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ORIGINAL ARTICLE

Docosahexaenoic Acid Supplementation Promotes Erythrocyte Antioxidant Defense and Reduces Protein Nitrosative Damage in Male Athletes

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Abstract The aim of this study was to determine the influence of long-term docosahexaenoic acid (DHA) dietary supplementation on the erythrocyte fatty acid profile and oxidative balance in soccer players after training and acute exercise. Fifteen volunteer male athletes (age 20.0 ± 0.5 years) were randomly assigned to a placebo group that consumed an almond-based beverage ($n = 6$), or to an experimental group that consumed the same beverage enriched with DHA ($n = 9$) for 8 weeks. Blood samples were taken in resting conditions at the beginning and after 8 weeks of nutritional intervention and training in resting and in post-exercise conditions. Oxidative damage markers (malonyldialdehyde, carbonyl and nitrotyrosine indexes) and the activity and protein level of antioxidant enzymes (catalase, superoxide dismutase, glutathione reductase and peroxidase) were assessed. The results showed that training increased antioxidant enzyme activities in erythrocytes. The experimental beverage increased DHA from 34.0 ± 3.6 to 43.0 ± 3.6 nmol/10^9 erythrocytes. DHA supplementation increased the catalytic activity of superoxide dismutase from 1.48 ± 0.40 to 10.5 ± 0.35 pkat/10^9 erythrocytes, and brought about a reduction in peroxidative damage induced by training or exercise. In conclusion, dietary supplementation with DHA changed the erythrocyte membrane composition, provided antioxidant defense and reduced protein peroxidative damage in the red blood cells of professional athletes after an 8-week training season and acute exercise.

Keywords DHA · Omega-3 · Erythrocyte · Exercise · Oxidative stress · Soccer

Abbreviations

ANOVA	Analysis of variance
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAT	Catalase
DNPH	2,4-Dinitrophenylhydrazine
DHA	Docosahexaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
FID	Flame ionization detector
GPx	Glutathione peroxidase
GRd	Glutathione reductase
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MUFA	Monounsaturated fatty acid(s)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PUFA	Polyunsaturated fatty acid(s)
ROS	Reactive oxygen species
SFA	Saturated fatty acids
SOD	Superoxide dismutase
TBAR	Thiobarbituric acid reactive substance
UCP	Uncoupling protein

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Introduction

Regular physical activity for a healthy body induces adaptation against elevated oxidant levels by increasing cellular and plasma antioxidant capability [1, 2]. However, with acute exercise, this adaptation is minimal, and antioxidant capability can be overwhelmed by oxidant production [3]. Reactive oxygen species (ROS) comprise both free radical and non-free radical oxygen intermediates such as hydrogen peroxide, superoxide, single oxygen, hydroxyl radical, lipid hydroperoxide and lipid radicals [4], and these species may damage cellular macromolecules through oxidative modification [5]. However, it is well established that ROS, when present at low or moderate levels, act as intracellular redox signaling molecules, as evidenced in HL60 cell cultures stimulated with an acute level of hydrogen peroxide or with a continuous low level production of hydrogen peroxide [6]. Long-term stimulation of the endogenous defense mechanism due to regular physical activity brings about the continuous presence of physiological oxidant stimuli, resulting in antioxidant adaptation and greater protection against oxidative challenges [6]. Moreover, extensive and repetitive exercise performed in extreme environmental conditions may cause muscle damage, due to overtraining, and result in reduced athletic performance [3]. Acute exercise involves a kinetic shift in antioxidant enzymes increasing their activities and gene expression which, however, may not be sufficient to restore an oxidant-antioxidant redox balance [7].

Erythrocytes are highly susceptible to oxidative stress and cell damage [8]. The high polyunsaturated fatty acid (PUFA) content of the erythrocyte membranes together with the high quantity of oxygen and heme iron in the erythrocyte are adequate conditions to produce the peroxidation of PUFA. Erythrocytes generate significant amounts of anion superoxide, hydrogen peroxide, simultaneously to the oxidative reaction of hemoglobin with oxygen [9]. Exercise induces a situation of increased oxygen transport, thereby enhancing the capabilities to produce oxidative damage in lipids of erythrocytes. The inability to repair damaged components caused by ROS can increase the rate of hemolysis. However, the ROS produced in erythrocytes can be eliminated by antioxidant defense system enzymes: catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GRd) and glutathione peroxidase (GPx) [7, 8]; as well as the oxidized/reduced glutathione system, and other low molecular weight antioxidants such as vitamins E and C [10].

Dietary intake of n-3 fatty acids, and specifically supplementation with docosahexaenoic acid (22:6n-3; DHA), increases blood levels of this fatty acid [11–14]. A potential increase of unsaturated fatty acids enhances oxidative susceptibility to producing lipid hydroperoxides and oxidative-derived products. Paradoxically, beneficial effects have been pointed out with a diet rich in n-3 fatty acids for promoting maintenance of the antioxidant status and reduction of oxidative damage [13, 15]. Moreover, the antioxidant effects of a mixture of n-3 PUFA have been reported to work by inhibiting lipid peroxidation in erythrocytes [16]. The effects of DHA supplementation on oxidative stress may depend on resting or post-exercise conditions [17], and on antioxidant intake taken together with DHA [18]. In resting conditions, biomarkers of oxidative stress decrease with DHA treatment [17], whereas in post-exercise conditions, the effect of supplementation on stress oxidative parameters is not clearly known. DHA supplementation may have different effects depending on immune cell type [19, 20]. Peripheral blood mononuclear cells (PBMC) and neutrophils respond to exercise stimuli by increasing the capacity to produce ROS [21, 22]. DHA diet supplementation has been seen to increase the PBMC protein levels of uncoupling protein 3 (UCP3) while reducing mitochondrial ROS production in a regular soccer training period, and also to reduce oxidative damage markers and increase Cu/Zn-SOD protein levels in response to acute exercise [19]. In neutrophils, DHA diet supplementation did not modify the adaptive response of the antioxidant system to training or ROS production induced by immune stimulation after acute exercise while training increased antioxidant defenses (CAT, GPx and GRd enzyme activities) and decreased oxidative damage markers (malonyldialdehyde, carbonyl and nitrotyrosine indexes) [20]. The effects of DHA, training season and acute exercise on the oxidative stress in erythrocytes are not clearly known.

There are several studies analyzing the effects of both DHA and eicosapentaenoic acid (20:5n-3; EPA) (together or individually) and fish oil supplementation on immune cell composition and function during exercise [23, 24]. The aim of the present study was to determine the effects of diet supplementation with DHA on the fatty acid composition of erythrocytes and oxidative balance after an 8-week training season and after acute exercise in soccer players. The influence of training season, acute exercise and DHA supplementation on erythrocyte antioxidant enzyme activities and levels and on the oxidative and nitrosative damage markers was determined.

