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Effects of chronic intermittent hypoxia and polyunsaturated fatty acids on infarct size and oxidative stress markers in cardiac ischemia reperfusion

Original article.

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Abstract.

Chronic intermittent hypoxia (CIH) induces structural and functional changes in heart, probably associated with ischemia-reperfusion (IR), a pathophysiological event linked with excessive reactive oxygen species generation. The cardioprotective effects of polyunsaturated fatty acids (PUFA) have not been well characterized in CIH. The aim of this study was to determine the effects of CIH and PUFA on infarct size (IS) and oxidative stress markers in cardiac IR.

Twenty-eight adult rats were randomly divided in 4 groups: normobaric normoxia (Nx); Nx + PUFA (0.3 $g.kg^{-1}d^{-1}$); hypobaric hypoxia (Hx); and Hx + PUFA. CIH was induced by 4 intercalate periods of hypoxia-normoxia with 96 hours intervals each, in a hypobaric chamber (428 tor; pO_2: 89.6 mmHg) during 32 days. In ex vivo model (Langendorff), hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion. The IS, TBARS, GSH/GSSG ratio and IL1beta levels were assessed in hearts.

Infart size decreased in 25.8% (Nx+PUFA), 38.8% (Hx) and 51.7% (Hx+PUFA), relative to Nx ($P<0.05$, all vs. Nx). In addition, TBARS (38,1%) and IL-1beta (23,8%) cardiac levels diminished in Hx rats relative to Nx ($P<0.05$). Hypoxia and PUFA supplementation are independently associated with lower levels of these stress markers and higher levels of GSH/GSSG ratio. These findings suggest that CIH and PUFA induce a cardioprotective effect associated with an enhanced antioxidant and anti-inflammatory status.

Key words: hypobaric hypoxia, oxidative stress, infarct size, polyunsaturated fatty acids.

1. Introduction.

Intermittent systemic hypoxia occurs in many common physiological and pathophysiological conditions in human life, caused by environmental factors (e.g. high altitude exposure), cardiopulmonary disorders (e.g. heart failure, chronic obstructive pulmonary disease and sleep apnea) and hematological diseases (e.g. anemia). In fact, intermittent exposure is much more frequent than chronic exposure to hypoxia [**1**]. Interestingly, a few controlled protocols of intermittent hypoxia have shown protective effects against myocardial infarction by a signaling mechanism that depends on the antioxidant status improvement [2]. In Chile, there is a less studied pattern of hypoxia in miners and astronomers, the chronic intermittent hypoxia (CIH), which involves alternating between normobaric normoxia and hypobaric hypoxia in a set cycle [**2**]. The CIH model has gathered interesting information about the characteristics of acclimatization to CIH, particularly in relation to the effects of hypoxia, but there is also evidence that hypobaria is playing a relevant role in the cardiovascular system [3]. For example, studies showing different heart rate variability responses in normobaric hypoxia versus hypobaric hypoxia suggest that these conditions are clearly not similar stimuli to the cardiovascular and respiratory systems [4]. The long term adjustments in CIH tend to resemble those in chronic hypoxia at the level of ventilatory and cardiovascular responses, red cell mass and cardiac β-adrenergic receptors, among others. However, considering exposure to the same altitude, there is a difference in the time needed to complete acclimatization, whereas acclimatization to chronic hypoxia is achieved in few months, CIH acclimatization is achieved in years, with stabilization of biomedical variables being observed after 18 months of exposure [5].

Exposure to high altitude may result in a pro-oxidant imbalance, enhanced generation of reactive oxygen species (ROS), and related damage to lipids, proteins and DNA [6]. The severity of oxidative injury is related to the degree of altitude and therefore the decrease in PO_2. Several sources of ROS are activated during exposure to high altitude, including the mitocondrial electron transport chain [7], xanthine oxidase, NADPH oxidase and nitric oxyde synthase [8]. Moreover, the enzymatic and non-enzimatic antioxidant systems are affected by exposure to high altitude, for example glutathione peroxidase activity and total antioxidant capacity diminution [9]. Some studies have shown that CIH increased lipid peroxidation and carbonyl derivates in skeletal muscle and plasma of rats [9-11]. In heart, CIH could induce left ventricular dysfunction and oxidative damage in contractile system [12]; however, the effects of CIH on cardiac function and structure are still unknown.

In vitro studies, animal experiments, observational studies, and randomized clinical trials have examined the cardiovascular effects of fish consumption and long-chain ω-3 polyunsaturated fatty acids (PUFAs). These PUFAs are composed by eicosapentaenoic acid (EPA; 20:5 n-3), docosahexaenoic acid (DHA; 22:6 n-3) and α linolenic acid (ALA; 18:3 n-3) [13]. In relation to chronic consumption, it has been reported that PUFAs are selectively incorporated into cardiac cell membranes, in a dose-related manner, after 8 weeks of supplementation [14]. Also, PUFAs can improve post-ischemic functional recovery in the Langendorff perfusion of rat hearts, suggesting the benefit of highly enriched PUFA content diet [15]. Regular intake can slow the heart rate, reduce myocardial oxygen consumption, and increase coronary reserve. These properties contribute to protective preconditioning-like effects on the myocardial IR damage and improved post-ischemic recovery.

Therefore, we proposed that CIH, in cycles mimicking the miners shifts, will alter the cardiac response to ischemia-reperfusion and PUFA supplementation will improve this response. The aim

of this work was to determine the effects of CIH and PUFA on infarct size (IS), lipid profile and oxidative stress markers in cardiac IR.

