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Abstract

This randomized and controlled trial investigated whether the increase in elite training at different altitudes altered the oxidative stress biomarkers of the nervous system. This is the first study to investigate four F_4-neuroprostanes and four F_2-dihomo-isoprostanes quantified in 24-hour urine. The quantification was carried out by Ultra High Pressure Liquid Chromatography-triple Quadrupole-Tandem Mass Spectrometry (UHPLC-QqQ-MS/MS). Sixteen elite triathletes agreed to participate in the project. They were randomized in two groups, a group submitted to Altitude Training (n=8) and a group submitted to Sea Level Training (n=8), with a Control group of non-athletes (n=8). After experimental period, the Altitude Training group triathletes gave significant data: 17-*epi*-17-F_{2t}-dihomo-IsoP (from 5.2 ± 1.4 µg/mL 24 h^{-1} to 6.6 ± 0.6 µg/mL 24 h^{-1}), *ent*-7(*RS*)-7-F_{2t}-dihomo-IsoP (from 6.6 ± 1.7 µg/mL 24 h^{-1} to 8.6 ± 0.9 µg /mL 24 h^{-1}), and *ent*-7-*epi*-7-F_{2t}-dihomo-IsoP (from 8.4 ± 2.2 µg/mL 24 h^{-1} to 11.3 ± 1.8 µg/mL 24 h^{-1}) increased, while, of the neuronal degeneration-related compounds, only 10-*epi*-10-F_{4t}-NeuroP (8.4 ± 1.7 µg/mL 24 h^{-1}) and 10-F_{4t}-NeuroP (5.2 ± 2.9 µg/mL 24 h^{-1}) were detected in this group. For the control group and sea level training groups, no significant changes had occurred at the end of the 2-weeks experimental period. Therefore, and as the main conclusion, the training at moderate altitude increased the F_4-NeuroPs- and F_2-dihomo-isoPs-related oxidative damage of the central nervous system (CNS) compared to similar training at sea level.

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Abstract

This randomized and controlled trial investigated whether the increase in elite training at different altitudes altered the oxidative stress biomarkers of the nervous system. This is the first study to investigate four F_4-neuroprostanes and four F_2-dihomo-isoprostanes quantified in 24-hour urine. The quantification was carried out by Ultra High Pressure Liquid Chromatography-triple Quadrupole-Tandem Mass Spectrometry (UHPLC-QqQ-MS/MS). Sixteen elite triathletes agreed to participate in the project. They were randomized in two groups, a group submitted to Altitude Training (n=8) and a group submitted to Sea Level Training (n=8), with a Control group of non-athletes (n=8). After experimental period, the Altitude Training group triathletes gave significant data: 17-*epi*-17-F_{2t}-dihomo-IsoP (from 5.2 ± 1.4 µg/mL 24 h^{-1} to 6.6 ± 0.6 µg/mL 24 h^{-1}) , *ent*-7(*RS*)-7-F_{2t}-dihomo-IsoP (from 6.6 ± 1.7 µg/mL 24 h^{-1} to 8.6 ± 0.9 µg /mL 24 h^{-1}), and *ent*-7-*epi*-7-F_{2t}-dihomo-IsoP (from 8.4 ± 2.2 µg/mL 24 h^{-1} to 11.3 ± 1.8 µg/mL 24 h^{-1}) increased, while, of the neuronal degeneration-related compounds, only 10-*epi*-10-F_{4t}-NeuroP (8.4 ± 1.7 µg/mL 24 h^{-1}) and 10-F_{4t}-NeuroP (5.2 ± 2.9 µg/mL 24 h^{-1}) were detected in this group. For the control group and sea level training groups, no significant changes had occurred at the end of the 2-weeks experimental period. Therefore, and as the main conclusion, the training at moderate altitude increased the F_4-NeuroPs- and F_2-dihomo-isoPs-related oxidative damage of the central nervous system (CNS) compared to similar training at sea level.

INTRODUCTION

The practice of training at an altitude is well-known among coaches and athletes, particularly elite athletes. At altitude, exposure to hypoxia is known to influence all functional systems of the body, including the central nervous system (CNS), the endocrine, respiratory, and cardiovascular systems, the blood oxygen-carrying capacity, and morphological and functional adaptations in skeletal muscle [1]. The nervous system is especially vulnerable to reactive oxygen species (ROS)-mediated injury some reasons are that the high oxygen consumption of the brain for high energy needs, that is, high O_2 consumption, results in excessive ROS produced. In addition, the neuronal membranes are rich in polyunsaturated fatty acids (PUFA), which are particularly vulnerable to free radical attack [2]. When exercising at altitude the body responds to the fall in the barometric pressure as well as physical exercise, other factor that contribute to increased oxidative stress (OS) according to literature [3,4]. The OS seems to be linearly related to the altitude: higher altitude leads to greater oxidative challenge to the body [5]. The effects of hypoxia in the brain may influence the training intensity and/or the physiological responses during training at altitude [1]. In addition, previous research indicated that the exercise-induced OS may alter the capacity of oxidation and anti-oxidation of brain tissue [6,7]. Nevertheless the OS related consequence of high altitude training is poorly known [8]. But there are evidence relatively consistent in human and animal studies that reporting that high altitude-associated hypoxia causes oxidative damage to lipids, proteins, and DNA. This damage can be due to the increased level of ROS production and/or decreased level of antioxidant capacity [9].

Lipid peroxidation generates a variety of end products, which can then be measured in biological fluid as an indirect index of OS [10-13]. The most representative end-products of fatty acids oxidation of the system nervous are the F_4-neuroprostanes (F_4-NeuroPs), from DHA (Docosahexaenoic Acid), and the F_2-dihomo-isoprostanes (F_2-dihomo-IsoPs), from AdA (Adrenic Acid) [10]. The PUFAs, DHA and AdA are highly localized in the nervous tissue and represent the main PUFAs in grey and white matter, respectively [10,14], although AdA also has a high presence in the adrenal gland and kidneys [15] and DHA likewise in adipose tissue, rectal epithelium, muscle, liver and spleen, heart and cheek, red blood cells, and sperm [16]. The quantification of F_4-NeuroPs and F_2-dihomo-IsoPs provides a highly sensitive index of oxidative neuronal injury, which likely represents a global measure of oxidant status in the CNS [17]. In the literature it is mentioned that some metabolites of F_4-NeuroPs could have biological activities (anti-arrhythmic activities) [10].

Currently, the detection of F_4-NeuroPs and F_2-dihomo-IsoPs is mainly performed in brain tissue and/or body fluids. They are used mainly in clinical trials to elucidate the role of OS in the diseases [18,19]. Nowadays, no attention has been paid yet concerning to the investigation of these CNS degradation markers and physical exercise and the influence of training at altitude or to sea level to analyze their OS generation. As noted earlier, the exposure to high altitude increased the level of ROS production or decreased level of antioxidant capacity, and then, can lead to oxidative damage to macromolecules [9]. Therefore, this randomized controlled-trial investigated whether training at different altitudes

(~2300 m and 400 m) can alter the OS linked to the nervous system in elite triathletes by analyzing the variations in the values of F_4-neuroPs/ F_2-dihomo-IsoPs excreted before and after of the experimental period at different altitudes. To the best of our knowledge, it is the first study concerning the assessment of these non-invasive biomarkers-NeuroPs and dihomo-IsoPs- in elite triathletes subjected to AT (altitude training) or SLT (sea level training).