Materials and Methods

Participants and Study Design

A double blind, randomized supplementation study was performed with 15 male soccer players, at the beginning of their annual sport season. Participants were randomly

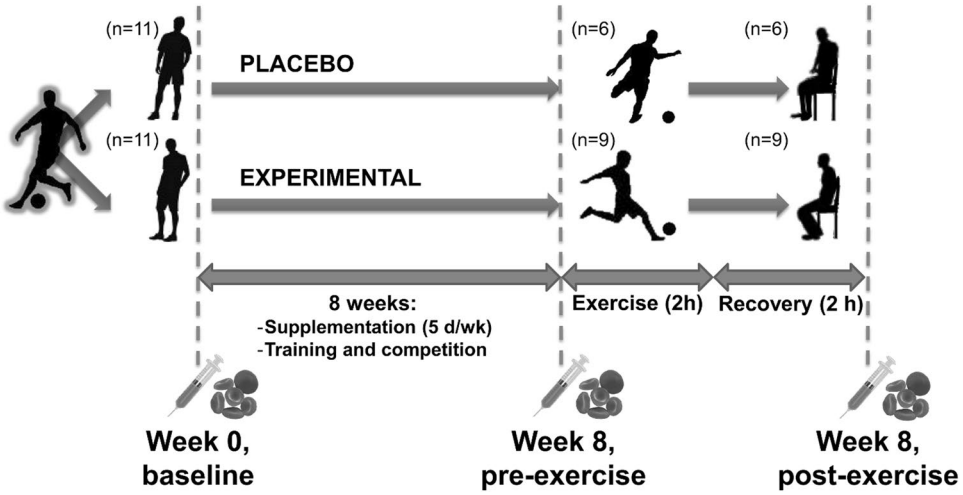

Fig. 1 Diagram of the treatment time line

assigned to be included in two groups: placebo ($n = 6$) and experimental ($n = 9$). Participants in the study had a daily intake each morning before the training session of 1 L of their respective beverages for 5 days weekly (excluding the match day and the day of rest), over a total period of 8 weeks (Fig. 1). Inclusion/exclusion criteria were: age (16–35 years), sex (male), non-smokers, balanced diet, body mass index (19–25 kg/m^2) and physical activity of 1–2 h daily 5–7 days/week. All the participants were informed of the purpose and demands of the study before giving their written consent to participate. The study protocol was in accordance with the Declaration of Helsinki for research on human participants and was approved by the Ethical Committee of Clinical Investigation of the Comunidad Autónoma de les Illes Balears No IB 994/08 PI (Palma de Mallorca, Balearic Islands, Spain). The project was registered at ClinicalTrials.gov (NCT02177383).

Drink Composition

The two drinks were made up of 3.0 wt% almond with water, lemon (40 mg/L) and cinnamon (200 mg/L) flavors, tocopherol acetate (50 mg/L) and the different oils (olive oil or algal vegetable oil) in functions of placebo and experimental beverage. The placebo was 0.8 wt% refined olive oil and the experimental was 0.6 wt% refined olive oil and 0.2 wt% DHA-S (DSM, Columbia, USA). DHA-S is an algal vegetable oil containing a minimum of 35 % 22:6n-3 fatty acid. The DHA-S ingredients are DHA algal oil (*Schizochytrium* sp.), high oleic sunflower oil (sunflower lecithin, rosemary extract), tocopherols and ascorbyl palmitate (as antioxidants). The DHA-S fatty acid profile used in the present study was: 14:0 (7.4 %), C16:0 (18.8 %), 18:0 (0.9 %), 18:1n-9 (7.9 %), 18:2n-6 (1.0 %), 20:4n6 (0.6 %), 20:5n-3 (1.4 %), 22:5n-6 (15.3 %), 22:6n-3 (39 %) and others (1.7 %). The two almond drinks were prepared by Liquats Vegetals S.A. (Girona, Spain) and were obtained by: bleaching and grinding almonds with water and then centrifuging the mixture to remove insoluble materials. Sucrose (6.0 wt%), lecithin (0.1 wt%), salt (0.02 wt%), tocopherol acetate (0.02 wt%), natural cinnamon (0.02 wt%), lemon flavors (0.004 wt%), and the respective oil for the experimental (olive oil plus DHA-S) or placebo (olive oil) treatment was added. Finally the beverages were sterilized and packed. The two beverages were

Table 1 Beverage composition

	Placebo (mg/100 mL)	Experimental (mg/100 mL)
16:0	64.5 ± 6.2	129 ± 22*
16:1	9.80 ± 0.83	21.0 ± 4.1*
18:0	45.4 ± 6.4	76.9 ± 16.7
18:1n-9	419 ± 102	890 ± 221
18:2n-6	214 ± 46	479 ± 118
18:3n-6	4.06 ± 1.48	11.6 ± 5.9
18:3n-3	15.4 ± 2.1	17.8 ± 1.7
20:0	4.03 ± 0.21	5.07 ± 0.31
20:1n-9	0.841 ± 0.060	1.55 ± 0.10*
20:2n-6	11.9 ± 0.9	12.2 ± 0.6
20:3	ND	0.896 ± 0.094*
20:4n-6	6.86 ± 0.42	5.96 ± 0.38
22:0	ND	3.60 ± 0.08*
22:5n-3	ND	78.5 ± 3.2*
22:6n-3	ND	160 ± 6*
α-Tocopherol (mg/L)	41.6 ± 17.8	45.7 ± 27.7

20:3 was the 20:3n-6 and 20:3n-3 mixture

Statistical analysis: Student's *t* test for unpaired data

ND not detected

* Significant differences between placebo and experimental, $p < 0.05$

identical in taste and appearance. The fatty acid composition and α-Tocopherol content of the beverages is shown in Table 1. 1 L of beverage had 490 kcal. Intake of 1 L of the experimental drink for 5 days a week provided 1.14 g DHA/day.

Experimental Procedure

For each participant, three different blood samples were obtained (Fig. 1). One blood sample was taken under resting conditions at the beginning of the nutritional intervention (week 0, baseline). Another two blood samples were taken at the end of the nutritional intervention, in resting (week 8, pre-exercise) and post-exercise conditions (week 8, post-exercise). 1 L of beverage, placebo or experimental, was consumed before the physical activity session at week 8. The exercise consisted of a 2-h usual physical training session. After a 15 min warm-up, the participants performed the Leger Boucher test [25]. After this, they did a recovery exercise of control-passing over 15 min. The main body of the training session was characterized by small-sided games [19, 20]. The exercise was planned to be performed at 70 % VO_{2max} for more than 50 % of the training session in order to induce oxidative stress [26].

Venous blood samples were obtained from the antecubital vein of participants with two vacutainers containing EDTA (ethylenediaminetetraacetic acid) as anticoagulant for hemogram analyses (2 mL) and to purify erythrocytes (6 mL) following an adaptation of the method described elsewhere [27, 28]. Erythrocyte fraction was obtained after centrifugation (900×*g*, 30 min, 4 °C). Then, erythrocytes were cleaned with phosphate buffered saline (PBS), centrifuged (900×*g*, 20 min, 4 °C) and lysed with water at the initial blood volume. Cell lysates were stored at −80 °C until biochemical analyses. Venous blood samples were obtained after 12 h, overnight, fasted conditions (basal sample), and 2 h after finishing training as this is coincident with an increment in circulating immune cells, with changes in antioxidant enzymes activities and in markers of oxidative damage [29, 30].

Hematological Analysis

Hematological parameters such as erythrocyte number, mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), were determined in an automatic flow-cytometer analyzer Technicon H2 (Bayer, Leverkusen, Germany) VCS system. Hemoglobin concentration was determined using Drabkin reagent to oxidize the heme group leading to the formation of methemoglobin which reacts with potassium cyanide forming cyanmethemoglobin, a stable pigment which can be detected spectrophotometrically at 540 nm.

Anthropometry Measurements

Height was determined using a mobile anthropometer (Kawe 44444, Asperg, Germany) to the nearest millimeter, with the participant's head placed in the Frankfurt plane. Body weight was determined to the nearest 0.1 kg using a digital scale (Tefal, sc9210, Rumilly, France). Participants were weighed with bare feet and wearing light underwear. Waist and hip circumference were measured to the nearest 0.1 cm, using a non-stretchable measuring tape (KaWe, 43972, France). Triceps, subscapular, biceps, iliac crest, supraspinal, abdominal, thigh, and leg skinfold thickness were measured using a Holtain skinfold calliper (Tanner/Whitehouse, Crosswell, Crymych, UK), and the mean of three measurements was used [31]. Participants were asked to stand erect in a relaxed position with both feet placed together on a flat surface during measurements.

Different anthropometric indexes were calculated using these measurements: body mass index [BMI = mass (kg)/squared height (m)]; waist-hip index [waist circumference (cm)/hip circumference (cm)]; fat free mass (FFM = 100 − BF). Body fat percentage was determined from skinfold thickness according to the Carter-Yuhasz equation [32]. All anthropometric measurements were performed by one observer to avoid inter-observer variation.