2. Methods

2.1 *Animals and surgical techniques.*

All animal care and 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), and were approved by the Faculty of Medicine Bioethics Committee. (Protocol number: CBA# 0627 FMUCH). All experiments were conducted on *ex vivo* heart model in adults male Wistar rats (n=28). The animals were randonmly divided in four equal groups: normobaric normoxia (~750 tor; pO_2 156 mmHg; Nx, n=7); Nx + PUFA (n=7), supplemented with PUFA, Acolest TG (720 mg, DHA:EPA = 1.1:1.0; 0.3 $gkg^{-1}d^{-1}$); hypoxic hypoxia (~428 tor; pO_2 90 mmHg; Hx, n=7) and, Hx + PUFA (n=7). The hypoxic groups were exposed in 4 cycles to 96 h of hypobaric hypoxia followed by 96 h of normobaric normobaria during 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 Hx (22◦C). The 4 groups received the same amount of daily food and drinking water (15 g of standard pellet meals; water *ad libitum*). The PUFA supplementation was maintained during the 32 days of the protocol.

Following the hypobaric or normobaric exposure cycles, the rats were anaesthetized with pentobarbital (50$mgkg^{-1}$ IP) for surgical intervention. Once confirm deep anaesthesia, a sternotomy was performed, heparin 100Ukg^{-1} intravenous (IV) was administered and heart was fastly excised.

2.2 Ex vivo heart and myocardial infarction.

Immediately after dissection, the heart was mounted in a temperature-regulated heart chamber and perfused retrograde via the ascending aorta using a peristaltic infusion pump (Gilson Minipuls3, France) at a constant flow of 10–14 ml min^{-1}. This generate an initial mean coronary (aortic) perfusion pressure of 60– 70mmHg with physiological modified Krebs Henseleit Buffer solution containing (in mM) NaCl (128.3), KCl (4.7), CaCl2 (1.35), NaHCO3 (20.2), NaH2 PO4 (0.4), MgSO4 (1.1), glucose (11.1) and pH 7.4 at 37 °C when equilibrated with a mixture of 95% O2 /5% CO2 . Perfusate and bath temperatures were maintained at 37 °C by a thermostatically controlled water circulator (B. Braun Thermomix 1420, Germany). Then, a latex balloon inserted in the left ventricle through the mitral valve was connected to a pressure transducer (Bridge Amp ML221 AD Instruments, Australia) and filled with normal saline to produce a left ventricle end-diastolic pressure (LVEDP) of 5– 10mmHg. The volume of the balloon was maintained constant throughout the experiment. After 15 min stabilization (basal conditions), hearts with a left ventricular developed pressure (LVDP) less than 60mmHg and a heart rate (HR) less than 180 bpm were excluded from the study. The remaining hearts were subject to 30 min of global ischaemia followed by 120 min reperfusion.

At the end of reperfusion period, heart weight measured immediately after the hemodynamic measurements. The heart was perfused with 15 mL 2,3,5-triphenyltetrazolium chloride 1%(Sigma Chemical) in phosphate buffer adjusted to pH 7.4, for 15 min at 37 °C. After overnight storage in 10% formaldehyde, five to seven 2mm slices were obtained, covered by a glass and digitally photographed. Each slice image was analyzed by planimetry using the Image J program (ImageJ2 is v2.0.0-beta-8, NIH, USA). In addition, the IS was measured and expressed as a percentage of the total ventricular volumen [17].

2.3 Lipid Profile

Blood samples were collected during surgery and centrifuged at 3500g for 15min to obtain serum. The serum levels of total colesterol, triglycerides (TG), and high-density lipoprotein cholesterol (HDL-c) were determined using commercially available kits (Sigma-Aldrich Chemie GmbH, Postfach, CH-9471, Buchs, Switzerland) according to the manufacturers' instructions. Low density lipoprotein cholesterol (LDL-c) was calculated according to the Friedwald formule: LDL-C = Total cholesterol - HDL-C - (TG/5) [18].

2.4 Oxidative stress markers and IL-1β levels.

The measurement of oxidized glutathione (GSSG) and reduced glutathione (GSH) in rat hearts was assessed by a *o*-phthalaldehyde as a fluorescent reagent. *N*-ethylmaleimide was used to prevent interference of GSH with measurement of GSSG [19]. Then, the GSH/GSSG ratio was calculated. Lipid peroxidation was assessed by the thiobarbituric acid reaction at pH 3.5, followed by solvent extraction with a mixture of n-butanol/pyridine (15:1, v/v). Tetramethoxypropane was used as the external standard, and the levels of lipid peroxides were detected spectrophotometrically at 532 nm and were expressed as mmol thiobarbituric acid reactive substances (TBARS) per milligram protein [20]. Interleukin-1β was determined by commercially available ELISA kits (eBioscience, San Diego, CA, USA) according to manufacturer’s instructions (500 μg protein per sample) [21].

2.5 Statistical analysis

A sample size calculation was performed on the basis of the IS reduction. The assumptions used for this purpose included an expected 35% of reduction in IS at the end of reperfusion in the Nx rats plus PUFAs, compared with rats that did not receive any supplementation (Nx, control group). The

sample size calculation was based on the differences in the mean value between two groups with equal sample size, prespecified 5% alpha error and 80% power. The resulting sample size was 7.1 rats in each treatment group (Nquery advisor 7.0). All data was expressed and mean ± SEM and compared using one-way ANOVA and Post-hoc test Tukey (Stata 10.0 for Windows). Differences were considered statistically significant when P values ≤0.05.

3. Results

3.1 Animals weight

All animals were incorporated to the study at similar age and body weights (232 ± 15 g). By the end of the exposure protocol, Nx animals showed a body weight of 331.2 ± 37 g. In contrast, Nx+PUFA group showed 29.1% (447.7 ± 49 g) increased weight relative to Nx (P=0.03). However, Hx rats markedly decreased body weight by 24.2% (261.8 ± 45 g) relative to Nx (P=0.03). This hypoxia-induced effect was fully reverted in the Hx+PUFA (Figure 1).

