differed (180 minutes in the present study, and 12 days in the NASA studies). Prolonged exposure to hyperbaric oxygen has previously shown to cause higher levels of oxidative stress (Benedetti et al., 2004), an observation that may suggest that the level of oxidative stress could be higher in the NASA studies compared to the present one. A difference in stress level could result in opposite SOD activity responses between the persistent NASA

studies and the acute present study, giving different outcomes in SOD concentration. These are speculations that require further investigations before any absolute conclusions can be drawn from the results of the present study.

Methodological considerations

Bubble detection

To measure the amount of vascular bubbles, visual two-dimensional (2D) ultrasound imaging was employed. This provides a view of both left and right heart chambers and bubbles present there. Recently, the technology of harmonic ultrasound was introduced to decompression physiology, which provides higher quality of images and better visualisation of smaller objects (Pollock, 2007). The question then remains if the lack of bubbles in the present study could be due to low sensitivity of the ultrasound apparatus used, and if small bubbles could be present without being detected. A study was performed to compare conventional 2D ultrasound images with 2D harmonic ultrasound images to see if harmonic ultrasound could detect bubbles that were previously invisible to conventional imaging (Blogg et al., 2014). A good agreement between paired images gave no significant difference in detection of bubbles between the two methods. However, a clear majority of mismatching pairs showed a higher bubble load with the use of harmonics. Regarding bubble measurements in the present study, gas bubbles might therefore have been present but not showing on the 2D ultrasound image. Although, if this was the case, the amount of bubbles would still be very low and the stress level observed from the rest of the experimental measurements would still match the bubble load.

Oxidative stress measurements

The most direct way of assessing oxidative stress is by measuring the level of free oxygen radicals, but methods for this is less used as free radicals are short-lived and highly reactive to a vast variety of molecules (Dotan et al., 2004). There are different types of oxidative stress, lipid peroxidation being one on them, but also oxidized proteins and fragmented DNA from DNA damage are indices of oxidative stress. Measurement of lipid peroxidation is however the most common way of assessing oxidative stress, including spectroscopic technique with the use of TBARS assays. As there are several types of oxidative stress and hundreds of methods to

measure them, it is hard to indicate oxidative stress in a sample based on one method alone. Thus, there would be most reasonable to use a combination of methods that each represents the different types of oxidative stress to define the overall oxidative stress or oxidative status in a sample. In the present study both assessment of lipid peroxidation and measurement of antioxidant concentrations (SOD and catalase) were performed. Regarding correlations between different markers of oxidative stress they often depend on pathophysiological conditions, e.g. correlation between MDA and a specific antioxidant enzyme could be positive in one situation, and negative in another. There seem to be a difference in activation of enzymes depending on the level of oxidative stress. A high level of stress may activate one enzyme while deactivating another, and the other way around. Also, different pathologies may be associated with distinct types of oxidative stress, and under certain circumstances some types of oxidative stress may dominate over others. Appropriate use of different indices is therefore important when evaluating oxidative stress.

Choice of assay for detection of oxidative stress

Choosing the proper methods when analysing experimental products is vital for the validity of the results. There are numerous of assays for detecting a variety of molecular products available, and using the wrong one may give a false impression of the reality. For example, the use of TBARS assays is one of the most common ways to measure lipid peroxidation, a method that is based on assessment of MDA as a marker of oxidised lipids (Devasagayam et al., 2003). However, a study performed to test the accuracy of the method found TBARS assays to be little specific towards MDA compared to high-performance liquid chromatography (HPLC) (Moselhy et al., 2013). TBARS assays tend to give an overestimation of plasma MDA levels because of TBAs inaccuracy to form only MDA adducts, as TBA also seem to react with other oxidised lipid products. Other, more specific, accurate and sensitive methods, like HPLC may therefore be a better choice when assessing lipid peroxidation. Several methods for detection of oxidative stress are available, each with disadvantages and advantages. As different errors can occur it is beneficial to use two or more methods to avoid wrong conclusions.

