High-Protein-Pufa Supplementation, Red Blood Cell Membranes, and Plasma Antioxidant Activity in Volleyball Athletes

Marco Malaguti, Marta Baldini, Cristina Angeloni, Pierluigi Biagi, and Silvana Hrelia

The authors evaluated the role of a high-protein, low-calorie, polyunsaturated fatty-acid (PUFA) -supplemented diet on anthropometric parameters, erythrocyte-membrane fatty-acid composition, and plasma antioxidant defenses of nonprofessional volleyball athletes. The athletes were divided in two groups: One (n = 5) followed the Mediterranean diet, and the other (n = 6) followed a high-protein, low-calorie diet with a 3-g/day fish-oil supplementation. All the athletes had anthropometric measurements taken, both at the beginning and at the end of the study, which lasted for 2 months. Body-mass index and total body fat were significantly diminished in the second group, while they remained unchanged in the first. Plasma total antioxidant activity (TAA) was significantly increased in the plasma of both groups, with no differences between the groups, suggesting that physical activity, not the different diets, is the main contributor to the increase of plasma TAA. The second group showed a significant increase in erythrocyte-membrane PUFA content and in the unsaturation index value (UI) because of the fish-oil supplementation. A high-protein, low-carbohydrate, fish-oil-supplemented diet seems to be useful only when the aim of the diet is to obtain weight loss in a short-term period. The significant increase in the UI of erythrocyte membranes indicates the potential for harm, because a high intake of PUFA might increase susceptibility to lipid peroxidation not counterbalanced by a higher increase in TAA. Adherence to the Mediterranean diet seems to be the better choice.

Keywords: physical activity, health, sport, nutrition

Many athletes follow different diets to improve their performances. Diets characterized by a higher protein:carbohydrate ratio than conventional recommended diets are among the most famous eating regimens marketed to improve athletic performances (Cheuvront, 1999). The professed health benefits of low-carbohydrate diets have been advocated intermittently over the last century and have enjoyed increasing popularity over the last decade. Although there have been no cross-sectional or longitudinal studies examining the potential health merit of adopting these diets, closely related peer-reviewed findings from scientific research cast strong doubt over the purported benefits of this eating regimen. When properly
evaluated, the theories and arguments of popular low-carbohydrate-diet books like *The Zone* rely on poorly controlled, non-peer-reviewed studies; anecdotes; and nonscientific rhetoric (Cheuvront, 2003).

In addition to reducing and qualitatively selecting carbohydrates, it seems that controlling eicosanoids and fatty acids is important to improve physical well-being (Fontani et al., 2005). The recommendation to supplement the diet with n-3 fatty acids (especially EPA or fish oil) is another well-known characteristic of the Zone Diet (Cheuvront, 2003). It is thought that n-3 polyunsaturated fatty acids (PUFAs), occurring mainly in fish oil (Jho, Cole, Lee, & Espat, 2004; Sanders, 2000), are an important anti-inflammatory agent able to reduce proinflammatory cytokines (Jho, Cole, Lee, & Espat, 2004) and improve multiple risk factors for cardiovascular disease (Murphy et al., 2007). Inhibitory effects of n-3 on tumorigenesis and a variety of inflammatory diseases have also been described (Fontani et al.). Murphy et al. have shown that a 6-month PUFA supplementation increased erythrocyte n-3 long-chain PUFAs, and Kamada, Tokuda, Aozaki, and Otsuji (1993) found that an increase in erythrocyte n-3 long-chain PUFAs is strictly correlated to erythrocyte-membrane fluidity, resulting in positive effects on oxygen diffusion, especially during exercise (Ribou, Vigo, & Salmon, 2004). Fatty-acid composition greatly affects membrane physical-chemical status and function, modulating microviscosity and activity of membrane proteins, enzymes, and receptors (Zamaria, 2004).

Exercise-induced hypoxemia has been associated with limited oxygen diffusion, and because of PUFAs’ role in modulating cell-membrane fluidity, it has been hypothesized that PUFA administration could reduce exercise-induced hypoxemia (Aguilaniu et al., 1995). The mechanisms of exercise-induced hypoxemia are still not clearly understood. Moreover, in physical training, modifications in membrane properties of red blood cells, which could contribute to alterations in gas exchange, have been reported (Nikolaidis & Mougios, 2004).

