CARDIOPROTECTIVE EFFECTS OF *LYCIUM BARBARUM* EXTRACT FROM OXIDATIVE/NITROSATIVE STRESS UNDER INTERMITTENT HYPOBARIC HYPOXIA EXPOSURE IN RAT

IRINA CAMELIA CHIŞ[1*], DOINA BALTARU[2], ANCA DUMITROVICI[3], ANDREI COŞERIU[4], FLORINA VLAD[4], REMUS MOLDOVAN[1], ADRIANA MUREŞAN[1]

[1] *Department of Physiology, "Iuliu Hatieganu" University of Medicine and Pharmacy, Clinicilor St., No. 1, Cluj district, Zip code 400023, Cluj-Napoca, Romania, irinnaus@yahoo.com*
[2] *"Constantin Papilian" Military Emergency Hospital, G-ral Traian Mosoiu Str., No. 22, Cluj district, Zip code 400132, Cluj-Napoca, Romania*
[3] *"I. Chiricuta" Oncologic Institute, Republicii Str., No. 34–36, Cluj district, Zip code 400015, Cluj-Napoca, Romania*
[4] *Student, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania*

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Exposure to high altitude, which is associated with decreased oxygen pressure, could result in oxidative/nitrosative stress, enhanced generation of reactive oxygen and nitrogen species (RONS) in heart tissue. RONS produced in excess cause myocardial injury, cardiomyocyte apoptosis, and cell necrosis. The fruits of *Lycium barbarum* (LBG), small red berries, have been used for thousands of years in traditional Chinese medicine for biological activities including antioxidative, anti-aging, anti-tumor, immunostimulating and cytoprotective activities. Recent studies have demonstrated that LBG extracts possess cardioprotective activities due to their antioxidants effects. The present study was to evaluate the cardioprotective effects of LBG extract in animals exposed to intermittent hypobaric hypoxia (IHH) and therefore exposed to oxidative/nitrosative stress. Sixty adult male Wistar rats were randomly assigned into six groups and were exposed to short-term (2 days) or long-term (4 weeks; 5 days/week) IHH in a hypobaric chamber or kept under normobaric normoxia (Nx). Some of the rats were treated with natural antioxidant LBG extract (30 mg/kg body weight) daily, before each IHH exposure; the remaining rats received physiological NaCl solution. Control rats were kept under Nx and treated in a corresponding manner. The cardiac tissue homogenate levels of some free radicals (malondialdehyde, MDA and carbonylated proteins, CP), nitrite plus nitrate (NOx) production, activity of antioxidant enzymes (superoxide dismutase, SOD and catalase, CAT) and inducible nitric oxide synthase (iNOS) protein expression were measured one day after the last exposure to IHH.

[*] Corresponding author (Irina Camelia Chis, MD, PhD, Lecturer, Department of Physiology, 1-3, Clinicilor Street, "Iuliu Haţieganu", University of Medicine and Pharmacy, RO 400023, Cluj-Napoca, Romania; Phone: +40-744-707080; Fax 0040-264-597257; E-mail: irinnaus@yahoo.com)

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In cardiac tissue homogenate, MDA and CP levels, NOx production and iNOS expression of IHH-exposed rats had increased and SOD and CAT activities had decreased significantly compared to those of the Nx-exposed rats. The treatment with LBG decreased the levels of MDA, CP, iNOS and NOx in rats exposed to IHH. However, LBG administration increased myocardial SOD and CAT activity in the IHH-exposed rats. These results suggest that the LBG can protect hypoxic myocardium and decrease the hypoxia-induced oxidative/nitrosative cardiac damage.

Keywords: cardioprotective, intermittent hypobaric hypoxia, *Lycium barbarum* extract, oxidative/ nitrosative stress.

INTRODUCTION

Hypoxic hypoxia met at high altitudes (HA) is known as altitude hypoxia or hypobaric hypoxia (HH). Exposure of the organism to HA is an oxinitrosative physiological stress which brings about an increased production of reactive oxygen and nitrogen species (RONS) by: altered mitochondria electron transport chains, activating enzymes: nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase/reductase, nitric oxide synthases (NOS), etc., and by the depletion of cellular enzymatic and non-enzymatic antioxidants (1-3). In high concentrations, RONS causes the peroxidation of lipids, proteins and oxidative damage to DNA, necrosis/apoptosis of the cells and the increased production of proinflammatory cytokines (IL-1β, IL-6, TNF-α, etc.) (4-7). Effects of exposure to HH depend on the degree and duration of exposure to HA. RONS produces excessive activation of higher expression of hypoxia inducible factor (HIF-1α), which then activates over 100 genes inclusively involved, including angiogenesis, erythropoiesis, apoptosis, increased expression of inducible NOS (iNOS), etc. (2, 7-9).