Rat model

Studies have shown that the amount of fat has an influence on bubble formation (Francis and Mitchell, 2003, Brubakk et al., 2007). Several gases are more soluble in lipid than aqueous tissues, so the percentage of body fat will therefore influence the loading of gases and bubble formation following decompression. More fat may then give a higher bubble load. Since fatty acids are targets of oxygen radicals, higher body fat may result in higher oxidative stress as well (Halliwell and Chirico, 1993, Nyska and Kohen, 2002). As previously stated, lipid peroxidation initiates a cascade of reactions that produces additional radicals (Klaassen and Casarett, 2008). Because of these radical interactions with fatty tissues, the weight of the rats is a parameter that is important to consider when interpreting results. The present experiment took place over a period of two weeks where four rats were treated each day. This gives enough time for the rats to gain a considerably amount of weigh for it to have an influence on the outcome of different measurements. To prevent big weight differences between diving rats and respective controls they were treated on the same week; HeO-1 and Ctrl-1 the first week, and HeO-4 and Ctrl-4 the second week. Despite the two-week time span of the experiment, all four groups of rats had quite similar weight range (Table 1, Appendix A). As there were little weight differences between groups, we can rule out excessive adipose tissue as a factor influencing the measured decompression stress and oxidative stress.

Anaesthesia

The influence of anaesthetization on rats remains an unclear factor in this experiment. All animals were anaesthetized in order to make the experimental conditions as similar as possible for all groups. Rats are animals that are quite sensitive to environmental changes, and it may be reasonable to suggest that anaesthetic injections could be stressful to the rats and thereby affect the outcome of the measured parameters. The effects of different types of anaesthesia are ought to be investigated in order to take into considerations the effects of anaesthesia when interpreting results.

CONCLUSION AND FUTURE PERSPECTIVES

The present study examined whether saturation diving gives oxidative stress by investigating markers of lipid peroxidation and antioxidant status after simulated dives in a rat model. A decrease in catalase concentration was evident post dive, but there were no significant differences between groups regarding lipid peroxidation or SOD concentration. These findings may indicate a low level of oxidative stress after the applied dive profile.

To unravel the reason for the observed decrease in catalase concentration after hyperbaric exposure, supplementary antioxidant measurements are required. In addition to the enzyme concentration it would be interesting to measure enzyme activity as well as expression of the genes that code for catalase. This is also relevant for SOD enzymes, as these measurements would reveal if a decrease, increase or unchanged concentration of the antioxidants is due to increased or decreased enzyme activity and/or increased or decreased gene expression.

To further investigate oxidative stress it would be interesting to look at a broader spectrum of antioxidants, as both enzymatic and non-enzymatic antioxidants deal with oxygen radicals. They play different roles in the handling of oxygen radicals, are found in different amounts in different cell types, and have different affinity for different radicals (Michiels et al., 1994). Therefore it would be advantageous to consider more than just SOD and catalase among antioxidants as markers of oxidative stress as there are numerous specialized antioxidant enzymes that reacts with and detoxifies oxidative compounds (Sies, 1997). Glutathione peroxidases (GSH-Px) are one of the major classes of antioxidant enzymes and protect biological membranes by preventing lipid peroxidation propagation (Michiels et al., 1994). They are, like catalase, important for the elimination of H_2O_2 (Figure 1), but have a higher affinity for the compound and may therefore be more efficient in managing detoxification of the non-radical oxygen derivate (Fridovich, 1998, Michiels et al., 1994). GSH-Px in particular would therefore be of interest to investigate further in comparison with other enzymatic antioxidants.

Further investigation could be done by comparing the present results with a group of rats exposed to the same dive profile, but to hyperbaric air instead of heliox. It would be interesting to see if composition of the gas mixture constitutes a big difference when it comes to oxidative stress following saturation diving, and also if air dives cause higher decompression stress in form of higher bubble loads.