3.2 Cardiac and infart size

Heart weight measured immediately after the hemodynamic measurements was markedly increased in Hx vs Nx group (0.43±0.12 vs 0.29±0.08 mg/g) (P=0.039) (Fig. 2A). The heart weight-to-body weight ratio showed an even more significant increase in the Hx group reflecting cardiac hypertrophy. These changes are attenuated by the PUFA supplementation (0.33±0.12 mg/g) (Figure 2A) (P=0.041)

Normobaric normoxic group presented an infart size of 63 ± 18% of the total ventricular volume. Interestingly, Hx group show a 37.8 % lower IS relative to Nx (P=0.032). Furthermore, the effect of PUFA supplementation show a reduction of IS in 27.8% (P=0.041) and 51.7% (P=0.028) in Nx and Hx groups, respectively, relative to Nx (Figure 2B).

3.2 Lipid profile, oxidative stress markers and IL-1β levels.

The lipids on serum in Nx were 0.81±0.21 mmol L^{-1}(TG), 0.47±0.05 mmol L^{-1} (LDL) and 0.37±0.07 mmol L^{-1} (VLDL), considered normal for this species [22]. Relative to these, Nx+PUFA rats show 39.3% (P=0.035), 47.1% (P=0.021) and 49.8% (P=0.027) lower levels of TG, LDL-c and VLDL, respectively. In contrast, the CIH exposure induced higher levels of TG (1.41±0.57) and VLDL (0.61±0.18). PUFA supplementation restores these changes in Hx+PUFA group, reducing TG by 30.4% (0.98±0.29) and VLDL by 32.2% (0.43±0.31) relative to Hx group. Moreover, HX-PUFA showed higher levels of TG (P=0.035) and VLDL (P=0.028) compared to Nx+PUFA group (Table 1).

Cardiac oxidative stress was assessed by TBARS and the GSH/GSSG ratios. Nx group TBARS were 10.75±1.80 nmol/mg prot. Relative to Nx, all groups showed a reduction in levels of TBARS, 31.1% (Nx-PUFA), 38.3% (Hx) and 39.4% (Hx-PUFA) (Figure 3A). In contrast, the GSH/GSSG ratio, showed an increased in 146.8% (51.4±8.7, Nx-PUFA) and 161% (56.4±11, Hx) and 235% (83.2±13.3, Hx+PUFA), relative to Nx (35.1±7.9) (Figure 3B).

The IL-1β levels, a proinflammatory cytokine, in Nx+PUFA and Hx groups showed a reduction in cardiac levels reaching to 64.3% (P=0.039) and 78.7% (P=0.027) relative to Nx baseline values (308.5±33). Similarly, Hx+PUFA group showed a reduction in 56.7% (P=0.019) levels of this interleukin relative to Nx group (Figure 4).

4. Discussion

The present study demonstrates, for the first time, that chronic intermittent hypoxia and polyunsaturated fatty acids omega 3(derivated from fish oil), protect the rat heart against global ischemia-reperfusion injury. Furthermore, these effects are associated with antioxidant and anti-inflammatory effects on the myocardial tissue.

High altitude exposure is considered a health risk [23]. Nowadays it is estimated that more than 140 million people inhabit worldwide at altitudes over 2500 m and an important percentage above 3500 m. [23,24]. When adding people travelling to high-altitude for tourism, sports or work, these numbers increased significantly, reason enough to consider life at highlands as a public health issue. High-altitude physiology and cardiorespiratory responses may be divided by the exposition period extension. Until now, much of the studies focus on acute response to hypoxia or long-term responses which involve acclimatization and adaptation. However, few studies have focus on the intermittent pattern and the long-lasting cardiovascular effects. Intermittent hypoxia, which is experienced by a great number of Andean workers (e.g. miners and observatory workers), is a model of exposure to hypoxia whereby periods of stay at higher altitude are interspersed with periods of stay at sea level. These periods may be as short as one day to several days [25]. This model is different from acute (sport and tourism) episodic (sleep apnoea) or chronic (permanent residence) exposure. Episodic and chronic hypoxia are characterized by marked cardiovascular effects to offset a global decrease in tissue oxygen supply, including polycythaemia and an overall sympathetic stimulation. In parallel, hypoxic pulmonary vasoconstriction leads to pulmonary hypertension and a right ventricular hypertrophy [26]. Moreover, CIH is associated with the development of systemic hypertension and left ventricular dysfunction [27,28], and further evidence is now emerging that the right side of the circulation may also be compromised [29,30]. At present, however, our understanding of the basic mechanisms linking CIH and cardiovascular

dysfunction is limited by the pathophysiological heterogeneity of hypoxic patients and the presence of multiple confounding and comorbid conditions, including obesity and previous cardiac function [31]. Consequently, there is a seriously need for the development of experimental models that allow the study of mechanisms leading to cardiovascular dysfunction in CIH.

Ischemic heart disease, following acute myocardial infarction, is the most prevalent health issue in the world and is a major cause of morbidity and mortality. Severe impairment of coronary blood supply produces a spectrum of clinical síndromes such as heart failure, arrhythmias and acute pulmonary edema. Primarily, no blood flow to the heart causes an imbalance between oxygen demand and supply, resulting in damage or dysfunction of the cardiac tissue [32]. Hypoxia is set when oxygen content is a limiting factor for the normal cellular processes, including the production of ATP.. Tissue hypoxic initial hypoxic insult is determined primarily by the magnitude and duration of the interruption in the oxygen supply, and the subsequent damage induced by reperfusion. Two major types of myocardial injury occur following myocardial reperfusion: (i) stunning – manifested by reversible contractile dysfunction and; (ii) infarction – myocardial challenges account for up 50% of IS. Considerable evidence attributes to ROS and oxidative stress as important determinats of these processes, produced either by the myocardium itself or infiltrating inflammatory cells. The accumulation of ROS can lead to cellular damage through different pathways including direct injury to biomolecules or indirect damage though the activation of pro-apoptotic pathways [34]. In this view, oxidative stress biomarkers are relevent to determine the magnitud of injury in the myocardium subjected to IR injury. Previous studies, in isolated rat hearts showed that the increased in lipid peroxidation and lower concentration of glutathione, the main intracelular compound, were associated with higher IS and ventricular dysfunction, in normobaric conditions [16, 35]. Various reports show that atrial glutathione content is lower in heart samples from rats subjected to regional and global ischaemia. Moreover, the drop of GSH

levels is in agreement with the occurrence of IR cycle, an increased oxidative stress and IS [36, 37]. This data are in agreement with our findings (Figure 3). . A non-expected and very interesting outcome was that chronic intermittent hypoxia, rather than increasing the infarct size, is protecting the cardiac integrity and improving the recovery after ischemia-reperfusion. Indeed, an estimated cardiac hypertrophy would be atenuated with PUFA in CIH rats (Figure 2A). Ventricular hypertrophy, as a compensatory phenomenon is a challenge objectified in other models of chronic hypoxia, which may decrease myocardial function [2,26].

