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Short-Term versus Long Term intermittent hypobaric hypoxia on cardiac fibrosis and cardioprotective effects of Natural antioxidants supplementation in rats hearts

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Abstract. Controversial effects of intermittent hypobaric hypoxia (IHH) such as cardiac damage or cardiac protection are still mysterious. It is unclear if short-term and long-term IHH challenges exert different changes of the pro-oxidant/antioxidant balance in the heart and throughout the body. It has been shown that natural antioxidants supplementation (Quercetin, *Lycium barbarum* and Chitosan) is effective in preventing the hypoxic stress. The aim of this study was to evaluate the protective effect of these natural antioxidants' administration in heart in animals exposed to IHH and therefore exposed to oxidative stress. One hundred male Wistar rats were randomized assigned into ten groups was exposed to short-term and long-term IHH (380 mmHg, 12% O_2, 8h/day) for different periods of time (2 days or 4 weeks) or kept in normoxia for 4 weeks. Some of the rats were administered natural antioxidants cu 30 minute before each IHH exposure of IHH. After short-term IHH or long-term IHH challenge, myocardial morphology was determined by histological analysis. Myocardial sections were examined histopathologically to determine the cardiomyocyte viability and morphometry. The results show a negative effect of IHH on the cardiomyocyte viability and cardio-protective effects of natural antioxidants' administration. This study suggests that treatment with Quercetin, *Lycium barbarum* or Chitosan alone substantially restored the myocardial architecture and acted like cardioprotectants.

Key words: oxidative stress, intermittent hypobaric hypoxia**,** Quercetin, *Lycium barbarum,* Chitosan.

INTRODUCTION

The reactive oxygen species (ROS) formed under physiological stress conditions, such as physical effort, hyperthermia or exposure to high-altitude may enhance the tolerance of the heart to ischemia/reperfusion injury by stimulation of cellular antioxidant defense systems that maintain optimal redox balance. Exposure to the short-term and long-term intermittent hypobaric hypoxia (IHH) is considered a physiological oxidative stress that affects the myocardial tissue through the production of ROS (1-3). The myocardial sources of ROS are being alterations of: mitochondrial respiratory chain, cellular membrane and cytosol, endothelial cells and lipid and protein perturbations (4). Intermittent hypoxia has been shown to protect the heart against acute ischemia/reperfusion-induced injury and attenuated ischemia/reperfusion-induced apoptosis (5-6). The mechanism underlying induction of the long-lasting protected phenotype of IHH adapted hearts is not precisely understood. It has been proposed that ROS generated during the adaptation period are important components of a signaling pathway leading to this form of cardio-protection as treatment of rats with an antioxidant eliminated the infarct size-limiting effect of IHH (7-8).

Quercetin (2-(3, 4-dihydroxyphenyl)-3, 5, 7-tryhydroxy-4H-chromen-4-one), a type of polyphenolic compound, has anti-inflammatory, antiproliferation, anti-histamine antioxidant effects (9). Quercetin exists in many types of vegetables and fruits. The recent studies showed the protective activities of Quercetin in different types of cells, including cardiomyocytes, neuronal cells, testis and renal cells, and liver cells in ischemia/reperfusion injury (10-14). Several recent reports have indicated that Quercetin reduces the oxidative stress caused by ischemia/reperfusion in cardiomyocytes by inhibiting the xanthine dehydrogenase/xanthine oxidase system. Quercetin can also scavenge the ROS and inhibit the activation of ERK or MAP kinas in ROS-induced cardiomyopathy (15). The protective mechanism in which the Quercetin plays a role in protecting cardiomyocytes from ischemia/reperfusion injury is still unknown.

