

Research Article

Blueberry Extracts Protect Testis from Hypobaric Hypoxia Induced Oxidative Stress in Rats

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Exposure to hypobaric hypoxia causes oxidative damage to male rat reproductive function. The aim of this study was to evaluate the protective effect of a blueberry extract (BB-4) in testis of rats exposed to hypobaric hypoxia. Morphometric analysis, cellular DNA fragmentation, glutathione reductase (GR), and superoxide dismutase (SOD) activities were evaluated. Our results showed that supplementation of BB-4 reduced lipid peroxidation, decreased apoptosis, and increased GR and SOD activities in rat testis under hypobaric hypoxia conditions ($P < 0.05$). Therefore, this study demonstrates that blueberry extract significantly reduced the harmful effects of oxidative stress caused by hypobaric hypoxia in rat testis by affecting glutathione reductase and superoxide dismutase activities.

1. Introduction

Berries are a recognized source of antioxidants since they contain phytochemicals, nonenzymatic factors of plant origin that significantly benefit health [1, 2]. Such extracts have proven to be effective in preventing the effects of oxidative stress under different pathological conditions [3–6]. Among the different species, there is a group classified as blueberries that have a dark color due to anthocyanins and polyphenols as principal pigments with antioxidant activities [3]. Phytochemicals have been demonstrated to be powerful inhibitors of lipid peroxidation when compared to other classic antioxidants [3, 7], and the protective effect of polyphenols against oxidative damage seems to be via glutathione system [8].

The enzymatic mechanism against oxidative stress is made of free radical scavengers like superoxide dismutase (SOD), catalase (CAT), and the glutathione-dependent enzymes such as glutathione peroxidase (GPx), glutathione S-transferase (GSH), and Glutathione reductase (GR) [9].

GR and enzymatic antioxidant mechanisms play an essential role in preventing oxidative damage in cells and tissues [10].

We have previously described that hypobaric hypoxia induced oxidative damage, decreased glutathione reductase activity and ascorbic acid, and had a protective role against oxidative stress [11]. The effect of a reduced spermatogenesis under hypobaric hypoxia [12] is accompanied by an increased vascularization and reactive oxygen species (ROS) in the testis [13, 14]. These vascular changes are induced by ROS via inhibition of prolyl hydroxylase domain (PHD) proteins [11]. The activity of PHD seems to be restored by a supplement of ascorbic acid [15] making it possible to generate strategies for administering antioxidants to prevent the effects of hypobaric hypoxia as previously suggested [14, 16, 17].

Previously, It has been demonstrated that enriched blueberries reduced the adverse effects of oxidative stress in rat neuron cell lines and brain tissues [18, 19]. Such extract has shown to cross the blood-brain barrier [19, 20]. Brain

homeostasis and spermatogenesis depend on blood-to-brain and blood-to-germ cells transport of metabolites and substances [21], therefore it was of interest to determine whether the protective effect can be induced in rat testis *in vivo* model. The aim of this work was to evaluate the protective effect of a blueberry-enriched polyphenol extract (BB-4) against oxidative stress in rat testis exposed to hypobaric hypoxia.

2. Materials and Methods

2.1. Experimental Design. Ten-week-old Sprague Dawley rats (*Rattus norvegicus* specie) were divided into six groups (5 rats per group): (1) normobaric conditions (Nx); (2) Nx plus administration of physiological solution (PS); (3) Nx plus blueberry extract (BB-4), rich in polyphenols, (BB-4); (4) hypobaric hypoxia (HH); (5) HH plus PS and (6) HH + BB-4. Rats were housed under a 12 hours of Light: 12 hours of Dark cycles and the humidity was $61 \pm 9\%$. BB-4 was administered intraperitoneally (10 mg dry extract/kg of body weight) or with physiological solution (1 mL of NaCl 9 mg/mL) at 96-hour intervals. The selection of the dose and route of administration of BB-4 was based on previous work reported [11, 14] where the protective effect of compounds did not affect the liver as analyzed by the presence of transaminases in the blood of animals. Groups 4, 5, and 6 were exposed to HH conditions for 96 hours in a hypobaric chamber (428 tor; pO₂: 89.6 mmHg) for a period of 32 days. The desired pressure inside the hypobaric chamber was achieved by pressure changes simulating altitude increases of 150 meters per minute. The animals in the Nx groups were lodged in the same room as the HH (22°C, 15 g of pellet meals per day and 250 mL of water per rat). All procedures complied with the principles of animal care outlined by the National Society Laboratory and the Medical Research, and the Guide for the Care and Use of Laboratory Animals (Institute of Animal Laboratory Resources, 1996).

