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Changes in Oxidative Stress and Vascular Reactivity of Thoracic and Abdominal Rat Aorta with Different Periods of Exposure to Hyperbaric Oxygenation

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ABSTRACT

Oxidative stress and the antioxidant defense always seek balance in aerobic systems. Therefore, when O_2 is added to an aerobic system during hyperbaric oxygenation, oxidative stress is induced and the antioxidant defense is stimulated. Dose-response curves were established for rat aorta exposed to different agonists. The aorta were from control rats and animals with 3, 10 or 20 HBO sessions. Parameters of vascular reactivity, oxidative stress and antioxidant defense were measured. With hyperbaric oxygenation, vascular reactivity was modified by the altered interaction between oxidative stress and antioxidant defense. The result was a low contractile response, possibly caused by endothelium dependent or independent pathways. Since, acetylcholine did not modify the vascular response, HBO seems to act by endothelium independent pathways related to nitric oxide. Hyperbaric oxygen therapy (HBO) led to an over 60% decrease in all parameters of oxidative stress and antioxidant defense (enzymatic and non-enzymatic) of thoracic aorta, an effect that lasted during the 20 sessions of HBO. This result indicates a probable drastic reduction in ROS and NO production, which could be explained by the capacity of an excess of oxygen to eliminate certain products that are normally involved in chain reactions leading to oxidative stress.

Key words: Hyperbaric oxygen therapy, vascular reactivity, thoracic aorta, abdominal aorta, oxidative stress, nitric oxide

INTRODUCTION

Hyperbaric oxygenation (HBO) therapy consists of exposing the patient to O_2 at a pressure of two atmospheres (2 atm, 100% above the normal barometric environmental conditions). Exposure of an individual to 2 atm of O_2 dissolves this gas in plasma (Henry's law), which increases the diffusion of oxygen to tissues (Edwards, 2010a).

Hyperbaric oxygenation (HBO) therapy was initially used mainly to treat carbon monoxide intoxication (Weaver *et al*., 2002; Zamani, 2013), diabetic foot and gaseous embolism (Branger *et al*., 2001), finding considerable improvement in patients. The use of HBO therapy has since expanded with 14 pathologies (Gill and Bell, 2004; Feldmeier, 2003), now approved by the Underwater and Hyperbaric Medical Society (UHMS), including osteomyelitis, heart attack (Feldmeier, 2003) and the decompression of divers (Zamani, 2013).

Although the mechanism of action is still not completely clear, the great majority of disorders treated with HBO are related to ischemic processes and therefore, to the cardiovascular system (Edwards, 2010b; Stephen, 2010; Williams, 2010). Some researchers have proposed that the mechanism of action of HBO therapy involves the release of nitric oxide (NO) and therefore vasodilation, which would be regulated by antioxidant enzymes (Allen *et al*., 2009; Demchenko *et al*., 2000).

Current controversy centers around whether or not NO can generate nitrosative stress (McGavock *et al*., 1999; Gregorevic *et al*., 2001). This type of stress is regulated by endogenous enzymes as well as non-enzymatic mechanisms

(Benedetti *et al*., 2004) that neutralize free radicals. For example, the superoxide anion (O_2^-) is a free radical that is regulated by the action of superoxide dismutase (SOD) (Kormanovski *et al*., 2010; Hink *et al*., 2006) and hydrogen peroxide (H_2O_2). The latter molecule is mediated by catalase (CAT) (Johansson and Borg, 1988; Subbotina, 2006). Glutathione peroxidase (GPX) and reduced glutathione (GSH) are also important antioxidants that neutralize free radicals (Kormanovski *et al*., 2010) and avoid cellular death by lipoperoxidation (Puzserova *et al*., 2008; Maragos *et al*., 1991; Thom *et al*., 2003).

It has been proposed that HBO therapy regulates different enzymes resulting in a release of O_2, which in turn may stimulate different pathways leading to the release of free radicals that could influence vascular reactivity. Hence, the aim of the present study was to analyze whether, alterations in vascular reactivity in rats exposed to HBO are related to changes in oxidative stress.

MATERIALS AND METHODS

Animals: Male Wistar rats (250-300 g) were maintained at room temperature (18-25°C) and on a 12 h light/dark cycle, with food and water provided *ad libitum*. Animals were handled in accordance with Mexican federal regulations for animal experimentation and care (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico City, Mexico) and the good practices of the CICUAL in regard to research with experimental animals.