The goji fruit *(Lycium barbarum),* belong to the Solanaceae family, and is heavily used in Traditional Chinese medicine in ameliorating obesity and diabetes (16-17). The *Lycium barbarum* (LBG) represent a rich source of vitamins, in particular riboflavin, thiamin and ascorbic acid; carotenoids; flavonoids; iron; selenium and germanium; and contains 18 types of amino acids, including taurine (a nonessential free amino acid); etc. Recent research showed that LBG extract has also many biologically beneficial effects, including anti-aging, hypotensive effect, anti-apoptotic activity, anti-tumor and cytoprotective properties, immunostimulatory effects, etc. Eyesight improvement, blood pressure control, cholesterol level lowering, a good adjunct to combat the adverse effects of chemotherapy and radiotherapy in various tumors, etc. have also been reported (17-20). Goji fruit possesses potent antioxidant capacity in multiple organs and stress models (16, 21). Recent researchs has demonstrated cardioprotective and neuroprotective effects of LBG extract (2, 22, 23).

Chitosan is a α-(1-4)-D-glucosamine polymer, a natural polysaccharide present in shellfish, clams, krill, oysters, fungi, etc (24). It has been shown to possess antilipidemic, antiulcerogenic, antiaging, membrane-stabilizing and antioxidant properties (25-26). Recent researches show that the cardioprotective effect of Chitosan on the myocardial defense system in a myocardial infarction is due to its antioxidant, hypolipidemic and membrane stabilizing properties (25). Because hypoxic stress caused by either short-term or long-term hypobaric hypoxia is one of the mechanisms underlying the initiation of cardiac damage, the aim of this study was to evaluate whether Quercetin, LBG extract or Chitosan supplementation is effective in preventing cardiac damage.

MATERIALS AND METHODS

Drugs and chemicals: The Chitosan used in this experiment were purchased from Sigma Chemical Company Inc., UK. The chemicals were of analytical grade. Quercetin and LBG were extracted, dosed and encapsulated at the "PROPLANTA" Applied Vegetal Biotechnologies Center in Cluj-Napoca, Romania. Quercetin, LBG extract and Chitosan were dissolved in saline solution.

Animals: The study involved 100 albino Wistar male rats (weight 170-200 g at 12 weeks old). All the animals were obtained from the Biobase of the "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca. The animals were cared for in the Biobase of the Physiology Department of the same University. They were isolated for 10 days prior to their introduction in the study for acclimatization. The animals received a standard diet and their access to water was not restricted. All the experiments were conducted in agreement with the protocols and recommendations of the University of Medicine and Pharmacy Cluj-Napoca, Ethics Committee.

The animals were randomly subdivided into ten experimental groups (n=10): 1st Group - unprotected normobaric normoxia (760 mmHg, 21% O_2 and 79%) rats (control group) - rats maintained in normobaric normoxia for 2 days ; 2nd Group - unprotected short-term intermittent hypobaric hypoxia (IHH) rats (control group); 3rd Group - treated short-

term IHH rats, rats treated with Quercetin; 4th Group- treated short-term IHH rats, rats treated with *Lycium barbarum* (LBG) extract; 5th Group- treated short-term IHH rats, rats treated with Chitosan; 6th Group - unprotected normobaric normoxia (760 mmHg, 21% O_2 and 79%) rats (control group) - rats maintained in normobaric normoxia for 4 weeks; 7th Group - unprotected long-term IHH rats (control group); 8th Group- treated long-term IHH rats, rats treated with Quercetin; 9th Group- treated long-term IHH rats, rats treated with LBG extract and 10th Group- treated long-term IHH rats, rats treated with Chitosan.

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 Physiology Department of the UMF ″Iuliu Haţieganu″ Cluj-Napoca), where temperature and humidity were maintained at 28ºC and 55-60%, respectively, for 2 days (short-term IHH, 380 mmHg, 12% O_2 and 88% N_2, for eight hours per day) and for 4 consecutive weeks (long-term IHH, 380 mmHg, 12% O_2 and 88% N_2, for eight hours per day, 5 days per week). The rats were taken out of the hypoxic chamber once after every 8h exposure for receiving food and water.