The structural and biochemical changes observed in the myocardium subjected to CIH could be associated with preconditioning mechanisms. Reduced IS, regarded as the ultimate indicator of cardioprotection by ischemic preconditioning (IP) [38], was observed in this study in Hx group. The reduced IS was comparable to that after PUFA suplementation in the Nx group, expressing a probable effect of pharmacological preconditioning. This ability of PUFA and IP to produce comparable cardioprotection was previously reported for the antiarrhythmic effects of both strategies in rats *in vivo* [39,40]. This type of IP induced at the final of CIH exposure, may be explained by the induction of systemic and myocardial antioxidant and anti-inflammatory effects. This hypothesis is supported by lower lipid peroxidation markers such as TBARS levels and increase in GSH/GSSG ratio, in Hx group. With respect to the preconditioning mechanism induced by CIH, the relaxing effects of hypoxia on arterial smooth muscle cells [41], the increases coronary flow and myocardial capillary angiogenesis, activation of ATP-sensitive K^+ channels and inhibition of mitochondrial permeability transition pores have been suggested in experimental findings [42,43]. However, there is a threshold in which the adaptation turns maldaptive and increased the cardiovascular risk (**ref**). The next question, then, is how long does the cardioprotective effects last in CIH. Further studies should be focus on decoding the involved mechanisms and clarify these doubts.

Our results also showed that the body weight was significantly lower in the Hx groups than in Nx groups. This is explained by the higher metabolic demands at hypobaric hypoxia, with more oxygen and energy consumption by the respiratory muscles (**ref**). In contrast,PUFA supplementation, prevented the body weight loss in hypoxia and enhanced the body weight in normoxia. Hence, considering that body and organ weights are the result of both anabolic and catabolic processes, it can be argued that PUFA comsumption was able to counteract some of the effects of hypoxia on metabolic balance in rats [44].

Clinical trials, including primary and secondary studies, have concluded that consumption of fatty fish, or pharmaceutical supplementation with PUFA is an effective strategy to reduce cardiovascular morbidity and mortality, as well as classic risk factors such as essential hypertension and dyslipidemia [45,46]. Omega 3 have been shown to improve a number of cardiac hemodynamic factors such as blood pressure [47], left ventricular diastolic filling, heart rate, and endotelial function [48]. The cardioprotective effects also include arrhytmia prevention [49], plasma triacylglycerol reduction [50], antiinflammatory response and enhancement of plaque stability [51, 52]. These metabolic effects are in agreement with serum prolifeshowed in Table 1. The effects of PUFA are due to the induction of changes in the properties of the cell membrane, such as the fluidity and excitability properties [53]. In this view, the incorporation of PUFA can generate a lower availability of arachidonic acid by cyclooxygenase and induce the antiinflamatory pathways during ischemic injury [54]. These data are in agreement with our results, in which PUFA supplementation increases the antioxidant and antiinflammatory effects induce by the intermittent hypobaric exposure. Moreover, in our model, PUFA could contribute to enhance the antioxidant status and mantain the cardiac structure due to long-term effects [16]. This mechanism is associated with the activation of genomic pathways, previously described in PUFA chronic consumption in rats [14,55].

In conclusion, the cardioprotective effects of CIH are associated with attenuation of pro-oxidant and pro-inflammatory effects in isolated heart of rats infarcted by global IR. The clinical implications of PUFA supplementation in this model of hypobaric hypoxia could be relevant for occupational health of high-altitude exposed workers or casual athletes. The molecular mechanisms of these effects are waiting to be delucidated in future studies.

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Leyends of figures.

1. Effect of CIH and PUFA on body weight at final of protocol: normobaric normoxia (Nx), intermittent hypoxia (Hx) and PUFA supplementation (Nx+PUFA; Hx+PUFA) groups, respectively. Variables are expressed as mean ± SD. Significant differences: $^{*}p<0.05$ vs Nx; $^{\sigma}$ $p<0.05$ vs Hx; ω $p<0.05$ vs Hx+PUFA; † $p<0.05$ vs Nx+PUFA.

2. Effect of CIH and PUFA on heart weight (2A) and infarct size (2B) at final of protocol. The heart rats were subjected to global ischemia of 30 min followed by 120 min of reperfusion. Normobaric normoxia (Nx), intermittent hypoxia (Hx) and PUFA supplementation (Nx+PUFA; Hx+PUFA) groups, respectively. Variables are expressed as mean ± SD. Significant differences: $^{*}p<0.05$ vs Nx; $^{\sigma}$ $p<0.05$ vs Hx; † $p<0.05$ vs Nx+PUFA.

3. Effect of CIH and PUFA on oxidative stress markers, TBARS (3A) and reduced glutathione (GSH)/ oxidized glutathione (GSSG) ratio (3B). The heart samples were obtained of rats subjected to global ischemia of 30 min followed by 120 min of reperfusion. Normobaric normoxia (Nx), intermittent hypoxia (Hx) and PUFA supplementation (Nx+PUFA; Hx+PUFA) groups, respectively. Variables are expressed as mean ± SD. Significant differences: $^{*}p<0.05$ vs Nx; $^{\sigma}$ $p<0.05$ vs Hx; † $p<0.05$ vs Nx+PUFA.