Exposure to hyperbaric oxygenation: Four groups of rats were formed (n = 6), including a control group (without exposure to HBO) and three experimental groups exposed to HBO during 3, 10 or 21 h sessions at a pressure of 2 atm in an experimental hyperbaric chamber (MISSA).

Obtaining biological samples and preparing aortic rings: After sacrificing animals by decapitation (previously anaesthetized with pentobarbital at 60 mg kg^{-1}), venous blood samples were taken from the vena cava and stored in eppendorf tubes (previously numbered). Samples were immediately placed on dry ice and later frozen at -80°C to await processing.

After taking blood samples, the thoracic and abdominal portions of the aorta were extracted. The thoracic aorta was excised from the diaphragm to the aortic arch and the abdominal aorta from the diaphragm to the iliac artery. Aortic segments were immediately submerged in cold Krebs solution to remove all adjacent connective tissue. Both thoracic and abdominal segments were cut into aortic rings (4-5 cm long), each mounted on two stainless steel hooks in an isolated organ chamber. One of the hooks was fixed to the bottom of the chamber and the other to a transductor linked to a Biopac System apparatus for registering changes in tension. The isolated organ chamber contained 10 mL of Krebs bicarbonate solution with the following composition (in mM): NaCl 11, KCl 4.7, KH2PO2 1.2, MgSO4 7, H_2O 1.2, $CaCl_2$, $2H_20$ 2.5, NaHCO3 25, dextrose 11.7 and calcium disodium EDTA 0.026. The chamber was maintained at a constant temperature of 37°C and pH of 7.4 and was continuously bubbled with a mixture of 95% O_2 and 5% CO_2.

Experimental design to evaluate vascular reactivity: A standard dose-response curve was constructed representing vascular reactivity to phenylephrine (1×10^{-9}-1×10^{-5}M) under normal conditions (without HBO treatment). To ascertain whether, there are changes in vascular relaxation dependent on endothelium tissue, a standard dose-response curve was constructed for the effect of acetylcholine (1×10^{-9}-1×10^{-5}M) on rings precontracted with phenylephrine (1×10^{-6} M). To explore possible changes in vascular reactivity independent of endothelium tissue, a standard dose-response curve was constructed for the response to sodium nitroprusside of rings precontracted with phenylephrine (1×10^{-3} M).

Evaluation of oxidative stress: Blood samples were processed in a solution of phosphate buffer (20 mmol) with 0.1% Triton detergent by homogenization with the Randox procedure to determine the levels of superoxide dismutase (SOD) (Branger *et al*., 2001; Gregorevic *et al*., 2001; Fattman *et al*., 2003; Marklund, 1980) and glutathione peroxidase (GPx) (Williams, 2010). On the other hand, the Cayman Chemical procedure was employed to evaluate the activity of catalase (CAT) (Branger *et al*., 2001; Fujimoto *et al*., 2001), the non-enzymatic antioxidant response represented by reduced glutathione (GSH) (Korkmaz *et al*., 2008) and the level of nitrates/nitrites (NO). Finally, the concentration of the products of lipoperoxidation, known as thiobarbituric acid reactive substances (TBARS) (Korkmaz *et al*., 2008; Armstrong and Browne, 1994; Yagi, 1998; Tompach *et al*., 1997), was assessed by the well-known procedure. The concentration of total protein was measured in tissue homogenates with a modified version of the Biuret method.

The level of the Total Antioxidant Status (TAS) was measured by the Randox procedure in the system that generates the ABTS® cation radical ($HX\text{-}Fe^{III}+H_2O_2$), with absorbance at 600 nm. The presence of antioxidants in stained tissue diminished absorbance. A synthetic antioxidant (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard. Thus, the level of TAS is expressed as nmol mg^{-1} of the total proteins of this standard. The other parameters are also expressed as nmol mg^{-1} of total protein, except SOD and CAT (expressed as U mg^{-1} of total protein).

Statistical analysis: All values represent the Mean±SEM of the rats in each group (n = 6). To obtain the maximum effect (E_{max}), a non-linear regression adjustment was made for concentration-response curves. The comparison between groups for the agonists was made with two-way ANOVA and the post-hoc Bonferroni test to determine statistical significance. For evaluation of the enzymatic activity of TAS, SOD, GPx, CAT, GSH, TBARS and NO, the GraphPadPrism statistical package was used. The comparison between groups for these parameters was made with two-way ANOVA and the post-hoc Dune test. In all cases, statistical significance was considered with $p<0.05$.