2.2. Blueberry Extracts. Six polyphenol-enriched blueberry extracts were obtained from fresh blueberries, locally harvested, using different solvents (with increasing polarity), and denominated BB-1–6, depending on the solvents employed (chloroform, acetone/water, ethanol, ethanol/water, water/acetone, or methanol). The most active extract was obtained with ethanol/water and was denominated BB-4. To obtain the enriched polyphenol extracts (BB), we used an Amberlite XAD-7 adsorber resin (Merck, Darmstadt, Germany) and an Amberlite XAD-2 adsorber resin (Supelpack 2; Sigma-Aldrich). The extract obtained had 1.5% polyphenols for each 100 g of fresh fruit, where the main components were rutin (0.34%) and isoquercetin (0.42%). The BB-4 extract was shown to be the most active in the preliminary activity screening test and it was then characterized to determine its polyphenol composition through HPLC techniques. The final BB-4 extract was dried and diluted in dimethylsulfoxide (DMSO) to a final concentration of

810 mg/liter. Different dilutions from 1:10 to 1:100,000 were prepared daily in external solution containing 5 mM CaCl₂, 100 mM NaCl, 45 mM tetraethylammonium chloride (TEACl), 10 mM HEPES, 5.5 mM KCl, and 10 mM glucose [18].

2.3. Organ Collection. The animals were weighed and sacrificed by cervical dislocation. The testes were removed and placed in a saline buffer PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM sodium phosphate monobasic, pH 7.2) (Sigma Chemicals, St Louis, MO, USA) for further studies.

2.4. Preparation of Tissue Homogenate and Protein Assay. The 100% of testis were completely homogenized in 0.5 mL of extraction buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, Tween-20 0.1% v/v pH 7.4, 100 µg/mL PMSF (Sigma Chemicals, St Louis, MO, USA) with a Potter glass homogenizer (Glass-Col K4424, CA, USA) at 50 rpm. Then the samples were centrifuged for 30 minutes at 4°C. The protein concentration was determined on the supernatant using the Coomassie blue method [22].

2.5. Glutathione Reductase Expression Determined by Western Blot (SDS/PAGE). Aliquots of tissue homogenate of testis containing equal concentration of proteins, 50 µg, were electrophoresed (120 mV) in a 12% SDS/PAGE gel as previously described by Farias et al. [11], using a primary anti-rat glutathione reductase antibody against rabbit (1:500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a secondary anti-rabbit antibody (Jackson Immune Research Laboratories, PA, USA). β-tubulin was used as a loading control in all Western blot assays. The bands obtained were analyzed with Image J Software (<http://rsbweb.nih.gov/ij/download.html>) and the integrated density values of the glutathione reductase bands were normalized by dividing by the value of the loading control band.

2.6. Histological Procedures. Testis from each animal was weighed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 24 hours at room temperature. The testicles were embedded in paraffin after dehydration in ascending alcohol concentrations. Five sections were cut from the equatorial zone toward the testicular apex and mounted on glass slides and stained with hematoxylin-eosin sections.

2.7. Determination of Cellular DNA Fragmentation. In order to detect apoptosis by DNA fragmentation, an *in situ* Oligo Ligation method (ApoTag ISOL, Q-BIOgene, UK) was carried out as described by Lesauskaite et al. [23]. This method is based upon the specificity of the enzyme T4 DNA ligase [24]. In these experiments, we utilized five 5 µm tissue sections that were obtained from rat testicles from the equatorial zone toward the testicular apex. The distance between the sections corresponded to 120 µm. This procedure was repeated for every condition tested.