A relevant subject of interest could be the effect of repetitive diving on oxidative stress. A study done by Obad and colleagues (2010) showed a decrease in total antioxidant capacity after a series of dives were performed (Obad et al., 2010). However, they could not find any significant difference when they compared pre and post measurements for one single dive. These results indicate an accumulation of oxidative stress over time that depletes the total antioxidant capacity, as it has to deal with the increased stress. It could be interesting to see if repetitive diving with the present dive profile would give similar results as in the previous study by Obad et al. (2010).

The use of animal models in translational research is necessary in order to map the mechanisms behind dive related illness in humans. This is an area of interest and importance as humans are performing underwater operations. Better knowledge about the subsequent challenges is essential to make a safer working environment.

APPENDIX

Appendix A. Body weight of rats

Table A1. Body weight for all four groups of rats presented with means and standard deviation (SD).

Group	Body weight (g)										Mean	SD
HeO-1	242	267	236	292	327	280	283	250	270	275	272.2	26.5
Ctrl-1	254	249	251	293	268	273	272	284	287	267	269.8	15.3
HeO-4	290	270	285	262	248	284	262	297	266	274	273.8	15.1
Ctrl-4	251	260	260	254	299	287	277	273	277	273	271.1	15.1

Appendix B. Raw data and standard curve for catalase

Table B1. Raw data for catalase concentrations. Measured optical density (OD) and correction for dilution factor 1:10.

Sample nr.	OD	Mean	Correction blank	ng/ml	Dilution 1:10	Sample nr.	OD	Mean	Correction blank	ng/ml	Dilution 1:10
1	0.459	0.459	0.367	6.9	69.4	21	0.461	0.445	0.353	6.6	65.9
	0.459						0.428				
2	0.567	0.579	0.487	10.2	102.0	22	0.514	0.520	0.428	8.5	85.0
	0.590						0.525				
3	0.421	0.432	0.340	6.3	62.9	23	0.451	0.442	0.350	6.5	65.2
	0.442						0.432				
4	0.573	0.567	0.475	9.9	98.5	24	0.608	0.587	0.495	10.4	104.5
	0.561						0.565				
5	0.516	0.552	0.460	9.4	94.0	25	0.416	0.419	0.327	6.0	60.0
	0.587						0.421				
6	0.561	0.585	0.493	10.4	104.0	26	0.420	0.466	0.374	7.1	71.0
	0.609						0.511				
7	0.468	0.505	0.413	8.1	80.9	27	0.405	0.408	0.316	5.8	57.6
	0.541						0.410				
8	0.539	0.549	0.457	9.3	93.1	28	0.380	0.406	0.314	5.7	57.2
	0.558						0.431				
9	0.515	0.480	0.388	7.5	74.6	29	0.486	0.501	0.409	8.0	80.0
	0.445						0.516				
10	0.500	0.497	0.405	7.9	79.0	30	0.473	0.467	0.375	7.1	71.3
	0.494						0.461				
11	0.440	0.457	0.365	6.9	68.9	31	0.437	0.424	0.332	6.1	61.1
	0.474						0.410				
12	0.577	0.607	0.515	11.1	110.7	32	0.495	0.510	0.418	8.2	82.2
	0.636						0.524				
13	0.552	0.558	0.466	9.6	95.7	33	0.422	0.407	0.315	5.8	57.5
	0.563						0.392				
14	0.580	0.574	0.482	10.1	100.6	34	0.649	0.662	0.570	12.9	129.3
	0.568						0.675				
15	0.546	0.539	0.447	9.0	90.2	35	0.472	0.450	0.358	6.7	67.2
	0.531						0.428				
16	0.550	0.577	0.485	10.1	101.4	36	0.485	0.465	0.373	7.1	70.7
	0.603						0.444				
17	0.476	0.494	0.402	7.8	78.1	37	0.465	0.437	0.345	6.4	64.1
	0.512						0.408				
18	0.490	0.509	0.417	8.2	82.0	38	0.466	0.473	0.381	7.3	72.7
	0.527						0.479				
19	0.483	0.471	0.379	7.2	72.2	39	0.505	0.490	0.398	7.7	77.0
	0.458						0.474				
20	0.508	0.517	0.425	8.4	84.1	40	0.509	0.506	0.414	8.1	81.3
	0.525						0.503				

Table B2. Measured optical density (OD) values for catalase standards.

