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Original Article

Hyperbaric oxygenation applied before or after mild or hard stress: effects on the redox state in the muscle tissue

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ABSTRACT The mechanism is unclear for the reported protective effect of hyperbaric oxygen preconditioning against oxidative stress in tissues, and the distinct effects of hyperbaric oxygen applied after stress. The trained mice were divided into three groups: the control, hyperbaric oxygenation preconditioning, and hyperbaric oxygenation applied after mild (fasting) or hard (prolonged exercise) stress. After preconditioning, we observed a decrease in basal levels of nitric oxide, tetrahydrobiopterin, and catalase despite the drastic increase in inducible and endothelial nitric oxide synthases. Moreover, the basal levels of glutathione, related enzymes, and nitrosative stress only increased in the preconditioning group. The control and preconditioning groups showed a similar mild stress response of the endothelial and neuronal nitric oxide synthases. At the same time, the activity of all nitric oxide synthase, glutathione (GSH) in muscle, declined in the experimental groups but increased in control during hard stress. The results suggested that hyperbaric oxygen preconditioning provoked uncoupling of nitric oxide synthases and the elevated levels of GSH in muscle during this study, while hyperbaric oxygen applied after stress showed a lower level of GSH but higher recovery post-exercise levels in the majority of antioxidant enzymes. We discuss the possible mechanisms of the redox response and the role of the nitric oxide in this process.

INTRODUCTION

Hyperbaric oxygenation (HBO) affects the production of nitric oxide (NO) and redox state in tissues, but the respective mechanisms are still not clear [1]. NO is involved in pro-oxidant and antioxidant activity. The latter function is related to the direct or indirect participation of NO in the multitude of metabolic signaling cascades, including the regulation of blood vessel tone [2]. However, contradictory data exist regarding the impact of HBO on the level of NO. Indeed, the main focus of most reports is the bioaccessibility of this free radical [3,4]. The enzymatic pathway for NO formation is based on nitric oxide synthase (NOS) isoforms. These have a dimeric structure and can be able to function properly in a coupled state and in the presence of different cofactors, essential is tetrahydrobiopterin (BH_4). The oxidation of such cofactors by hyperoxia, hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and iron (Fe^{3+}) causes the uncoupling of NOS [5,6], resulting in a drop in NO production and an increase in the generation of the superoxide anion radical ($^-O_2^•$) [2]. The reaction of NO with $^-O_2^•$ affords $ONOO^-$, a strong oxidant that can intensify the decrease in NO levels and nitrosative stress.

NO as a free radical has a lifetime of only a fraction of a second. The possible modes of transport and donation at a distance, discussed in a recent review [2], involve S-nitrosylation. This is the covalent aggregation of NO to a protein cysteine thiol group to form S-nitrosothiols (RSNO), the structure responsible for the

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transfer of signaling over long distances. In the same review, the authors discuss the role of the S-nitrosylation of various thiol-containing molecules, including glutathione (GSH). This low-molecular-weight thiol participates in the process of S-nitrosylation that yields S-nitrosoglutathione (GSNO), known to be stable for hours in water and capable of modulating the effects of NO at a distance. The relation between NO and GSH, demonstrated many years ago, is discussed in another recent review [7]. GSH can be mobilized from its reserves in an emergency, mainly located in the liver. The evidence of an inverse relation between the concentration of GSH and NO in mouse liver during endotoxemia was attributed to NO's effect on the synthesis of GSH [8].

Many studies have shown the protective effect of HBO preconditioning (HBOP) against oxidative stress in tissues during diverse types of stress, including pathologies [9,10]. In a model of trained mice, our group has found gender differences in the redox response of many tissues (e.g., muscle and liver) at basal levels and after subjecting animals to mild stress (fasting) or hard physical stress (prolonged exercise), either with or without HBOP [11,12]. Regarding the behavior of basal parameters in muscle tissue during an HBO session, only females exhibited a sharp decrease in NO and antioxidant enzymes and an increase in GSH. On the other hand, they displayed a significant decline in the level of GSH in the liver, without any change in its degree of oxidation [12]. Using the same model of trained female mice, the current contribution aimed to evaluate the redox response in muscle tissue caused by HBO sessions, which were carried out prior to the stress event (HBOP) or afterward (HBOA). Three areas were explored: 1) the hypothesis of the uncoupling of NOS during an HBOP session; 2) the possible protective mechanism of HBOP against oxidative stress; and 3) the different response of animals to HBOP and HBOA groups.

METHODS

Animal model

Two-month-old female Balb/C mice (n = 126), all taken from the same breeding lot, were provided by the bioterium of the Escuela Superior de Medicina (Instituto Politécnico Nacional, Mexico City, Mexico). They were maintained in transparent plastic cages with food and water available *ad libitum*, at 20°C–25°C and on a 12-h light/dark cycle. The animals were sacrificed by injecting pentobarbital sodium (60 mg/kg of body weight) immediately after the respective procedure.

All mice were housed, monitored, and cared for humanely and all procedures were under the ARRIVE (animal research reporting *in vivo* experiments) guidelines [13] and Mexican Federal Regulations for Animal Experimentation and Care (NOM-062-ZOO-1999; Ministry of Agriculture, Mexico City, Mexico). This national regulation is in accordance with the international ethics standards of scientific journals. The current protocol was approved by the Institutional Committee on the Use and Care of Laboratory Animals (CICUAL, 04/14-12-2016) of the Escuela Superior de Medicina, Instituto Politécnico Nacional. Due to the distinct redox response of male and female mice during HBO and stress in a previous study with the same animal model, females were employed presently.

Study design

The training was designed to strengthen the antioxidant response to its maximum capacity. All animals were initially submitted to a 2-week adaption program consisting of non-forced swimming sessions that gradually increased in duration (from 10 to 60 min). Subsequently, they underwent a 12-week training program of non-forced swimming (3 times/week, 60 min/session). For the swimming sessions, a transparent tank was divided into cells (25 × 25 cm), and one animal was placed in each cell containing water at 32°C ± 2°C. Finally, the mice were dried and returned to their cages following an exercise session.