MATERIALS AND METHODS

Physical characteristics of participants and dietary intake

Sixteen elite triathletes (12 male and 4 female) from the University of Alicante (Spain) agreed to participate in the project. They were randomized in two groups, a group subjected to Altitude Training (AT, n=8) and a group subjected to Sea Level Training (SLT, n=8). The Control group (Cg, n=8) were non-athletes, with similar anthropometric characteristics and the same age range and healthy lifestyle as the triathletes, they have remained at sea level in all study. In the three groups were included 6 men y 2 women for to avoid variations in the analysis. All the volunteers were sea level residents, non-smokers, had stable food habits, and did not receive any medication during the experimental procedure (prescription or over-the counter medication). None had made a trip to high altitude in the three months before the intervention program. The study was approved by the Bioethics Committee of the University Hospital of Murcia and all participants provided written, informed consent to a protocol approved by the institution.

The physical parameters (Table 1) and dietary habits (Table 2) of the triathletes were controlled before the onset of the assay and after the experimental period- AT or SLT- according to their biological and physiological characteristics. Regarding to Cg, their physical parameters and dietary habits also were evaluated at the same time that the triathletes. The anthropometric measurements were made according to the International Society for the Advancement of Kinanthropometry (ISAK) and were performed by the same, internationally certified anthropometrist (level 2 ISAK) in order to decrease technical errors. The body composition was determined by GREC Kinanthropometry consensus [20], using a model consisting of: total fat by Withers´s formula [21]; lean weight by the procedure described by Leet et al. [22]; and residual mass by the difference in the weight (Table 1).

. The triathletes consumed a constant, equal diet (Table 2) from two weeks before the onset of the study until its conclusion, to avoid any interference with urinary analyses. The calculation of the dietary parameters and calorific intake was accurately designed and overviewed during the experimental intervention by nutritionists, using specific software for the calculation (data calculated by the software available on the website:http://www.easydiet.es),with the additional assistance of the Spanish and USDA databases (http://www.bedca.net/) and http://www.nal.usda.gov/fnic/foodcomp/search/). All the food for the study was prepared and weighed to achieve the desired and constant calorific and nutrient intake for each triathlete.

Training load

The training load quantification was performed using the Objective Load Scale (ECOs) developed by Cejuela Anta and Esteve-Lanao[23]. Variations in ECOs were recorded as training loads, which were measured and slightly modified daily and weekly to ensure the homogeneity of the training program, taking into account the variable physical characteristics of each athlete during the study (Table 1). The method used allowed the quantification of the training loads in triathlon (swim, bike, run, and transitions). The training loads developed by elite triathletes in the present work were similar to those found in other studies [24-26].

Experimental design

The study was a randomized controlled trial (Fig. 1) where the athletes were randomly divided into two groups, a group subjected to AT (n=8) and a group undergoing SLT (n=8), during an experimental period of 2-weeks, supervised their training. The hypoxia exposure was carried out in the "Centro de Alto Rendimiento de Sierra Nevada (CAR)" (2320 m altitude; Sierra Nevada, Alpujarra and Valle de Lecrín, Spain). Before the onset of the experimental period, the training load and diet of the two groups were kept similar for two weeks. The experimental training period for the athletes started with an increase of the effort loads, keeping the ECOs during the two weeks. The Cg maintained their lifestyle at sea level (400 m) throughout the assay. The first urine sample was collected 24-hour at the beginning the experimental period y the second urine sample was collected 24-hour at the end of the experimental period. The samples of the Cg were collected at the same time than of the triathletes. Urine samples were aliquoted immediately of its collection and were stored at -80 ºC until further analysis.

Chemicals and Standards

Six F_4-neuroprostanes (F_4-NeuroPs) were studied, 4(*RS*)-4-F_{4t}-NeuroP, 4-F_{4t}-NeuroP, 4-*epi*-4F_{3t}-NeuroP, 4-F_{3t}-NeuroP, 10-*epi*-10-F_{4t}-NeuroP, and 10-F_{4t}-NeuroP as well as four F_2-dihomo-isoprostanes (F_2-dihomo-IsoPs): 17-*epi*-17-F_{2t}-dihomo-IsoP, 17-F_{2t}-dihomo-IsoP, *ent*-7(*RS*)-7-F_{2t}-dihomo-IsoP, and *ent*-7-*epi*-7-F_{2t}-dihomo-IsoP. Three deuterated internal standards (d_4-4(RS)-F_{4t}-NeuroP, d_4-10-epi-10-F_{4t}-NeuroP, and d_4-10-F_{4t}-NeuroP) were used too (Fig. 2). All standards were synthesized by Durand's team at the Institut des Biomolecules Max Mosseron (IBMM) (Montpellier, France). The β-glucuronidase, type H2 from *Helix pomatia*, and bis-(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All LC-MS grade solvents were obtained from J.T. Baker (Phillipsburg, New Jersey, USA). Strata SPE cartridges (Strata X-AW, 100 mg 3 mL^{-1}) were purchased from Phenomenex (Torrance, California, USA).

Sample collection and preparation

A complete clinical analysis - consisting of hematology, chemistry, and urine chemistry analysis - was performed at the onset of and after the experimental period. All samples (blood and urine) were collected by a nurse from the subjects early in the morning, under fasting conditions. Blood samples at rest were obtained by venipuncture, at the beginning and at the end experimental period and were placed in different tubes according to the analytical procedures. Samples were processed within 1 h of collection and stored at -

80ºC for the analytical determinations. The hematological parameters were recorded using an automated hematological analyzer (CellDyn 3700 and 4000, Abbott, IL, USA) at the Clinical Analysis Service of the Hospital Virgen de la Arrixaca (Murcia, Spain).

Twenty-four-hour urine samples were collected before and after the 2-weeks training period. They were collected in sterile, dark polystyrene tubes with screw caps. The urine analyses were also performed in a modular analyzer (Roche Diagnostic, Mannheim, Germany). The 24-h^{-1} urine was used for the absolute calculation of the amounts of F_4-NeuroPs and F_2-dihomo-IsoPs excreted. The urinary F_4-NeuroPs and F_2-dihomo-IsoPs were analyzed using our previously-described method [27] .

UHPLC-QqQ-MS/MS analyses

The separation of the F_4-NeuroPs and F_2-dihomo-IsoPs in the urine was performed using a UHPLC coupled with a 6460 QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany), using the set-up described previously Medina, et al. [27]. Data acquisition and processing was performed using MassHunter software version B.04.00 (Agilent Technologies, Walbronn, Germany). The qualitative and quantitative analysis of F_4-NeuroPs and F_2-dihomo-IsoPs was performed using the authentic markers synthesized by Durand's team. Three deuterated anal ytes were used as internal standards (Fig. 2).