2.8. Determination of Lipid Peroxidation in the Testis. The protocol for the thiobarbituric acid (TBA) (Sigma Chemicals, St Louis, MO, USA) assay was carried out as described by Draper and Hadley [25]. 5 mg of tissue homogenates were centrifuged at 10 000 g for 10 min at 4°C to sediment mitochondria and cell debris. The sediments were suspended in PBS pH 7.4, mixed with BHT-TCA solution (1% w/v BHT dissolved in 20% w/v TCA), and centrifuged at 1000 g for 5 min. Supernatant was then mixed with 0.5 N HCl and 120 mM TBA in 26 mM Tris and heated in a water bath at 80°C for 10 min. After cooling, the absorbance of the resulting chromophore was determined at 532 nm using a OPTIZEN UV-Visible spectrophotometer (3220UV) and MDA production was determined by using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. The results were expressed as nmol of MDA equivalents/mg tissue.

2.9. Determination of Glutathione Reductase Activity in the Testis. To measure glutathione reductase activity, homogenates were thawed at room temperature and centrifuged at 700 ×g for 10 min, after which 20 μl of supernatant was added to quartz cuvettes containing a fresh solution of 0.44 mM GSSG, 0.30 M EDTA, in 0.1 M phosphate buffer—pH 7.0—and 0.036 M NADPH was added just before the enzymatic determination as the starting reagent. The assay was run at 340 nm for 4 min with absorbance readings taken every 30 s. Glutathione reductase activity was estimated using NADPH extinction coefficient of $6.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ and expressed as U/mg of protein [11, 26].

2.10. Determination of Superoxide Dismutase Activity in the Testis. Superoxide dismutase activity was measured by the autoxidation of pyrogallol method as described by S. Marklund and G. Marklund [27]. The activity of superoxide dismutase was assayed in 1000 ×g supernatants of testis homogenates. One unit of superoxide dismutase was defined as the amount of protein that caused 50% pyrogallol autoxidation inhibition. Superoxide dismutase activity was expressed as U/mg protein.

2.11. Evaluation of Hematocrit, Body Weight, and Testicular Mass Relative to Body Weight. Blood samples were obtained with a needle from the left ventricle of the rat once sacrificed. The percentage of hematocrit was determined by centrifugation of the capillary tube with heparinized blood in a microhematocrit centrifuge (IEC Model MB, GSR Technical Sales, Canada). The animals were weighed and the testicular mass relative to body weight was determinate at the end of 32 days: $(\text{Testicular mass/body weight}) \times 100 = (\%)$.

2.12. Statistical Analysis. The results were analyzed as previously described by Farias et al. [11] by the two-way ANOVA in order to determine the presence of a significant interaction between the environmental factors (sea level and high altitude) and the injection factors (without injection, vehicle and dose), making it possible to determine whether the treatment with BB-4 under IHH produces any significant effect on the variables different to that produced in Nx.

Also, the two-way ANOVA enabled to determine whether at least one level of each factor (environmental or injection) affected the results and to determine whether the changes in the barometric pressure or the administration of BB-4 produced any significant effect on the results. The statistical significance was established to $P < 0.05$ for all analyses and a Bonferroni test was performed to compare treatments. Data were analyzed using the Graph Pad Prism Software v4.0 (San Diego, CA, USA). The results are presented in graphs with standard deviation of the mean (SD).

3. Results

The effect of hypobaric hypoxia exposure on testicular mass, testicular mass relative to body weight, diameter of seminiferous tubule, and height of epithelium was reversed with treatment with BB-4 ($P < 0.05$). Indeed, all these parameters came back to similar levels to those obtained in Nx (Figures 1(a), 1(b), and 1(c); Table 1). The hypoxia hypobaric condition induced apoptotic DNA fragmentation in spermatogenic cells in rats (Figure 1(d); $P < 0.05$). However, in rats subjected to hypobaric hypoxia and treated with BB-4, the apoptotic index significantly decreased ($P < 0.05$). On the other hand, lipid peroxidation (TBARS) was significantly higher ($P < 0.05$) under hypobaric hypoxia as compared to normoxic conditions in the testis as shown in Figure 2(a). The blueberry extract (BB-4) did not affect rats exposed under normoxia; however, this substance reduced lipid peroxidation in treated rats with the extract ($P < 0.05$). BB-4 seemed to protect the testis only under hypobaric hypoxic conditions.