Trained female mice were randomly distributed into the control, HBOP, and HBOA groups. Each group of mice was divided into three subgroups (n = 7): basal, fasting (mild stress, 4 h), and prolonged exercise (hard stress, 4 h). This time was chosen for prolonged exercise because the time of exhaustive swimming was approximately five hours in this animal model. The parameters of each subgroup were measured in muscle tissue and plasma immediately after the stress event and/or HBO session and upon completion of a 24-h recovery period. A scheme of the time course of events for the 24-h experiment is provided (Table 1).

The fasting subgroup was included for the following reason: The animals fasted for 4 h, thus eliminating the possible effects of fasting by comparing post-exercise with post-fasting levels (net effect of exercise). According to the study design, both experimental groups corresponding two basal subgroups show similar initial and post-recovery levels of the parameters. On the other hand, the values were different between the basal subgroups of the experimental groups and the control (without HBO). The animals of HBOP group were exposed to the treatments (fasting or exercise) 30 min after the HBO session. The HBO session of HBOA group was administered 30 min after the stress treatments. All HBO sessions were carried out for 1.5 h (15 min pressurization, 60 min exposure, and 15 min depressurization) in a hyperbaric chamber for small animals with an oxygen pressure of 2 ATA. For the HBOP group, the animals were returned to their cages with food and water available during 30 min before the treatments.

Sample processing

Skeletal muscle was extracted from the hind leg (vastus lateralis) and placed in 30 μM cold phosphate buffer solution (pH 7.2), and 0.1% of Triton 100 was added. For each mg of tissue, 10 μl

Table 1. The time course of events during study

2 weeks	12 weeks	Procedure	Control			HBOP			HBOA		
Adaptation, increasing swimming from 10–60 min/session	Training, with 60-min sessions					HBO session					
		Samples	x	x	x	x	x	x			
		Subgroups	B	F	E	B	F	E	B	F	E
		Samples	x	x	x	x	x	x	HBO session		
		Samples							x	x	x
		Subgroups	B24	F24	E24	B24	F24	E24	B24	F24	E24
		Samples	x	x	x	x	x	x	x	x	x

A hyperbaric oxygenation (HBO) session was administered before the stress event (HBOP group) or afterwards (HBOA group). The three groups (control, HBOP, and HBOA) were each divided into three subgroups: basal (B), fasting (F), and prolonged exercise (E). Measurements each group were made before and immediately after the stress event (B, F, and E) and following a 24-h recovery period (B24, F24, and E24). Tissue samples were taken where the box is marked with an “x”.

of buffer was used. Tissues were homogenized and centrifuged at 10 000 rpm for 15 min and supernatants were stored at –75°C. Plasma was separated from blood samples and stored at the same temperature.

Assay kits (Cayman Chemical, Ann Arbor, MI, USA) were employed for the measurement of total proteins (TP, No. 704002), NO (nitrate/nitrite colorimetric assay kit, No. 780001), total reduced GSH (glutathione assay kit, No. 703002), glutathione reductase (GR, No. 703202) and catalase (CAT assay kit, No. 707002). The Randox chemical assay kits (Randox, Crumlin, UK) were adapted in order to quantify the activity of total superoxide dismutase (SOD, No. SD125), total glutathione peroxidase (GPx, RS504), in tissue homogenates. The values of NO and GSH are expressed as nmol/mg of TP/ml, corresponding to a μmol concentration, and those of enzymes as U/mg of TP/ml. The level of 3-nitrotyrosine (3NT) was established in homogenates by the enzyme-linked immunosorbent assay (ELISA) with the 3-nitrotyrosine ELISA kit (No. ab116691; Abcam, Cambridge, MA, USA), is expressed as ng/mg of proteins. The level of BH_4, also determined by ELISA (No. E-EL-0110; Elabscience, Houston, TX, USA), is expressed as pg/mg of proteins. The plasma concentration was quantified for GSH (pGSH, nM/ml) with the same kit (703002) and for estradiol (pg/ml) by ELISA (No. 0460818; Assay Designs, Ann Arbor, MI, USA).

Western blot

The activity of inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) was assessed by Western blot. Every subgroup sample was separated into three parts to be analyzed at different times. Briefly, 100 μg of protein were subjected to 10% SDS-PAGE under non-reducing conditions and then transferred to polyvinylidene fluoride membranes (Immobilon PVDF, 0.45 μm; MilliporSigma, Burlington, MA, USA). The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline (TBS) and 0.1% Tween 20 (TBS-T, pH 7.4) for 2 h. Subsequently, they were washed 3 times with TBS-T and incubated with the primary antibody for eNOS (Cat. SC-5302) at 4°C for 18 h under constant agitation. After adequately washing the membranes with TBS-T, they were incubated with the secondary antibody (Cat. SC-516102) for 2 h under constant agitation. Detection was then carried out by the enhanced chemiluminescence method (Western Blotting Luminol Reagent, Cat. 2048; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Membranes were photographed and the image was digitalized to conduct densitometric analysis on Imagen Studio Lite software (LI-COR Biosciences, Lincoln, NE, USA). The relative presence of each protein was normalized with β-actin as the housekeeping protein.

Descriptive statistical analysis was performed on SPSS version 21 (IBM Co., Armonk, NY, USA). Data are expressed as the mean ± SD. Parameters were examined with analysis of variance (ANOVA) followed by Tukey’s *post-hoc* test. The Pearson correlation formula calculated bivariate correlation coefficients (r) between parameters. In all cases, significance was considered at $p < 0.05$.

RESULTS

Effects of HBOP

For the control and experimental subgroups the response of the NO-related parameters was evaluated by quantifying the levels of iNOS, eNOS, nNOS, BH_4, NO, and 3NT in muscle tissue presented in Fig. 1.