Some of the rats received Quercetin (30 mg/kg/day, dissolved in saline solution) or *Lycium barbarum* extract (LBG, 30 mg/kg/day, dissolved in saline solution) via an intragastric tube (0.6 ml/rat) for two days or for consecutive weeks 30 minutes before each IHH exposure. Other groups were given intra-peritoneal injections of Chitosan (0, 30-0, 35 microg/animal/day, dissolved in saline solution) for a period of 2 days or consecutive weeks 30 minutes before each IHH exposure. The control groups were treated with saline solution (0.6 ml/rat) via an intragastric tube (control groups). After normoxic or hypoxic exposure, rats were weighed and decapitated (with sodium pentobarbital, 60 mg/rat ip). The hearts of animals were rapidly excised, cleaned with cold saline and weighed. The left and right ventricular walls and the septum were dissected and used for analysis. The heart was sectioned transversally to the ventricles, to about 1/3 of the apex, parallel to the cord into sections of about 4 mm thickness. The wall thickness of the left ventricle (LV) and right (RV) and interventricular septum (S) were also measured. For the histopathological examination the myocardial tissues were fixed in 10% neutral buffered formalin and, after proper fixation, were dehydrated in graded series of alcohol, cleared in Xilene and embedded in paraffin wax. Multiple longitudinal and transversal sections from each block were prepared at 4 µm, and stained with Haematoxylin and Eosin (H&E).

The Haematoxylin and Eosin staining (H&E) was used because it allows a good observation of different cell types. For measuring the cardiomyocyte diameter (morphological study) we used the technique of Aiello et al. (27). Tissue analysis was performed using an Olympus system for image acquisition and analysis, respectively an Olympus BX51 microscope equipped with Olympus Cell B software. 50 cardiomyocytes were measured from each section, from at least 10 different regions, with 200x magnification. Statistical analysis and all morphological data were performed using Shapiro-Wilk normality test, followed by the two-sample t-test, using R' software (R Development Core Team, 2010).

RESULTS AND DISCUSSIONS

Cardiac changes of rats under short-term versus long-term intermittent hypobaric hypoxia: The heart weights were not significantly increased following 2 days short-term intermittent hypobaric hypoxia (Table I). After 4 weeks of intermittent hypobaric hypoxia significant cardiac hyperthrophy was observed due to increased heart weight-to-body weight ratio (mean±SD; 2.71±0.14 $x10^3$ v.s. 3.78±0.42 $x10^3$, $P<0.05$) (Table I). Rats treated with Quercetin, LBG extract and Chitosan, respectively and exposed to long-term intermittent hypobaric hypoxia showed a significant decrease in heart-weight-to-body weight (Table I).

In order to quantify cardiac wall thickness we made a cross-section of whole heart and carried out histopathological analysis of ventricular tissue stained with Haematoxylin and Eosin (H&E) using a technique previously described (28). Therefore, multiple measurements were performed for each section (12 per section) from the internal endocardium to the external epicardium for the right and left ventricle open to the interventricular septum (Figure 1).

Trabeculae were excluded from measurements. For the histopathological exam samples of myocardium were freshly harvested and processed immediately after killing rats included by means of the wax technique. Longitudinal and transverse sections of myocardium from each rat in the middle cardiac ventricular wall including right, left and interventricular were stained with Haematoxylin and Eosin (H&E) and examined by light microscopy.

Table 1

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

	1st Group	2nd Group	3rd Group	4th Group	5th Group	6th Group	7th Group	8th Group	9th Group	10th Group
Initial body weight (g)	208± 24.95	173.9± 23.64	188.3± 10.03	176.6± 18.1	182.7± 20.89	208± 24.95	187± 8.22	176.4± 10.53	178.7± 13.73	189± 9.07
Final body weight (g)	208.9± 24.54	177.6± 27.17	188.9± 12.07	172.6± 12.31	180± 17.45	208.5± 24.91	202.43 ± 6.62*	178.42 ± 11.38[a]	193.6± 12.45[a]	205± 8.85[a]
Final whole heart weight (g)	0.59± 0.06	0.51± 0.07	0.51± 0.03	0.51± 0.06	0.51± 0.06	0.59± 0.06	0.76± 0.07*	0.50± 0.03[a]	0.63± 0.16[a]	0.63± 0.03[a]
Final whole heart weight/ final body weight ($x10^{-3}$)	2.70± 0.24	2.81± 0.30	2.90± 0.47	2.91± 0.27	2.90± 0.17	2.71± 0.14	3.78± 0.42*	2.90± 0.17[a]	3.00± 0.35[a]	3.12± 0.17[a]