Statistical analyses

All the statistical analyses were performed using the SPSS 17.0 software package (LEAD Technologies Inc., Chicago, USA). Quantitative data are presented as mean ± SD (standard deviation). The amounts excreted of F_4-NeuroPs and F_2-dihomo-IsoPs were calculated as µg/mL per 24-h^{-1} urine. The normality was analyzed by the Shapiro–Wilk test and the test of equal variances by Levene's test. Specific differences between the hematological, serum, and urinary parameters of SLT and AT triathletes before and after training period were determined by *t*-test. The different amounts excreted of F_4-NeuroPs and F_2-dihomo-IsoPs within the same group, AT or SLT, as a result of the training at different altitudes were examined by paired *t*-test (before /after). Results were considered to be statistically significant when $P< 0.05$.

RESULTS

Anthropometric variables and training performance

The kinanthropometric measurements, performed following the ISAK, did not yield representative differences between the experimental groups. The training loads of the triathletes (SLT and AT) were at the onset of the training period ranged from 458 to 727 ECOs and after the experimental period from 766 to 1021 ECOs (Table 1).

Hematology, chemistry, and urine chemistry modifications

Regarding the results of the tests and profiles, significant variation in the hematology, chemistry, and urine chemistry was detected between the AT and SLT triathletes, after the training program. In this study, we only mentioned the parameters that showed significant changes according to the different tests and profiles of the clinical analyses and that could be relevant - in biochemical terms - to the F_4-NeuroPs and F_2-dihomo-IsoPs, according to previous investigations [28-32] (Table 3). Regarding the clinical urinary parameters (urinary density (g mL^{-1}), pH, proteins (mg dL^{-1}), calcium (mg dL^{-1}), phosphorus (mg dL^{-1}), uric acid (mg dL^{-1}), urea (mg dL^{-1}), creatinine (mg dL^{-1}), and potassium (mEq L^{-1})), no significant differences between the AT and SLT groups of triathletes before and after the experimental period were observed.

Qualitative analysis of F_4-neuroprostanes and F_2-dihomo-isoprostanes

Ten biomarkers were screened in the urine of volunteers. Their identification was confirmed according to their molecular masses, the characteristic MS/MS fragmentation product ions, and the retention time relative to the corresponding standard. The mass spectral information on the F_4-NeuroPs and F_2-dihomo-IsoPs is summarized in our previously reported [27].

The NeuroPs deriving from DHA were not detected in the samples of the triathletes in the SLT and Cg groups, under any condition- perhaps because they were present at very low levels, below the limit of detection and/or quantification (LOD / LOQ). Only two NeuroPs were detected in the urine of the AT group after training (10-*epi*-10-F_{4t}-NeuroP, 10-F_{4t}-NeuroP) (Table 4). The analytes deriving from AdA were detected in triathletes and non-triathletes at the beginning and end of the experimental period. In the present study, F_3-NeuroPs (4-*epi*-4-F_{3t}-NeuroP and 4-F_{3t}-NeuroP) formed by the oxidation of docosapentaenoic acid were analyzed, but were below the LOD/ LOQ. Therefore, these data are not shown.

Quantification of F_4-neuroprostanes and F_2-dihomo-isoprostanes

A total of six biomarkers were quantified in the triathlete's urine, as described in Table 4. All of the urinary biomarkers were normalized to the total 24-h excretion volume. The values are presented as the mean (± SD) total urinary excretion at the onset of and after the experimental period for all groups (µg/mL 24 $h^{-1)}$. In the AT group, only the analytes 10

epi-10F_{4t}-NeuroP and 10-F_{4t}-NeuroP (8.4 ± 2.1 and 5.2 ± 1.2 µg/mL 24 h^{-1}, respectively) were detected, after physical training during exposure to altitude.

On the other hand, the markers of lipid peroxidation derived from AdA were quantified in all groups, and showed statistically-significant variation in the AT group. The 2-weeks exposure to moderate altitude produced significant increases in the urinary levels of 17-*epi*-17-F_{2t}-dihomo-IsoP, *ent*-7(*RS*)-7-F_{2t}-dihomo-IsoP, and *ent*-7-*epi*-7-F_{2t}-dihomo-IsoP, compared to their corresponding concentrations at the start of the training period – according to the paired *t*-test (Table 4). The urinary excretion of F_2-dihomo-IsoPs in the Cg and SLT group had not changed significantly after two weeks of the experimental period at sea level.

DISCUSSION

When comparing the urinary excretion of F_4-NeuroPs and F_2-dihomo-IsoPs at the onset of and after the experimental period in all three groups, four points emerged primarily:

1) The markers of lipid peroxidation derived from DHA were analyzed but were detected under the LOD and LOQ in the urine samples of Cg and SLT triathletes. The LOD are as follows: 4(*RS*)-4F_4-NeuroP: 5.90 ng/mL, 4-F_4-NeuroP: 5.90 ng/mL, 10-*epi*-10-F_4-NeuroP: 0.15 ng/mL, and 10-F_4-NeuroP: 0.10 ng/mL). The LOQ are as follows: 4(*RS*)-4F_4-NeuroP: 11.81 ng/mL, 4-F_4-NeuroP: 11.81 ng/mL, 10-*epi*-10-F_4-NeuroP: 0.34 ng/mL, and 10-F_4-NeuroP: 0.15 ng /mL. Both the LOD and LOQ were based on the method by [27]. This fact suggested that our healthy people, as well as athletes in physical training at sea level, did not show changes in their F_4-NeuroPs values.

2) In our study, after two weeks of AT, the analytes 10-*epi*-10-F_{4t}-NeuroP (8.4 ± 2.1 µg/mL 24 h^{-1} urine) and 10-F_{4t}-NeuroP (5.2 ± 1.2 µg/mL 24 h^{-1} urine) - derived from DHA - were detected only in this group. This suggests that 10-*epi*-10-F_{4t}-NeuroP and 10-F_{4t}-NeuroP may be potential biomarkers of lipid peroxidation physical exercise under hypoxia (low levels of oxygen) at moderate altitude (2320 m altitude). The effects of hypoxia in the brain may influence the training intensity and/or the physiological responses during training at altitude [1]. In cerebral cortex of newborn pigs, an increase in the levels of F_4-NeuroP and other OS markers, after hypoxia and resuscitation with supplementary oxygen (reoxygenation), was detected [33]. Other study in cerebral tissue, observed that DHA seems to be more damaged by ischemia (restriction in blood supply to tissues) compared with hypoxia, suggesting that the increase of F_4-NeuroPs could represent a specific marker for ischemia damage mainly [32]. In human research was indicated that acute hypoxia on Rett syndrome patients, increases plasma levels of F_4-NeuroPs by two orders of magnitude, as compared to those of healthy controls [34]. These results supported to previous studies *in vivo*, about possible hypoxic state links and an increased NeuroPs values.