The basal and post-fasting values of iNOS displayed a significant 6-fold increase in muscle tissue in the HBOP group relative to the control. The control animals only showed a boost in the level of iNOS during exercise, while the HBOP group exhibited a decrease in this parameter compared to post-fasting levels (net response) at both measurement times. Following the 24-h recovery period, the level of iNOS was higher in all HBOP subgroups than in the corresponding control subgroups.

The eNOS response was similar to iNOS in the HBOP group

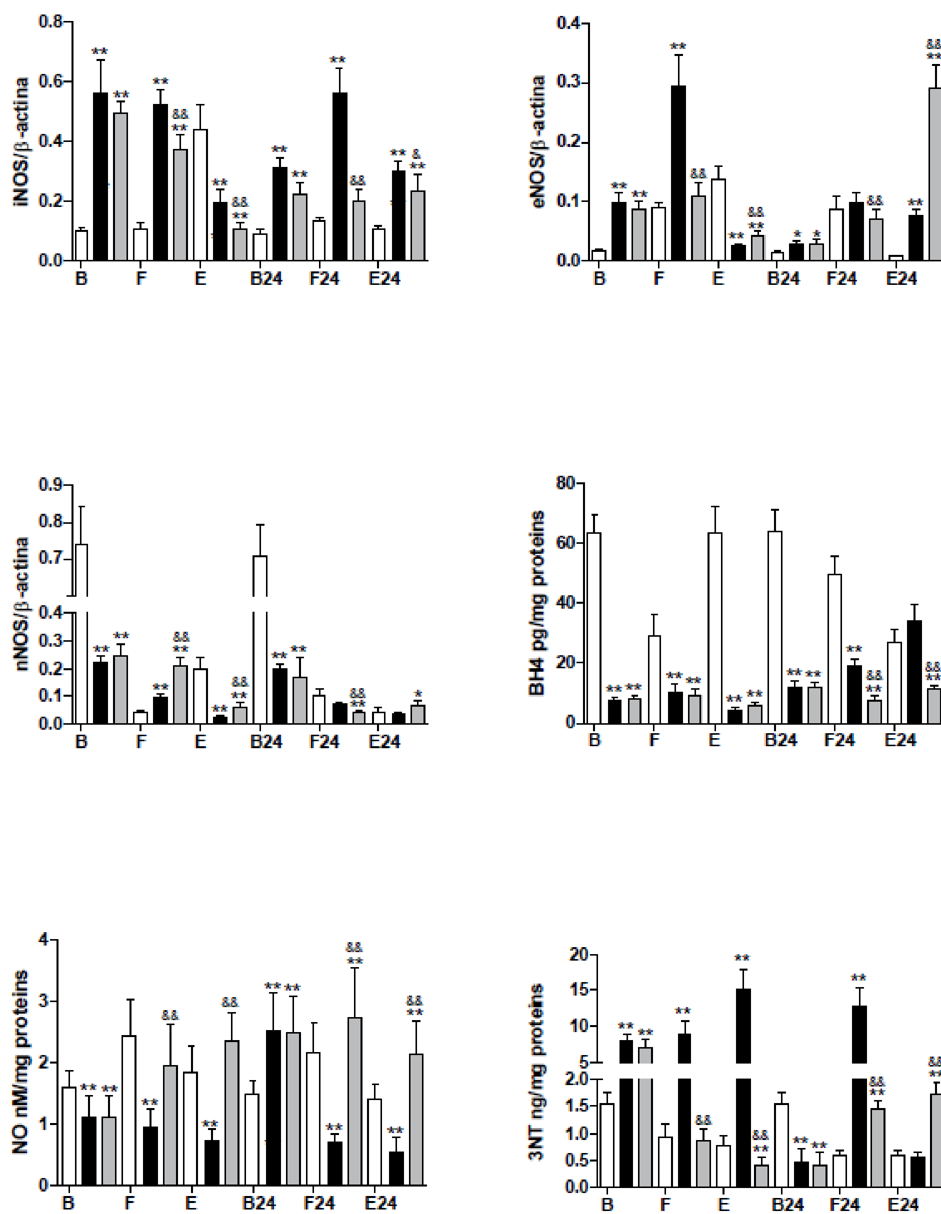

Fig. 1. Basal and post-stressed parameters related to the NO production were measured in muscle tissue of mice immediately and after 24 h of the recovery period. The three groups were the control (white columns), hyperbaric oxygen preconditioning (HBOP, black columns), and hyperbaric oxygen session after the stress event (HBOA, grey columns). Subgroups: B, basal; F, fasting (mild stress); E, prolonged exercise (hard stress). B24, F24, and E24: corresponding recovery subgroups. Values are expressed as the mean ± SD. NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; eNOS, endothelial NOS; nNOS, neuronal NOS; BH_4, tetrahydrobiopterin; 3NT, 3-nitrotyrosine. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the corresponding control inside each subgroup. $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ compared to the other experimental group inside each subgroup.

when measured immediately after the stress: an enhanced basal and post-fasting level and a reduced post-exercise level compared to the control group. Regarding the post-recovery values, however, the basal and post-fasting levels of the HBOP group dropped to the basal level of the control, while the HBOP post-exercise levels were higher in the control group. The nNOS in control group presented a 7-fold greater basal level than the other NOS. The basal level of nNOS was 3-fold lower, both initially and after the 24-h recovery period, in the HBOP versus the control group. Due to the stress treatments, nNOS diminished to a minimal level in the control and experimental groups, where it remained for at least 24 h. The post-exercise nNOS level was higher in the control group versus HBOP group (similar to iNOS and eNOS). During the recovery period, the two stressed subgroups of HBOP mice maintained a lower level of nNOS than its basal subgroup.

Concerning BH_4, a 6-fold decrease was found in the basal subgroup of the experimental animals compared to the initial elevated level in the basal subgroup of control mice, indicating the probable oxidation of BH_4 by HBOP. The level of BH_4 was similar and elevated in the post-fasting and post-exercise subgroups of the control but lower in the corresponding HBOP subgroups. During the recovery period, the level of BH_4 declined in the control stress subgroups, while the post-exercise level of this parameter rose in the HBOP subgroups, demonstrating partial recovery within 24 h.