*Values are means±SD (n=10 in each group). *P<0.05 as compared to the 1st Group and 6th Group (control groups); [a]P<0.05 as compared to the 2nd Group and 7th Group (control groups).*

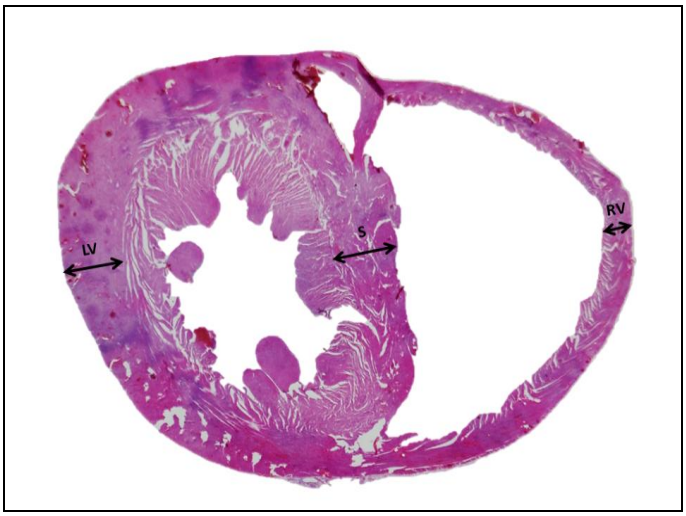

Fig. 1 Cross-section through the rat heart. The wall thickness was measured for the left ventricular (LV) and the right (RV) and the interventricular septum (S).

An H & E staining examination was completed with a magnifying glass.

As shown in Table 1, the animals in the control groups (the 1st Group and 6th Group) had the lowest values observed in the cardiac wall thickness. In both control Groups maintained at IHH (short-term IHH- 2nd Group and long-term IHH- 7th Group), the values recorded in the thickness of the left ventricle, interventricular septum law and were significantly higher than those of Groups of control rats maintained in normobaric normoxia (1st Group and 6th Group).

In rats treated with Quercetin, both after short-term IHH exposure (3rd Group) and after long -term IHH exposure (8th Group), there was a protective effect on cardiac hypertrophy from the results obtained in the two Control Groups (2nd Group and 7th Group) .

The treatment with LBG extract and with Chitosan, respectively mitigated the degree of cardiac hypertrophy as much in the rats exposed to short-term IHH as with those exposed to long-term IHH, but to a lesser extent than treatment with Quercetin .

Table 2

The cardiac wall thickness (mm) of the rats in the 10 experimental groups (Mean±SD)

	1st Group	2nd Group	3rd Group	4th Group	5th Group	6th Group	7th Group	8th Group	9th Group	10th Group
Left ventricle	1.72± 0.14	1.87± 0.22*	1.76± 0.09[a]	1.83± 0.32	1.84± 0.21	1.68± 0.14	1.86± 0.27*	1.75± 0.19[a]	1.8± 0.24	1.79± 0.17
Right ventricle	0.73± 0.05	0.87± 0.09*	0.78± 0.04[a]	0.83± 0.12	0.81± 0.15	0.69± 0.07	0.88± 0.14*	0.76± 0.13[a]	0.81± 0.22	0.82± 0.08
Interven-tricular septum	1.53± 0.11	1.69± 0.13*	1.61± 0.19[a]	1.65± 0.24	1.64± 0.14	1.52± 0.09	1.68± 0.08*	1.59± 0.24[a]	1.65± 0.13	1.62± 0.12

Values are means±SD (n=10 in each group). *P<0.05 as compared to the 1st Group and 6th Group (control groups); [a]P<0.05 as compared to the 2nd Group and 7th Group (control groups).