Dietary Intake

Dietary habits were assessed using a 7-day dietary record completed at the beginning of the study. A qualified dietician verified and quantified the food records. All food items consumed were transformed into nutrients using a computerized program based on the European and Spanish food composition tables [33, 34].

Fatty Acid Determination

Erythrocyte and beverage (250 µL) lipid content was extracted with chloroform/methanol (2:1, by vol) by a modified method of Folch et al. [35, 36], containing 0.01 % butylated hydroxyanisole (BHA) as antioxidant and 20 µL of *n*-heptadecanoic acid (15 mM) as the internal standard. The resultant organic phase was evaporated under a nitrogen stream at 55 °C. The dry residue was dissolved in 225 µL of *n*-hexane and 25 µL of Meth-Prep™ II (Grace Davison Discovery Sciences, Columbia, MD, USA) and the derivatization reagent was added. An aliquot of 1 µL was injected into the gas chromatograph with helium as a mobile phase at 2.17 mL/min flux, measured at 150 °C in head column. The gas chromatograph was an Agilent 5890 model (Agilent Technologies, Santa Clara, CA, USA) with a flame ionization detector (FID) and the column was a Supelcowax® 10 Capillary GC column, 30 m × 0.53 mm,

d_f 0.50 µm (Supelco, Bellefonte, PA, USA). The temperature curve began at 150 °C with a temperature gradient of 4 °C/min up to 260 °C which was maintained for 15 min. The injector was at 280 °C and the FID at 300 °C. Individual fatty acids and a mix of fatty acid methyl esters (Supelco®) were used for the identification of the chromatography peaks.

Quantification was performed from the internal standard (17:0) and the responses of the fatty acids in the FID was corrected by a response factor calculated from the areas of the standard fatty acids of different chromatograms with different fatty acid concentrations and the area of the internal standard obtained from the same chromatograms. The coefficient of variation was calculated to be 15 %.

α-Tocopherol Determination

α-Tocopherol was determined in the placebo and experimental drinks. The extraction of lipid soluble vitamins was carried out using *n*-hexane after deproteinization with ethanol containing 0.2 % butylated hydroxytoluene (BHT). α-Tocopherol concentration was determined after drying the samples under nitrogen current and dissolving in methanol. The mobile phase consisted of acetonitrile/tetrahydrofuran/water (550:370:80, by vol.). The HPLC was a Shimadzu (Canby, OR, USA) with a diode array detector and the column was a Nova Pak, C_{18}, 3.9 × 150 mm. α-Tocopherol was determined at 290 nm. Quantification was carried out with an external patron (Sigma-Aldrich).

Malondialdehyde Determination

Malondialdehyde (MDA) as a marker of lipid peroxidation was analyzed in 1/100 diluted erythrocytes using a colorimetric assay kit (Calbiochem). Briefly, samples and standards were placed in 1.5 mL tubes containing *n*-methyl-2-phenylindole (10.3 mM) in acetonitrile/methanol (3:1, by vol.). HCl 12 N was added and the samples were incubated for 1 h at 45 °C. Absorbance was measured at 586 nm. The method used is specific for MDA determination [37, 38].

Assay of Nitrotyrosine and Protein Carbonyls

Protein carbonyl derivatives and nitrotyrosine (N-Tyr) were determined by immunological methods using the OxiSelect™ Protein Carbonyl Immunoblot Kit (Cell Biolabs, INC) and OxiSelect™ Nitrotyrosine Immunoblot Kit (Cell Biolabs, INC) following the manufacturer's instructions. Total protein concentrations were measured by the method of Bradford [39]. Initially, erythrocyte samples (10 or 150 µg of protein for carbonyl or N-Tyr, respectively) were transferred onto a nitrocellulose membrane by the dot blot method. For carbonyl determination, the membrane was incubated in the presence of 2,4-dinitrophenylhydrazine (DNPH) after transference. Then the membrane was incubated with the primary antibody, specific to DNP moiety proteins in the case of carbonyl determination, or rabbit anti-N-Tyr antibody for N-Tyr determination. This step was followed by incubation with a horseradish peroxidase-antibody (goat anti-rabbit IgG) conjugate directed against the primary antibody. The membrane was then treated with luminol, which is converted to a light-emitting form at wavelength 428 nm by the antigen/primary antibody/secondary antibody/peroxidase complex. The light was visualized and detected by short exposure to a Chemidoc XRS densitometer (Bio-Rad Laboratories). Image analysis was performed using Quantity One-1D analysis software (Bio-Rad Laboratories). The coefficient of variation has been calculated to be 10 % for carbonyl index and 12 % for N-Tyr index.

Enzymatic Determinations

Erythrocyte catalase (CAT) activity was measured by the spectrophotometric method of Aebi [40]. Results are expressed referring to the constant K of the first order reaction of decomposition of hydrogen peroxide ($K = 3.4 \times 10^7$ L/mol/s for pure catalase from human erythrocytes) [41]. Superoxide dismutase (SOD) activity was measured in erythrocytes by an adaptation of the method of McCord and Fridovich [42]. Glutathione reductase (GRd) activity was measured in erythrocytes by a modification of the Goldberg and Spooner spectrophotometric method [43]. Glutathione peroxidase (GPx) activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler [44]. All activities were determined in erythrocyte samples with a Shimadzu UV-2100 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at 37 °C.

Western Blot Analysis

Antioxidant enzyme protein levels of erythrocyte samples were determined by Western blot. Protein extracts were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). CAT (20 µg of protein), Cu/Zn-SOD (10 µg of protein) and GPx (200 µg of protein) were loaded on a 12 % SDS-PAGE gel, whereas GRd (10 µg of protein) was loaded on a 15 % SDS-PAGE gel. Following electrophoresis, samples were transferred onto a nitrocellulose membrane and incubated with a primary monoclonal antibody: anti-CAT antibody (Calbiochem), anti-Cu/Zn-SOD antibody (Sigma) and anti-GRd antibody and anti-GPx antibody (Santa Cruz). Then incubation with a secondary peroxidase-conjugated antibody was performed. Protein bands were visualized by Immun-Star® Western C® Kit reagent

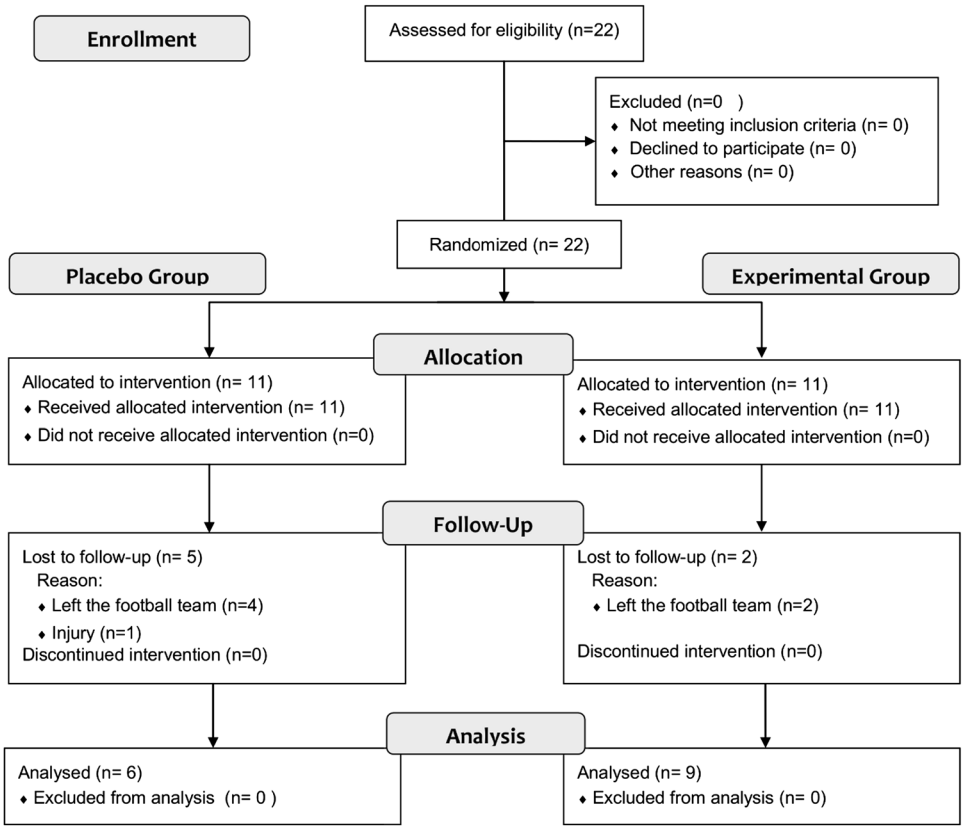

Fig. 2 Representation of a consort flow diagram showing the movement of participants in the study

(Bio-Rad Laboratories) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed with Quantity One-1D Software (Bio-Rad Laboratories).