The HBOP mice showed a reduction at the initial basal measurement of NO but a 2-fold higher level after the recovery period than the control group. On the other hand, the stressed HBOP mice had lower NO levels than the stressed control animals at both measurement times. Furthermore, compared to the mini-

mal levels of 3NT (an indicator of nitrosative stress) in the control group throughout the study, the HBOP group initially exhibited enhanced basal and post-stress levels. However, by the end of the recovery period, the basal and post-exercise levels of nitrosative stress in the HBOP group decreased to a minimal level.

The antioxidant response was evaluated for the control and experimental subgroups (Fig. 2) by quantifying the GSH, GR, GPx, CAT, and SOD levels in muscle tissue and the concentration of GSH estradiol in plasma. The last hormone expressed also direct and/or indirect antioxidant action.

Regarding the basal level of GSH, the initial value was approximately 2-fold greater in the HBOP versus control group, but the post-recovery value decreased in the HBOP mice to the level of the control. However, the level of GSH was higher in the HBOP versus control group after both stress events and upon completion of the 24-h recovery period (except similar post-exercise level), confirming a positive effect of HBOP on the nonenzymatic antioxidant capacity of muscle tissue, which lasted for at least 24 h.

The initial basal level of CAT was 3-fold lower in the HBOP versus the control group, a low level also found at the two post-stress measurement times for the fasting and exercise subgroups. However, the HBOP basal level of CAT reached that of the control by the end of the recovery period. The level of SOD was lower in the HBOP versus control subgroups throughout the study, showing a significant difference immediately after exercise and upon completion of the 24-h recovery period for the basal and fasting animals. Compared to the control, the basal levels of GPx and GR in the HBOP group were two-fold greater (contrary to CAT and SOD), coinciding with the elevated basal level of GSH. The level of GPx was higher in HBOP than control mice for all subgroups at the initial and post-recovery measurement, while GR showed higher post-exercise levels only.

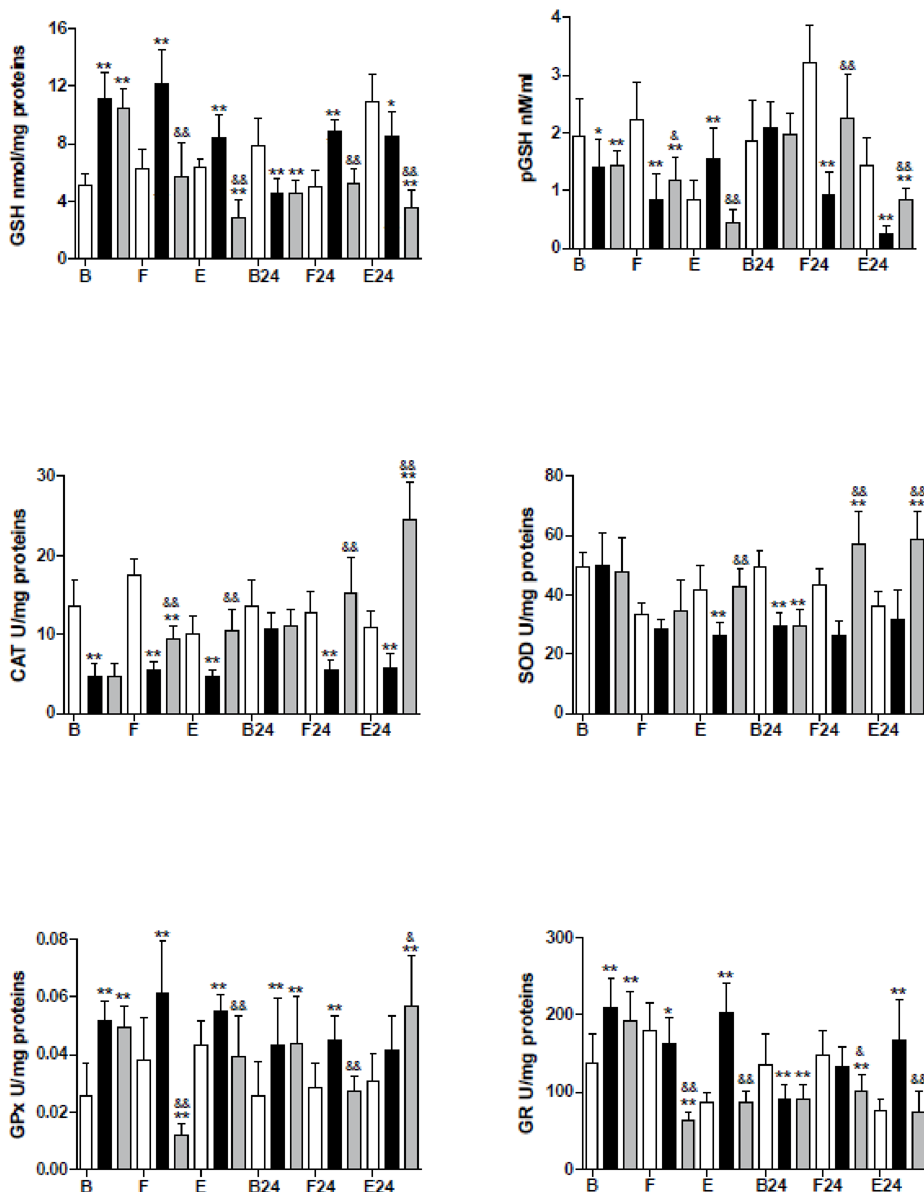

Fig. 2. Basal and post-stressed parameters related to the antioxidant response were measured in muscle tissue of mice immediately and after 24 h of a recovery period. The three groups were the control (white columns), hyperbaric oxygen preconditioning (HBOP, black columns), and hyperbaric oxygen session after the stress event (HBOA, grey columns). Subgroups: B, basal; F, fasting (mild stress); E, prolonged exercise (hard stress). B24, F24, and E24: corresponding recovery subgroups. Values are expressed as the mean ± SD. GSH, glutathione; pGSH, plasma GSH; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase. *$p < 0.05$ and **$p < 0.01$ compared to the corresponding control inside each subgroup. $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ compared to the other experimental group inside each subgroup.