Furthermore, for the cardiomyocyte diameter measurement (morphometric study), the applied technique was that of Aiello et al. (27). According to the author, 5 μm thick sections were evaluated both transversally and longitudinally (Fig. 3 and 4) To calculate the diameter of cardiomyocytes, only round or oval cells with a round and visible nucleus were studied.

Table 3

Cardiomyocyte diameter in the 10 experimental groups (Mean±SD, μm)

	1st Group	2nd Group	3rd Group	4th Group	5th Group	6th Group	7th Group	8th Group	9th Group	10th Group
Cardio-myocyte diameter	15.7	16.6*	15.3[a]	15.9	16.0	16.1	16.2*	15.8[a]	16.5	15.6
Standard deviation	0.87	1.01*	1.74[a]	0.89	1.62	2.04	0.56*	1.41[a]	1.25	1.80

Values are means±SD (n=10 in each group). *P<0.05 as compared to the 1st Group and 6th Group (control groups); [a]P<0.05 as compared to the 2nd Group and 7th Group (control groups).

50 cardiomyocytes were measured from each section, from at least 10 different regions, with 200x magnification After the measurement of cardiomyoctes' diameters, the results were expressed in μm (Table III. and Figure 4.). There were no significant morphometric differences

observed between the cardiomyoctes' diameters environment in the animals of the six treated experimental groups. However, there is a growing trend of cardiomyoctes' diameters in control groups maintained at IHH (short-term IHH- 2nd Group and long-term IHH- 7th Group), which is mitigated by administration of Quercetin (3rd Group and 8th Group).

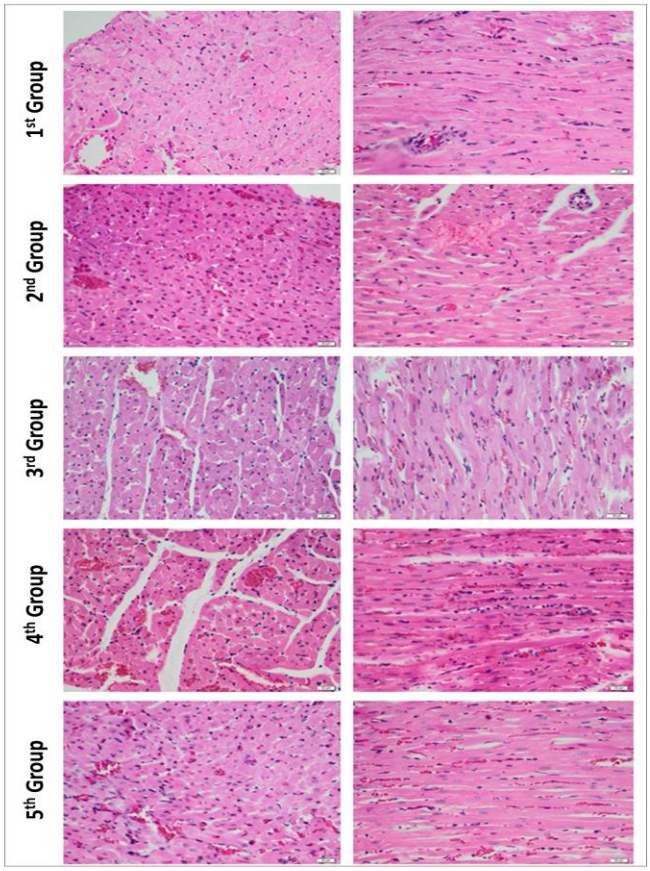

Fig. 2 Cardiac changes of Wistar rats exposed to normoxia, short-term IHH, long-term IHH and treated short-term or long-term IHH. Representative examples of histopathological analysis in the transverse sections (left) and longitudinal sections (right) from the myocardium stained with haematoxylin and eosin (H&E). The images were magnified 200 times. The 1st Group and 3rd Group: no histological changes regarding cardiomyocytes or capillaries; 2nd Group, 4th Group and 5th Group: moderate congestion of the intermuscular capillaires.