Hypobaric hypoxia severely affects the structural and functional integrity of the heart by virtue of RONS (5, 6). Myocardium is one of the most sensitive organs to hypoxic stress, because overproduced RONS induce cardiomyocyte injury. Hypoxia leads to cardiac apoptosis by the expression and accumulation of pro-apoptotic proteins, followed by cardiac remodeling, fibrosis biventricular ventricular hypertrophy, left ventricular dysfunction and heart failure (5, 10-12).

Nitric oxide (NO) is a free radical gas that is an important intracellular and intercellular signaling molecule involved in the regulation of physiological and pathological mechanisms in cardiovascular, nervous and immune systems. Reactive oxygen and nitrogen species, but especially NO, play a crucial role in the regulation of cardiac activity. NO is generated by the conversion of L-arginine to L-citruline in the presence of the family of nitric oxide synthase (NOS) isoenzymes. Three isoforms of NOS – neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) – have been found in different cell types that generate NO in various areas (13-16); eNOS and nNOS are constitutive isoforms that are responsible for the generation of NO physiological concentration, while iNOS is

involved in the production of high levels of NO in various types of cells, such as cardiomyocytes, monocytes/macrophages, mast cells, endothelial cells, hepatocytes, smooth muscle cells, Kupffer cells, fibroblasts, etc. (13, 14).

Alteration of NO production by the action of iNOS isoenzyme is a critical factor in heart injury to hypoxia/reoxygenation. NO produced in excess in the myocardium, under the action of hypoxia-induced iNOS, rapidly reacts with superoxide anion to form peroxynitrite (ONOO-), a more toxic metabolite that causes myocardial injury and endothelial dysfunction (17, 18).

Barbary wolfberry fruit (Goji berry), which is the dried fruit of *Lycium barbarum* (LBG), has long been used to nourish various organs and maintain the healthy state of body as an anti-aging herbal medicine in the Eastern world. Different biological activities of LBG have been demonstrated, including anti-aging, anti-apoptotic, anti-tumor, cytoprotective and immunostimulating and antioxidative activities (19-23). Lycium barbarum has been used for many decades for ameliorating obesity and diabetes (24, 25). Recent research shows the biologically beneficial cardioprotective and neuroprotective effects of LBG extract (26-29).

In the present study we evaluated the protective effect of LBG extract supplementation in hypoxic cardiac tissue in animals that were exposed to IHH and therefore, to oxidative/nitrosative stress. We focused on the correlation between LBG extract administration in cardiomyocytes and the cardioprotective role of LBG conducted by decreasing oxidative/nitrosative stress induced in the heart by intermittent hypobaric hypoxia exposure.

MATERIALS AND METHODS

DRUGS AND CHEMICALS

Lycium barbarum (LBG) fruits were extracted, dosed and encapsulated at the "PROPLANTA" Applied Vegetal Biotechnologies Center in Cluj-Napoca, Romania. They were standardized by their chemical composition using the high-performance liquid chromatography (HPLC) method to ensure batch-to-batch consistency. For this study, LBG extract was suspended in physiological NaCl solution (PS) as a vehicle.

EXPERIMENTAL DESIGN AND SAMPLING

Sixty healthy Wistar albino male rats (three-months-old) were used in this study. Rats were purchased from the Experimental Animal House of the Faculty of Medicine of "Iuliu Hatieganu" University of Medicine and Pharmacy of Cluj-

Napoca, Romania. Their mean weight was 239±49.49 grams (g) at the start of the trials. All animals used in the experiment were kept for ten days to acclimatize in the Physiology Department Animal House Laboratory conditions before introduction in the study. They received a standard diet and their access to water was not restricted. All the experiments were performed according to the approved animal-care protocols of the Ethical Committee on Animal Welfare of the "Iuliu Hatieganu" University in accordance with the Romanian Ministry of Health and complying with the Guiding Principles in the Use of Animals in Toxicology.