RESULTS

Changes in vascular reactivity with HBO treatment: After HBO session 10, the contractile effect of phenylephrine on both thoracic and abdominal portions of the aorta was significantly lower (0.3549 g) than without this treatment (1.137 g) (Fig. 1). Contrarily, HBO treatment did not significantly ($p>0.05$) modify the relaxant effect of acetylcholine on rings from either the thoracic or abdominal segment (compared to the control group; Fig. 2). Finally, only with thoracic aorta was there a significant difference between the control and HBO groups in regard to the vasodilation induced by sodium nitroprusside (Fig. 3). The latter effect can be appreciated as of the third HBO session and declined from the tenth to twentieth session.

Changes in oxidative stress with HBO treatment: Basal values of all parameters were higher in the thoracic than abdominal portion of the aorta (Fig. 4). The results indicate that basal levels of oxidative stress, as well as the enzymatic and non-enzymatic antioxidant capacity are between 2 and 3-fold greater in the thoracic than abdominal segment. In thoracic aorta, all parameters of oxidative stress were significantly lower with than without HBO treatment.

Two parameters of oxidative stress measured in the present study are TBARS and NO (indicating lipoperoxidation via ROS and NO, respectively). Regarding basal values (Table 1), the level of TBARS was similar in both aortic segments but the level of NO was two-fold greater in the thoracic portion. After the first three HBO sessions, with thoracic aorta there was an important decrease in TBARS and NO (up to 51 and 64%, respectively) and with the abdominal segment a 19% increase in TBARS and no significant change in NO.

The integral parameter of the antioxidant defense (TAS) decreased by 68% in the thoracic and 48% in the abdominal

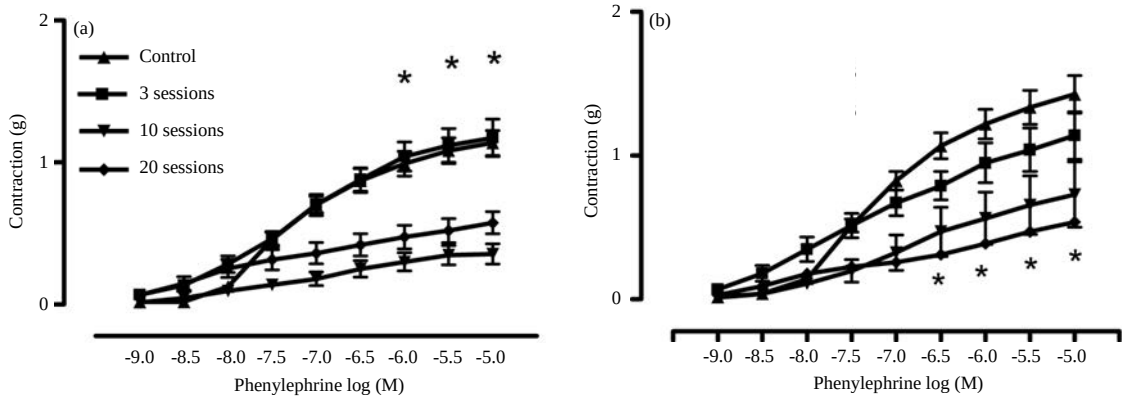

Fig. 1(a-b): Dose-response curves of, (a) Thoracic and (b) Abdominal aorta to phenylephrine (1×10^{-9}-1×10^{-5}) with HBO (n = 6), *p<0.05