Compared to the control, the HBOP group showed an initial moderate decrease in the basal level of plasma GSH, and reached a similar value upon completion of the 24-h recovery period. There was a reduced level of plasma GSH in the HBOP versus control subgroup regarding fasted mice. For exercised (versus fasted) animals, the level of plasma GSH was lower for the control, higher for the HBOP group immediately after the stress event, and lower for the HBOP group after the recovery period.

Compared to the control, the plasma level of estradiol in HBOP mice displayed an increase in the basal subgroup, a decrease in to control basal level with fasting, and an even more significant reduction with exercise in immediately response. On the other hand, the plasma concentration of estradiol dropped drastically in the fasted and exercised control mice (versus basal), and was 7-fold lower than in the fasted HBOP animals. Furthermore, estradiol level after the 24-h recovery period in the stressed subgroups of the control returned to initial basal level, a pattern not found in the experimental groups.

Effects of HBOA

The levels of iNOS following fasting and exercise were lower for HBOA than HBOP mice, but higher o similar for HBOA than control animals. The post-exercise changes were similar in both experimental groups, with lower levels shown by HBOA mice at both measurement times. However, compared to control, experimental exercised animals showed lower initial and higher recovered post-exercise levels. Immediately after fasting and at the end of the recovery period, the level of eNOS was similar in the HBOA and control groups but was much lower than the initial post-fasting level of the HBOP group. The post-exercise level of eNOS was reduced in both experimental groups compared to the control (similar to iNOS). Although the pattern of changes in the post-exercise eNOS level was similar at both measurement times for both experimental groups, there was a sharp increase in this recovery parameter for the exercised HBOA mice. The level of nNOS was similar in the fasted and basal subgroups of HBOA mice, being higher than the corresponding subgroups of the HBOP and control animals. Immediately after exercise, the level of nNOS was higher in control than in experimental groups, but at the end of the recovery period the level of nNOS was more significant in the HBOA group than in other groups. The pattern of changes in post-exercise levels in experimental groups was similar for all NOS isoforms.

The post-stress levels of BH_4 in the HBOA group were minimal during the study, similar to the basal levels at both measurement times. The inhibitory effect of HBO on the levels of BH_4 lasted at least 24 h in the HBOA group. Immediately after fasting and following the recovery period, the levels of NO and 3NT were similar in the control and HBOA groups, but the exercise stress levels of NO were higher compared to the control and HBOP groups, while the same levels of 3NT were minimal in control and HBOA group and maximal in HBOP group. Immediately after the fasting event and following the recovery period, the value of GSH was similar in the fasted HBOA and control subgroups at both measurement times (similar to NO) while being lower in the exercised animals of the HBOA versus control and HBOP groups (contrarily to NO).

The post-stress level of GSH was significantly lower (approximately 50%) in the HBOA versus HBOP group throughout the study. The post-stress levels of CAT were similar to the control group, except for a drastic increase for the exercised HBOA subgroup during the recovery period (similar to eNOS and nNOS), and higher at both measurement times compared to the HBOP group. The HBOA mice exhibited SOD values close to those of the control immediately after the stress events. At the end of the 24-h recovery, the fasting and exercised HBOA animals displayed elevated levels of this parameter, as they did for eNOS, nNOS and CAT. The post-stress values of GPx and GR showed a similar pattern of changes in both experimental groups, except for the elevated recovery post-exercise level in the HBOA group. Immediately after fasting and upon completion of the recovery period, these levels were lower in the HBOA versus HBOP and control groups.

Compared to the control, both experimental groups showed a significant reduction of plasmatic GSH immediately after fasting. There was an increase in the post-fasting level of GSH in plasma during the recovery period only in the HBOA group. Compared to the control, the experimental groups exhibited distinct post-exercise levels of plasma GSH, with a lower value for the HBOA in both measurements and a higher immediately value and minimal post-recovery value in the HBOP group.

On the other hand, the pattern of behavior of plasmatic estradiol in the HBOP and HBOA groups during the study was similar ($r = 0.978$, $p < 0.001$), but different from that of the control. The minimal estradiol levels in the stressed subgroups of control mice returned to the initial basal level after 24 h of recovery. However, the HBOA group showed higher immediately post-stress and similar recovery levels than the control group.

Correlation analysis

Multiple correlations were detected between various parameters during study (18 subgroups, sequence similar to the Figs. 1 and 2) (Table 2).

There were seven parameters with a major quantity of significant correlations (six): iNOS, NO, 3NT, CAT, GSH, GPx, and GR (five). Basal levels of these parameters suffered significant change during hyperbaric session of experimental groups. This data confirmed an increase in iNOS associated with an increase in nitrosative stress, GSH, pGSH, and GPx levels, and a decrease in NO and CAT levels. Finally, a decrease in NO was associated with the increase in GSH, GR and nitrosative stress, and a decrease in plasmatic GSH and CAT, confirmed strong relation in a pattern

Table 2. Matriz of Pearson´s bivariate correlation coefficients detected between measured parameters in 18 subgroups of study (sequence similar the Figs. 1 and 2)

iNOS	nNOS	eNOS	BH4	NO	3NT	GSH	CAT	SOD	GPx	GR	pGSH	EST	Parameters
				–0.52*	0.55*	0.75**	–0.66**		0.56*		–0.51*		iNOS
			0.57*										nNOS
									0.67**				eNOS
									–0.51*				BH4
					–0.67**	–0.74**	0.75**			–0.60**	0.58*		NO
						0.72**	–0.66**		0.54*	0.66**			3NT
							–0.74**		0.50*	0.77**			GSH
								0.57*		–0.51*			CAT
													SOD
											–51*		GPx
												–0.50*	GR
													pGSH
													EST

NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; eNOS, endothelial NOS; nNOS, neuronal NOS; BH_4, tetrahydrobiopterin; 3NT, 3-nitrotyrosine; GSH, glutathione; pGSH, plasma GSH; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; EST, plasma estradiol. *p < 0.05 and **p < 0.01.