Statistical Analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS v.21.0 for Windows). Results are expressed as means ± SEM. and $P < 0.05$ was considered statistically significant. The Shapiro–Wilk W test was applied to assess the normal distribution of the experimental data. To test the effects of supplementation and training or supplementation and acute exercise, a two-way analysis of variance (ANOVA) was performed. When significant effects were found, one-way ANOVA was used to determine the differences between the groups.

Results

Twenty-two participants were initially recruited but seven dropped out of the study (six of them left the soccer team and one had an injury) as explained in the consort flow diagram (Fig. 2). Athletes' nutritional intake before the nutritional intervention was similar in both groups. The reported energy intake was for active people, whose energy and protein intake should be 20 % higher than the general population [45]. Participants' protein intake was greater than the general recommendations while carbohydrate and fiber intakes were lower [45]. Participant diets had a high quantity of cholesterol and lipids with a poor balance of saturated to unsaturated fats in relation to dietary guidelines, a pattern observed in the general Balearic Island population [46]. Diet supplementation with the placebo and experimental beverages increased PUFA intake. DHA intake by participants was lower than recommendations [15].

The fatty acid composition of the experimental and placebo beverages is shown in Table 1. The experimental drink contained significantly higher concentrations of the fatty acids 20:3 (20:3n-6 and 20:3n-3 fatty acids mixture), 22:0, 22:5 and 22:6n-3, whereas they were undetected in the placebo drink. The experimental beverage also contained significantly higher concentrations of 16:0, 16:1, and 20:1n-9 fatty acids compared to the placebo beverage, when the results are expressed as mg/100 mL. There was no significant difference in α-Tocopherol concentration between the two drinks.

There were no differences in anthropometric characteristics or physical activity capabilities between the placebo

Table 2 Anthropometric parameters of the athletes

	Placebo ($n = 6$)	Experimental ($n = 9$)
Age (years)	19.3 ± 0.4	20.4 ± 0.5
Weight (kg)	76.5 ± 1.8	76.4 ± 3.5
Height (cm)	179 ± 2	180 ± 3
Waist circumference (cm)	78.2 ± 0.8	78.5 ± 1.1
Hip circumference (cm)	97.0 ± 1.0	96.6 ± 1.4
Systolic blood pressure (mmHg)	117 ± 8	122 ± 3
Diastolic blood pressure (mmHg)	56.7 ± 5.9	66.7 ± 3.5
Body Mass Index (BMI, kg/m^2)	24.0 ± 0.6	23.5 ± 0.5
Waist-hip ratio (WHR)	0.805 ± 0.012	0.814 ± 0.012
Body fat (%)	7.53 ± 0.24	7.21 ± 0.25
Fat-free mass (%)	92.5 ± 0.2	92.8 ± 0.3
VO_{2max} (mL/kg min)	60.4 ± 1.8	62.0 ± 0.9
Intense physical activity time (min/day)	96.4 ± 57.9	50.4 ± 13.1
Moderate physical activity time (min/day)	68.6 ± 17.1	63.2 ± 14.6

Statistical analysis: Student's *t* test for unpaired data

* Significant differences between placebo and experimental, $p < 0.05$

Table 3 Daily nutrient intake of the athletes before supplementation

	Placebo ($n = 6$)	Experimental ($n = 9$)
Energy (kcal)	2,518 ± 226	2,215 ± 210
Water (mL)	1,880 ± 287	1,254 ± 173
Proteins (g)	108 ± 10	105 ± 12
Carbohydrates (g)	282 ± 37	225 ± 26
Fiber (g)	18.8 ± 2.3	13.9 ± 0.9*
Lipids (g)	106 ± 9	99.2 ± 8.1
Saturated fatty acids (g)	37.0 ± 3.1	36.5 ± 4.4
Monounsaturated fatty acids (g)	46.1 ± 4.1	42.6 ± 3.1
Polyunsaturated fatty acids (g)	11.9 ± 1.5	10.7 ± 0.9
Cholesterol (mg)	430 ± 21	428 ± 62
Alcohol (g)	NI	NI
Protein energy (%)	17.5 ± 0.8	18.8 ± 0.7
Carbohydrate energy (%)	44.7 ± 3.8	40.5 ± 2.1
Lipid energy (%)	37.8 ± 2.9	40.7 ± 1.7
Saturated fatty acids energy (%)	13.1 ± 1.0	14.8 ± 0.9
Monounsaturated fatty acids energy (%)	16.3 ± 1.1	17.6 ± 1.1
Polyunsaturated fatty acids energy (%)	4.41 ± 0.80	4.42 ± 0.27
Alcohol energy (%)	NI	NI
Sodium (mg)	2,931 ± 446	2,293 ± 182
Potassium (mg)	2,860 ± 219	3,037 ± 376
Magnesium (mg)	282 ± 15	288 ± 42
Phosphorous (mg)	1,538 ± 91	1,543 ± 219
Calcium (mg)	821 ± 42	876 ± 169
Iron (mg)	22.6 ± 8.6	13.7 ± 1.7
Copper (mg)	2.05 ± 0.11	2.64 ± 0.34
Selenium (μg)	94.8 ± 13.1	99.5 ± 12.4
Iodine (μg)	102 ± 14	76.4 ± 11.2
Zinc (mg)	13.1 ± 1.3	12.6 ± 2.5
Retinol (μg)	396 ± 43	1,421 ± 783
Carotenes (μg)	1,905 ± 276	2,534 ± 751
Vitamin A (retinol equivalents)	787 ± 56	1,882 ± 890
Thiamine (mg)	1.44 ± 0.08	1.53 ± 0.24
Riboflavin (mg)	1.88 ± 0.05	2.17 ± 0.40
Vitamin B_6 (mg)	2.44 ± 0.29	2.07 ± 0.35
Vitamin B_{12} (μg)	13.7 ± 3.2	17.9 ± 4.5
Vitamin C (mg)	80.2 ± 12.3	89.2 ± 25.0
Vitamin D (μg)	2.43 ± 0.40	1.88 ± 0.43
Vitamin E (mg)	8.06 ± 0.70	6.99 ± 0.57
Niacin (mg)	27.2 ± 1.3	26.5 ± 5.5
Pantothenic acid (mg)	5.52 ± 0.18	6.45 ± 1.23
Folic acid (μg)	262 ± 11	255 ± 44

Statistical analysis: Student's *t* test for unpaired data

NI non-ingested

* Significant differences between placebo and experimental, $p < 0.05$

and experimental group of athletes at the beginning of the study (Table 2). No differences were observed in nutrient intake except for fiber between the placebo and experimental groups at baseline (Table 3). The intake of polyunsaturated lipids was about 11 g daily in both groups before the nutritional intervention. Diet supplementation with 1 L per day of the beverage provided an increment of about 12 % of the total lipid intake. Experimental group intakes were approximately 1.07, 4.64 and 3.93 g/day of additional saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and PUFA, respectively, whereas the placebo group intake of additional SFA, MUFA and PUFA amounted to 0.57, 2.21 and 1.28 g/day, respectively. DHA consumption from the diet ranged from 68.5 to 150 mg/day according to the 7-day dietary record analysis.