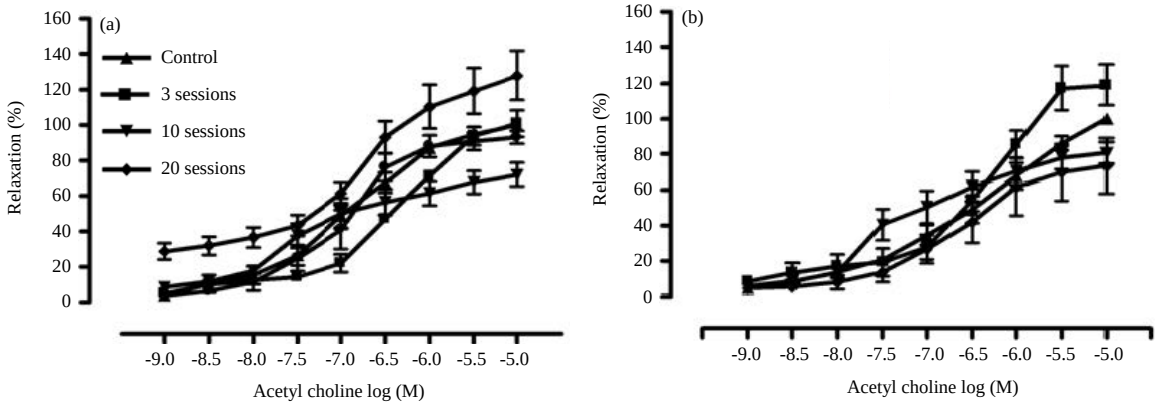

Fig. 2(a-b): Dose-response curves of, (a) Thoracic and (b) Abdominal aorta to acetylcholine (1×10^{-9}-1×10^{-5}) with HBO (n = 6), *p<0.05

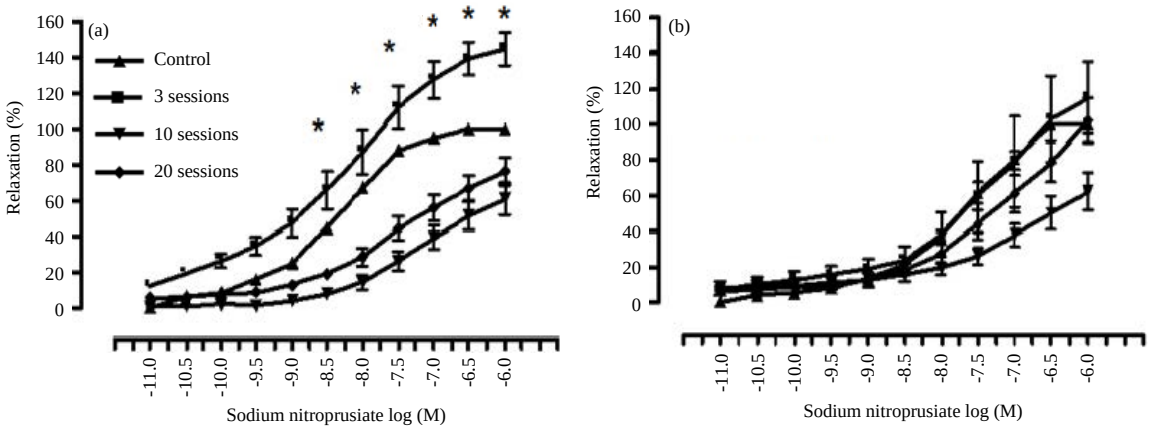

Fig. 3(a-b): Dose-response curves of (a) Thoracic and (b) Abdominal aorta to sodium nitroprusiate (1×10^{-11}-1×10^{-6}), with HBO (n = 6), *p<0.05

segment after 3 HBO sessions. In the thoracic aorta, there was also a decrease in all measured enzymes involved in the antioxidant defense (GPx, SOD and CAT; 90, 80 and 62%, respectively) as well as the non-enzymatic response (GSH, 81%). In the abdominal aorta there was a decrease in SOD (53%) and GSH (67% as of the fifth session). The level of GPx showed no significant change, while CAT showed a tendency to increase. After 10 HBO sessions, all parameters of oxidative stress and the antioxidant response decreased significantly in both aortic segments, with the exception of CAT and NO.

DISCUSSION

Hyperbaric oxygenation (HBO) therapy has been used for the treatment of diverse diseases related to ischemia, based on the idea that the inhalation of pure oxygen at higher than normal atmospheric pressure should allow for an elevated partial pressure of arterial and venous oxygen. This result would facilitate the diffusion of oxygen to the cells, thus favoring the recovery of an ischemic zone and avoiding cell death (Tompach *et al*., 1997).

The effects that have been observed with HBO therapy, include a reduction in heart rate (from 10-15%) (30), stimulation of neoangiogenesis (Muhonen *et al*., 2004), regulation of the release of NO (Demchenko *et al*., 2000) and an increase in the scavenging of ROS. However, to date there have been scarce reports on vascular reactivity in thoracic and abdominal portions of the aorta (Branger *et al*., 2001; Hink *et al*., 2006; Thom *et al*., 2003). Hence, the current contribution employed a rat model to explore the effects of HBO therapy on the contractile and vasodilatory response of the thoracic and abdominal aorta, the corresponding modification of oxidative stress and the antioxidant response (both enzymatic and non-enzymatic).