According to the literature, F_4-NeuroPs not only might be biomarkers of lipid peroxidation, but also could have anti-arrhythmic effects [10]. Therefore, the detection of 10-*epi*-10F_{4t}-NeuroP and 10-F_{4t}-NeuroP, besides indicating an increase in lipid peroxidation in athletes submitted to altitude, also suggests a role for lipid metabolism against arrhythmias induced by altitude. Since arrhythmia starts at an altitude of about 2000 m, an immediate increase in ventilation, mediated by peripheral chemoreceptors, is observed according to the literature [35]. In addition, arrhythmia is one of the physiological responses to hypoxia exposure, caused by ventilator and circulatory responses that are accompanied by an increase in the sympathetic activity and local vasoregulatory effects. Therefore, these are undoubtedly key mechanisms improving oxygen delivery to tissues [1,35]. The increase in the amount of NeuroPs in urine is also evidence that the hard work involved in elite training results in OS in tissues where DHA is present - like muscle, as well as adipose tissue, rectal epithelium, liver and spleen, heart and cheek, red blood cells, and sperm. Further research is required to elucidate the biological role of the NeuroPs in triathletes.

In ours AT athletes, 17-*epi*-17-F_{2t}-dihomo-IsoP, *ent*-7(*RS*)-7-F_{2t}-dihomo-IsoP, and *ent*-7-*epi*-7-F_{2t}-dihomo-IsoP increased compared with the amount before the short-term training at moderate altitude (Table 4). F_2-dihomo-IsoPs reflect the oxidative status of brain white matter, but also could reflect the OS of the other organs where they are present [18]. Altitude training stimulates the adrenergic nervous system responsible for the acute cardiovascular response to hypoxia, playing a crucial role in the adaptation to acute hypoxia during exercise [36]. Dosek, et al. [5], they mentioned that physical exercise at high altitude could further increase the altitude-induced oxidative stress- as can be seen in this study- and the associated oxidative damage although the OS seems to be linearly related to the altitude: higher altitude leads to greater oxidative challenge to the body. On the other hand, the excretion of F_2-dihomo-IsoPs by Cg and SLT triathletes had not changed significantly after two weeks. This result indicates that an acute increase in training at sea level for elite athletes using ECOs did not influence the urinary excretion of F_2-dihomo-IsoPs. An increase of the OS biomarkers followed to aerobic and anaerobic acute physical exercise has been shown in numerous investigations, but also is mentioned that in well-training athletes, this is not always fulfilled [37]. In studies developed, *in vivo* , it was found that chronic exercise could increase the resistance against OS, providing enhanced protection [38-40] for this reasons is mentioned that some athletes do not show changes in its levels of biomarkers of OS, since is associated with an adaptive process[5,40].

4) The last point concerns anthropometric, biochemical, and hematological parameters and their connection with F_4-NeuroPs and F_2-dihomo-IsoPs, since some of them have been associated with an increase or decrease in lipid peroxidation. As regards the anthropometric parameters and their relation to an increase in lipid peroxidation, a study carried out by Ohmori and co-workers reported that body mass index (BMI) in humans is related to an increase of lipid peroxidation [31], but in this sense, in our volunteers (triathletes and Cg) no changes in BMI were found during the study and there were no statistical differences between the two groups. On the other hand, the activities of the pancreatic enzymes (amylase and lipase) were increased after two weeks at altitude. The pancreatic lipases are involved in the mobilization of fatty acids from fat deposits - for example, during stress - and play a role in lipolysis, which, together with the biogenic monoamines, could influence in the process peroxide oxidation of natural lipid [29]. Another biochemical parameter in plasma that showed significant changes was ferritin, the concentration of which decreased. In our study the plasma iron remained constant whilst ferritin levels declined. Previous study demonstrated, in cerebral cortex from rats with hypoxia-ischemia, an increase in desferoxamine-chelatable free iron that could have induced cerebral OS [32]. These authors reported increases in F_2-IsoPs and F_4-NeuroPs concomitant with that of iron, suggesting a dual interaction in relation to this oxidative damage.

Normal physiological increases in the red blood cells count occur at high altitudes or after strenuous physical training [41]. According to literature 2-weeks at moderate altitude exposition the hemoglobin concentrations and hematocrit increased their values in elite athlete [42,43]. Although the AT group showed higher hemoglobin concentration values after training, these have not been significant in the statistical tests due to the variability of the

results (Table 3). But AT group showed significant increases in ACV (averaged corpuscular volume) and ACH (averaged corpuscular hemoglobin), compared to the baseline determinations. Hence, these data reflect a general activation of erythropoiesis in response to presumed renal tissue hypoxia for the AT athletes. Red blood cell morphology is an important biosensor for OS imbalance and chronic hypoxemia (low oxygen in the blood), in neurodegenerative diseases [14,28]. Moreover, the leukocyte concentrations in peripheral blood after the AT effort also exhibited significant modifications. An earlier study mentioned that leukocyte antioxidants, in patients with type 2 diabetes, are related to lipid peroxidation [30]. Whether due to physical exercise in altitude and/or exposure altitude, influenced significantly in to alter metabolic process and lipid peroxidation of the AT group, however it still not clear whether they are causative or associative, is necessary further investigation to clarify these results in elite athletes.

In conclusion**,** our study F_2-dihomo-IsoPs and F_4-NeuroPs have been detected for the first time in the urine of elite triathletes subjected to two weeks of training at altitude or sea level. The F_4-NeuroPs were only detected in the group training at moderate altitude, suggesting that the altitude factor could be related to their production from DHA, through lipid peroxidation. The F_2-dihomo-IsoPs also showed increases in their urinary excretion in athletes subjected to AT, *versus* their baseline amounts. Therefore, and as the main conclusion, the training at moderate altitude increased the F_4-NeuroPs- and F_2-dihomo-isoPs-related oxidative damage of the CNS compared to similar training at sea level.

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The authors declare that they have no conflict of interest.

REFERENCIES

[1] Rusko HK, Tikkanen HO, Peltonen JE. Altitude and endurance training. J Sports Sci 2004;22(10):928-44; discussion 945.

[2] Joseph F. Why Is the Nervous System Vulnerable to Oxidative Stress? In: Natan Gadoth HHG, editor. Oxidative Stress and Free Radical Damage in Neurology. illustrated ed. New York, NY 10013, USA: Springer Science & Business Media; 2010. p 323

[3] Askew EW. Work at high altitude and oxidative stress: antioxidant nutrients. Toxicology 2002;180(2):107-19.

[4] Calbet JA, Lundby C. Air to muscle O2 delivery during exercise at altitude. High Alt Med Biol 2009;10(2):123-34.

[5] Dosek A, Ohno H, Acs Z, Taylor AW, Radak Z. High altitude and oxidative stress. Respir Physiol Neurobiol 2007;158(2-3):128-31.