4. Effect of CIH and PUFA on *IL-1β levels* of heart rats subjected to global ischemia of 30 min followed by 120 min of reperfusion. Normobaric normoxia (Nx), intermittent hypoxia (Hx) and PUFA supplementation (Nx+PUFA; Hx+PUFA) groups, respectively. Variables are expressed as mean ± SD. Significant differences: $^{*}p<0.05$ vs Nx; $^{\sigma}$ $p<0.05$ vs Hx.

6. References

[1]. Dumitrascu R, Heitmann J, Seeger W, Weissmann N, Schulz R (2013) Obstructive sleep apnea, oxidative stress and cardiovascular disease: lessons from animal studies. Oxid Med Cell Longev. 2013 (2013):234631.

[2]. Farias JG, Jimenez D, Osorio J, Zepeda AB, Figueroa CA, Pulgar VM (2013) Acclimatization to chronic intermittent hypoxia in mine workers: a challenge to mountain medicine in Chile. Biol Res 46 (1):59-67.

[3]. Povea C, Schmitt L, Brugniaux J, Nicolet G, Richalet JP, Fouillot JP (2005) Effects of intermittent hypoxia on heart rate variability during rest and exercise. High Alt Med Biol 6 (3):215-225.

[4]. Zupet P, Princi T, Finderle Z (2009) Effect of hypobaric hypoxia on heart rate variability during exercise: a pilot field study. Eur J Appl Physiol 107 (3):345-350.

[5]. Richalet JP, Donoso MV, Jiménez D, Antezana AM, Hudson C, Cortès G, Osorio J, Leòn A. (2002) Chilean miners commuting from sea level to 4500 m: a prospective study. High Alt Med Biol 3 (2):159-166.

[6]. Wozniak A, Drewa G, Chesy G, Rakowski A, Rozwodowska M, Olszewska D (2001) Effect of altitude training on the peroxidation and antioxidant enzymes in sportsmen. Med Sci Sports Exerc 33(7):1109-1113.

[7]. Chen J, Gao Y, Liao W, Huang J, Gao W (2012) Hypoxia affects mitochondrial protein expression in rat skeletal muscle. OMICS 16(3):98-104.

[8]. Siques P, López de Pablo AL, Brito J, Arribas SM, Flores K, Arriaza K, Naveas N, González MC, Hoorntje A, León-Velarde F, López MR (2014) Nitric oxide and superoxide anion balance in rats exposed to chronic and long term intermittent hypoxia. Biomed Res Int 2014:610474.

[9]. Radak Z, Lee K, Choi W, Sunoo S, Kizaki T, Oh-ishi S, Suzuki K, Taniguchi N, Ohno H, Asano K (1994) Oxidative stress induced by intermittent exposure at a simulated altitude of 4000 m decreases mitochondrial superoxide dismutase content in soleus muscle of rats. Eur J Appl Physiol Occup Physiol 69 (5):392-395.

[10]. Radak Z, Asano K, Lee KC, Ohno H, Nakamura A, Nakamoto H, Goto S (1997) High altitude training increases reactive carbonyl derivatives but not lipid peroxidation in skeletal muscle of rats. Free Radic Biol Med 22(6):1109-1114.

[11]. Kumar D, Bansal A, Thomas P, Sairam M, Sharma SK, Mongia SS, Singh R, Selvamurthy W (1999) Biochemical and immunological changes on oral glutamate feeding in male albino rats. Int J Biometeorol 42 (4):201-204.

[12]. Ding W, Zhang X, Huang H, Ding N, Zhang S, Hutchinson SZ, Zhang X (2014) Adiponectin Protects Rat Myocardium against Chronic Intermittent Hypoxia-Induced Injury via Inhibition of Endoplasmic Reticulum Stress. PLoS One. 9 (4):e94545.

[13]. Rodrigo R, Prieto JC, Castillo R (2013) Cardioprotection against ischaemia/reperfusion by vitamins C and E plus n-3 fatty acids: molecular mechanisms and potential clinical applications. Clin Sci (Lond) 124 (1):1-15.

[14]. Xie N, Zhang W, Li J, Liang H, Zhou H, Duan W, Xu X, Yu S, Zhang H, Yi D (2011) α -Linolenic acid intake attenuates myocardial ischemia/reperfusion injury through anti-inflammatory and anti-oxidative stress effects in diabetic but not normal rats. Arch Med Res 42 (3):171-181.

[15]. Al-Khalifa A, Maddaford TG, Chahine MN, Austria JA, Edel AL, Richard MN, Ander BP, Gavel N, Kopilas M, Ganguly R, Ganguly PK, Pierce GN (2007) Effect of dietary hempseed intake on cardiac ischemia-reperfusion injury. Am J Physiol Regul Integr Comp Physiol 292 (3):R1198-R1203.

[16]. Castillo RL, Arias C, Farías JG (2014) Omega 3 chronic supplementation attenuates myocardial ischaemia-reperfusion injury through reinforcement of antioxidant defense system in rats. Cell Biochem Funct 32 (3):274-281.

[17]. Collins TJ (2007) ImageJ for microscopy. Biotechniques 43 (1 Suppl): 25–30.

[18]. Salaj R, Stofilová J, Soltesová A, Hertelyová Z, Hijová E, Bertková I, Strojný L, Kružliak P, Bomba A (2013) The effects of two Lactobacillus plantarum strains on rat lipid metabolism receiving a high fat diet. Scientific World Journal 29 (2013): 135142.

[19]. Hissin PJ, Hilf R (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 74 (1): 214–226.

[20]. Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95 (2): 351–358.

[21]. Chen L, Lin SX, Agha-Majzoub R, Overbergh L, Mathieu C, Chan LS (2006) CCL27 is a critical factor for the development of atopic dermatitis in the keratin-14 IL-4 transgenic mouse model. Int Immunol 18 (8):1233-1242.

[22]. Kim JG, Mandal PK, Choi KD, Pyun CW, Hong GE, Lee CH (2014) Beneficial dietary effect of turmeric and sulphur on weight gain, fat deposition and lipid profile of serum and liver in rats. J Food Sci Technol 51 (4):774-779.