The animals were randomly subdivided into six experimental groups (n=10): 1st Group (control group) – rats maintained in normobaric normoxia (Nx) conditions (760 mm Hg, 21% O_2 and 79% N_2) for 2 days (short-term) and treated with PS (STNx+PS); 2nd Group (control group) – rats maintained in short-term hypobaric hypoxia (STIHH) conditions and treated with PS (STIHH+PS); 3rd Group – rats maintained in STIHH conditions and treated with LBG extract (STIHH+LBG); 4th Group (control group) – rats maintained in normobaric normoxia (Nx) conditions (760 mm Hg, 21% O_2 and 79% N_2) for 4 weeks (long-term) and treated with PS (LTNx+PS); 5th Group – rats maintained in long-term hypobaric hypoxia (LTIHH) conditions and treated with PS (LTIHH+PS); 6th Group – rats maintained in LTIHH conditions and treated with LBG extract (LTIHH+LBG). The animals were weighed at the beginning and at the end of the experiment.

The animals were exposed to a simulated altitude of 5500 m in a barochamber (in the Animal House Lab. Physiology Department of the Faculty of Medicine of "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania) for 2 days (short-term IHH, 380 mm Hg, 12% O_2 and 88% N_2, for 8 hours per day) and for 4 consecutive weeks (long-term IHH, 380 mm Hg, 12% O_2 and 88% N_2, for 8 hours per day, 5 days per week). The rats were taken out of the hypoxic chamber once after every 8 hours exposure in order to receive food and water. The Nx rats were kept in the same environment as the IHH rats with free access to water and food, except hypoxic exposure. Throughout the entire period of the experiment, all animals were maintained in special cages artificially illuminated (12 h dark/12 h light cycle), at a temperature of 21-23°C and 50-60% humidity, in an animal room.

Some of the rats received LBG extract (30 mg/kg/day, dissolved in PS) *via* an intragastric tube (0.6 mL/rat) for two days or for consecutive weeks 30 minutes before each IHH exposure. The control groups were treated with vehicle (0.6 mL of PS) *via* an intragastric tube.

After 24 hours of the last exposure to normoxia or hypoxia, all rats were anaesthetized by intraperitoneal administration of sodium pentobarbital (60 mg/rat) and sacrificed by cervical dislocation, and their heart was quickly dissected, washed in ice cold saline, dried on filter paper, and weighed immediately.

Frozen heart tissues were placed into plastic tubes, homogenized at a concentration of 100 mg tissue per mL of 25 mM buffer (pH 7.4) composed of Tris-HCl 10, NaCl 137, Na_2EDTA 1, dithiotreitol (DTT) 0.5 and sucrose 250 (in mmol/L) on ice, using a Polytron homogenizer (Brinkman Kinematica Switezerland). The homogenate was centrifuged for 10 min at 9000 g, and the cellular debris was discarded (33). The supernatant was allocated into separate tubes, stored at -80°C and used for biochemical assays.

BIOCHEMICAL ASSAYS AND ANALYSIS

The cardiac tissue homogenate levels of oxidative and nitrosative stress were determined.

The cardiac tissue homogenate levels of oxidative stress were estimated by measuring free radical production: lipid peroxides and protein carbonylation. Lipid peroxidation was determined by measuring the malondialdehyde (MDA) concentrations (by fluorescein dosage, Conti method) (30), and results were expressed in nmol/mg protein. Protein carbonylation was estimated by measuring the carbonylated protein (CP) levels; CPs as products of the reaction between reactive oxygen species and proteins were determined using the hydrochloric guanidine method (31), and results were expressed in nmol/mg protein.

The myocardial antioxidant enzymes activity was estimated by measuring the levels of superoxide dismutase (SOD) and catalase (CAT) in the cardiac tissue homogenate. The myocardial SOD activity was assayed as described by Flohe *et al.* (32); a unit of enzyme activity was defined as the enzyme reaction giving 50% inhibition of NBT reduction in 1 min under the assay conditions and expressed as specific activity in U/mg protein. The myocardial CAT activity was determined using the method proposed by Pippenger *et al.* (33), which consists in following the change in absorbance of a solution of H_2O_2 10 mM in potassium phosphate buffer 0.05 M, pH 7.4, at 240 nm; one unit of CAT is defined as the amount of enzyme which induces a reduction in absorbance of 0.43 at 25°C for 3 minutes; CAT activity was also expressed as units per milligram of protein (U/mg protein).