[6] Camiletti-Moirón D, Aparicio VA, Aranda P, Radak Z. Does exercise reduce brain oxidative stress? A systematic review. Scandinavian Journal of Medicine & Science in Sports 2013;23(4):e202-e212.

[7] Li JIE, Wang Y. Effect of different methods of hypoxic exercise training on free radical oxidation and antioxidant enzyme activity in the rat brain. Biomedical Reports 2013;1(6):925-929.

[8] Bakonyi T, Radak Z. High altitude and free radicals. J Sports Sci Med 2004;3(2):64-9.

[9] Radak Z, Acs Z, Bori Z, Taylor AW, Yang H. The effects of high-altitude exposure on reactive oxygen and nitrogen species. Systems Biology of Free Radicals and Antioxidants2014. p 407-416.

[10] Galano JM, Mas E, Barden A, Mori TA, Signorini C, De Felice C, Barrett A, Opere C, Pinot E, Schwedhelm E and others. Isoprostanes and neuroprostanes: total

synthesis, biological activity and biomarkers of oxidative stress in humans. Prostaglandins Other Lipid Mediat 2013;107:95-102.

[11] Hsiu-Chuan Y. Detection of F2-isoprostanes and F4-neuroprostanes in clinical studies. Journal of Biomedical Laboratory Science 2010;22(1):1-10.

[12] Halliwel BG, Gutteridge JMC.Free radical in biology and medicine. New York:Oxford University Press1999.

[13] Roberts LJ, 2nd, Montine TJ, Markesbery WR, Tapper AR, Hardy P, Chemtob S, Dettbarn WD, Morrow JD. Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid. J Biol Chem 1998;273(22):13605-12.

[14] Signorini C, De Felice C, Durand T, Oger C, Galano J-M, Leoncini S, Pecorelli A, Valacchi G, Ciccoli L, Hayek J. Isoprostanes and 4-Hydroxy-2-nonenal: Markers or Mediators of Disease? Focus on Rett Syndrome as a Model of Autism Spectrum Disorder. Oxidative Medicine and Cellular Longevity 2013;2013:10.

[15] VanRollins M, Woltjer RL, Yin H, Morrow JD, Montine TJ. F2-dihomo-isoprostanes arise from free radical attack on adrenic acid. J Lipid Res 2008;49(5):995-1005.

[16] Arterburn LM, Hall EB, Oken H. Distribution, interconversion, and dose response of n−3 fatty acids in humans. The American Journal of Clinical Nutrition 2006;83(6):S1467-1476S.

[17] Musiek ES, Cha JK, Yin H, Zackert WE, Terry ES, Porter NA, Montine TJ, Morrow JD. Quantification of F-ring isoprostane-like compounds (F4-neuroprostanes) derived from docosahexaenoic acid in vivo in humans by a stable isotope dilution mass spectrometric assay. J Chromatogr B Analyt Technol Biomed Life Sci 2004;799(1):95-102.

[18] Durand T, De Felice C, Signorini C, Oger C, Bultel-Ponce V, Guy A, Galano JM, Leoncini S, Ciccoli L, Pecorelli A and others. F(2)-Dihomo-isoprostanes and brain white matter damage in stage 1 Rett syndrome. Biochimie 2013;95(1):86-90.

[19] Montine TJ, Quinn JF, Milatovic D, Silbert LC, Dang T, Sanchez S, Terry E, Roberts LJ, 2nd, Kaye JA, Morrow JD. Peripheral F2-isoprostanes and F4-neuroprostanes are not increased in Alzheimer's disease. Ann Neurol 2002;52(2):175-9.

[20] Alvero Ramón JRCA, M.D.; Herrero de Lucas, A.; Martínez Riaza, L.; Moreno Pascual, C.; Porta Manzañido, J.; Sillero Quintana, M.; Sirvent Belando, J.E. Protocolo de valoración de la composición corporal para el reconocimiento médico-deportivo. Documento de Consenso del Grupo Español de Cineantropometría (GREC) de la Federación Española de Medicina del Deporte (FEMEDF). Archivos de Medicina del Deporte 2010;27(139):330-44.

[21] Withers RT, Craig NP, Bourdon PC, Norton KI. Relative body fat and anthropometric prediction of body density of male athletes. Eur J Appl Physiol Occup Physiol 1987;56(2):191-200.

[22] Lee RC, Wang Z, Heo M, Ross R, Janssen I, Heymsfield SB. Total-body skeletal muscle mass: development and cross-validation of anthropometric prediction models. Am J Clin Nutr 2000;72(3):796-803.

[23] Saugy JJ, Schmitt L, Cejuela R, Faiss R, Hauser A, Wehrlin JP, Rudaz B, Delessert A, Robinson N, Millet GP. Comparison of "Live High-Train Low" in Normobaric versus Hypobaric Hypoxia. PLoS ONE 2014;9(12):e114418.

[24] Lucia A, Esteve-Lanao J, Olivan J, Gomez-Gallego F, San Juan AF, Santiago C, Perez M, Chamorro-Vina C, Foster C. Physiological characteristics of the best Eritrean runners-exceptional running economy. Appl Physiol Nutr Metab 2006;31(5):530-40.

[25] Medina S, Dominguez-Perles R, Cejuela-Anta R, Villano D, Martinez-Sanz JM, Gil P, Garcia-Viguera C, Ferreres F, Gil JI, Gil-Izquierdo A. Assessment of oxidative stress markers and prostaglandins after chronic training of triathletes. Prostaglandins Other Lipid Mediat 2012;99(3-4):79-86.

[26] Medina S, Domínguez-Perles R, García-Viguera C, Cejuela-Anta R, Martínez-Sanz JM, Ferreres F, Gil-Izquierdo A. Physical activity increases the bioavailability of flavanones after dietary aronia-citrus juice intake in triathletes. Food Chemistry 2012;135(4):2133-2137.

[27] Medina S, Miguel-Elizaga ID, Oger C, Galano JM, Durand T, Martinez-Villanueva M, Castillo ML, Villegas-Martinez I, Ferreres F, Martinez-Hernandez P and others. Dihomo-isoprostanes-nonenzymatic metabolites of AdA-are higher in epileptic patients compared to healthy individuals by a new ultrahigh pressure liquid chromatography-triple quadrupole-tandem mass spectrometry method. Free Radic Biol Med 2015;79:154-63.

[28] Ciccoli L, De Felice C, Paccagnini E, Leoncini S, Pecorelli A, Signorini C, Belmonte G, Valacchi G, Rossi M, Hayek J. Morphological changes and oxidative damage in Rett Syndrome erythrocytes. Biochim Biophys Acta 2012;1820(4):511-20.

[29] Gureeva NV. Pancreatic lipase activation, inhibition, and relationship to peroxide oxidation of lipids. Pharmaceutical Chemistry Journal 2009;43(11):619-624.

[30] Kurtul N, Bakan E, Aksoy H, Baykal O. Leukocyte lipid peroxidation, superoxide dismutase and catalase activities of type 2 diabetic patients with retinopathy. Acta Medica (Hradec Kralove) 2005;48(1):35-8.