PUFAs are prone to oxidation, with increased susceptibility to oxidation being linked to the degree of unsaturation. The more unsaturated n-3 PUFAs, EPA and DHA, are thought to be more susceptible to oxidation than n-6 PUFA arachidonic acid, but recent findings also suggest that n-3 PUFAs or fish oil might have a positive effect in countering oxidative stress (Barbosa et al., 2003; Nakamura et al., 2005). Physical activity is known to modulate redox balance. It leads to an increase in the production of reactive oxygen species, but mild, regular exercise has been found to stimulate endogenous antioxidants to counteract the new condition of stress and to maintain the balance between oxidants and antioxidants (Sen, 1995). In particular, induction of some antioxidant enzymes after exercise has been demonstrated (Oztasan et al., 2004; Ramel, Wagner, & Elmadfa, 2004). People who undertake mild but regular exercise show higher antioxidant enzyme levels in their muscles, especially in Type I and Type IIa fibers, than sedentary people, and they are more resistant to exercise-induced oxidative stress (Ji, 2002; Sen) because increased enzyme activity and antioxidant gene expression (Metin et al., 2003).

The current study was performed to evaluate whether a high-protein, low-carbohydrate diet supplemented with PUFAs influences body mass index (BMI) and percent body fat, red blood cell membrane composition, and plasma antioxidant activity in nonprofessional volleyball athletes.
Materials and Methods

Materials

Trolox, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and lipid standards were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals and solvents were of the highest analytical grade.

Study Participants

Eleven nonprofessional male volleyball athletes, playing on the same team, were recruited. All the athletes signed an informed-consent form. The study was carried out at the beginning of the season so that for 4 weeks before the experiments, participants could abstain from exercise training.

The participants were divided in two groups, according to dietary treatment. Group A ($n = 5$; age $28.8 \pm 4.7$ years, height $1.86 \pm 0.07$ m) followed a conventional diet (55% carbohydrates, 15% proteins, 30% lipids) low in saturated fat; high in monounsaturated fat, mainly from olive oil; high in complex carbohydrates from legumes; and high in fiber. Group B ($n = 6$; age $31.7 \pm 4.08$ years, height $= 1.86 \pm 0.03$ m) followed a low-calorie, high-protein diet (40% carbohydrates, 30% proteins, 30% fats) with pharmacological supplementation of PUFA as fish oil (3 g/day, ESKIM 1,000 mg, SIGMA TAU, containing $\geq 85\%$ ethylic esters of EPA + DHA; EPA:DHA ratio was between 0.9 and 1.5). The mean ($\pm$ SD) caloric intake was $2,750 \pm 95$ kcal in Group A and $2,450 \pm 83$ kcal for Group B. Both groups were asked to consume the same kind and amount of fruits and vegetables five times a day.

The players in the study trained three times a week, and each training session lasted 2 hr. Sessions included both aerobic and anaerobic exercises and were designed to improve strength, power, speed, passing, serving, and blocking technique, as well as game tactics and positioning skills. Generally the aerobic sessions lasted about 70–90 min, and the intensity of the exercises was designed to train the athletes at 65% of their VO$_{2\text{max}}$. The anaerobic sessions lasted about 50–30 min. Moreover, athletes played a match per week and, in a 2-month period, each athlete played almost the same number of minutes, according to their roles and skills.

Both at the beginning and at the end of the study, anthropometric measurements, height, weight, and total body fat were evaluated.

All anthropometric measurements were performed by the same operator according to the Anthropometric Standardization Reference Manual (Lohman & Martorell, 1988). Weight was measured to the nearest 100 g, and height, to the nearest 0.1 cm, using an electronic balance and a stadiometer, respectively. BMI was calculated as weight/height$^2$ (kg/m$^2$). Skinfold thicknesses (biceps, triceps, subscapular, and suprailiac) were measured to the nearest millimeter using calipers on the right side of the body. All skinfold measurements were repeated three times, and the three values were averaged. Percent body fat was calculated according to Durnin and Womersley’s (1974) method for biceps, triceps, subscapular, and suprailiac skinfolds.
Blood was drawn by venipuncture from the cubital vein from all groups, with heparin used as an anticoagulant. Ten milliliters of venous blood were collected from every athlete, both at the beginning and at the end of the study, which lasted for 2 months.