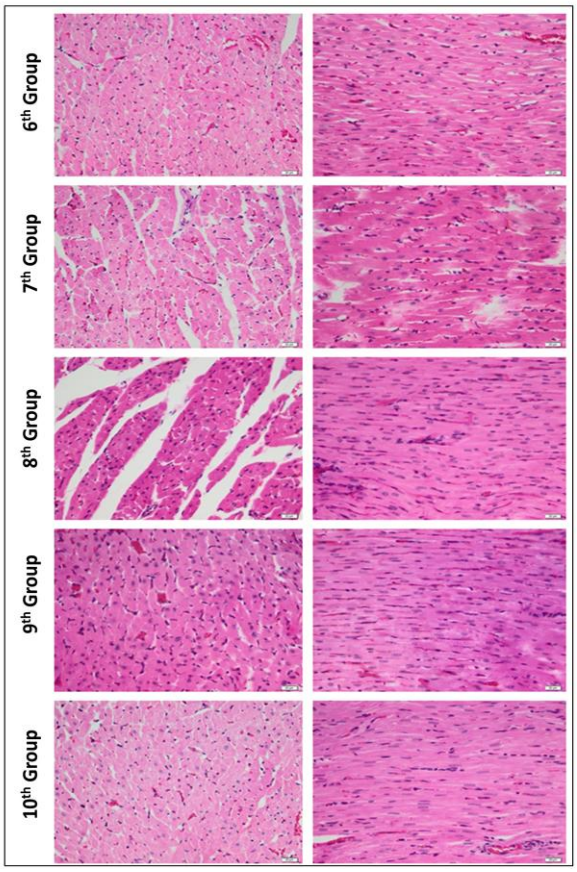

Fig. 3 Cardiac changes of Wistar rats exposed to normoxia, short-term IHH, long-term IHH and treated short-term or long-term IHH. Representative examples of histopathological analysis in the transverse sections (left) and longitudinal sections (right) from the myocardium stained with haematoxylin and eosin (H&E). The images were magnified 200 times. The 6th Group, 8th Group and 9rd Group: no histological changes regarding cardiomyocytes or capillaries; 7th Group, 9th Group and 10th Group: moderate congestion of the intermuscular capillaires

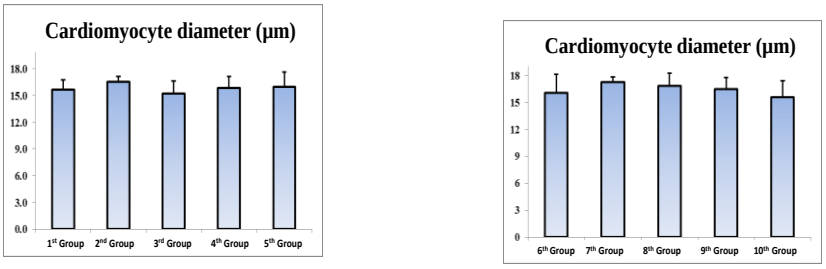

Fig. 4 Quantification of cardiomyocytes diameters in the 10 experimental Wistar rats groups (Mean ± SD). Left: experiment short-term IHH; Right: experiment long-term IHH.

The mitigation of the growth process in cardiomyoctes' diameters induced exposure at IHH was less evident when administering LBG extract (Group 4th and 9th Group) or Chitosan (5th Group and 10th Group). In addition to the changes mentioned above, a slight congestion of the capillaries of muscle fibers was observed in the majority of the animals from the control Groups maintained at IHH as well as the groups treated with LBG extract or Chitosan (Figures 2. and 3).

This change was not observed in any animal from the control Groups maintained in normobaric normoxia and only sporadically in those treated with Quercetin.