The nitrosative stress markers in cardiac tissue homogenate were determined by measuring the NO and iNOS levels. NO production was indirectly detected by measuring the degradation products of NO, the total nitrite level, after a prior conversion of nitrates into nitrites under the action of nitrate reductase. The nitrite plus nitrate (NOx) production was determined by measuring the nitrite, a stable end-product of NO metabolism, using the Griess reaction (34). The heart tissue homogenate was mixed with an equal volume of Griess reagent followed by spectrophotometric measurement at 543 nm. Nitrite concentrations in the heart tissue homogenate were estimated by comparison with a sodium nitrite standard curve. The NOx levels are expressed as nanomoles per milligram of protein (nmol/mg protein).

The cardiac tissue homogenate levels of iNOS were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN). They are expressed in nanograms per milligram of protein (ng/mg protein).

STATISTICAL ANALYSIS

Results were expressed as mean value standard deviation of means (SD). Statistical significance was tested at 95% confidence interval (CI) ($p<0.05$). Data were analyzed using the Statistical Package for the Social Sciences version 17.0 (SPSS-17). All experimental parameters measured were analyzed by one-way analysis of variance (ANOVA) followed by the Scheffe multiple comparisons post-test.

RESULTS

Cardiac changes of rats under short-term *versus* long term intermittent hypobaric hypoxia

The heart weights did not increase significantly following 2 days short term IHH (STIHH) (Table 1). After 4 weeks of IHH, a significant cardiac hypertrophy was observed due to increased heart weight-to-body weight ratio (mean±SD; $4.18\pm0.11 \times 10^3$ *vs.* $3.65\pm0.51 \times 10^3$, $P<0.05$) (Table 1). Rats treated with LBG extract and exposed to long term IHH (LTIHH) showed a significant decrease in heart-weight-to-body weight (Table 1).

Table 1

Effects of short-term (2 days) and long term (4 weeks) intermittent hypobaric hypoxia on rat heart

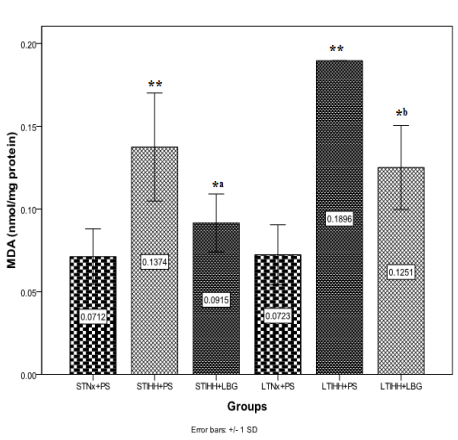
	1st Group (STNx+PS)	**2nd Group (STIHH+PS)**	**3th Group (STIHH+LBG)**	**4th Group (LTNx+PS)**	**5th Group (LTIHH+PS)**	**6th Group (LTIHH+LBG)**
Initial body weight (g)	195± 18.5	197± 14.5	190± 12.1	194± 16.5	264± 27.5	273± 42.6
Final body weight (g)	195± 18.5	197± 14.5	184± 11.3	194± 16.5	275± 21.4*	285± 42.3[a]
Final whole heart weight (g)	0.72± 0.15	0.75± 0.08	0.73± 0.15	0.71± 0.16	1.15± 0.25*	1.04± 0.03[a]
Final whole heart weight/final body weight (x10^3)	3.69± 0.81	3.80± 0.55	3.96± 1.32	3.65± 0.51	4.18± 0.11*	3.74± 0.35[a]

Values are means±SD (n=10 in each group). *$P<0.05$ as compared to the 1st Group and 4th Group (control groups); [a]$P<0.05$ as compared to the 2nd and 5th Group, respectively (control groups).