Table 4 illustrates erythrocyte fatty acid composition comparing the baseline (initial) and week 8 pre-exercise (final) conditions. SFA, MUFA, PUFA and total fatty acids in erythrocytes were unchanged by the nutritional intervention in either group. The main effect found was for the erythrocyte content of DHA, which significantly increased in the experimental group, reaching values 1.3 times higher than placebo group. DHA supplementation significantly increased the concentration of the 20:3 mixture of fatty acids, although this value was also different between groups at the beginning of the study. Exercise had little influence on erythrocyte fatty acid composition, irrespective of the placebo or experimental group, comparing the

Table 4 Effect of nutritional intervention on basal fatty acid erythrocyte composition in placebo and experimental groups

	Initial (nmol/10^9 erythrocytes)	Final (nmol/10^9 erythrocytes)	ANOVA		
			S	*T*	*S* × *T*
16:0					
Placebo	62.7 ± 4.2	59.6 ± 5.8	0.789	0.378	0.773
Experimental	62.8 ± 5.8	56.8 ± 3.2			
16:1					
Placebo	2.02 ± 0.13	1.67 ± 0.17	0.233	0.308	0.521
Experimental	1.63 ± 0.23	1.55 ± 0.19			
18:0					
Placebo	122 ± 10	108 ± 13	0.601	0.104	0.750
Experimental	130 ± 10	110 ± 6			
18:1n-9					
Placebo	41.3 ± 2.2	42.1 ± 3.2	0.694	0.839	0.995
Experimental	39.9 ± 4.5	40.6 ± 2.5			
18:2n-6					
Placebo	51.3 ± 3.0	56.4 ± 3.7	0.120	0.308	0.956
Experimental	44.2 ± 5.6	48.7 ± 3.8			
18:3n-6					
Placebo	4.39 ± 0.39	4.44 ± 0.51	0.735	0.727	0.826
Experimental	4.16 ± 0.43	4.39 ± 0.26			
18:3n-3					
Placebo	12.5 ± 1.4	11.9 ± 1.6	0.342	0.964	0.628
Experimental	13.3 ± 1.5	14.1 ± 1.5			
20:0					
Placebo	1.79 ± 0.2	1.36 ± 0.18	0.138	0.193	0.519
Experimental	1.98 ± 0.18	1.83 ± 0.25			
20:1n-9					
Placebo	3.24 ± 0.34	3.53 ± 0.32	0.263	0.246	0.722
Experimental	2.70 ± 0.29	3.25 ± 0.42			
20:2n-6					
Placebo	5.17 ± 0.52	4.15 ± 0.46	0.762	0.146	0.458
Experimental	4.69 ± 0.53	4.35 ± 0.16			
20:3					
Placebo	8.28 ± 1.37	8.98 ± 1.38	**0.007**	0.631	0.814
Experimental	5.67 ± 0.78	5.91 ± 0.34*			
20:4n-6					
Placebo	117 ± 8	128 ± 12	0.550	0.394	0.972
Experimental	110 ± 14	120 ± 8			
22:0					
Placebo	0.827 ± 0.080	1.22 ± 0.18	0.082	0.316	0.592
Experimental	1.42 ± 0.35	1.54 ± 0.2			
22:5n-3					
Placebo	7.05 ± 0.40	7.77 ± 0.80	0.218	0.226	0.796
Experimental	5.92 ± 0.61	7.03 ± 0.89			
22:6n-3					
Placebo	29.0 ± 1.3	33.6 ± 3.1	**0.035**	**0.045**	0.511
Experimental	34.0 ± 3.6	43.0 ± 3.6*			

Table 4 continued

	Initial (nmol/10^9 erythrocytes)	Final (nmol/10^9 erythrocytes)	ANOVA		
			S	*T*	*S* × *T*
SFA					
Placebo	187 ± 14	170 ± 19	0.757	0.169	0.750
Experimental	196 ± 16	170 ± 9			
MUFA					
Placebo	46.6 ± 2.6	47.3 ± 3.6	0.608	0.815	0.953
Experimental	44.3 ± 4.9	45.5 ± 3.0			
PUFA					
Placebo	235 ± 14	255 ± 19	0.487	0.200	0.696
Experimental	210 ± 26	247 ± 14			
Total					
Placebo	468 ± 29	472 ± 42	0.757	0.860	0.927
Experimental	451 ± 46	463 ± 26			

Placebo ($n = 6$) and experimental ($n = 9$). 20:3 was the 20:3n-6 and 20:3n-3 mixture

Statistical analysis: Two-way ANOVA, data points in bold are significant, $p < 0.05$

One-way ANOVA, $p < 0.05$

S effect of supplementation, *T* effect of time comparing the baseline and pre-exercise condition, (*S* × *T*) interaction between both factors

* Significant differences between placebo and experimental, # Significant differences between initial and final training period

8-week pre- and post-exercise conditions (Table 5). However, a significant increase in 18:3n-3 fatty acid concentration (about 63 %) after exercise was detected in erythrocytes of the placebo group. Differences in DHA content after the supplementation period were maintained after acute exercise. The 22:0 fatty acid concentration significantly increased only in the experimental group after exercise. The concentration of 18:3n-3 and the 20:3 mixture of fatty acids were significantly lower (about 63 and 34 % respectively) in the experimental group in resting conditions, whereas the concentration of 22:0 and 22:6n-3 fatty acids were about 63 and 39 % significantly higher in the experimental group after exercise. EPA levels in erythrocytes were below detection limits, probably due to the low fish intake of soccer players.

Neither nutritional intervention nor training affected erythrocyte characteristics (Table 6). Erythrocyte counts, hemoglobin, hematocrit, MCV, MCH, MCHC and RDW maintained the basal initial values after 8 weeks of nutritional intervention in both the placebo and experimental groups. Acute exercise and dietary supplementation induced a higher MCHC value after exercise in the experimental group.

Supplementation with DHA did not affect MDA erythrocyte levels (an indicator of lipid peroxidation), carbonyl index (an indicator of protein modification) or N-Tyr index (an indicator of nitrosative damage) (Table 7). However, MDA levels significantly increased 1.5 times in both the placebo and experimental group after the training season. The N-Tyr index significantly decreased at the end of the training season in both groups. Acute exercise and DHA diet supplementation did not affect MDA levels or carbonyl index. The N-Tyr index significantly increased in the placebo group after exercise.

DHA dietary supplementation and training significantly affected the activity and protein levels of antioxidant enzymes in erythrocytes (Table 8a, b). Figure 3 shows a representative blot of different erythrocyte antioxidant protein levels in groups and conditions studied. CAT activity was significantly increased at the end of the training period in both groups. An interaction between DHA supplementation and training was observed in the activity of SOD and GPx. SOD activity was higher in the experimental group at the end of the training season, whereas GPx activity was higher in the placebo group. Protein levels of CAT, Cu/Zn-SOD and GPx were not affected by supplementation or training. GRd activity increased significantly at the end of the study in both groups; whereas its respective protein levels showed higher values in the experimental group at the end of training.

Table 8a, b shows the effects of acute exercise on antioxidant enzyme activities and protein levels at the end of the nutritional intervention. Erythrocytes in the experimental group had lower CAT and GPx activities than the placebo group in resting conditions; however, the basal values of SOD and GR activities were similar in placebo and experimental groups. Acute exercise and diet supplementation significantly changed GPx activity and maintained CAT, SOD and GR basal activities after exercise. GPx activity decreased after exercise in the experimental group.

