

ORIGINAL ARTICLE

Blueberries prevent the effect of intermittent hypobaric hypoxia in rat epididymisA. B. Zepeda¹, G. M. Calaf^{2,3}, C. A. Figueroa¹ & J. G. Farías¹¹ Departamento de Ingeniería Química, Facultad de Ingeniería, Ciencias y Administración, Universidad de La Frontera, Temuco, Chile;² Instituto de Alta Investigación, Universidad de Tarapacá, Arica, Chile;³ Center for Radiological Research, Columbia University Medical Center, New York, NY, USA**Keywords**

Epididymis—hypobaric hypoxia—infertility—oxidative stress—polyphenols

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Summary

Intermittent hypobaric hypoxia (IHH) induced a decrease in sperm count and oxidative damage in epididymis. We have previously demonstrated that a blueberry-enriched polyphenol extract (BB-4) reduced the adverse effects of oxidative stress in rat testis under hypobaric hypoxia. The aim of this study was to evaluate whether BB-4 could reverse oxidative stress in epididymis. To evaluate the protective role of BB-4 in epididymis, male rats were exposed to IHH. Lipid peroxidation, (LPO) expression and activity of glutathione reductase (GR) were evaluated. Our results showed a reduction in LPO and a decrease in GR activity in rat epididymis exposed to IHH. These results suggest that BB-4 can prevent the effects of IHH in rat epididymis.

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Introduction

The development of work activity in Chile has forced a great number of workers to be constantly exposed to high altitude for prolonged and intermittent periods of time, named intermittent hypobaric hypoxia (IHH; Germack *et al.*, 2002; Farias *et al.*, 2013). We previously described that hypobaric hypoxia induced oxidative stress in rat testis and epididymis (Farias *et al.*, 2010) as well as DNA damage in the rat testicular cells resulting from oxidative stress. This situation induced the production of defective spermatozooids and decreased sperm count (Farias *et al.*, 2012; Zepeda *et al.*, 2012a). The phytochemicals have demonstrated to be powerful inhibitors of lipid peroxidation (LPO) compared with other classic antioxidants (Kong *et al.*, 2003; Duthie *et al.*, 2006). The cells respond to polyphenols mainly through direct interactions with receptors or enzymes involved in signal transduction, which may result in modification of the redox status of the cell and may trigger a series of redox-dependent reactions (Forman *et al.*, 2002). As antioxidants, polyphenols may improve cell survival, and such biological effects of polyphenols may extend well beyond the modulation of oxidative stress (Pietta *et al.*, 1998; Scalbert *et al.*, 2005). On the other hand, we found that BB-4, polyphenol-

enriched blueberry extract, reduced the effects of hypobaric hypoxia in rat testis, as well as the apoptotic DNA fragmentation in spermatogenic cells in rats (Zepeda *et al.*, 2012b). Therefore, blueberries constitute an interesting alternative in the diet of people subjected to IHH as protector of oxidative damage in male reproductive system. The objective of this work was to evaluate the protective role of blueberry-enriched polyphenol extract in epididymis of rats exposed to IHH.

Materials and methods**Experimental design**

Ten-week-old Sprague–Dawley rats (*Rattus norvegicus* species) were divided into six groups (five rats per group): (i) normobaric conditions (Nx); (ii) Nx plus administration of physiological solution (PS); (iii) Nx plus blueberry extract rich in polyphenols (BB-4); (iv) IHH; (v) IHH plus PS; and (vi) IHH + BB-4. Rats were housed under 12 h of light cycles and then 12 h of dark cycles, and the humidity was 61 ± 9%. Groups 4, 5 and 6 were exposed to hypobaric hypoxia conditions for 96 h in a hypobaric chamber (428 tor; pO₂: 89.6 mmHg) and then remained 96 h in normobaric condition. All these

procedures were carried out for a period of 32 days. The desired pressure inside the hypobaric chamber was achieved by pressure changes simulating altitude increases of 150 m min^{-1} . The animals in the Nx groups were lodged in the same room as the IHH ($22 \text{ }^\circ\text{C}$, 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).