[31] Ohmori K, Ebihara S, Kuriyama S, Ugajin T, Ogata M, Hozawa A, Matsui T, Tsubono Y, Arai H, Sasaki H and others. The relationship between body mass index

and a plasma lipid peroxidation biomarker in an older, healthy Asian community. Ann Epidemiol 2005;15(1):80-4.

[32] Signorini C, Ciccoli L, Leoncini S, Carloni S, Perrone S, Comporti M, Balduini W, Buonocore G. Free iron, total F-isoprostanes and total F-neuroprostanes in a model of neonatal hypoxic-ischemic encephalopathy: neuroprotective effect of melatonin. J Pineal Res 2009;46(2):148-54.

[33] Solberg R, Longini M, Proietti F, Vezzosi P, Saugstad OD, Buonocore G. Resuscitation with supplementary oxygen induces oxidative injury in the cerebral cortex. Free Radic Biol Med 2012;53(5):1061-7.

[34] De Felice C, Ciccoli L, Leoncini S, Signorini C, Rossi M, Vannuccini L, Guazzi G, Latini G, Comporti M, Valacchi G and others. Systemic oxidative stress in classic Rett syndrome. Free Radic Biol Med 2009;47(4):440-8.

[35] Burtscher M, Mairer K, Wille M, Gatterer H, Ruedl G, Faulhaber M, Sumann G. Short-term exposure to hypoxia for work and leisure activities in health and disease: which level of hypoxia is safe? Sleep Breath 2012;16(2):435-42.

[36] Favret F, Richalet JP. Exercise and hypoxia: the role of the autonomic nervous system. Respir Physiol Neurobiol 2007;158(2-3):280-6.

[37] Fisher-Wellman K, Bloomer RJ. Acute exercise and oxidative stress: a 30 year history. Dyn Med 2009;8:1.

[38] Lin C, Wu CJ, Wei IH, Tsai MH, Chang NW, Yang TT, Kuo YM. Chronic treadmill running protects hippocampal neurons from hypobaric hypoxia-induced apoptosis in rats. Neuroscience 2013;231:216-24.

[39] Liu J, Yeo HC, Overvik-Douki E, Hagen T, Doniger SJ, Chyu DW, Brooks GA, Ames BN. Chronically and acutely exercised rats: biomarkers of oxidative stress and endogenous antioxidants. J Appl Physiol (1985) 2000;89(1):21-8.

[40] Nikolaidis MG, Kyparos A, Vrabas IS. F(2)-isoprostane formation, measurement and interpretation: the role of exercise. Prog Lipid Res 2011;50(1):89-103.

[41] Bailey DM, Davies B. Physiological implications of altitude training for endurance performance at sea level: a review. Br J Sports Med 1997;31(3):183-90.

[42] Heinicke K, Heinicke I, Schmidt W, Wolfarth B. A three-week traditional altitude training increases hemoglobin mass and red cell volume in elite biathlon athletes. Int J Sports Med 2005;26(5):350-5.

[43] Wehrlin JP, Hallen J. Linear decrease in .VO2max and performance with increasing altitude in endurance athletes. Eur J Appl Physiol 2006;96(4):404-12.

FIGURE CAPTIONS

Figure 1 Study design. The triathletes were randomized in two groups, a group subjected to Altitude Training (AT, n=8) and a group subjected to Sea Level Training (SLT, n=8). The Control group (Cg, n=8) were non-athletes. For two weeks, the training loads and diet were kept similar. Urine samples were collected before and after the training period (SLT or AT) and at the same times for the Cg.

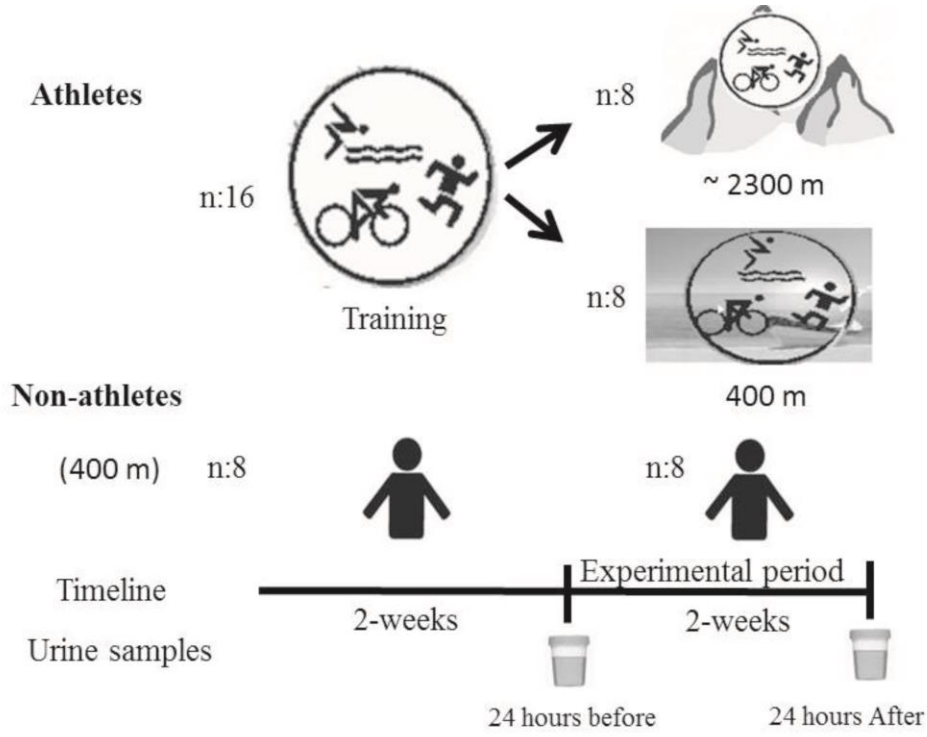

Figure 2 Chemical structures of F_{4t}-neuroprostanes, F_{2t}-dihomo-isoprostanes, and deuterated internal standards. A: F_{4t}-Neuroprostanes, B: F_{2t}-dihomo-isoprostanes.