Table 5 Effect of acute exercise on erythrocyte fatty acid composition in placebo and experimental groups after nutritional intervention

	Pre-exercise (nmol/10^9 erythrocytes)	Post-exercise (nmol/10^9 erythrocytes)	ANOVA		
			S	*E*	*S* × *E*
16:0					
Placebo	59.6 ± 5.8	53.4 ± 9.5	0.978	0.506	0.605
Experimental	56.8 ± 3.2	56.0 ± 2.4			
16:1					
Placebo	1.67 ± 0.17	1.52 ± 0.24	0.741	0.900	0.342
Experimental	1.55 ± 0.19	1.76 ± 0.13			
18:0					
Placebo	108 ± 13	100 ± 18	0.677	0.630	0.828
Experimental	110 ± 6	107 ± 6			
18:1n-9					
Placebo	42.1 ± 3.2	37.6 ± 6.7	0.589	0.741	0.344
Experimental	40.6 ± 2.5	42.9 ± 1.4			
18:2n-6					
Placebo	56.4 ± 3.7	48.8 ± 9.1	0.477	0.484	0.376
Experimental	48.7 ± 3.8	49.6 ± 1.8			
18:3n-6					
Placebo	4.44 ± 0.51	4.81 ± 0.31	0.441	0.619	0.531
Experimental	4.39 ± 0.26	4.35 ± 0.24			
18:3n-3					
Placebo	11.9 ± 1.6[a]	19.4 ± 1.7[b]	0.266	**0.015**	**0.012**
Experimental	14.1 ± 1.5[a]	14.0 ± 0.1[a]			
20:0					
Placebo	1.36 ± 0.18	1.35 ± 0.24	0.353	0.373	0.375
Experimental	1.83 ± 0.25	1.60 ± 0.07			
20:1n-9					
Placebo	3.53 ± 0.32	3.44 ± 0.68	0.899	0.739	0.582
Experimental	3.25 ± 0.42	3.62 ± 0.19			
20:2n-6					
Placebo	4.15 ± 0.46	4.01 ± 0.70	0.440	0.894	0.810
Experimental	4.35 ± 0.16	4.39 ± 0.16			
20:3					
Placebo	8.98 ± 1.38	7.87 ± 1.59	**0.017**	0.669	0.449
Experimental	5.91 ± 0.34*	6.22 ± 0.36			
20:4n-6					
Placebo	128 ± 12	113 ± 21	0.949	0.601	0.479
Experimental	120 ± 8	122 ± 6			
22:0					
Placebo	1.22 ± 0.18	1.02 ± 0.13	**0.032**	0.831	0.461
Experimental	1.54 ± 0.20	1.66 ± 0.24*			
22:5n-3					
Placebo	7.77 ± 0.80	6.67 ± 0.76	0.747	0.437	0.586
Experimental	7.03 ± 0.89	6.86 ± 0.54			
22:6n-3					
Placebo	33.6 ± 3.1	29.0 ± 5.3	**0.011**	0.346	0.789
Experimental	43.0 ± 3.6	40.4 ± 3.0*			

Table 5 continued

	Pre-exercise (nmol/10^9 erythrocytes)	Post-exercise (nmol/10^9 erythrocytes)	ANOVA		
			S	*E*	*S* × *E*
SFA					
Placebo	170 ± 19	156 ± 28	0.555	0.427	0.992
Experimental	170 ± 9	166 ± 8			
MUFA					
Placebo	47.3 ± 3.6	42.5 ± 7.6	0.634	0.803	0.352
Experimental	45.5 ± 3.0	48.2 ± 1.5			
PUFA					
Placebo	255 ± 19	234 ± 38	0.854	0.635	0.614
Experimental	247 ± 14	248 ± 10			
Total					
Placebo	472 ± 42	432 ± 72	0.690	0.539	0.729
Experimental	463 ± 29	462 ± 17			

Placebo ($n = 6$) and experimental ($n = 9$). 20:3 was the 20:3n-6 and 20:3n-3 mixture

Statistical analysis: Two-way ANOVA, data points in bold are significant, $p < 0.05$

One-way ANOVA, $p < 0.05$

S effect of supplementation, *E* effect of exercise comparing the 8-week pre- and post-exercise conditions, (*S* × *E*) interaction between both factors

* Significant differences between placebo and experimental, # Significant differences between basal and post-exercise

The protein levels of these antioxidant enzymes were not significantly affected by dietary supplementation or acute exercise, with the exception of GRd protein levels. The experimental group had significantly higher erythrocyte GRd protein levels after exercise than the placebo group.

Discussion

Nutritional Intervention

Supplemental intake with the experimental beverage of DHA (1.14 g/day) was lower than that used in nutritional intervention studies with the general population [12, 24] or trained men [13, 14, 18] but was similar to that used in studies with healthy people [11, 47]. The daily intake of the experimental drink increased DHA levels in erythrocyte membranes, in accordance with other studies [11, 12], although no changes were observed in SFA, MUFA, total PUFA and total fatty acids. Changes in the lipid composition of erythrocytes indicated that the participants followed the prescribed beverage intake during the trial and this was effective in incorporating DHA into erythrocytes. The beverages, experimental more than placebo, did present long chain n-3 fatty acids other than DHA; however, none of these other fatty acids were significantly increased in erythrocytes over the supplementation period. The potential contribution of these other long chain fatty acids to the results obtained cannot be ruled out, but at their dosage in the experimental beverages they are unable to alter the fatty acid composition of erythrocytes.

Effects of Exercise on the Fatty Acid Composition of Erythrocytes

Membrane fatty acid composition is a dynamic system and its control and regulation is not clearly understood [48]. Dietary fatty acid intake is known to significantly affect the incorporation of fatty acids into cell membranes, thereby influencing their function [12]. Exercise, regardless of intake, can modify cell fatty acid profiles in different tissue types [49]. An increased fluidity of red blood cell membranes has been observed after chronic exercise [50], and depending on the intensity and sport-type, erythrocyte fatty acid composition may be altered [48, 50]. We found a significant increase in 18:3n-3 in erythrocyte membranes after acute exercise. This is the first time that a rapid change in membrane composition has been observed following exercise in humans. However, several studies performed with rats showed an effect of acute exercise on the fatty acid composition of erythrocytes, which was influenced by aging, training status and body temperature [51, 52].

Effect of Supplementation, Training, and Exercise on Oxidative Stress

EPA and DHA dietary supplementation can modulate erythrocyte membrane deformability and the capacity of

Table 6 Erythrocyte characteristics in placebo and experimental groups

	Initial basal	Final basal	ANOVA		
			S	*T*	*S* × *T*
A					
Erythrocytes (10^6/μL)					
Placebo	5.43 ± 0.21	5.20 ± 0.16	0.344	0.395	0.462
Experimental	5.19 ± 0.11	5.17 ± 0.10			
Hemoglobin(g/dL)					
Placebo	16.0 ± 0.3	15.4 ± 0.4	0.411	0.248	0.569
Experimental	15.5 ± 0.3	15.4 ± 0.3			
Hematocrit (%)					
Placebo	46.9 ± 0.8	45.1 ± 1.1	0.723	0.252	0.427
Experimental	45.9 ± 0.9	45.5 ± 0.8			
Mean corpuscular volume (MCV) (fL)					
Placebo	86.9 ± 2.6	87.0 ± 2.3	0.425	0.944	0.912
Experimental	88.4 ± 0.9	88.1 ± 1.0			
Mean corpuscular hemoglobin (MCH) (pg)					
Placebo	29.7 ± 1.0	29.8 ± 1.0	0.873	0.926	0.755
Experimental	30.0 ± 0.4	29.7 ± 0.5			
Mean corpuscular hemoglobin concentration (MCHC) (g/dL)					
Placebo	34.1 ± 0.2	34.3 ± 0.3	0.117	0.852	0.393
Experimental	33.9 ± 0.1	33.7 ± 0.2			
Red blood cell distribution width (RDW) (%)					
Placebo	11.7 ± 0.1	11.9 ± 0.1	0.218	0.480	0.468
Experimental	11.6 ± 0.1	11.6 ± 0.2			
	Basal	Post-exercise	ANOVA		
			S	*E*	*S* × *E*
B					
Erythrocytes (10^6/μL)					
Placebo	5.20 ± 0.16	5.29 ± 0.20	0.460	0.906	0.605
Experimental	5.17 ± 0.10	5.12 ± 0.10			
Hemoglobin(g/dL)					
Placebo	15.4 ± 0.4	15.9 ± 0.4	0.491	0.332	0.651
Experimental	15.4 ± 0.3	15.5 ± 0.3			
Hematocrit (%)					
Placebo	45.1 ± 1.1	45.7 ± 1.1	0.859	0.974	0.554
Experimental	45.5 ± 0.8	44.9 ± 0.9			
Mean corpuscular volume (MCV) (fL)					
Placebo	87.0 ± 2.3	86.7 ± 2.3	0.480	0.830	0.989
Experimental	88.1 ± 1.0	87.8 ± 0.9			
Mean corpuscular hemoglobin (MCH) (pg)					
Placebo	29.8 ± 1.0	30.3 ± 1.0	0.996	0.391	0.875
Experimental	29.7 ± 0.5	30.4 ± 0.4			
Mean corpuscular hemoglobin concentration (MCHC) (g/dL)					
Placebo	34.3 ± 0.3	35.0 ± 0.25	0.093	**0.003**	0.642
Experimental	33.7 ± 0.2	34.6 ± 0.21[#]			
Red blood cell distribution width (RDW) (%)					
Placebo	11.9 ± 0.1	11.6 ± 0.1	0.122	0.169	0.698
Experimental	11.6 ± 0.2	11.4 ± 0.1			