[23]. Penaloza D, Arias-Stella J (2007) The heart and pulmonary circulation at high altitudes: healthy highlanders and chronic mountain sickness. Circulation 115 (9): 1132-1146.

[24]. Farias JG, Osorio J, Soto G, Brito J, Siques P, Reyes JG (2006) Sustained acclimatization in Chilean mine workers subjected to chronic intermittent hypoxia. High Alt Med Biol 7(4):302-306.

[25]. Wang Z, Li AY, Guo QH, Zhang JP, An Q, Guo YJ, Chu L, Weiss JW, Ji ES (2013) Effects of cyclic intermittent hypoxia on ET-1 responsiveness and endothelial dysfunction of pulmonary arteries in rats. PLoS One 8 (3): e58078.

[26]. Wang Z, Chesler NC (2013) Pulmonary vascular mechanics: important contributors to the increased right ventricular afterload of pulmonary hypertension. Exp Physiol 98(8):1267-1273.

[27]. Peppard PE, Young T, Palta M, and Skatrud J (2000) Prospective study of the association between sleep-disordered breathing and hypertension. N Engl J Med 342 (19): 1378–1384.

[28]. Campen MJ, Shimoda LA, O'Donnell CP (2005) Acute and chronic cardiovascular effects of intermittent hypoxia in C57BL/6J mice. J Appl Physiol (1985) 99 (5):2028-2035.

[29]. Kasai T, Bradley TD (2011) Obstructive sleep apnea and heart failure: pathophysiologic and therapeutic implications. J Am Coll Cardiol 57 (2):119-127.

[30]. Douglas RM, Bowden K, Pattison J, Peterson AB, Juliano J, Dalton ND, Gu Y, Alvarez E, Imamura T, Peterson KL, Witztum JL, Haddad GG, Li AC (2013) Intermittent hypoxia and hypercapnia induce pulmonary artery atherosclerosis and ventricular dysfunction in low density lipoprotein receptor deficient mice. J Appl Physiol (1985) 115 (11):1694-1704.

[31]. Badran M, Ayas N, Laher I (2014) Cardiovascular complications of sleep apnea: role of oxidative stress. Oxid Med Cell Longev 2014:985258.

[32]. N.S. Dhalla, H.K. Saini, P.S. Tappia, R. Sethi, S.A. Mengi, S.K. Gupta (2007) Potential role and mechanisms of subcellular remodeling in cardiac dysfunction due to ischemic heart disease J. Cardiovasc Med 8 (4): 238–250.

[33]. Kwasiborski PJ, Kowalczyk P, Mrówka P, Kwasiborski M, Cwetsch A, Przybylski J (2012) Selected biochemical markers of hypoxia. Przegl Lek 69 (3): 115–119.

[34]. Vanden Hoek TL, Li C, Shao Z, Schumacker PT, Becker LB (1997) Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. J Mol Cell Cardiol 29 (9): 2571–2583.

[35]. Senthamizhselvan O, Manivannan J, Silambarasan T, Raja B (2014) Diosmin pre-treatment improves cardiac function and suppresses oxidative stress in rat heart after ischemia/reperfusion. Eur J Pharmacol S0014-2999(14)00311-2.

[36].Ahmed LA, Salem HA, Attia AS, Agha AM (2012) Comparative study of the cardioprotective effects of local and remote preconditioning in ischemia/reperfusion injury. Life Sci 90(7-8):249-56.

[37].Carnes CA, Janssen PM, Ruehr ML, Nakayama H, Nakayama T, Haase H, Bauer JA, Chung MK, Fearon IM, Gillinov AM, Hamlin RL, Van Wagoner DR (2007) Atrial glutathione content, calcium current, and contractility. J Biol Chem 282 (38):28063–28073.

[38].Liu Y, Downey JM (1992) Ischemic preconditioning protects against infarction in rat heart. Am J Physiol 263(4 Pt 2):H1107-H1112.

[39]. Al Makdessi S, Brandle M, Ehrt M, Sweidan H, Jacob R (1995) Myocardial protection by ischemic preconditioning: the influence of the composition of myocardial phospholipids. Mol Cell Biochem 145 (1): 69–73.

[40]. Abdukeyum GG, Owen AJ, McLennan PL (2008) Dietary (n-3) long-chain polyunsaturated fatty acids inhibit ischemia and reperfusion arrhythmias and infarction in rat heart not enhanced by ischemic preconditioning. J Nutr 138(10):1902-1909.

[41]. Hurtado A (1960) Some clinical aspects of life at high altitudes. Ann Intern Med 53:247-258.

[42]. Zhang Y, Zhong N, Gia J, Zhou Z (2004) Effects of chronic intermittent hypoxia on the hemodynamics of systemic circulation in rats. Jpn J Physiol 54 (2):171-174.

[43]. Zhu WZ, Xie Y, Chen L, Yang HT, Zhou ZN (2006) Intermittent high altitude hypoxia inhibits opening of mitochondrial permeability transition pores against reperfusion injury. J Mol Cell Cardiol 40 (1):96-106.

[44]. Farias JG, Puebla M, Acevedo A, Tapia PJ, Gutierrez E, Zepeda A, Calaf G, Juantok C, Reyes JG (2010) Oxidative stress in rat testis and epididymis under intermittent hypobaric hypoxia: protective role of ascorbate supplementation. Androl 31 (3):314-321.

[45]. Mozaffarian D, Geelen A, Brouwer IA, Geleijnse JM, Zock PL, Katan MB (2005) Effect of fish oil on heart rate in humans: a meta-analysis of randomized controlled trials. Circulation 112 (13):1945-1952.

[46]. Marchioli R, Marfisi RM, Borrelli G, Chieffo C, Franzosi MG, Levantesi G, Maggioni AP, Nicolosi GL, Scarano M, Silletta MG, Schweiger C, Tavazzi L, Tognoni G (2007) Efficacy of n-3 polyunsaturated fatty acids according to clinical characteristics of patients with recent myocardial infarction: insights from the GISSI-Prevenzione trial. J Cardiovasc Med (Hagerstown) 8:S34-S37.