There was a significant increase in glutathione reductase and superoxide dismutase activities under hypobaric hypoxia in comparison to normoxic groups ($P < 0.05$) as shown in Figures 2(b) and 2(c). BB-4 seemed to protect the testis under hypobaric hypoxic conditions when the enzymes SOD and GR increased. The specific activities of these enzymes significantly increased ($P < 0.05$) in rats treated with blueberry extract and subjected to hypobaric hypoxia ($P < 0.05$). There were no significant differences in glutathione reductase protein expression under hypoxia in comparison to normoxic groups ($P > 0.05$), as observed in Figure 2(d). The hematocrit was significantly greater ($P < 0.05$) in groups subjected to hypobaric conditions when compared to normoxic groups (Figure 3(b)). The blueberry extract did not have any effect on the polycythemia that usually characterizes exposure to hypobaric hypoxia as a compensatory mechanism to the drop in arterial pressure of oxygen. Our results showed a significant decrease ($P < 0.05$) in body weight of hypobaric hypoxia groups in comparison to normoxic groups (Figure 3(a)). However, blueberry extract treatment did not affect this parameter under any environmental conditions.

4. Discussion

These results showed that the levels of lipid peroxidation in all groups subjected to hypobaric hypoxia induced

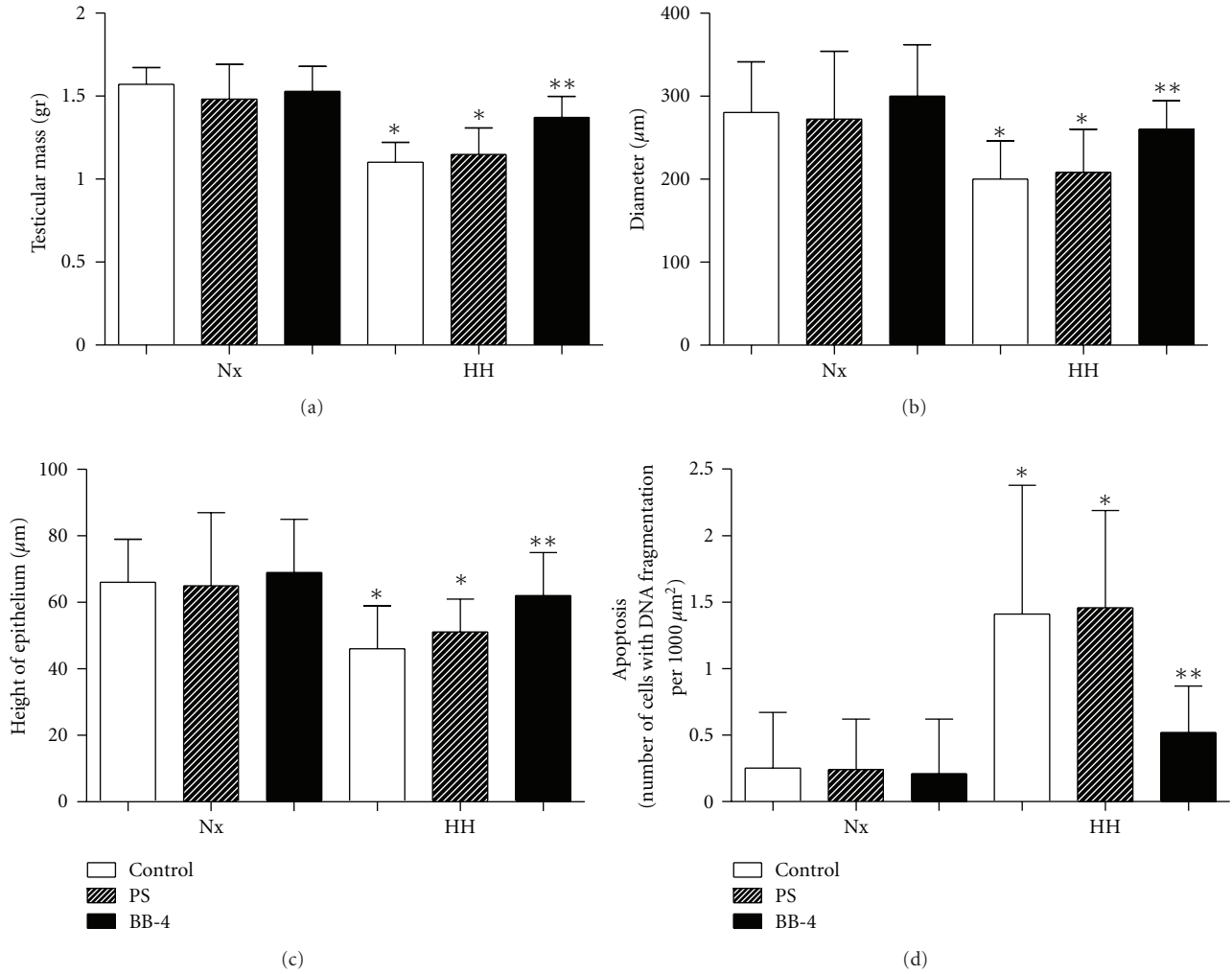


FIGURE 1: Effect of intermittent hypobaric hypoxia and BB-4 on (a) testicular mass (gr), (b) diameter (μm), epithelium height, (c) and (d) apoptotic index (number of cells with fragmented DNA per $1000 \mu\text{m}^2$). Rats were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) conditions, with or without treatment of blueberry extract (BB-4). PS: rats treated with physiological NaCl solution. Experiment: 32-day treatment. Bars indicate the mean \pm SD ($n = 5$). * $P < 0.05$ (HH versus Nx control); ** $P < 0.05$ (HH + BB-4 versus HH control).