Standard (ng/ml)	OD	Mean	Correction blank
100	1.841	1.932	1.840
	2.022		
50	1.319	1.348	1.256
	1.377		
25	0.952	1.003	0.911
	1.054		
12.5	0.572	0.600	0.507
	0.625		
6.25	0.334	0.325	0.233
	0.316		
3.12	0.199	0.222	0.130
	0.245		
1.56	0.161	0.168	0.076
	0.174		
0	0.092	0.092	0.000

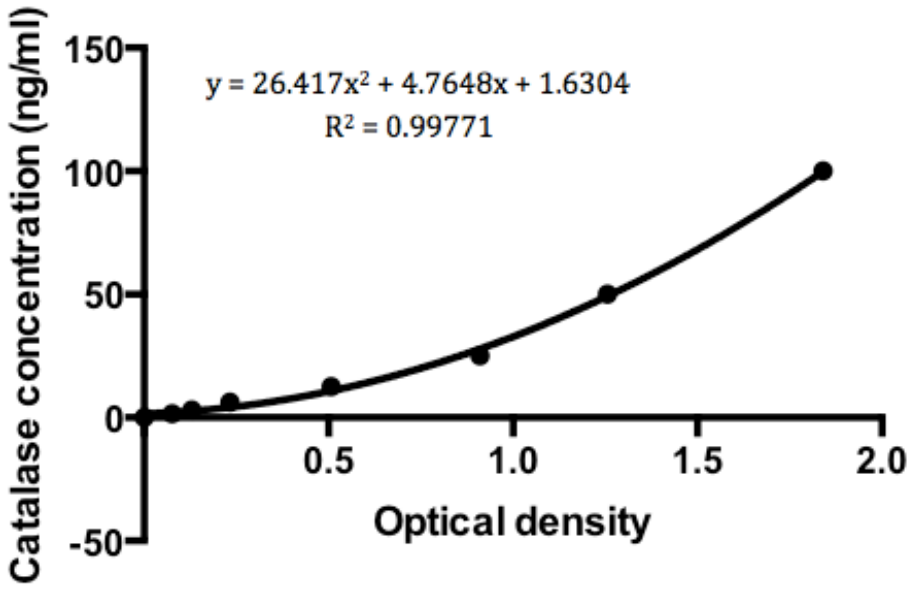

Figure B1. Catalase standard curve.

Appendix C. Raw data and standard curve for TBARS

Table C1. Raw data for TBARS concentrations. Measured optical density (OD) and correction for dilution factor 1:2.