Table 3. The bivariate correlation coefficients (Pearson's r) between various parameters within each group (control, HBOP, and HBOA)

Control	r	HBOP	r	HBOA	r
iNOS-GPx	0.841*	GSH-nNOS	0.802*	eNOS-iNOS	0.901**
eNOS-SOD	0.849*	GSH-GPx	0.790*	eNOS-NO	–0.788*
eNOS-3NT	0.951**	GSH-CAT	–0.807*	nNOS-CAT	0.852*
eNOS-BH_4	0.798*	pGSH-BH4	–0.798*	nNOS-3NT	0.792*
nNOS-NO	0.865*	pGSH-NO	0.820*	3NT-SOD	0.802*
nNOS-GPx	0.804*	eNOS-SOD	0.874*	3NT-CAT	0.800*
SOD-BH_4	0.947*	NO-CAT	0.912**		
CAT-GR	0.934**				

HBO, hyperbaric oxygenation; HBOP, HBO preconditioning; r, correlation coefficient; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; eNOS, endothelial NOS; nNOS, neuronal NOS; BH_4, tetrahydrobiopterin; 3NT, 3-nitrotyrosine; GSH, glutathione; pGSH, plasma GSH; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase. *p < 0.05; **p < 0.01.

of behavior of NO and GSH during study. The response of plasma estradiol was not showed anything significant correlations during study except a negative correlation with GR.

No significant bivariate correlation existed between the behavior of estradiol and the other parameters in any of the three groups, indicating the absence of any direct relation. However, a different bivariate correlation was detected between various parameters in each group (Table 3).

In the control group, the different NOS isoforms showed positive correlations with most parameters, evidencing the close relation between NO production and the antioxidant response. Particularly notable is the positive correlation between nNOS and NO and the absence of correlation of GSH with any other parameter in muscle tissue. Contrarily in the HBOP group, GSH is correlated with nNOS, GPx (positive), and CAT (negative), while NO has a high positive correlation with catalase. Interestingly, a negative correlation of plasma GSH with BH_4 and a positive correlation with NO was only found in the HBOP group. In the HBOA group, a positive correlation was detected between eNOS and iNOS and a negative correlation between eNOS and NO. A positive correlation was also identified for nNOS with CAT and 3NT, and 3NT with SOD and CAT, confirmed relation of nitrosative stress with response of antioxidant enzymes.

Response to mild and hard stress

A summary is presented (Table 4) of the significant changes in each parameter in response to mild stress (fasting) and hard stress (prolonged exercise), measured immediately after the stress event and at the end of the recovery period. Each post-fasting level is compared to the corresponding basal level, and each post-exercise level to the corresponding post-fasting level (net response).

Response to mild stress

There was a significant response to mild stress in the control and HBOP group only regarding NO-related parameters. The similar changes involved an increased level of eNOS and

Table 4. The response of the parameters to mild stress (fasting) and hard stress (prolonged exercise)

Mild stress								Hard stress							
NO production				Antioxidants				NO production				Antioxidants			
Groups	C	HP	HA	Groups	C	HP	HA	Groups	C	HP	HA	Groups	C	HP	HA
Immediately after stress															
iNOS				GSH				iNOS	I	D	D	GSH	I	D	D
eNOS	I	I		CAT				eNOS	I	D	D	CAT	D		
nNOS	D	D		SOD	D	D	D	nNOS	I	D	D	SOD			
NO				GPx				NO				GPx			
BH_4				GR				BH4				GR	D	I	
3NT				pGSH				3NT				pGSH	D	I	
				EST	D	I	I					EST	I	D	D
After the recovery period															
iNOS		I		GSH		I		iNOS		D		GSH	D		
eNOS	I	I		CAT		D	I	eNOS	D		I	CAT			I
nNOS	D	D		SOD			I	nNOS	0			SOD			I
NO	I	D		GPx			I	NO	D			GPx			I
BH_4	D	I		GR				BH4	D	I		GR	D		
3NT				pGSH	I	D	I	3NT	0	I	D	pGSH	D	D	D
				EST	I							EST	D	I	I

Significant changes are indicated as an increase (I), decrease (D), or no change (unmarked). Each post-fasting level is compared to the corresponding basal level, and each post-exercise level to the corresponding post-fasting level (net response). NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; eNOS, endothelial NOS; nNOS, neuronal NOS; BH_4, tetrahydrobiopterin; 3NT, 3-nitrotyrosine; GSH, glutathione; pGSH, plasma GSH; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; EST, plasma estradiol; C, control; HP, the HBOP group; HA, the HBOA group.

decreased level of nNOS at the both measurement times. The increased recovery levels of iNOS and BH_4 and decreased NO level in the HBOP group contrasted with an increase of NO and a decrease of BH_4 in control group. All groups displayed a decline in SOD and increased plasma estradiol (experimental groups) and a decrease (control) in the immediate response. During the recovery period, the level of GSH in muscle tissue increased and the levels of CAT and plasma GSH decreased in the HBOP group, while the levels of CAT, SOD and plasma GSH increased in the HBOA group. The control group showed an increase of plasma GSH and estradiol only.

Response to hard stress

The activity of all NOS isoforms declined in the two experimental groups and rose in the control during hard stress. The quantification of these parameters at the end of the recovery period revealed a reduced level of iNOS and an enhanced level of BH_4 and 3NT in the exercised HBOP mice, and a boost in the level of eNOS and a decrease of 3NT in the exercised HBOA animals. The control group showed a decrease in eNOS, NO and BH_4. Concerning the immediate antioxidant response to hard stress, the level of GSH in muscle and plasma estradiol increased in control and decreased in experimental groups, and the levels of GR and plasma GSH decreased in control and increased in the HBOP group. After the recovery period, experimental groups showed a reduced plasma GSH and an enhanced estradiol level, while the HBOA group also had increased levels of antioxidant enzymes. The control group presented decreased levels of GSH, GR in muscle, and GSH and estradiol in plasma.