[47]. Mozaffarian D, Gottdiener JS, Siscovick DS (2006) Intake of tuna or other broiled or baked fish versus fried fish and cardiac structure, function, and hemodynamics. Am J Cardiol 97 (2):216-222.

[48]. Goodfellow J, Bellamy MF, Ramsey MW, Jones CJ, Lewis MJ (2000) Dietary supplementation with marine omega-3 fatty acids improve systemic large artery endothelial function in subjects with hypercholesterolemia. J Am Coll Cardiol 35 (2):265-270.

[49]. Rodrigo R, Korantzopoulos P, Cereceda M, Asenjo R, Zamorano J, Villalabeitia E, Baeza C, Aguayo R, Castillo R, Carrasco R, Gormaz JG (2013) A randomized controlled trial to prevent post-operative atrial fibrillation by antioxidant reinforcement. J Am Coll Cardiol 62 (16):1457-1465.

[50]. Harris WS. n-3 fatty acids and serum lipoproteins: human studies (1997) Am J Clin Nutr 65(5 Suppl):1645S-1654S.

[51]. Thies F, Garry JM, Yaqoob P, Rerkasem K, Williams J, Shearman CP, Gallagher PJ, Calder PC, Grimble RF (2003) Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomised controlled trial. Lancet 361(9356):477-485.

[52]. Castillo R, Rodrigo R, Perez F, Cereceda M, Asenjo R, Zamorano J, Navarrete R, Villalabeitia E, Sanz J, Baeza C, Aguayo R (2011) Antioxidant therapy reduces oxidative and inflammatory tissue damage in patients subjected to cardiac surgery with extracorporeal circulation. Basic Clin Pharmacol Toxicol 108 (4):256-262.

[53]. Leaf A, Xiao YF, Kang JX, Billman GE (2005) Membrane effects of the n-3 fish oil fatty acids, which prevent fatal ventricular arrhythmias. J Membr Biol 206 (2):129-139.

[54]. Brahmbhatt V, Oliveira M, Briand M, Perrisseau G, Bastic Schmid V, Destaillats F, Pace-Asciak C, Benyacoub J, Bosco N (2013) Protective effects of dietary EPA and DHA on ischemia-reperfusion-induced intestinal stress. J Nutr Biochem 24 (1):104-111.

[55]. Lluís L, Taltavull N, Muñoz-Cortés M, Sánchez-Martos V, Romeu M, Giralt M, Molinar-Toribio E, Torres JL, Pérez-Jiménez J, Pazos M, Méndez L, Gallardo JM, Medina I, Nogués MR (2013) Protective effect of the omega-3 polyunsaturated fatty acids: Eicosapentaenoic acid/Docosahexaenoic acid 1:1 ratio on cardiovascular disease risk markers in rats. Lipids Health Dis 1;12:140.

Table 1. Lipid profile in blood samples in protocol rats at final of CIH and normobaric exposure.

Blood lipids (mmol L^{-1})	**Nx** (n=7)	**Nx+PUFA** (n=7)	**Hx** (n=7)	**Hx+PUFA** (n=7)
Triglycerides	0.84 ± 0.31	0.49 ± 0,21*	1.41 ± 0.57*,†	0,98 ± 0.29$^{\dagger,\sigma}$
LDL-c	0.49 ± 0.07	0.25 ± 0.09*	0.51 ± 0.11†	0.56 ± 0.27$^{\sigma}$
HDL	0.59 ± 0.22	0.55 ± 0.18	0.49 ± 0.17	0.53 ± 0.23
VLDL	0.39 ± 0.08	0.19 ± 0.07*	0.61 ± 0.18*,†	0.43 ± 0.31$^{\dagger,\sigma}$

LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein. Variables are expressed as mean ± SD. Significant differences:

*P<0.05 vs Nx

†P<0.05 vs Nx + PUFA

$^{\sigma}$P<0.05 vs Hx

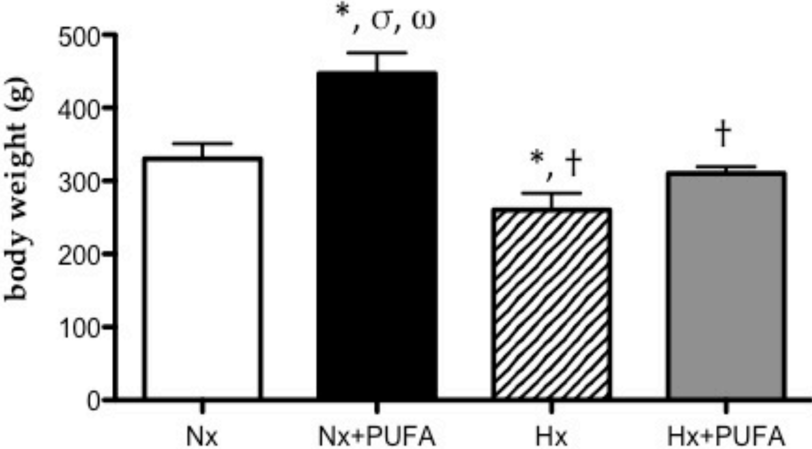


Figure 1.