Sample nr.	Pre OD	Pre mean	Post OD	Post mean	Post minus pre	uM	Dilution 1:2
1	0.041	0.044	0.051	0.058	0.014	0.03	0.06
	0.046		0.064				
2	0.036	0.037	0.074	0.072	0.035	0.23	0.47
	0.037		0.069				
3	0.041	0.040	0.087	0.087	0.047	0.34	0.69
	0.039		0.086				
4	0.043	0.042	0.223	0.224	0.183	1.65	3.31
	0.040		0.225				
5	0.040	0.041	0.223	0.224	0.184	1.66	3.33
	0.041		0.225				
6	0.043	0.039	0.316	0.320	0.281	2.60	5.20
	0.035		0.323				
7	0.040	0.039	0.334	0.335	0.296	2.75	5.49
	0.038		0.336				
8	0.040	0.041	0.339	0.339	0.299	2.77	5.54
	0.041		0.339				
9	0.039	0.038	0.058	0.060	0.022	0.10	0.21
	0.037		0.061				
10	0.039	0.030	0.058	0.059	0.029	0.18	0.35
	0.021		0.060				
11	0.042	0.042	0.097	0.105	0.064	0.51	1.02
	0.041		0.113				
12	0.038	0.041	0.206	0.203	0.162	1.45	2.90
	0.044		0.199				
13	0.040	0.040	0.239	0.241	0.201	1.83	3.66
	0.039		0.242				
14	0.039	0.038	0.311	0.311	0.273	2.53	5.05
	0.036		0.310				
15	0.017	0.027	0.327	0.329	0.303	2.81	5.62
	0.036		0.331				
16	0.040	0.041	0.360	0.352	0.312	2.90	5.79
	0.041		0.344				
17	0.040	0.036	0.062	0.063	0.027	0.15	0.30
	0.032		0.063				
18	0.039	0.042	0.076	0.079	0.038	0.26	0.51
	0.044		0.082				
19	0.039	0.039	0.138	0.144	0.105	0.91	1.82
	0.038		0.149				
20	0.043	0.040	0.220	0.220	0.181	1.63	3.27
	0.036		0.220				
21	0.031	0.036	0.252	0.251	0.216	1.97	3.94
	0.040		0.250				
22	0.040	0.040	0.318	0.322	0.283	2.62	5.23
	0.039		0.326				
23	0.033	0.035	0.318	0.329	0.294	2.72	5.45
	0.037		0.339				
24	0.038	0.029	0.340	0.334	0.305	2.83	5.67
	0.020		0.328				
25	0.048	0.047	0.075	0.075	0.028	0.17	0.33
	0.045		0.074				
26	0.040	0.041	0.066	0.064	0.023	0.12	0.24
	0.042		0.062				
27	0.044	0.040	0.140	0.139	0.099	0.84	1.69
	0.036		0.137				
28	0.037	0.038	0.181	0.179	0.141	1.25	2.51
	0.039		0.177				
29	0.042	0.040	0.243	0.250	0.210	1.91	3.83
	0.038		0.256				
30	0.040	0.039	0.311	0.315	0.277	2.56	5.12
	0.037		0.319				
31	0.042	0.042	0.322	0.322	0.280	2.59	5.19
	0.041		0.321				
32	0.039	0.039	0.338	0.339	0.300	2.78	5.56
	0.039		0.339				
33	0.036	0.039	0.065	0.067	0.029	0.17	0.34
	0.041		0.069				
34	0.039	0.037	0.075	0.076	0.039	0.27	0.53
	0.035		0.076				
35	0.038	0.040	0.144	0.143	0.103	0.89	1.78
	0.041		0.141				
36	0.042	0.034	0.177	0.175	0.141	1.25	2.50
	0.026		0.172				
37	0.037	0.040	0.275	0.270	0.230	2.11	4.21
	0.043		0.264				
38	0.038	0.038	0.292	0.294	0.256	2.36	4.71
	0.038		0.295				
39	0.030	0.032	0.342	0.342	0.311	2.89	5.78
	0.033		0.342				
40	0.035	0.036	0.346	0.341	0.305	2.83	5.67
	0.037		0.336				

Table C2. Measured optical density (OD) values for TBARS standards.

Standard (uM)	Pre OD	Pre mean	Pre minus blank	Post OD	Post mean	Post minus blank	Post minus pre
16.7	0.024	0.022	0	1.744	1.752	1.729	1.730
	0.020			1.759			
8.35	0.024	0.026	0.001	0.929	0.929	0.906	0.905
	0.027			0.929			
4.18	0.028	0.029	0.004	0.476	0.476	0.453	0.449
	0.029			0.476			
2.09	0.028	0.029	0.004	0.254	0.255	0.232	0.228
	0.029			0.255			
1.04	0.036	0.031	0.006	0.135	0.136	0.136	0.130
	0.025			0.137			
0.52	0.030	0.027	0.003	0.085	0.083	0.060	0.057
	0.024			0.080			
0.26	0.026	0.026	0.001	0.053	0.054	0.031	0.030
	0.025			0.054			
0	0.023	0.025		0.020	0.023		0
	0.026			0.026			