Placebo ($n = 6$) and experimental ($n = 9$)

Statistical analysis: two-way ANOVA, data points in bold are significant, $p < 0.05$

One-way ANOVA, $p < 0.05$

S effect of supplementation, *T* Effect of time, ($S \times T$) interaction between supplementation and time, *E* effect of exercise, ($S \times E$) interaction between supplementation and exercise

* Significant differences between placebo and experimental

[#] Significant differences between initial and final or significant differences between basal and post-exercise

[a] Effect of nutritional intervention on basal erythrocyte characteristics. Initial and final nutritional intervention data

[b] Effects of exercise on erythrocyte characteristics of placebo and experimental groups in basal and post-exercise conditions

Table 7 Erythrocytes oxidative damage markers in placebo and experimental groups

	Initial basal	Final basal	ANOVA		
			S	*T*	*S* × *T*
A					
MDA (μmol/10^6 erythrocytes)					
Placebo	2.30 ± 0.09	3.50 ± 0.21	0.585	**0.000**	0.752
Experimental	2.33 ± 0.12	3.63 ± 0.15			
Carbonyl index (%)					
Placebo	100 ± 29	150 ± 13	0.759	0.074	0.342
Experimental	112 ± 14	156 ± 14			
N-Tyr index (%)					
Placebo	100 ± 29	29.3 ± 10.5[#]	0.291	**0.000**	0.846
Experimental	123 ± 16	44.9 ± 7.1[#]			
	Basal	Post-exercise	ANOVA		
			S	*E*	*S* × *E*
B					
MDA (μmol/10^6 erythrocytes)					
Placebo	3.50 ± 0.21	3.74 ± 0.20	0.494	0.194	0.952
Experimental	3.63 ± 0.15	3.84 ± 0.14			
Carbonyl index (%)					
Placebo	100 ± 13	113 ± 3	0.817	0.073	0.092
Experimental	104 ± 8	126 ± 4			
N-Tyr index (%)					
Placebo	100 ± 36[a]	247 ± 33[b]	0.608	**0.007**	**0.020**
Experimental	153 ± 24[a]	166 ± 18[a]			

Placebo ($n = 6$) and experimental ($n = 9$). Statistical analysis: two-way ANOVA, data points in bold are significant, $p < 0.05$

One-way ANOVA, $p < 0.05$

S effect of supplementation, *T* effect of time, *S* × *T* interaction between supplementation and time, *E* effect of exercise, *S* × *E* interaction between supplementation and exercise

* Significant differences between placebo and experimental

[#] Significant differences between initial and final or significant differences between basal and post-exercise

[a] Effect of nutritional intervention on basal erythrocyte oxidative damage markers. Initial and final nutritional intervention data

[b] Effects of exercise on erythrocyte oxidative damage markers of placebo and experimental groups in basal and post-exercise conditions

O_2 transport [53]; similarly, PUFA dietary intake adapts mitochondria to use fatty acids as a fuel and increases their energy efficiency [54], thereby enhancing exercise performance [53, 55, 56]. Oxidative damage is more prominent in red cells, probably due to their high iron and PUFA content, their role as an oxygen transporter, and their protection of the host by neutralizing exogenous and endogenous free radicals [8]. Oxidative damage in the lipid fraction of erythrocytes significantly increased after the dietary intervention in both groups, although no additional change after the acute exercise at week 8 was observed. This increment in oxidative damage occurred over the soccer season with its accumulation of workouts and matches [57]. It has been pointed out that regular exercise in young athletes may be beneficial in reducing the amount of lipid peroxidation and increasing the activity of antioxidant enzymes [58]. The observed antioxidant enzyme activity increases in erythrocytes after training did not prevent oxidative damage of the erythrocyte lipid fraction. In a study performed with handball athletes over 6 months, erythrocyte antioxidant enzymes were significantly increased in a similar magnitude as in the present results. Moreover, the chronic adaptations to training demonstrated a significant protective effect against oxidative stress in erythrocytes, evidenced with decreased thiobarbituric acid reactive substances (TBAR) and carbonyl index [59]. Diet supplementation with DHA did not alter the pattern of oxidative damage in erythrocytes observed in the athletes during or after the training season. The absence of oxidative damage in lipids and proteins after exercise could be a consequence of the training status or the intensity of the training session. Intense exercise such as a mountain cycling or maximal exercise testing has

Table 8 Erythrocyte antioxidant enzyme activities and protein levels in placebo and experimental groups

	Initial basal	Final basal	ANOVA		
			S	*T*	*S* × *T*
A					
Enzyme activities					
Catalase (K/10^9 erythrocytes)					
Placebo	0.893 ± 0.262	8.38 ± 0.49[#]	0.472	**0.000**	0.163
Experimental	0.880 ± 0.220	7.14 ± 0.80[#]			
SOD (pkat/10^9 erythrocytes)					
Placebo	1.07 ± 0.07[a]	7.52 ± 0.85[b]	**0.001**	**0.000**	**0.010**
Experimental	1.48 ± 0.40[a]	10.5 ± 0.35[c]			
GRd (nkat/10^9 erythrocytes)					
Placebo	0.679 ± 0.125	4.39 ± 1.46[#]	0.547	**0.001**	0.652
Experimental	0.871 ± 0.264	3.46 ± 0.89[#]			
GPx (nkat/10^9 erythrocytes)					
Placebo	2.83 ± 0.26[a]	20.5 ± 2.6[b]	**0.049**	**0.000**	**0.048**
Experimental	2.87 ± 0.30[a]	13.3 ± 1.7[c]			
Western blot protein levels					
Catalase (%)					
Placebo	100 ± 29	139 ± 50	0.628	0.710	0.194
Experimental	106 ± 36	151 ± 62			
Cu/Zn-SOD (%)					
Placebo	100 ± 14	120 ± 32	0.968	0.908	0.617
Experimental	117 ± 29	105 ± 40			
GRd (%)					
Placebo	100 ± 22[a]	157 ± 46[a]	**0.012**	**0.003**	**0.041**
Experimental	127 ± 22[a]	400 ± 77[b]			
GPx (%)					
Placebo	100 ± 11	99.7 ± 13.7	0.236	0.727	0.739
Experimental	126 ± 18	114 ± 15			
	Basal	Post-exercise	ANOVA		
			S	*E*	*S* × *E*
B					
Enzyme activities					
Catalase (K/10^9 erythrocytes)					
Placebo	8.38 ± 0.49	9.68 ± 0.83	**0.049**	0.158	0.732
Experimental	7.14 ± 0.80	7.93 ± 0.58			
SOD (pkat/10^9 erythrocytes)					
Placebo	7.52 ± 0.85	7.90 ± 1.47	0.289	0.349	0.224
Experimental	10.5 ± 0.35	7.69 ± 1.43			
GRd (nkat/10^9 erythrocytes)					
Placebo	4.39 ± 1.46	7.44 ± 1.76	0.519	0.062	0.965
Experimental	3.46 ± 0.89	6.37 ± 1.63			
GPx (nkat/10^9 erythrocytes)					
Placebo	20.5 ± 2.6[a]	10.2 ± 1.0[b]	**0.042**	**0.001**	**0.037**
Experimental	13.3 ± 1.7[b]	10.3 ± 1.1[b]			
Western blot protein levels					
Catalase (%)					
Placebo	100 ± 36	91.6 ± 25.5	0.643	0.967	0.797
Experimental	108 ± 44	120 ± 36			