**Food Frequency Questionnaire**

Nutrient and food intake were measured using the Willett Food Frequency Questionnaire (Rimm et al., 1992; Willett et al., 1985), which has been validated in a wide range of ages (Martin-Moreno et al., 1993). Full instructions for completing the questionnaire were given, together with a list of 120 foods in which each food was characterized by a full description of usual serving size. Food preparation was taken into account to include all the ingredients used. Participants were asked to keep a detailed record of weekly food consumption, and they filled out the questionnaire three times during the study, at the beginning, after the first month, and at the end. They were also required to record the amount of food consumed and methods of food preparation. To estimate the portion size each participant was provided with pictures of standard meal and portion sizes.

All completed questionnaires were checked by a nutritionist for accuracy and completeness. Data of questionnaires were evaluated using a database for nutritional analysis (Medimatica S.r.l. 2004, Italy).

**Plasma Total Antioxidant Activity**

Plasma was separated from the cells by centrifugation at $2,000 \times g$ for 15 min at $4 ^\circ C$ and stored at $-80 ^\circ C$ until assay. Plasma total antioxidant activity (TAA) was measured using the method of Re et al. (1999), based on the ability of the antioxidant molecules in the sample to reduce the radical cation of ABTS, determined by the decolorization of $\text{ABTS}^+ \text{o}$ and measured as quenching of absorbance at 740 nm. Values obtained for each sample were compared with the concentration-response curve of a standard Trolox solution and expressed as millimoles of Trolox equivalents.

**Erythrocyte-Membrane Fatty-Acid Composition**

Plasma was separated from the cells by centrifugation at $2,000 \times g$ for 15 min at $4 ^\circ C$. Erythrocytes were washed three times with isotonic phosphate-buffer solution (PBS). The cells were then lysed and the erythrocyte membranes were isolated according to the method of Dodge, Mitchell, and Hanahan (1963). Lipids were extracted from ghosts according to the method of Folch, Lees, and Sloane Stanley (1957), and fatty-acid methyl esters were prepared from all samples according to the method of Stoffel, Chu, and Ahrens (1959).

The fatty-acid composition of erythrocyte-membrane total lipids was determined by gas chromatography. Methyl esters dissolved in n-hexane were gas-chromatographed on a Carlo Erba model 4160 (Milan, Italy) equipped with a capillary column (30 m $\times$ 0.25 mm i.d.) filled with a thermostable stationary phase (SP 2340, 0.10- to 0.15-µm film thickness), at a programmed temperature (160–210 $^\circ C$, with a 8 $^\circ C$/min gradient), with He as carrier gas at a flow rate of 2 ml/min as previously reported (Biagi et al., 1993). The gas-chromatographic peaks were identified on the basis of their retention time ratio relative to methyl stearate and
predetermined on authentic samples. Gas-chromatographic traces and quantitative evaluations were obtained using a Spectra Physics (San José, CA, USA) model 4100 computing integrator. The unsaturation index (UI) value was calculated by multiplying the relative molar content of each fatty acid by the number of double bonds and adding up all the scores of each fatty-acid methyl ester.

**Statistical Analysis**

All data are reported as $M \pm SD$. Statistical analysis was performed by Student’s $t$ test and two-way ANOVA with Bonferroni’s posttest to evaluate the presence of significant differences between the two groups.

**Results**

**Anthropometric Parameters**

Table 1 reports the BMI and the percent body fat of the participants of the two groups before and after the dietetic treatments and the physical training. Statistical analysis showed a significant decrease of the percent body fat after dietary treatment for participants of Group B, whereas participants of Group A did not show any variation of this parameter.

**Plasma TAA**

Figure 1 represents the plasma TAA expressed as mmol Trolox equivalent per liter for each participant of the two groups. In both the groups TAA was significantly increased ($p < .01$) at the end of the 2-month dietary treatment. Statistical analysis revealed no significant differences between the two groups either before or after the dietary treatments.

**Erythrocyte-Membrane Fatty-Acid Composition**

Gas-chromatographic analysis of methyl esters of red blood cell membranes is reported in Figures 2 and 3. The fatty-acid patterns of the erythrocyte membranes of the two groups were similar at the beginning of the trial (Figure 2), and the statistical analysis did not show any difference between the two groups. Figure 3 demonstrates significant differences between the two groups at the end of the study. In particular, Group B showed a significant decrease in linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) and a significant increase in eicosapentenoic acid (20:5n-3) and docosahexenoic acid (22:6n-3) compared with Group A.