Compared to the control group, there was a significantly lower contractile response to phenylephrine in both thoracic and abdominal aorta after the tenth session of HBO therapy. This suggests that HBO therapy possibly modulates the contractile effect through endothelium dependent and/or independent pathways (Fujimoto *et al*., 2001; Demchenko *et al*., 2000). Since, HBO therapy did not significantly affect the relaxant effect of acetylcholine on thoracic or abdominal aorta, it seems that the effect of HBO treatment is endothelium independent. Treatment of aorta with sodium nitroprusside aimed to confirm the idea of an endothelium independent relaxation of aorta, finding that only in the thoracic segment was there a significant modification of the vascular response. Accordingly, an increased relaxant effect was found in this segment as of the third HBO session, an effect that declined after the tenth session.

NO levels were determined in order to explore the endothelium independent pathway of vascular modulation. The basal value of NO was higher in the thoracic than abdominal aorta and only in the former segment was there a significant change in NO with hyperbaric oxygenation (a decrease as of the third session). The results indicate that there is a regional difference in the response of the aorta.

There are other reports describing an elevated level of oxygen in plasma after HBO treatment, an effect that should favor the enzymatic and non-enzymatic production of NO and therefore lead to vasodilation (Thom *et al*., 2003). The enzymatic form of NO results from the reversible reaction of L-citruline, which is catalyzed by Nitric Oxide Synthase (NOS) in the presence of oxygen. The non-enzymatic form of NO can be produced by the reaction of the substrate L-arginine with the superoxide anion (O_2) and hydrogen peroxide (H_2O_2). It is known that HBO therapy promotes the formation of reactive oxygen species, which could increase the production of NO_2. Accordingly, it has been reported that after exposure