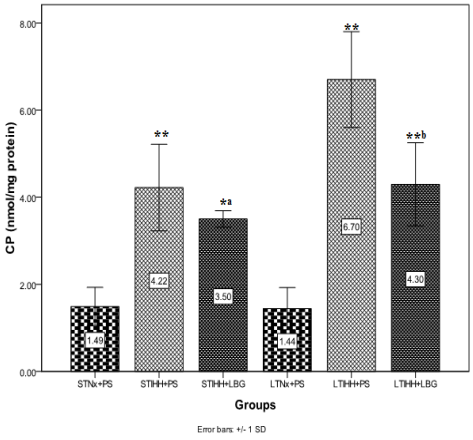
The effect of LBG extract on decreased oxidative stress in the cardiac tissue homogenate of rats exposed to short term or long term intermittent hypobaric hypoxia

***Lipid peroxidation*.** The MDA level in te cardiac tissue homogenate of rats exposed to STIHH or LTIHH was significantly higher ($P<0.005$) than that of controls (STNx+PS and LTNx+PS, respectively), the growth having higher statistical significance ($P<0.005$) after exposure to LTIHH (Fig. 1a). LBG treatment reduced ($P<0.05$) the MDA level in the heart tissue homogenate exposed to IHH for 2 days (STIHH+LBG) or 4 weeks (LTIHH+LBG), comparatively with animals exposed to IHH for 2 days or 4 weeks and treated only with vehicle (PS) (Fig. 1a).

***Protein carbonylation*.** The carbonylated proteins (CP) levels in cardiac tissue homogenate of rats exposed to STIHH or LTIHH were significantly higher ($P<0.005$) than those of controls (STNx+PS and LTNx+PS, respectively) (Fig. 1b). In contrast to the rats exposed to STIHH (STIHH+PS), those exposed to LTIHH (LTIHH+PS) showed an increase ($P<0.005$) in CP levels. The rats exposed to STIHH and treated with LBG (STIHH+LBG) presented a significantly lower ($P<0.05$) CP level than controls (STIHH+PS). The LBG treatment of rats exposed to LTIHH (LTIHH+LBG) decreased ($P<0.005$) the CP level comparatively with controls (LTIHH+PS) (Fig. 1b).



(a)



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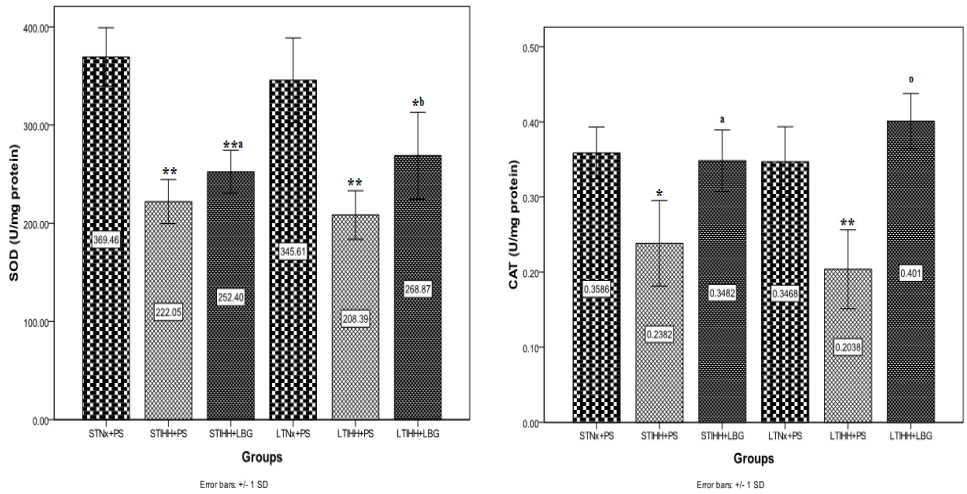


(c) **(d)**

Fig. 1 – The effect of short term and long term intermittent hypobaric hypoxia and LBG treatment on: (a) lipid peroxidation (MDA) (nmol/mg protein), (b) protein carbonylation (CP) (nmol/mg protein), (c) superoxide dismutase (SOD) activity (U/mg protein) and (d) catalase (CAT) activity (U/mg protein). Rats were submitted to short term (2 days) or long term (4 consecutive weeks) intermittent hypobaric hypoxia (STIHH or LTIHH) or normobaric normoxia (Nx) conditions, with treatment of physiological NaCl solution (PS) or *Lycium barbarum* (LBG) extract. Results are the means±SD for 10 animals in each group. Significant differences *P< 0.05 and **P<0.005 (STIHH and LTIHH *vs*. STNx+PS and LTNx+PS respectively); ^{a}P< 0.05 (STIHH+LBG *vs*. STIHH+PS); ^{b}P< 0.05 (LTIHH+LBG *vs*. LTIHH+PS).