Table 8 continued

	Basal	Post-exercise	ANOVA		
			S	*E*	*S* × *E*
Cu/Zn-SOD (%)					
Placebo	100 ± 27	76.9 ± 13.5	0.856	0.998	0.385
Experimental	87.4 ± 33.3	91.7 ± 20.9			
GRd (%)					
Placebo	100 ± 29	168 ± 6	**0.049**	0.523	0.615
Experimental	255 ± 49*	263 ± 56*			
GPx (%)					
Placebo	100 ± 14	85.5 ± 14.7	0.089	0.619	0.583
Experimental	117 ± 15	117 ± 10			

Placebo ($n = 6$) and experimental ($n = 9$)

Statistical analysis: Two-way ANOVA, data point in bold are significant, $p < 0.05$

One-way ANOVA, $p < 0.05$

S effect of supplementation, *T* effect of time, (*S* × *T*) interaction between supplementation and time, *E* effect of exercise, (*S* × *E*) Interaction between supplementation and exercise

* Significant differences between placebo and experimental

[#] Significant differences between initial and final or significant differences between basal and post-exercise

[a] Effect of nutritional intervention on basal erythrocyte antioxidant enzyme activities and Western blot protein levels. Initial and final nutritional intervention data

[b] Effects of exercise on erythrocyte antioxidant enzyme activities and Western blot protein levels of placebo and experimental groups, in basal and post-exercise conditions

induced oxidative damage in erythrocytes [8, 60], whereas in submaximal exercise, no significant differences have been obtained in MDA levels [60].

Neither training and exercise nor DHA supplementation affected protein oxidative damage measured as carbonyl index. The efficiency of dietary fish oil to reduce *in vivo* oxidative damage of proteins in rats, may differ due to variations in EPA/DHA content of studies [61]. Protein carbonylation is an important irreversible modification that increases during oxidative stress [62]. ROS formed in exercise initiates PUFA peroxidation to form lipid peroxidation end products such as MDA, which could result in the formation of carbonyl groups on the protein. Therefore, the formation of carbonylated proteins could be unrelated to the formation of MDA. The absence of changes in carbonylated proteins, together with the slight increase in MDA, indicates increased susceptibility to oxidation by lipids; and the MDA produced is too scarce for reaction with the proteins. The results also suggest that the production of ROS was not enough to directly oxidize the proteins in the presence of the erythrocyte antioxidant.

N-Tyr index was similarly decreased in both groups after the training season, although acute exercise increased the N-Tyr index in the placebo group, suggesting that DHA dietary supplementation reduced the nitrosative modifications induced by exercise. Superoxide anion produced in the erythrocytes can react with nitric oxide to form peroxynitrite, which can react with peptide-bound tyrosine to form N-Tyr [63]. In a situation of increased erythrocyte production of nitric oxide after exercise [28], the elimination of superoxide anions is of great importance in order to reduce peroxynitrite and N-Tyr production. SOD eliminates the anion superoxide that is necessary for the formation of peroxynitrite and N-Tyr. The increased SOD observed after activity training in the DHA supplemented group could explain the low N-Tyr index in erythrocytes at the end of the training period, as well as the maintenance of the value after acute exercise.

Erythrocyte antioxidant enzyme activities increased as a result of training, in accordance with preventive erythrocyte antioxidant protection induced by regular exercise [58]. DHA dietary supplementation exerted a differential influence on basal and post-exercise erythrocyte antioxidant enzyme activities. DHA dietary supplementation enhanced the effect of training on erythrocyte SOD activity, but diminished the training effect on GPx and CAT activities. A previous study with long distance skiers revealed a decrease in erythrocyte SOD activities in the blood following an acute bout of exercise [64]. However, it was also reported that no changes were found in erythrocyte SOD activity in trained individuals following a duathlon competition [7]. Antioxidant enzyme activities in erythrocytes are modulated by acute exercise [7] and this modulation is influenced by the presence of low molecular weight

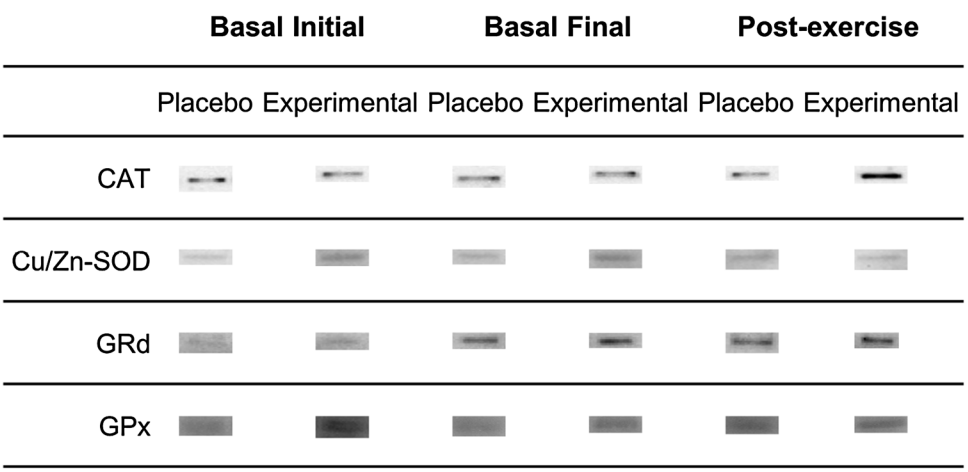

Fig. 3 Representative blots of catalase (CAT), Cu/Zn-superoxide dismutase (Cu/Zn-SOD), glutathione reductase (GRd) and glutathione peroxidase (GPx) determined by Western blot

antioxidants such as vitamin C [10]. For SOD, GPx and CAT enzymes, the increased enzyme activity did not parallel enzyme protein content, suggesting direct activation of the existing enzymes [65]. These changes in enzymatic capability could be related to the effects of ROS and low molecular weight antioxidants on the enzymatic protein [7, 57]. No evidence for a direct action of DHA on the catalytic activity of antioxidant enzymes was found, but a reduction in CAT, GPx and GRd catalytic activities and an increase in SOD catalytic activity was evidenced in the DHA supplemented group. An important increase in the protein levels of GRd was appreciated as a result of training, mainly in the DHA supplemented group. This could be related to GRd turnover during the maturation process of reticulocytes to erythrocytes, as this maturation has been reported to cause a significant decrease in antioxidant enzyme activities such as GRd, GPx, glutathione S-transferase, glucose-6-phosphate dehydrogenase, and CAT [66]. DHA dietary supplementation may induce higher GRd production in reticulocytes or possibly the GRd of reticulocytes was protected from degradation in the maturation process.

Conclusion

In summary, the consumption of a DHA-enriched almond drink for 8 weeks by professional athletes changed erythrocyte membrane composition without altering lipid oxidative damage markers. The enhanced erythrocyte SOD activity induced by DHA supplementation during the training season paralleled the reduction of peroxidative damage of erythrocyte proteins induced by training or acute exercise. The effects on erythrocyte antioxidant enzymes activities are not attributed to changes in the protein level of antioxidant enzymes but to catalytic capabilities. However, GRd increased its protein levels as a result of the interaction between training and DHA supplementation, pointing to an influence of DHA on GRd turnover during the maturation of reticulocytes to erythrocytes. DHA dietary supplementation promoted greater erythrocyte antioxidant defenses and less protein peroxidative damage in professional athletes during the training season and after acute exercise.

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Conflict of interest The authors declare that they have no conflict of interest.

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