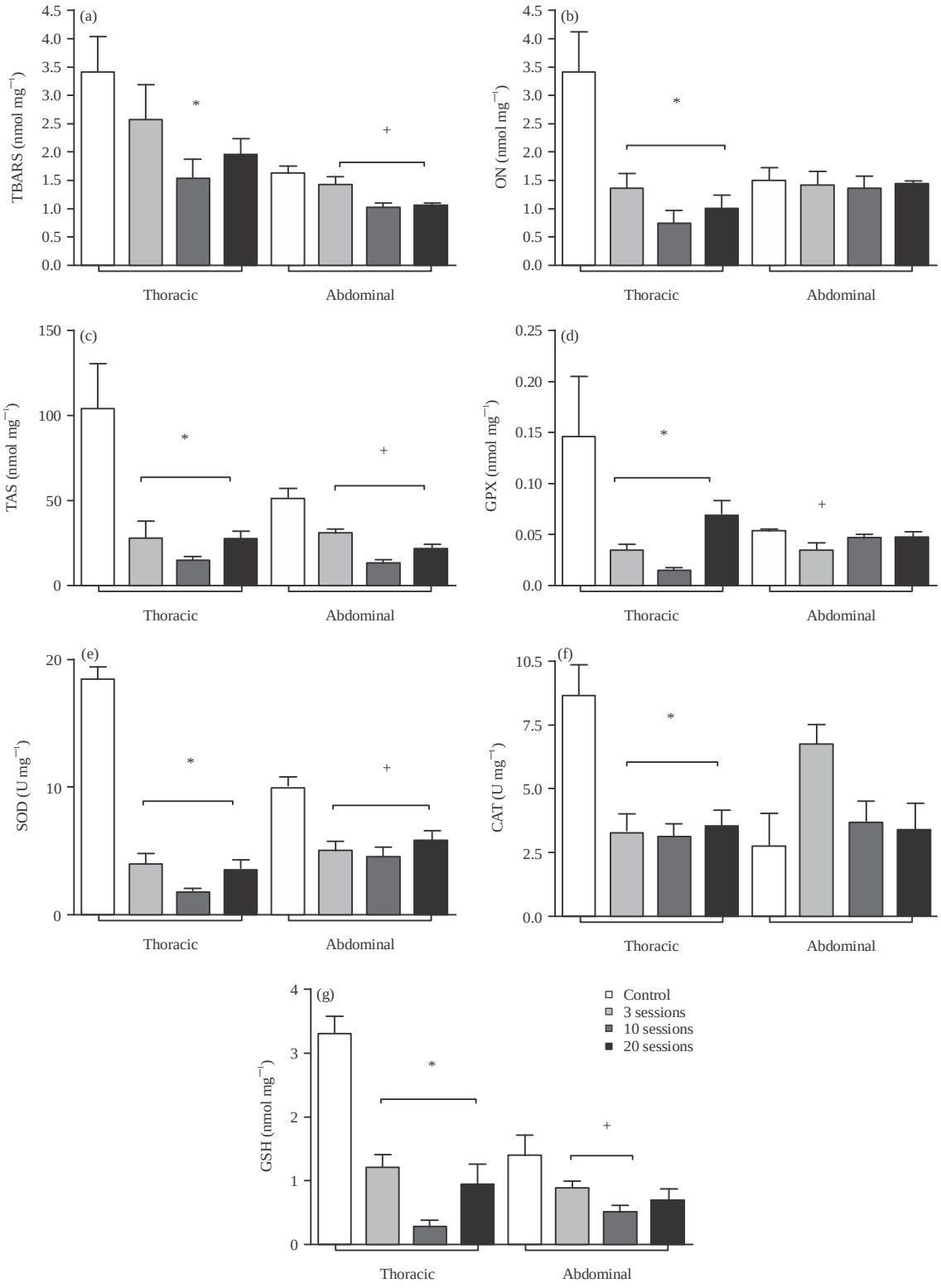

Fig. 4(a-g): Parameters of oxidative stress and the antioxidant defense in thoracic and abdominal aorta with HBO (n = 6), (a) TBARS: Thiobarbituric acid, (b) NO: Nitric oxide, (c) TAS: Total antioxidant status, (d) GPX: Oxidized glutathione peroxidase, (e) SOD: Superoxide dismutase, (f) CAT: Catalase and (g) GSH: Reduced glutathione, *$p<0.05$

to HBO therapy at 3 atm for 120 min, the level of NO increases 4-5 fold. It seems that there is an increase in NO under certain conditions of HBO therapy and a decrease under other conditions (Subbotina, 2006; Thom *et al*., 2003).

Hence, it is important to analyze the changes in the oxidant and antioxidant status of the thoracic and abdominal portion of the aorta in order to gain insights into the modification of the vascular response. Some studies have described that under normal conditions oxygen activates NADPH, giving rise to the formation of the superoxide anion (O_2). This anion is catabolized by SOD and GPx to form hydrogen peroxide (H_2O_2), which can form the hydroxyl radical (OH) or be catabolized by catalase to form H_2O and O_2 (Allen *et al*., 2009; Sen, 2001; Villanueva and Kross, 2012).

However, under the hyperbaric conditions of the present study the thoracic aorta showed an important decrease (over 60%) in all parameters of oxidative stress and antioxidant defense (both enzymatic and non-enzymatic), an effect that lasted during the 20 sessions of HBO. This suggests a probable drastic reduction in ROS and NO production (Villanueva and Kross, 2012) in the thoracic segment as of the third HBO session, which in turn would cause a decrease in the antioxidant defense. Perhaps the excess of oxygen caused the elimination of certain products that are normally involved in chain reactions leading to oxidative stress. In favor of this hypothesis is the fact that all parameters of oxidative stress and the antioxidant defense showed a similar degree of decrease in the thoracic segment (Kormanovski *et al*., 2010).

The increase in TBARS in the abdominal aorta (as of the third HBO session) evidenced an increase in oxidative stress, which was not accompanied by a decrease in the level of NO. Nevertheless, the values of the parameters of the antioxidant response were similar to those of the thoracic aorta. That is, in the abdominal segment the moderate decrease found in the antioxidant defense coincides with the moderate increase in oxidative stress, the latter of which is a logical result of increased oxygen levels due to HBO therapy.

CONCLUSION

Regional differences found in the vascular response and levels of oxidative stress suggest that many factors participated in the overall result. All parameters of oxidative stress and the antioxidant defense decreased significantly and to approximately the same degree in the thoracic segment. During the first ten HBO sessions, there was apparently an overall protective effect on blood vessels through the modulation of vascular tone (remaining within physiological conditions). The current results strongly suggest that a pathway other than NO was involved in this putative protective effect of HBO sessions. It is possible that HBO therapy increased the antioxidant response to a greater degree than it augmented oxidative stress.

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