TABLE 1: Testicular mass relative to body weight (%).

Nx	Nx + PS	Nx + BB-4	HH	HH + PS	HH + BB-4
0.44 ± 0.02	0.41 ± 0.03	0.43 ± 0.02	$0.40 \pm 0.01^*$	$0.40 \pm 0.04^*$	$0.48 \pm 0.03^{**}$

Rats were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) conditions, with or without treatment of blueberry extract (BB-4). PS: rats treated with physiological NaCl solution. (Testicular mass/body weight) $\times 100 = (\%)$. Mean \pm SD. * $P < 0.05$ (HH versus Nx control); ** $P < 0.05$ (HH + BB-4 versus HH control).

oxidative stress causing a decrease in testicular mass, arrest of spermatogenesis, and an increase in apoptosis. In previous studies, we reported that hypobaric hypoxia caused oxidative damage and histological changes in testis [11–13].

The production of reactive oxygen species at the testicular level can be especially accentuated by the presence of a basal hypoxic microenvironment and abundance of polyunsaturated fatty acids [28, 29]. On the other hand, the activities of glutathione reductase and superoxide dismutase were significantly reduced. We previously found that enzyme

expression did not change in rat testis under hypobaric hypoxia [11].

Several studies have demonstrated the beneficial effects of blueberries against oxidative stress. Blueberries have the ability to neutralize oxygen-containing free radicals [30]. Phenolic compounds with strong antioxidant properties are found in the diet and can prevent oxidative damage as a result of their ability to scavenge reactive oxygen species [31]. It has been reported that blueberries have protective effects against oxidative damage in animals [19, 32]. Animals exposed to