The protective role of Quercetin, *Lycium barbarum* extract (LBG extract) and Chitosan against oxidative stress in rats exposed to intermittent hypobaric hypoxia (IHH) was analyzed in this study. Oxidative stress can be triggered by a series of endogenous and exogenous factors, exposure to IHH being one of them (29). Exposure to high altitudes is associated with an increase in the production of reactive oxygen species which are generated during the phase of reoxygenation of IHH and contributes to the physiological responses. Exposure to IHH can be induced ischemic tolerance in the heart, a condition characterized by attenuation of contractile dysfunction, ventricular arrhythmias and cell death due to ischemia/reperfusion (7).

The protection of the heart against long-lasting myocardial ischemia/reperfusion that trigger or mimic intrinsec defense mechanisms may be of potential therapeutic significance. Recent researches has shown that IHH applied seven days after permanent coronary artery ligation reduces infarct size, mitigates myocardial fibrosis and improves cardiac performance.

The mechanisms involved in the cardioprotective effects are: activating mitochondrial ATP-sensitive potassium (KATP) channels, inhibiting the opening of the mitochondrial permeability transition pore, protecting mitochondrial ATP synthase which contributes to the preservation of mitochondrial function and left ventricle contraction following ischemia/ reperfusion; reducing severe Ca^{2+} overload; preserving intracellular pH; inducing nitric oxide production; activating prosurvival kinases; etc. (7). Hypoxia and oxidative stress were shown to be potential inducers of cardiac hypertrophy (30).

In the present our study, cardiac hypertrophy was found in rats with with four weeks long-term IHH and ventricular wall thickness and abnormal myocardial architecture were observated after long-term IHH. However, no significant changes of heart weight index and myocardial architecture have been found in rat heart after two days short-term IHH exposure. Our major findings are in accordance with recent researches (5, 7, 30) and implay that short-term and long-term intermittent hypobaric hypoxia exerted oppsing effects, protective and dteleterious effects on rat hearts. In the current study, the cardiac hypertrophy and abnormal

myocardial architecture and increased interstitial space were observed after four weeks of long-term IHH exposure and less significant results after two days of short-term IHH exposure. Therefore, we speculate that changes of cardiac hypertrophy and myocardial architecture under intermittent hypobaric hypoxia are tightly time-course dependent.

Quercetin is a polyphenolic compound which is frequently used in altitude illness. Its cardioprotective effects on ischemic lesions in rats were shown in recent researches (10, 15) as being due to a reduction of the oxidative stress and an increase of antioxidant enzymes. In a recent study, we observed that rats exposed to chronic hypobaric hypoxia and treated with

Quercetin presented a restored myocardial structure: cardiomyocytes' diameters did not decrease too much and there was a decrease in capillary densities compared to the control rats (2). In the current study, the rats treated with Quercetin and exposed to short-term IHH and long-term IHH presented a significantly restored myocardial architecture. We interpreted this as a result of Quercetin's antioxidant effect, which decreased the oxidative stress induced by intermittent hypobaric hypoxia exposure.

Different biological activities of LBG have been demonstrated, including anti-aging, anti-cancerous, immunostimulatory or cardio- and neuroprotective effects, and many studies suggested that the protective effect of LBG mainly depends on its anti-oxidative action (2, 17-20, 31). In the present study, we observed that rats exposed to short-term IHH or long-term IHH and treated with LBG extract presented myocardial architectural restoration but in a more moderate way than with Quercetin, this is a result of antioxidant properties.

Recent studies (25-26, 32) showed that Chitosan plays a role in the recovery of post-ischemic myocardial lesions by decreasing ROS, attracting chemokines and maintaining a normal level of antioxidant enzymes, thus proving its cardioprotective effects. Our study clearly shows that Chitosan administration in rats exposed to short-term IHH or long-term IHH restored the myocardial architecture although in an inferior way to that of Quercetin.

CONCLUSION

This study demonstrated that Quercetin, *Lycium barbarum* and Chitosan supplementation plays a protective role in heart in animals exposed to intermittent hypobaric hypoxia.

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