Blueberry extracts and treatment

The polyphenol-enriched blueberry extracts were obtained from fresh blueberries locally harvested using as solvent ethanol/water, and it was denominated BB-4. To obtain the enriched polyphenol extracts (BB-4), we used an Amberlite XAD-7 adsorber resin (Merck, Darmstadt, Germany) and an Amberlite XAD-2 adsorber resin (Supelpack 2; Sigma-Aldrich, Bellefonte, PA, USA), and we obtained an extract having 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 highly active in the preliminary activity screening test and was therefore characterised to determine its polyphenol composition through HPLC techniques. The final BB-4 extract was dried and diluted in dimethyl sulphoxide (DMSO) to a final concentration of 810 mg l^{-1} . Different dilutions from 1 : 10 to 1 : 100 000 were prepared daily in a solution containing (in mM): 5 CaCl_2 , 100 NaCl, 45 TEA-Cl, 10 HEPES, 5.5 KCl and 10 glucose (Fuentealba *et al.*, 2011). The selection of the dose and route of administration of BB-4 were based on previous work (Farias *et al.*, 2010; Farias *et al.*, 2012) where the protective effect of compounds did not affect the liver as analysed by the presence of transaminases in the blood of animals (i.p.: 10 mg dry extract per kilogram of body weight at 96-h intervals; Zepeda *et al.*, 2012a).

Preparation of tissue homogenates and protein assay

Epididymides were excised post-mortem, and then, tissues were homogenised in 0.5 ml of extraction buffer [Tris

50 mM, NaCl 100 mM, EDTA 1 mM, EGTA 2.5 mM, Tween-20 0.1% (pH 7.4), phenylmethylsulphonyl fluoride (PMSF) 100 mg ml^{-1} ; Sigma-Aldrich] with a Potter homogeniser (Glas-Col K4424; Glas-Col, Terre Haute, IN, USA). The samples were then centrifuged at 7000 g for 30 min at $4 \text{ }^\circ\text{C}$. Protein concentrations were determined using the Bradford method (Bradford, 1976).

Thiobarbituric acid-reactive substance (TBARS) assay

This assay permits to quantify malondialdehyde concentration by spectrophotometry (LPO indicator) when the substance reacts with the thiobarbituric acid generating a coloured product. The assay was carried out as described by Draper & Hadley (1990).

Glutathione reductase expression and activity

The appropriate diluted primary antibodies include rabbit anti-rat glutathione reductase (GR; 1 : 500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a secondary anti-rabbit antibody (1 : 1000; Jackson Immune Research Laboratories, West Grove, PA, USA). β -tubulin (1 : 1000; Santa Cruz Biotechnology) was used as loading control. GR activity was estimated using NADPH extinction coefficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as U mg^{-1} of protein. Western blot and activity assays were carried out as described previously (Farias *et al.*, 2010).

Statistical analysis

The results were analysed by the two-way ANOVA followed by a Bonferroni test. Data were analysed using the GRAPH-PAD PRISM software v4.0 (GRAPH-PAD PRISM, San Diego, CA, USA). The results are presented as the mean \pm SD.

Results

Measurement of LPO by TBARS

Lipid peroxidation was significantly higher under IHH in comparison with control ($P < 0.05$ versus Nx groups in epididymis). The BB-4 did not have any effect in rats exposed to normobaric condition (Nx groups); however,

Table 1 Effect of intermittent hypobaric hypoxia (IHH) and BB-4 on LPO

Nx	Nx + PS	Nx + BB-4	IHH	IHH + PS	IHH + BB-4
2.91 ± 0.48	3.29 ± 0.61	3.26 ± 0.36	$6.42 \pm 0.38^*$	$5.94 \pm 0.60^*$	$3.51 \pm 0.61^{*,\dagger}$

PS, physiological solution; LPO, lipid peroxidation.

Lipid peroxidation (malondialdehyde concentration, nmol mg^{-1} protein). Animals were submitted to IHH or normobaric (Nx) conditions, with or without treatment of blueberry extract (BB-4). PS, animals treated with physiological NaCl solution. Mean \pm SD. $N = 5$. $^*P < 0.05$ (IHH versus Nx control); $^\dagger P < 0.05$ (IHH + BB-4 versus IHH control).

BB-4 reduced LPO in epididymis under IHH (Table 1). An increase in LPO was observed in epididymis in all groups subjected to IHH.

GR activity

There was no significant difference in GR expression under IHH in comparison with normoxic groups ($P > 0.05$) as seen in Fig. 1. GR activity significantly ($P < 0.05$) decreased under hypobaric hypoxia condition when compared to control groups, while BB-4 treatment increased GR activity in epididymis of rats exposed to hypobaric hypoxia (Table 2).