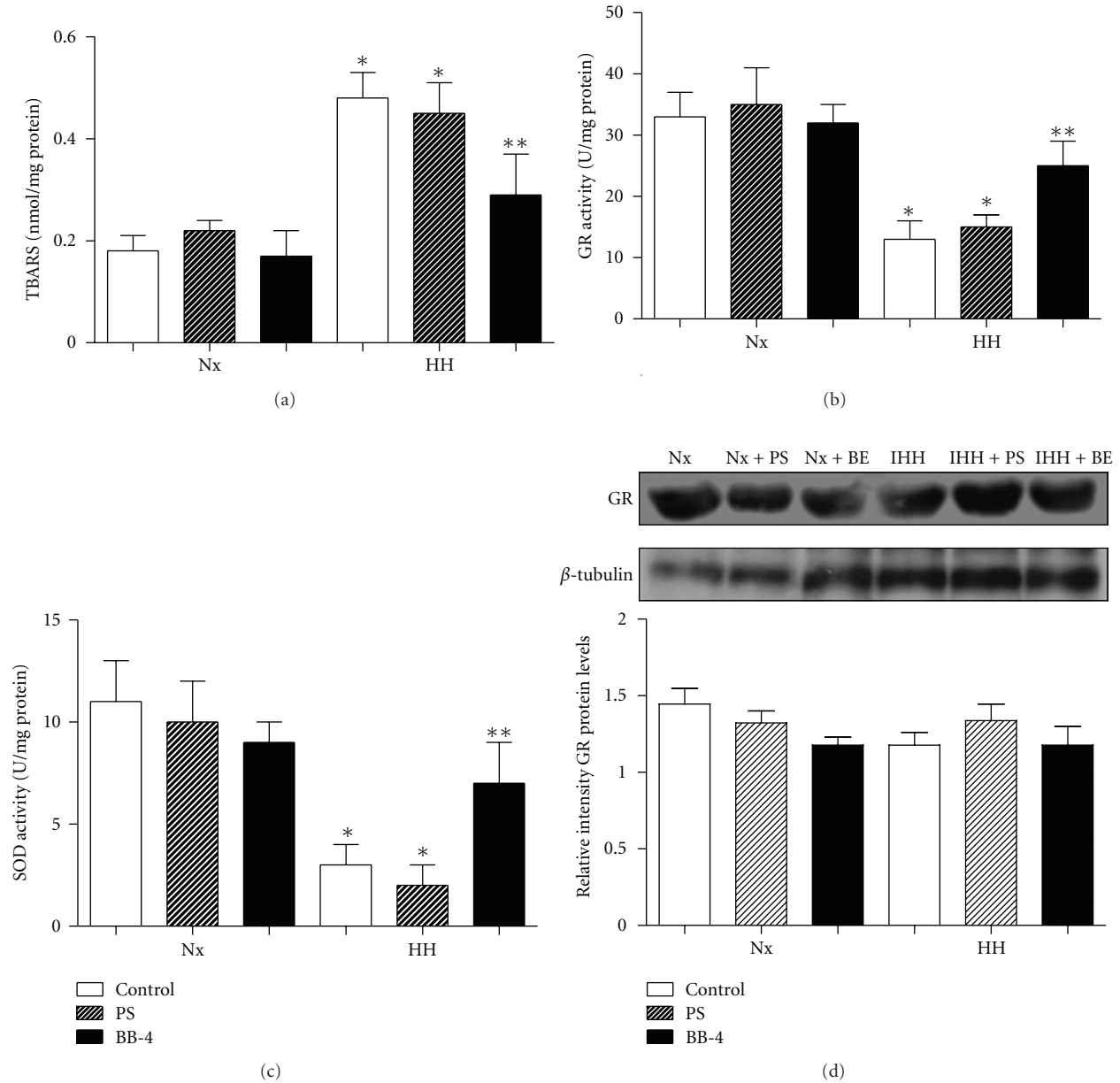


FIGURE 2: Effect of intermittent hypobaric hypoxia and BB-4 on (a) lipid peroxidation (TBARS) (nmol/mg protein), (b) glutathione reductase (GR) activity (U/mg protein), (c) superoxide dismutase (SOD) activity, and (d) glutathione reductase protein expression. Rats were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) conditions, with or without treatment of blueberry extract (BB-4). PS: rats treated with physiological NaCl solution. Experiment: 32-day treatment. Bars indicate the mean \pm SD ($n = 5$). * $P < 0.05$ (HH versus Nx control); ** $P < 0.05$ (HH+BB-4 versus HH control).

hypobaric hypoxia and treated with blueberry extract (BB-4) showed a significant decrease in lipid peroxidation in rat testis reaching levels similar to normoxic condition. On the other hand, blueberry extract prevented the effects of hypobaric hypoxia on testicular mass, arrest of spermatogenesis, and apoptosis. The effect of blueberry extract on spermatogenic cells can be attributed to a possible passage through the hematotesticular barrier and thus protecting this tissue from oxidative stress generated by hypobaric hypoxia. Our results indicated that there were no changes in GR expression. However, the enzyme activity was significantly restored in

animals subjected to hypobaric hypoxia and treated with blueberry extract suggesting that these compounds could activate the powerful endogenous antioxidant defenses by chemically reducing oxidized glutathione [33].