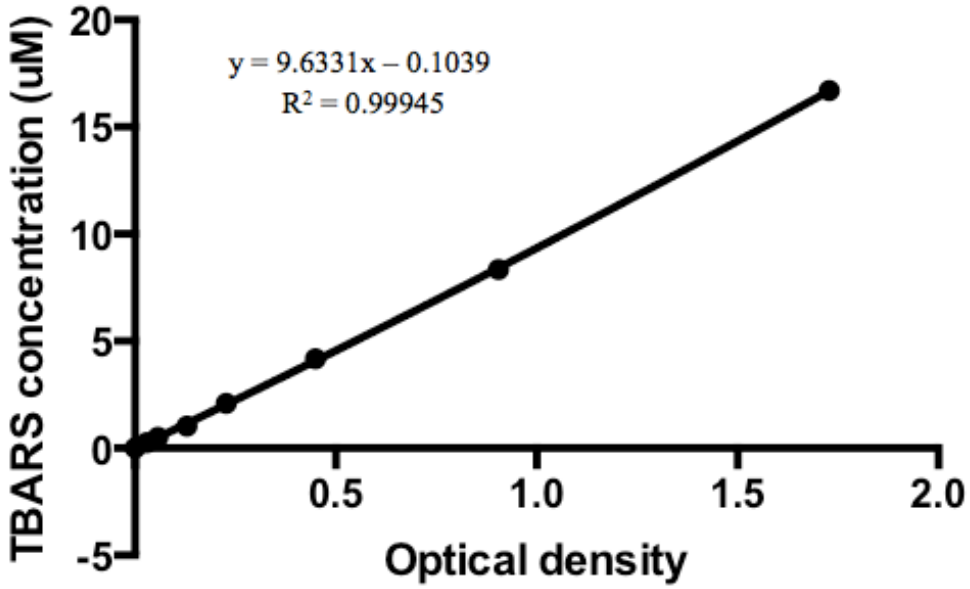

Figure C1. TBARS standard curve.

Appendix D. Raw data and standard curve for SOD

Table D1. Raw data for SOD concentrations. Measured optical density (OD) and correction for dilution factor 1:2000. SOD measurements for sample nr. 35 are missing due to lack of serum.

Sample nr.	OD	Mean	Correction blank	pg/ml	Dilution 1:2000	ug/ml
1	0.437	0.613	0.528	1280.016	2560031.759	2.56
	0.788					
2	0.528	0.538	0.453	1097.005	2194010.882	2.19
	0.548					
3	0.685	0.639	0.554	1345.290	2690580.964	2.69
	0.593					
4	0.613	0.599	0.514	1245.569	2491137.330	2.49
	0.584					
5	0.725	0.717	0.632	1537.958	3075916.647	3.076
	0.709					
6	0.552	0.616	0.531	1288.632	2577263.464	2.58
	0.680					
7	0.531	0.609	0.524	1271.402	2542803.293	2.54
	0.687					
8	0.507	0.617	0.532	1289.863	2579725.400	2.58
	0.726					
9	0.476	0.526	0.441	1067.596	2135191.733	2.14
	0.576					
10	0.567	0.564	0.479	1160.791	2321582.995	2.32
	0.561					
11	0.668	0.625	0.540	1309.563	2619125.376	2.62
	0.581					
12	0.648	0.588	0.503	1219.750	2439500.520	2.44
	0.528					
13	0.668	0.630	0.545	1323.111	2646222.675	2.65
	0.592					
14	0.720	0.641	0.556	1350.221	2700441.271	2.70
	0.562					
15	0.655	0.626	0.541	1312.026	2624051.562	2.62
	0.596					
16	0.536	0.530	0.445	1076.172	2152343.385	2.15
	0.523					
17	0.384	0.459	0.374	903.742	1807484.606	1.81
	0.534					
18	0.584	0.570	0.485	1175.524	2351048.097	2.35
	0.556					
19	0.567	0.579	0.494	1197.632	2395263.600	2.40
	0.591					
20	0.559	0.569	0.484	1171.840	2343680.929	2.34
	0.578					
21	0.581	0.642	0.557	1352.686	2705371.820	2.71
	0.703					
22	0.642	0.644	0.559	1357.617	2715233.713	2.72
	0.646					
23	0.495	0.507	0.422	1019.846	2039691.826	2.04
	0.518					
24	0.463	0.440	0.355	856.165	1712330.164	1.71
	0.416					
25	0.686	0.647	0.562	1365.014	2730028.535	2.73
	0.608					
26	0.573	0.603	0.518	1255.408	2510816.164	2.51
	0.632					
27	0.837	0.759	0.674	1640.795	3281590.653	3.28
	0.680					
28	0.603	0.592	0.507	1229.584	2459168.248	2.46
	0.581					
29	0.547	0.516	0.431	1043.102	2086204.862	2.09
	0.485					
30	0.899	0.801	0.716	1746.346	3492692.650	3.49
	0.703					
31	0.667	0.570	0.485	1175.524	2351048.097	2.35
	0.473					
32	0.536	0.464	0.379	914.729	1829457.602	1.83
	0.391					
33	0.486	0.462	0.377	909.846	1819691.165	1.82
	0.437					
34	0.635	0.561	0.476	1152.200	2304399.414	2.30
	0.486					
36	0.479	0.513	0.428	1034.533	2069065.704	2.07
	0.546					
37	0.550	0.595	0.510	1236.961	2473921.821	2.47
	0.640					
38	0.567	0.688	0.603	1464.996	2929991.556	2.93
	0.808					
39	0.583	0.567	0.482	1166.930	2333858.964	2.33
	0.550					
40	0.408	0.455	0.370	893.979	1787957.549	1.79
	0.502					