The effect of LBG treatment on increased myocardial antioxidant enzymes activity of rats exposed to short term or long term intermittent hypobaric hypoxia

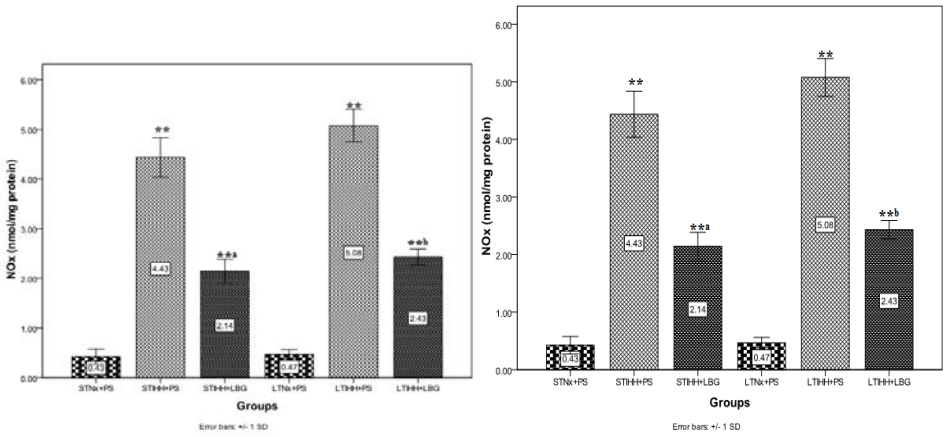
The myocardial SOD and CAT activity was significantly lower (P<0.05) after exposure to STIHH or LTIHH than those of controls (STNx+PS and LTNx+PS, respectively), without a significant statistical difference between the two types exposed to IHH (Fig. 1d and Fig. 1e). The rats treated with LBG and exposed to STIHH (STIHH+LBG) presented a significant growth (P<0.05) of the myocardial SOD and CAT activity comparatively with controls (STIHH+PS). Animals exposed to LTIHH and treated with LBG (LTIHH+LBG) showed an increase (P<0.05) of the myocardial SOD and CAT activity when compared to controls (LTIHH+PS) (Fig. 1d and Fig. 1e).

The effect of LBG treatment on decreased nitrosative stress in the cardiac tissue homogenate of rats exposed to short term or long term intermittent hypobaric hypoxia

***Nitrite plus nitrate production*.** The nitrate plus nitrite (NOx) level in the cardiac tissue homogenate of rats exposed to STIHH or LTIHH was significantly

higher (P<0.005) than that of controls (STNx+PS and LTNx+PS respectively), with a significantly higher level (P<0.005) after exposure to LTIHH (Fig. 2a). The rats exposed to STIHH and treated LBG (STIHH+LBG) presented a decreased (P<0.005) NOx level compared with controls (STIHH+PS). The animals exposed to LTIHH showed a significantly lower (P<0.005) NOx level after treatment with LBG (LTIHH+LBG) than controls (LTIHH+PS) (Fig. 2a).

Inducible nitric oxide synthase (iNOS) protein expression. The iNOS expression in the cardiac tissue homogenate of rats exposed to STIHH or LTIHH was significantly higher (P<0.05) than that of controls (STNx+PS and LTNx+PS, respectively), with growth rates being more statistically significant (P<0.05) after exposure to LTIHH (Fig. 2b). In contrast to animals exposed to STIHH (STIHH+PS), those exposed to LTIHH (LTIHH+PS) presented an insignificant increase in the iNOS levels. The LBG treatment of rats exposed to IHH for 2 days (STIHH+LBG) showed a significant decrease (P<0.05) of the iNOS level compared to controls (STIHH+PS). A significant decline (P<0.05) in the iNOS level was observed in the rats exposed to IHH for 4 weeks and treated with LBG (LTIHH+LBG) compared with their controls (LTIHH+PS) (Fig. 2b).