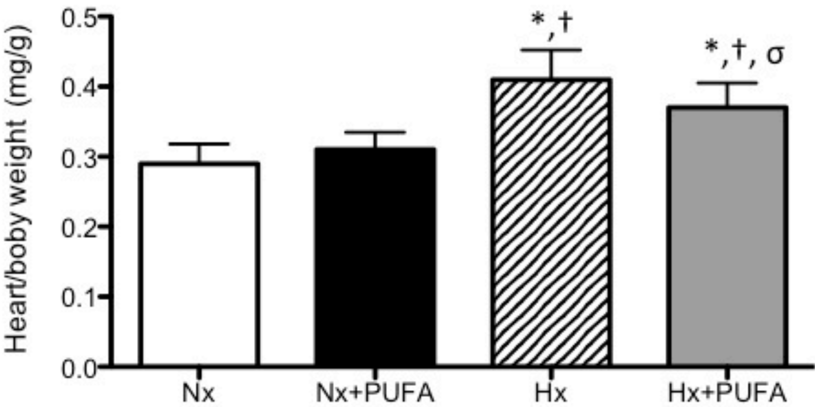
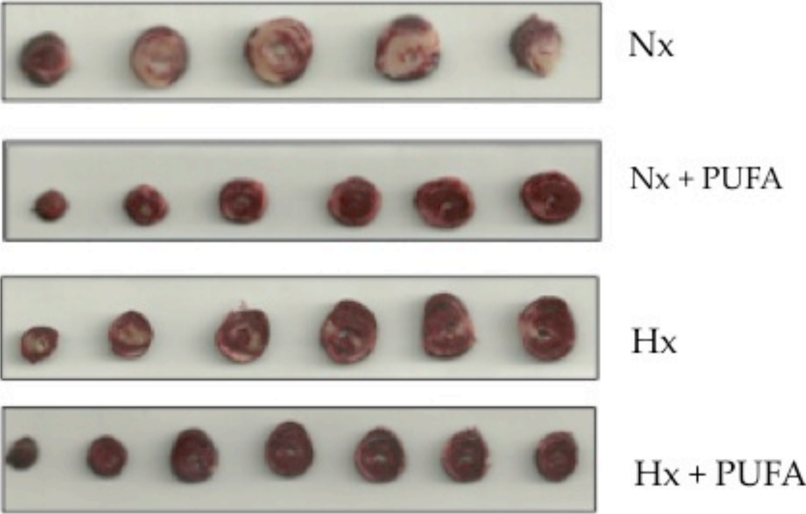
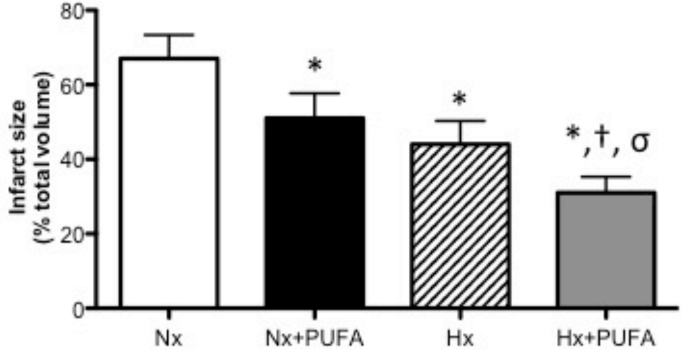


Figure 2A.





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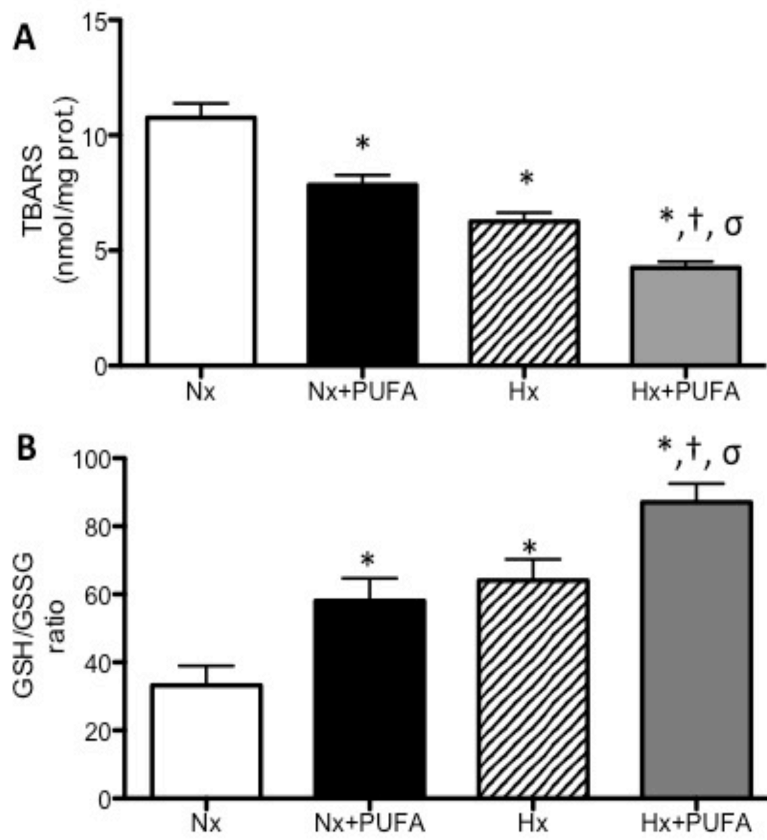

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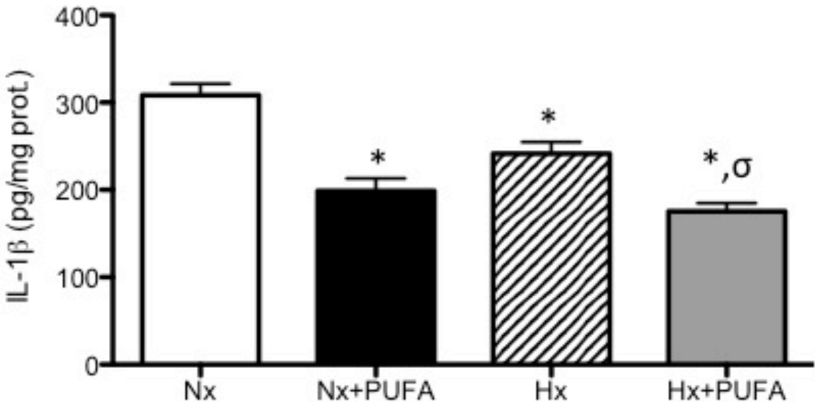

Figure 4