Table D2. Measured optical density (OD) values for SOD standards.

Standard (pg/ml)	OD	Mean	Correction blank
4000	1.654	1.680	1.595
	1.705		
2000	0.978	0.934	0.849
	0.889		
1000	0.511	0.485	0.400
	0.458		
500	0.258	0.251	0.166
	0.244		
250	0.163	0.161	0.076
	0.158		
125	0.120	0.125	0.040
	0.130		
62.5	0.115	0.121	0.036
	0.126		
0	0.080	0.085	0
	0.090		

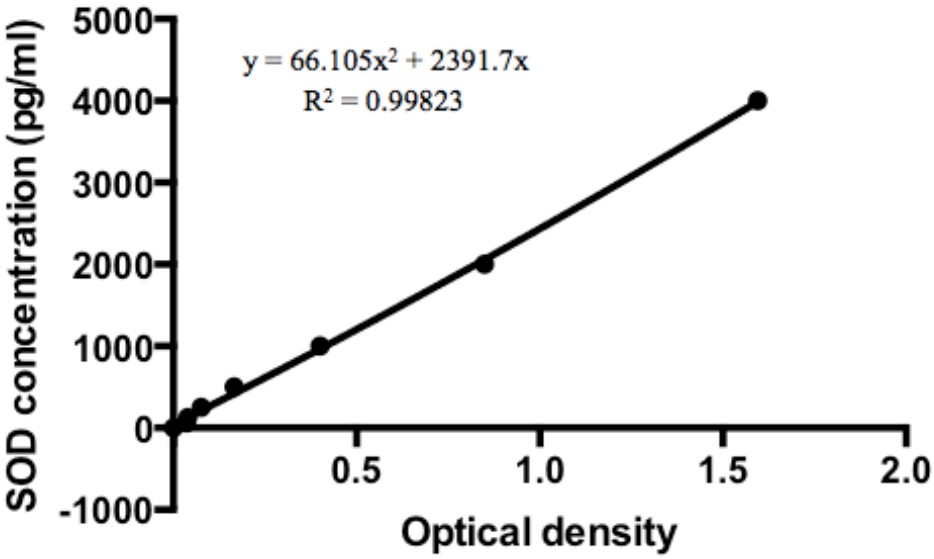

Figure D1. SOD standard curve.

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