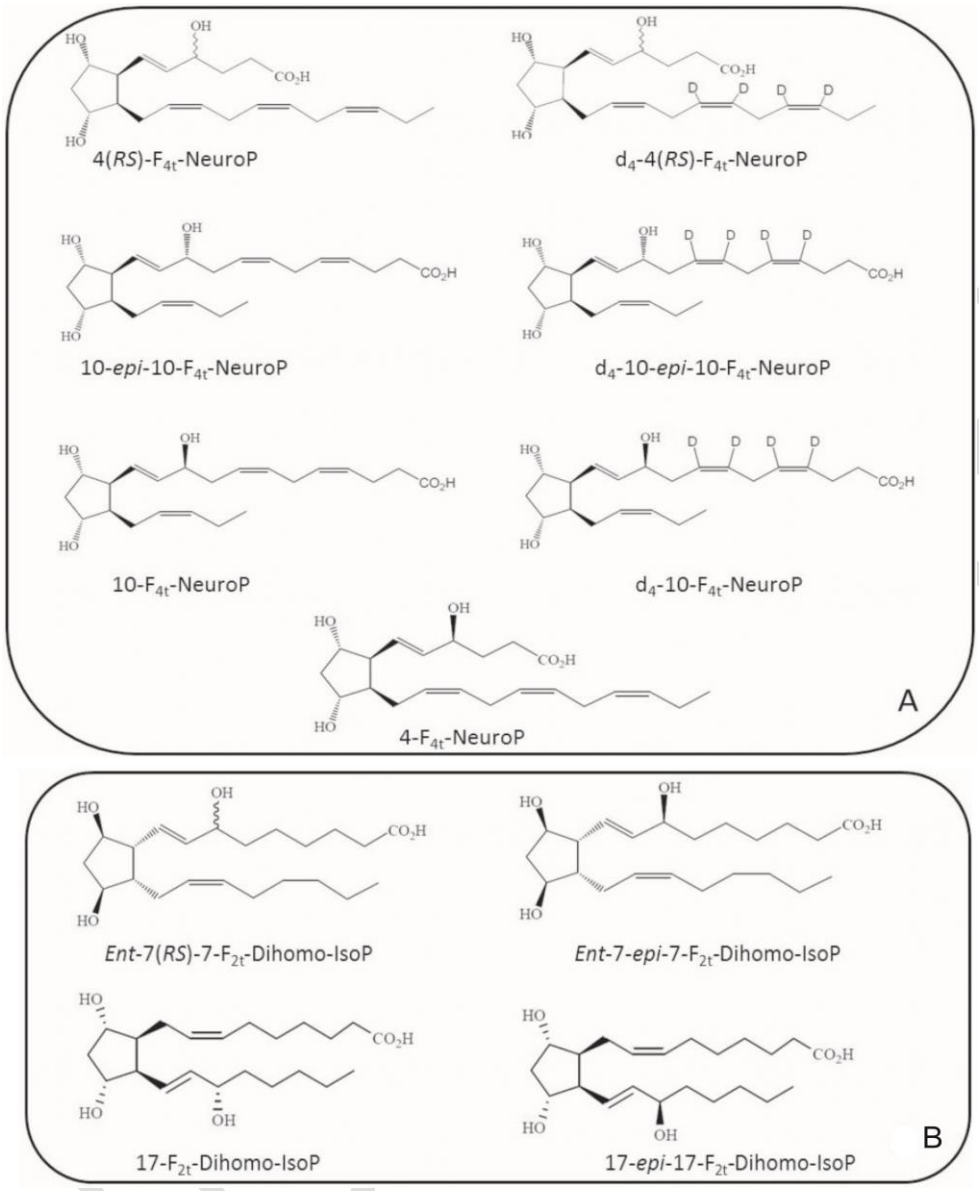

Figure 3 F_4-neuroprostanes and F_2-dihomo-isoprostanes (µg/mL 24 h^{-1}) determined in the urine of the three groups before and after the experimental period. The values shown are mean ± standard deviation. A) Cg: Control group (n=8), B) SLT: Elite triathletes training at sea level (n=8), and C) AT: Elite triathletes training at altitude (n=8).The average volume of urine excreted by volunteers was 1560.62 ± 627.32 mL per 24 h in the two periods. The level of statistical significance was set at P <0.05 (*) and P <0.001 (†), by the paired t-test. DHA: docosahexaenoic acid and AdA: adrenic acid.

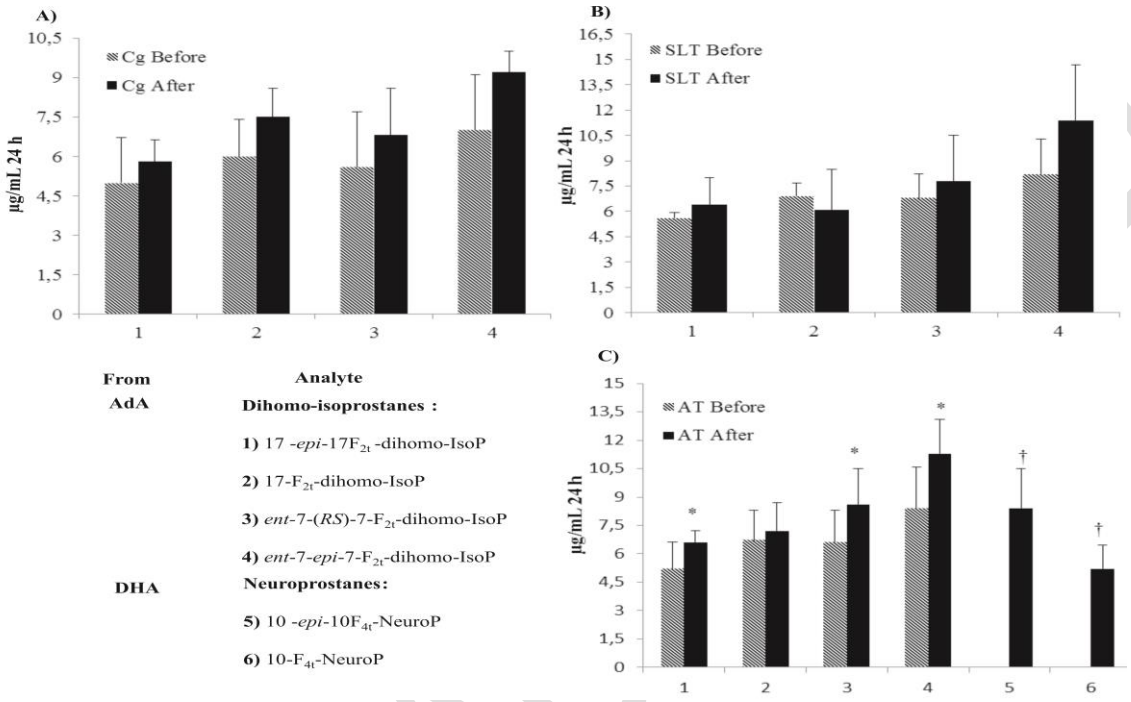

TABLE 1 Physical parameters and metabolic characteristics at the beginning and at the end of the experimental period both of control group (n=8) and of the triathletes according to their altitude level training (n=16, divided in two, at sea level n=8 and at altitude level n=8) y with their objective load scale (ECOs)