**Table 1  Effect of the Different Diets on the Percent Body Fat and Body-Mass Index of Volleyball Athletes**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A ($n = 5$)</th>
<th>Group B ($n = 6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Body-mass index (kg/m²)</td>
<td>25.0 ± 0.9</td>
<td>24.9 ± 1.0</td>
</tr>
<tr>
<td>% body fat</td>
<td>18.5 ± 2.5</td>
<td>18.7 ± 3.1</td>
</tr>
</tbody>
</table>
Figure 1 — Effect of the different diets on plasma total antioxidant activity (TAA) of volleyball athletes.

Figure 2 — Total lipid fatty acid composition (mol/100 mol) of erythrocyte membranes of volleyball athletes belonging to group A and B before the beginning of the dietary treatments and physical training, $M \pm SD$.

Figure 4 shows the UI of the two groups before and after the dietary treatments. The UI value is calculated by multiplying the relative molar content of each fatty acid for the number of double bonds and adding all the scores of each fatty-acid methyl ester. The UI was similar at the beginning of the study in the two groups and was significantly increased after the treatment only in Group B.
Figure 3 — Total lipid fatty acid composition (mol/100 mol) of erythrocyte membranes of volleyball athletes belonging to group A and B after 60 days of dietary treatments and physical training, $M \pm SD$. *$p < .05$.

Figure 4 — Unsaturation Index (U.I.) of fatty acids of erythrocyte membranes of volleyball athletes, $M \pm SD$. *$p < .05$.

Discussion

Diets characterized by lower carbohydrates, higher proteins, and higher n-3 fatty acids than conventional diets are a phenomenon representing a new generation of eating regimens followed by athletes to improve their athletic performance.
The scientific literature disputes the purported benefits of adopting a high-protein:carbohydrate-ratio diet for improved health (Cheuvront, 2003), whereas there is a great deal of evidence on the positive effects of n-3 polyunsaturated fatty-acid supplementation (Fontani et al., 2005). PUFAs reduce triglycerides, have anti-inflammatory effects, reduce insulin response to glucose, and reduce risk of cardiovascular disease and cancer (Donaldson, 2004; Holness, Greenwood, Smith, & Sugden, 2003; Jho et al., 2004; Mata López & Ortega, 2003), but, because they are prone to oxidation, they might lead to lower antioxidant capacity and increased consumption of antioxidants in the body (Thorlaksdottir et al., 2006). On the basis of these controversial reports, our aim was to analyze the effects of physical activity and two different dietary approaches on volleyball athletes. In particular, we evaluated the modification of erythrocyte-membrane fatty-acid composition and plasma antioxidant activity. The participants were divided in two groups; one followed a conventional diet, and the other followed a low-calorie, high-protein diet with pharmacological supplementation of PUFAs as fish oil (3g/day). At the end of the trial, participants of the second group showed a significant reduction in percent body fat, probably caused by the low-calorie diet, while the first group, thanks to their higher calorie intake, did not show any variation of the anthropometric parameters. The plasma TAA increased in both groups, and statistical analysis revealed that TAA increase is significantly correlated only with physical activity. Although physical activity leads to an increase in the production of reactive oxygen species, mild but regular physical activity has been found to stimulate the body’s antioxidant defenses to adapt themselves to the new condition of stress and to maintain the balance between oxidants and antioxidants; in particular, the induction of some antioxidant enzymes after exercise, as superoxide dismutase, has been demonstrated (Oztasan et al., 2004; Ramel et al., 2004). People who undertake mild but regular exercise show higher antioxidant enzyme levels in muscles, especially in Type I and Type IIa fibers, than sedentary people, and they are more resistant to exercise-induced oxidative stress (Ji, 2002; Sen, 1995) because of increased enzyme activity and antioxidant gene expression (Metin et al., 2003). Evelson et al. (2002) observed higher antioxidant defenses in rugby players’ plasma than sedentary controls,’ and Fatouros et al. (2004) demonstrated that endurance training can attenuate basal and exercise-induced lipid peroxidation and increase protection against oxidative stress. The lack of influence of fish-oil supplementation on plasma TAA is in accordance with results from two intervention studies that showed no effect of dietary fish-oil supplements (Yaqoob, Pala, Cortina-Borja, Newsholme, & Calder, 2000) or DHA supplementation (Wheaton, Hoffman, Locke, Watkins, & Birch, 2003) on plasma TAA. Our results are not in line with the data of Barbosa et al. (2003) showing that fish-oil n-3 PUFAs increase the antioxidant status in ulcerative colitis patients, or with a study demonstrating a positive correlation between plasma TAA and n-3-PUFA-rich diet in Icelandic women (Thorlaksdottir et al., 2006). The discrepancies between our study and those studies, although not easy to explain, could be ascribed to the fish-oil supplement in association with the usual medication for ulcerative colitis (2 g/day sulfasalazine) in Barbosa et al.’s study and to the gender of the participants (Icelandic women) in the latter.
The fish-oil-supplemented high-protein diet caused a modification of the erythrocyte-membrane fatty-acid composition, with a significant increase of PUFA n-3 relative molar content; a decrease, although not significant, in palmitic and stearic acids; and a significant decrease in linoleic and arachidonic acids. Moreover, participants in Group B had, after the treatment, a significantly higher UI than the participants in Group A.