DISCUSSION

The redox state response during physical stress in tissues of trained rodents in a short-term study depends on at least three key factors: 1) the basal levels of the antioxidant defense and NO; 2) the capacity of the mobilization of endogenous non enzymatic antioxidant resources to the strengthening of antioxidant defense and the bioaccessibility of NO; 3) possible modulation of antioxidant response by physiological effects of NO. Hyperbaric oxygen preconditioning affected the basal levels of antioxidant defense and NO production in muscle of trained female mice.

Response of basal levels during HBO session

Compared to the control group, the HBOP group exhibited a decreased initial basal level of NO in muscle tissue, despite a sharp increase in the basal levels of iNOS and eNOS. Under normal conditions, the most significant quantity of NO is generated by iNOS (versus eNOS and nNOS) under normal conditions. The current results suggest the drastic inhibition of the basal production of NO by the NOS isoforms during the HBO session. BH_4, an essential cofactor responsible for the normal functioning of the NOS isoforms in a coupled state, underwent a drastic reduction in the basal subgroup during the HBO session. Hence, the drop

in the basal levels of BH_4 and NO shreds of evidences of the uncoupling of NOS during session leads to an increased generation of $^-O_2^{\bullet}$ [5,6]. The reaction of $^-O_2^{\bullet}$ with NO forms peroxynitrite ($ONOO^-$), a strong oxidant that eliminates NO and generates to a higher level of nitrosative stress, reflected in an increased basal level of 3NT in proteins. However, SOD converts $^-O_2^{\bullet}$ into H_2O_2, thus explaining the striking reduction in basal level of CAT and a moderate decline in SOD in the HBO groups. The basal level of only one NOS isoform, nNOS, dropped during the HBO session and remained low in experimental subgroups for at least 24 h.

The basal levels of the parameters in the two experimental groups, which were of course similar, displayed the entire set of indicators related to the uncoupling of NOS during the short HBO session. There were lower levels of BH_4 and NO as primary indicators, and a drastic decrease in CAT a moderate decline in SOD, and an increase in 3NT as secondary indicators.

According to various reports, NO maintains homeostasis in the NO-ROS balance during exercise by protecting against oxidative stress. The regulation of this balance in the heart has mainly been attributed to nNOS [14-16]. On the other hand, various authors have attributed the harmful effects in patients with hypertension to the uncoupling of eNOS [17]. Hence, the therapeutic use of BH_4 has been contemplated for diabetic patients with hypertension [18].

The possible modes of NO transport and donation at a distance, discussed in a recent review [2], involve S-nitrosylation. One study contemplated advantage of RSNO, including GSNO, as transporters and donors of NO, discussing potential mechanisms of its release in therapy for endothelial dysfunction [19]. Interestingly, several researchers have ascribed the uncoupling of eNOS by BH4- and GSNO-related mechanisms, and discuss the role of BH_4 and GSNO in regulating the redox state in cells [20].

The concept of the bioaccessibility of NO is proposed to resolve the controversy over the concentration of NO and its observed effect described in various publications, taking into account that GSH is a transporter of NO in the conjugate of GSNO, which is stable in water for over an hour [21]. Therefore, the bioaccessibility of NO can be increased by the greater efficiency of its transport, thus compensating for its diminished production.

In the current study, a similar basal antioxidant response was found immediately after the HBO session, which included a sharp increase in the level of GSH and two related antioxidant enzymes, GPx and GR. The principal question of interest is the mechanism responsible for the higher basal level of GSH in muscle tissue, because it is a mobile, nonenzymatic, and potent endogenous antioxidant. The 50% increase in GSH in muscle, a tissue that represents 20%–30% of body mass, during a 1.5-h session of HBOP could be explained as more remarkable synthesis, the enhanced capacity of muscle tissue to capture GSH from the blood, and/or the mobilization of this antioxidant from the liver into the bloodstream. However, the synthesis of GSH is improbable since the HBOP session is short and is itself a stress factor.

Considering the decrease in the basal level of GSH in plasma during the HBO session of the HBOP group, an enhanced capacity for the uptake of this antioxidant from the blood is likely. The type of transport of GSH across the plasma membrane is still not clear, but recent reports describe the existence of ATP-dependent massive transport of GSH or its conjugates by means of multidrug resistance-associated proteins, and the transport of amino acids that are constitutive of GSH [22,23].

At the end of the 1990's, the relation between NO production and the level of GSH in cells was investigated. A rise in the NO level, provided by its donors, stimulates the synthesis of GSH in cell cultures [24,25]. Meanwhile, *in vivo* studies have shown the opposite relation between NO and GSH in the liver [7,26]. The potential role of NO in the modulation of GSH synthesis has been explored, including its effects on the activity of some related enzymes involved in the synthesis of GSH. Low NO levels stimulate the synthesis of GSH in the endothelium [27,28]. Hence, the following sequence of events is likely during an HBO session: an enhanced uptake of GSH from the bloodstream is caused by the drop in NO production, leading to a decreased concentration of GSH in the blood, which in turn triggers the release of GSH from the liver (or all events occur at the same time be the result of a decrease NO level in both tissues during session).

The mobilization of GSH is a likely mechanism for the enhanced level of this parameter in the muscle tissue of the HBOP group for various reasons, one of which is the high gradient of GSH levels between the liver and blood (200 *vs.* 1 μM) and muscle tissue in control mice (200 *vs.* 7 μM), previously demonstrated with the same animal model and protocol. During HBOP session the basal level of NO and GSH was observed to diminish significantly, the latter without changing GSH degree of oxidation, and to increase its level in muscle and other tissues [11,12]. The current data do not contradict this possibility. The HBO-induced increase in the basal level of GSH in muscle tissue fortifies the basal antioxidant defense, thus explaining the protective effect of HBOP against oxidative stress found in numerous studies [9,10,29-31]. Furthermore, the abundance of GSH reduces the rate of depletion of GPx and GR by the boost presently detected in the basal levels of these enzymes.