Hypobaric hypoxia caused a significant loss in body weight. The effect of hypoxia on body weight has been described by various authors who have indicated that mammals exposed to different hypoxia exposure experienced weight loss, dehydration, fat loss, and muscular mass reduction [34–36]. The blueberry extract did not have a protective effect on body weight. Our data suggested that blueberry

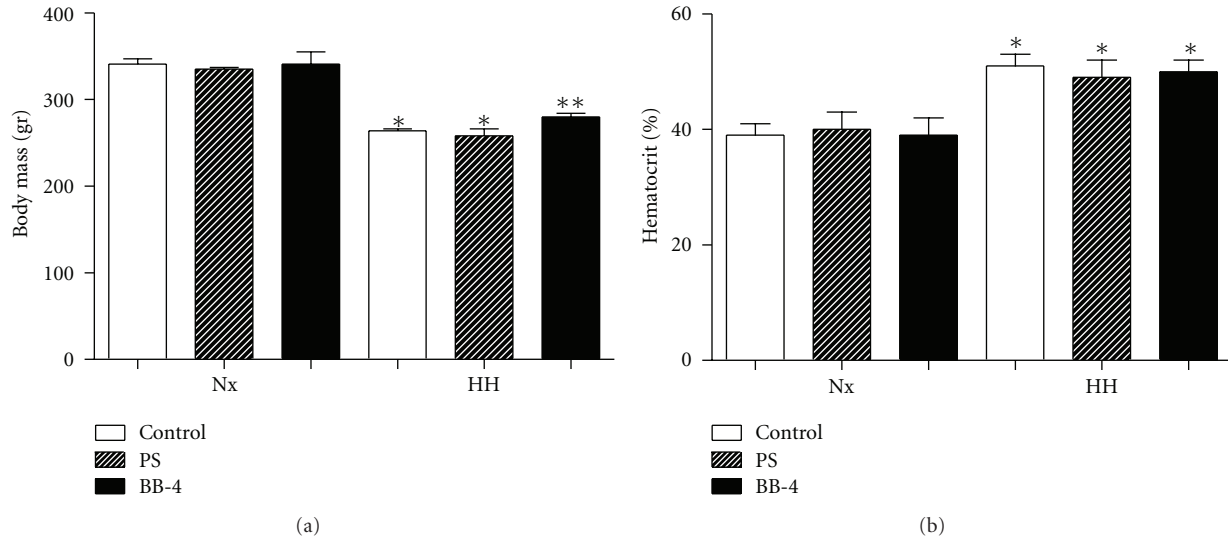


FIGURE 3: Effect of intermittent hypobaric hypoxia and BB-4 on (a) body weight and (b) hematocrit (%). Rats were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) conditions, with or without treatment of blueberry extract (BB-4). PS: rats treated with physiological NaCl solution. Experiment: 32-day treatment. Bars indicate the mean \pm SD ($n = 5$). * $P < 0.05$ (HH versus Nx control); ** $P < 0.05$ (HH+BB-4 versus HH control).

extract may blunt the oxidative stress induced by hypobaric hypoxia as showed by the decreased lipid peroxidation and the increased glutathione reductase and superoxide dismutase activities with BB-4 compared to control. The dose of BB-4 did not affect polycythemia, a characteristic of exposure to hypobaric hypoxia as a compensatory mechanism to the drop in arterial PO_2 . A significant increase in erythrocytes was observed in animals exposed to intermittent hypobaric hypoxia, which indicated that BB-E had no effect on erythropoietin; therefore, it did not affect one of the mechanisms of acclimatization to high altitudes [37].

These results corroborate previous studies demonstrating the beneficial effects of polyphenols present in natural and enriched foods [18]. Blueberry extract presented a protective effect against oxidative stress induced by hypobaric hypoxia by recovering glutathione reductase and superoxide dismutase activities. It can be concluded that extracts of natural origin can be effective in the prevention of oxidative stress induced by hypobaric hypoxia, and it opens the possibility of generating additional health benefits to people who live under conditions of oxidative stress.

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