These data are in agreement with the results of Cao, Schwichtenberg, Hanson, and Tsai (2006) showing an increase in n-3 PUFA content in erythrocyte membranes of male and female participants after a 8-week fish-oil supplementation, and also with the data of Murphy et al. (2007), who demonstrated a significant increase in n-3 long-chain PUFA content of erythrocyte membranes of participants consuming n-3-enriched food (to achieve an EPA + DHA intake of 1g/day) for 3 months. In our study, the fish-oil supplementation lasted for 60 days because that is the supplementation time used by Palozza et al. (1996) and Poppitt, Kilmartin, Butler, and Keogh (2005) to observe significant modifications in RBC lipid-membrane composition, with no alteration in calories or macronutrient percentage.

Erythrocytes are essential for the transport of oxygen to human cells. This function requires single unaggregated erythrocytes, with diameters of 7.5 µm, to pass through the microcirculation. Vessel diameters are often less than that of the erythrocytes, at 5 µm. It is therefore crucial that cells not be aggregated, and furthermore, the cells must be able to achieve considerable distortion and folding. Erythrocytes deformability and aggregation are thus very important features of the cells (Ho, Maple, Bancroft, McLaren, & Belch, 1999).

Dietary supplementation of fish oil has been associated with an increase of n-3 PUFAs in erythrocyte membrane, simultaneously increasing membrane fluidity (Kamada et al., 1986) and improving cell deformability (Cartwright, Pockley, Galloway, Greaves, & Preston, 1985). Ho et al. (1999) demonstrated a reduction in erythrocyte aggregation after dietary supplementation with n-3 and n-6 PUFAs in healthy volunteers. Guezennec, Nadaud, Satabin, Leger, and Lafargue (1989) reported that dietary supplementation of fish oil rich in n-3 PUFAs improved the hemorrheological response of athletes to exercise performed under hypoxic conditions.

In theory, although still open to debate, n-3 PUFAs could enhance oxygen delivery to contracting muscle and VO$_{2max}$, ultimately resulting in improved exercise performance (Jeukendrup & Aldred, 2004).

Even though this is an important blood rheological effect, PUFA peroxidation also has to be considered. It has been postulated that incorporating n-3 PUFAs into erythrocyte membrane might increase the potential for lipid peroxidation (Pedersen et al., 2003), and this is much more evident in participants who undergo high oxidative stress as athletes (Alessio, 1993).

The significant increase in the UI of erythrocyte membranes of participants following the high-protein fish-oil-supplemented diet also indicates the potential for harm, because a high intake of DHA might increase susceptibility to lipid peroxidation (Allard, Kurian, Aghdassi, Muggli, & Royall, 1997).

In conclusion, high-protein, low-carbohydrate diets seem to be useful only when the aim is to lose weight in a short-term period, but further studies are still needed to solve the open debate on the safety of long-term high protein intake.
Moreover, pharmacological fish-oil supplementation in physically active participants leads to an increase in erythrocyte-membrane PUFA content, related to improved hemorrheological response of athletes to exercise, but it is still not clear if the pro-oxidant effects of fish-oil supplementation could unfavorably affect these benefits. The balance between positive and harmful effects of PUFAs intake might depend on the dose.

It thus appears that the better choice for an athlete’s everyday diet is to provide the muscles with substrates to fuel the training program and to allow optimal adaptation to ultimately enhance performance. All these requirements can be fulfilled by the Mediterranean diet.

References


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