Variable	Control Group (<400 m)		Triathletes			
			Sea level (<400 m)		Altitude (~2320 m)	
	1	2	1	2	1	2
Age Year (yr)	21.9 (6.2)	21.9 (6.2)	20.8 (2.0)	20.8 (2.0)	20.3 (1.8)	20.3 (1.8)
Weight (kg)	64.4 (8.2)	64.7 (8.1)	68.4 (13.1)	65.4 (11.5)	68.1 (7.1)	67.2 (7.1)
Height (m)	1.7 (0.1)	1.7 (0.07)	1.7 (0.1)	1.7 (0.1)	1.8 (0.1)	1.8 (0.1)
BMI (kg m^{-2})	22.0 (0.9)	22.2 (0.5)	21.4 (2.5)	21.8 (0.1)	21.3 (0.6)	21.2 (0.7)
Total fat (kg)	9.3 (1.6)	9.5 (1.7)	8.4 (1.3)	8.7 (0.8)	6.2 (1.2)	6.0 (1.2)
Lean weight (kg)	26.7 (6.3)	26.3 (5.9)	29.1 (7.2)	27.3 (6.7)	32.2 (7.3)	30.1 (4.2)
Subscapular skinfold (mm)	11.5 (1.5)	11.8 (1.1)	10.6 (2.0)	11.0 (1.0)	7.8 (1.6)	7.7 (1.7)
Tricipital skinfold (mm)	12.9 (5.2)	12.7 (5.0)	12.3 (6.8)	12.8 (5.2)	7.4 (3.4)	8.2 (3.7)
Bicippital skinfold (mm)	4.6 (1.3)	5.3 (0.6)	4.7 (1.4)	5.0 (1.1)	4.6 (1.7)	3.7 (1.0)
Ileocrestal skinfold (mm)	14.1 (0.8)	14.4 (0.09)	13.9 (4.7)	15.9 (3.4)	10.4 (3.4)	8.9 (2.3)
Supraespinal skinfold (mm)	10.8 (0.6)	10.8 (0.7)	10.5 (3.6)	11.2 (2.8)	7.3 (1.9)	6.7 (1.9)
Abdominal skinfold (mm)	18.1 (3.7)	18.3 (3.7)	17.7 (7.1)	19.3 (4.9)	10.5 (4.6)	9.5 (3.8)
Thigh skinfold (mm)	17.8 (5.8)	17.7 (3.3)	16.1 (9.3)	17.9 (9.2)	10.4 (4.3)	10.5 (4.3)
Calf skinfold (mm)	12.3 (3.5)	12.6 (5.3)	10.2 (3.8)	10.6 (2.6)	7.1 (2.9)	7.4 (3.2)
Fat mass (%)	14.0 (10.2)	14.2 (10.5)	13.7 (3.4)	13.6 (5.7)	10.25 (2.2)	10.4 (1.8)
Training loads (ECOs)			553(95)	870(104)	933(88)	651(76)

Values are Mean (Standard Deviation).1= data corresponding to before experimental period; 2= data corresponding to after experimental period. BMI: Body Mass Index

TABLE 2. Dietary parameters and calorific intake

	Control group		Triathletes	
	Male (n=6)	Female (n=2)	Male (n=12)	Female (n=4)
Energy intake (kcal d $^{-1}$)	2913.1 (601.6)	2289.7 (270.7)	3483.5 (673.6)	2585.0 (376.4)
Carbohydrate (g d $^{-1}$)	309.4 (62.4)	257.0 (7.6)	409.1 (79.1)	324.3 (31.8)
Dietary fiber (g d^{-1})	29.5 (9.3)	25.2 (11.9)	26.6 (4.9)	26.4 (8.6)
Sugar (g d $^{-1}$)	125.7 (37.7)	147.8 (16.0)	169.8 (68.0)	147.6 (11.97)
Proteins (g d $^{-1}$)	130.8 (32.0)	97.6 (28.4)	145.7 (33.3)	109.9 (34.7)
Total lipids (g d^{-1})	127.9 (27.3)	96.8 (14.0)	140.4 (25.69)	94.1 (12.2)
SFA[a] (g d^{-1})	38.1 (11.0)	29.36 (5.8)	36.5 (10.5)	28.5 (6.7)
MUFA[b] (g d^{-1})	64.5 (15.4)	47.3 (4.0)	73.8 (14.0)	46.6 (2.4)
PUFA[c] (g d^{-1})	17.2 (3.9)	10.8 (0.4)	19.2 (1.7)	10.4 (1.0)
Vitamin C (mg d^{-1})	160.9 (101.2)	42.8 (12.5)	254.4 (69.6)	153.6 (63.1)
Vitamin E (mg d^{-1})	24.0 (10.8)	9.65 (0.3)	29.5 (8.42)	14.9 (1.0)
Vitamin D (mg d^{-1})	6.3 (4.1)	2.5 (3.4)	5.7 (3.2)	3.6 (2.6)
Iron (mg d^{-1})	24.7 (9.6)	17.0 (7.14)	26.9 (5.1)	24.6 (1.3)
Selenium (mg d^{-1})	100.5 (68.5)	133.3 (8.2)	235.1 (91.2)	168.4 (73.6)

[a] Saturated fatty acids

[b] Monounsaturated fatty acids

[c] Polyunsaturated fatty acids

Values are Mean (SD)

TABLE 3. Hematology, chemistry, and urine chemistry parameters of the elite triathletes before and after training at different altitude (n = 8 each group)

Variable	Before training			After training		
	SLT (<400 m)	**AT (~2320 m)**	**Sign**	**SLT (<400 m)**	**AT (~2320 m)**	**Sign**
Pancreatic amilase (U L^{-1}) [a]	22.33 (5.01)	25.25 (7.65)		22.50 (4.46)	30.13 (7.83) †	
Lactate dehydrogenase (U L^{-1}) [a]	179.5 (38.87)	213.63 (36.25)		157.83 (32.98)†	203.50 (25.00)	*
Lipase (U L^{-1})[a]	25.33 (6.22)	26.88 (8.34)		22.67 (4.97)	32.63 (10.17) †	*
C-Reactive Protein mg/dL [a]	0.10 (0.09)	0.11 (0.21)		0.12 (0.10)	0.03 (0.0)	
Ferritin (µg L^{-1}) [a]	48.33 (10.44)	55.88 (17.16)		58.00 (17.44)	38.50 (14.76) ††	*
Iron (µg L^{-1}) [a]	79.50 (28.65)	100.75 (20.81)		112.00 (62.03)	109.63 (72.99)	
Hemoglobin (g dL^{-1})	15.00 (1.31)	14.83 (1.13)		15.08 (0.86)	19.01 (10.78)	
Hematocrit (%)	45.60 (4.50)	44.81 (3.82)		44.83 (2.4)	45.79 (4.24)	
PDW (%)	16.57 (1.05)	17.29 (1.65)		16.38 (0.44)	15.85 (0.37)†	*
ACV (fL)	82.22 (2.86)	91.64 (2.22)		89.12 (2.81)	93.84 (2.21)†	
ACH (pg $cell^{-1}$)	29.40 (1.51)	30.39 (0.94)		30.23 (1.16)	31.30 (1.04)†	

Values are means (SD). Abbreviation: ACV, averaged corpuscular volume; ACH, averaged corpuscular hemoglobin; PDW: Platelet distribution width (it analyzed in blood)

[a] These parameters were analyzed in serum.

*Significant differences at $p<0.05$ measured at different altitudes at matched time points.

†Significant differences at $p<0.05$ between measurements before and after training periods at the same altitude.