Discussion

The present study corroborated the beneficial effects of polyphenols present in natural and enriched foods as it has been previously described on male reproductive system (Zepeda *et al.*, 2012a,b). The animals exposed to

hypobaric hypoxia and treated with blueberry extract showed a significant decrease in LPO in rat testis reaching levels similar to normoxic condition (Zepeda *et al.*, 2012b). Our results showed an increase in the LPO in epididymis in all groups subjected to IHH. Epididymis is a specialised organ in the production of reactive oxygen species (ROS), and it has a microenvironment with physiological ROS levels necessary to perform functions related to sperm maturation (Drevet, 2006). Epididymis being enriched with antioxidant system protects spermatozoa and facilitates their maturation process (Vernet *et al.*, 2004). A drop in the oxygen concentrations could eventually put at risks the sperm maturation functions and concentration (Cikutovic *et al.*, 2009; Reyes *et al.*, 2012). On the other hand, sperm plasma membrane, being rich in polyunsaturated fatty acids (PUFA), is highly susceptible to ROS attacks (Saradha & Mathur, 2006). The hypobaric hypoxia induced LPO in epididymis and a decreased sperm cell count (Farias *et al.*, 2010). The animals exposed to hypobaric hypoxia and treated with blueberry extracts showed a significant decrease in LPO in epididymis, reaching levels similar to normoxia condition. The effect of blueberry extracts on spermatogenic process may be attributed to the possible crossing of the tissue barriers and thus protecting to epididymis from the oxidative stress generated by hypobaric hypoxia. Our results indicated that there were no changes in GR expression in testis and epididymis under hypobaric hypoxia. However, enzyme activity was significantly restored in animals subjected to hypobaric hypoxia and treated with blueberry extracts, suggesting that these compounds could activate the powerful endogenous antioxidant defences by chemically reducing oxidised glutathione. The polyphenols have been shown to activate nuclear factor-erythroid-2-related factor 2, which stimulates the activities of antioxidant enzymes such as glutathione peroxidase (GPx), glutathione S-transferase, catalase, NAD(P)H/quinone oxidoreductase-1 (NQO1) and/or phase II enzymes (Hu, 2011). In conclusion, our results showed a protective role of polyphenols on male reproductive system and corroborated previous studies in testis, demonstrating the beneficial effects of blueberries present in natural and enriched foods (Fuentelba *et al.*, 2011; Zepeda *et al.*, 2012b). It can be concluded that extracts of natural origin rich in

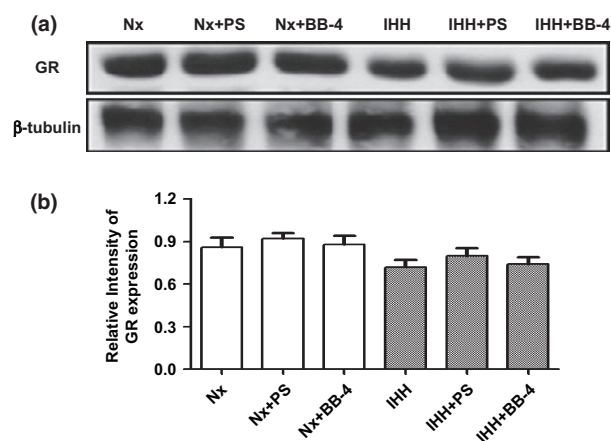


Fig. 1 Glutathione reductase expression (GR). (a) Immunoblot and (b) relative intensity of GR protein levels [(GR intensity of bands)/ β -tubulin (intensity of bands)]. The anti- β -tubulin antibody was used as a protein-loading control in Western blots. Animals were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) conditions, with or without treatment of blueberry extract (BB-4). PS, animals treated with physiological solution. $P > 0.05$ Nx versus IHH groups. PS, physiological solution.

Table 2 Glutathione reductase activity (U mg^{-1} protein)

Nx	Nx + PS	Nx + BB-4	IHH	IHH + PS	IHH + BB-4
0.08 ± 0.004	0.07 ± 0.003	0.07 ± 0.006	$0.03 \pm 0.03^*$	$0.02 \pm 0.03^*$	$0.06 \pm 0.003^{*,\ddagger}$

PS, physiological solution.

Animals were submitted to intermittent hypobaric hypoxia (IHH) or normobaric (Nx) conditions, with or without treatment of blueberry extract (BB-4). PS, animals treated with physiological NaCl solution. Mean \pm SD. $N = 5$. $^*P < 0.05$ (IHH versus Nx control); $^{\ddagger}P < 0.05$ (IHH + BB-4 versus IHH control).

polyphenols can be effective in the prevention of oxidative stress induced by hypobaric hypoxia and it opens the possibility to generate additional benefits to the health of people who live under oxidative stress conditions (Farias et al., 2005).

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