(a) **(b)**

Fig. 2 – The effect of short term and long term intermittent hypobaric hypoxia and LBG treatment on: (a) nitrite plus nitrate (NOx) production (nmol/mg protein) and (b) inducible nitric oxide synthase (iNOS) protein expression (ng/mg protein). Rats were submitted to short-term (2 days) or long term (4 consecutive weeks) intermittent hypobaric hypoxia (STIHH or LTIHH) or normobaric normoxia (Nx) conditions, with treatment of physiological NaCl solution (PS) or *Lycium barbarum* (LBG) extract. Results are means±SD for 10 animals in each group. Significant differences *P< 0.05 and **P<0.005 (STIHH and LTIHH *vs*. STNx+PS and LTNx+PS, respectively); [a]P< 0.05 (STIHH+LBG *vs*. STIHH+PS); [b]P< 0.05 (LTIHH+LBG *vs*. LTIHH+PS).

DISCUSSION

Exposure to long-term or short-term intermittent hypobaric hypoxia (IHH) has been reported to induce cardiac damage or cardiac protection in different studies with various conditions. Exposure to IHH is similar with ischemia/ reperfusion injury – the growth of RONS production during the phase of reoxygenation of IHH (4). Exposure to short-term IHH induced myocardial protection against ischemia/reperfusion-induced injury by reduced myocardial infarction, improved recovery of contractile function and limitation of ventricular arrhythmias (5-12). Controversial effects of IHH on cardiac damage or cardiac protection are still incompletely understood.

The cardioprotective role of LBG extract against oxidative/nitrosative stress in rats exposed to short-term or long-term IHH was analyzed in this study. Oxidative/nitrosative stress can be triggered by a series of endogenous and exogenous factors, exposure to IHH being one of them (4). Hypoxia appears to affect enzymatic and non-enzymatic antioxidants such as SOD, CAT and reduced glutathione, which are usually reduced. Recently, studies have demonstrated LBG interactions with many physiological processes and diseases (19-29). The effects of LBG could depend on physiological processes of the organs when these are subjected to environmental stress like IHH.

The present study demonstrated that short-term or long-herm IHH exposure induces oxidative stress in the heart, which is proved through the growth of lipid peroxidation and protein carbonylation in cardiac tissue homogenate. Our results are in accordance with those of other researchers (4, 6, 7, 35). After exposure to long term IHH, MDA and CP levels in the cardiac tissue homogenate increased more significantly ($P<0.05$) as against exposure to short term IHH. Our findings showed that the administration of LBG extract to rats exposed to IHH caused a significant decrease in oxidative stress in the heart. These results are in accordance with those of a previously report published by us, where LBG extract had a protective role in cardiomyocytes of rats subjected to IHH (3). Our findings indicate that SOD and CAT activity in cardiac tissue homogenate decrease significantly after short-term or long-term IHH exposure, and rats treated with LBG extract have a significantly restored SOD and CAT activity in cardiac tissue homogenate in animals subjected to IHH. The results of the present study have also shown that LBG had a protective effect on cardiac tissue through the lowering of oxidative stress in the heart. These results are in concordance with other reports that found anti-oxidative actions of LBG, especially cardioprotective effects, after IHH exposure (19, 25).

The present study demonstrated that short-term or long-term IHH exposure induced an increase of nitrosative stress in the heart, proved the growth of iNOS levels and NOx production in the cardiac tissue. This is in accordance with the results obtained by other researchers (35). After exposure to long-term IHH, the

iNOS and NOx levels in the cardiac tissue homogenate increased more significantly ($P<0.05$) as opposed to exposure to short-term IHH. These results demonstrate that oxidative/nitrosative stress is higher after exposure to long-term IHH. Rats treated with LBG and exposed to IHH showed a significant decline in nitrosative stress in the heart. The present results highlight that the LBG extract has cardioprotective effects through the lowering of oxidative/nitrosative stress in cardiac tissue.

CONCLUSION

In conclusion, our data demonstrate that short-term or long-term intermittent hypobaric hypoxia exposure induces the growth of oxidative/nitrosative stress in the heart, and LBG supplementation preceding exposure to hypoxia may have a beneficial cardioprotective potential on hypoxia-induced oxidative/nitrosative stress damage in the cardiac tissue.

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