After the recovery period, the control group showed minimal changes in the basal levels of all parameters. On the other hand, the experimental groups exhibited a drastic decrease in nitrosative stress, BH4, iNOS, and eNOS, and increased levels de NO and CAT confirmed at least a partial recovery of the coupled state NOS during 24 h recovery.

Response to mild stress

HBOP promoted the sensibility of NO-related parameters to mild stress at both measurements, while there was stability of this parameters in the HBOA group throughout the experiment. In the control and HBOP groups, fasting caused a similar increase

in eNOS and decrease in nNOS, which continued to change in the same direction during the recovery period. A decrease in the post-fasting level of NO during recovery in the HBOP group coincided with an elevated post-recovery level of iNOS in the HBOP group only, probably confirming possible compensation for a decrease in NO production an increase in iNOS activity. An increase in the post-fasting level of BH_4 in the same group confirms the partial recuperation of the average basal function of NOS in the HBOP group.

The recovery level of GSH of HBOP group only increased in muscle tissue and decreased in plasma which probably reflected an enhanced capacity for the uptake of GSH from the blood flow by muscle. A decrease in the post-fasting recovery level of CAT in the HBOP group probably reflects the maintenance of the production of H_2O_2 through the uncoupling of NOS yet.

In contrast, the HBOA and control groups showed increased post-recovery levels of plasma GSH, likely due to its release from other tissues (principally the liver) into the bloodstream. Meanwhile, the level of GSH did not change in muscle tissue in control and HBOA groups, which probably have a lower capacity for the uptake of GSH from the blood during mild stress. The positive effect in the HBOA group was the significant increase, compared to the control and HBOP groups, in CAT, SOD, and GPx during the recovery period. A similar hormonal response was observed in the HBOP and HBOA groups: an increase found immediately after fasting and no change during recovery. These results demonstrate that the differences in response to mild stress found between the experimental groups were independent of the hormonal response.

Response to hard stress

By comparing the recovery response of the post-exercise levels in both experimental groups, it is possible to draw some conclusions. In both cases, uncoupling of NOS exists (understood by the decreased level of BH_4) that relates to the generation of $^{-}O_2^{\bullet}$. However, in the HBOP group, this increase in oxidative and nitrosative stress is counteracted by the conversion of $^{-}O_2^{\bullet}$ into H_2O_2 (SOD), the latter of which is eliminated by CAT in the presence of the elevated levels of GSH and is accompanied by an increase in levels of BH_4 to control levels. On the other hand, in the HBOA group, this increase in oxidative and nitrosative stress is counteracted by the drastic increase in post-recovery levels of eNOS and all antioxidant enzymes, with minimal participation of GSH and without the recuperation of $BH_{4.}$ However, the increase in BH_4 in the HBOP group can be explained as follows: the capacity of HBOP group to reduce oxidized BH_4 (BH_2) was more significant than the rate of oxidation by $ONOO^-$. Although, based on some reports, the reduction of BH_2 to BH_4 may be a rapid process (salvage pathway) related to folic metabolism [32-34]. The present study was limited to a duration of 24 h, which probably accounts for the lack of recovery of BH_4 in the HBOA group. Contrarily to the control group, a similar reduction in the three NOS isoforms and estradiol in experimental groups during hard stress was probably related to its elevated post-fasting levels than the control. During the recovery period, the post-exercise level of iNOS decreased in the HBOP group only (compared to the post-recovery fasting level).

The level of GSH in muscle tissue decreased immediately after prolonged exercise (versus fasting) in experimental groups only that confirmed the importance of its elevated levels in antioxidant response during hard stress. Contrarily, to the control and HBOA group, immediately after prolonged exercise, HBOP animals exhibited an enhanced level of plasma GSH, related to the more significant contribution of GSH to the bloodstream from the liver.

Following the recovery period after hard stress, a similar decrease in plasma GSH was observed in all groups, resulting in a minimal value of post-exercise level in the HBOP group, which probably reflected a decrease in accessibility to reserves of this antioxidant in liver in this time.

The correlations detected in the three groups do not contradict the hypothesis about the close relationship between the levels of GSH and NO. In the control, a close relation was found between the three NOS isoforms and the enzymatic antioxidant response, confirming the connection between NO production and the antioxidant response. The HBOP group showed a close relation of muscle tissue GSH with plasma GSH and parameters related to NO production. This group displayed multiple indicators of the uncoupling of NOS throughout the experiment. In the HBOA group, there was an important role for eNOS that coincided with a drastic increase of principal antioxidant enzymes during the recovery period.

The results suggested that hyperbaric oxygen preconditioning provoked uncoupling of nitric oxide synthases and maintained the elevated levels of GSH in muscle during this study, while hyperbaric oxygen applied after stress showed a lower level of GSH but higher recovery post-exercise levels in eNOS and antioxidant enzymes. It is possible that during HBO session GSH can be mobilized from its reserves, mainly in the liver, and NO has important paper in this process, explaining the protective effect of HBOP against oxidative stress found in numerous studies. In the use of multiples HBO sessions it is possible the use of precursors of GSH synthesis to avoid the exhaustion of this reserves. Finally, the negative effects of HBOP during session in muscle, related to uncoupling of NOS (an increase of oxidative and nitrosative stress, and a decrease in NO production), were commenced completely by an increase of GSH level that is potent antioxidant and its conjugate (GSNO) is potential transporter and donor of NO at a distance.

The limiting factor during the experiment was the monitoring of the redox state for only 24 h. Therefore, it is necessary to amplify the time of observation. The contrarily response of nNOS during HBO session, compared to eNOS and iNOS, demanding the use of specific NOS inhibitors in future study.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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