Association between copper plasma concentration and copper-dependent metaloproteins in elite athletes

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ABSTRACT

Copper is a trace element essential in several biological processes, some of them important for physical activity, such as energy metabolism, iron homeostasis and antioxidant protection through the plasma ceruloplasmin, erythrocyte Cu-Zn superoxide dismutase (Cu-Zn SOD) and metallothionein. However, copper also participates in oxidative reactions releasing free radicals, which may adversely affect cell integrity and function. Physical activity is known to affect copper homeostasis and may interfere in the copper antioxidant capacity. Intense physical activity results in higher oxygen consumption, which favors the release of free radicals and may cause irreversible damage to the body when the natural mechanisms of protection, including those copper-dependent, are not properly stimulated. Few studies related exercise with plasma copper level and copper-dependent metalloproteins in elite athletes. The present study aimed at evaluating the association between different levels of plasma copper and copper-dependent metalloproteins in male elite athletes (n = 50). The biochemical indices studied were plasma copper and ceruloplasmin, and erythrocyte Cu-Zn superoxide dismutase and metallothionein by validated methods. The results showed that 32% of the athletes had plasma copper levels lower than 11 µmol/L, 38% between 11-13 µmol/L and 30% higher than 13 µmol/L. Plasma copper was associated with plasma ceruloplasmin level (r = 0.31, p = 0.004), and with Cu-Zn SOD (r = –0.32, p = 0.02); metallothionein erythrocyte were associated with Cu-Zn SOD (r = 0.73, p = 0.001) and with ceruloplasmin (r = 0.40, p = 0.006). These results suggest that both plasma and erythrocyte antioxidant capacity favor homeostatic adjustments in agreement with plasma copper levels in elite athletes.

Keywords: Metalloproteins. Metallothionein. Ceruloplasmin. Cu-Zn superoxide dismutase.

INTRODUCTION

Copper is a component of a great number of proteins and enzymes which play crucial biological functions to the cells and which contribute to the maintenance of its homeostasis[1]. Copper is an essential metal to living beings; however, it is also potentially toxic to the cells due to its easiness to suffer alterations of the oxidation as a free ion. Therefore, in order to have the copper-dependent metalloproteins satisfactorily playing their essential functions, such as the antioxidants, the copper ion needs to be suitably compartmentalized and present in intra and extracellular suitable concentrations. Both deficiency and excess of this mineral may do harm to the cellular integrity and functionality[1-3].

Suitable copper intake is important in order to guarantee good performance of athletes. Generally, athletes from different modalities overtake the copper dietetic recommendations[4-6]. However, other nutrients such as iron and zinc, when ingested in excess, may cause side effects on the copper homeostasis and possibly harm the maintenance of its antioxidant function[1]. The use of nutritional supplements which do not suitably contemplate the copper supply is very common among athletes, which could harm copper's essential role during physical activity.

Intense physical exercise promotes the release of reactive oxygen species[7] and may influence in the copper homeostasis[8]. The metalloproteins, serum ceruloplasmin, Cu-ZnSOD and erythrocyte metallothioneins, are copper-dependent metalloproteins and keep the intra and extracellular antioxidant protection, respectively[1,9-10]. Several factors influence the metalloproteins amounts, among them the serum copper, physical activity[1,8], use of zinc and ascorbic acid supplements, contraceptives, antibiotics, pregnancy and presence of inflammatory processes[11-13].

Considering that the studies on the influence of the serum copper concentration over the copper-dependent metalloproteins in the literature have been performed in female athletes[4], collegiate female athletes[6] and male adolescents[14], the aim of this research to evaluate the association of different serum plasma levels with copper-dependent antioxidant metalloproteins in adult male elite athletes is justified.

METHODS

Sample

The protocol of this study was approved by the Ethics Committee of Pedro Ernesto University Hospital (Rio de Janeiro, Brazil). Adult male elite athletes (n = 50), participated in the study after having received explanations about its aims and provided consent. The athletes were randomly selected by members of each federation among those who were most outstanding. All athletes participated in national and international competitions. None of the athletes took vitamin or mineral supplement during the study's development.

Samples collection

Blood samples were collected on the day posterior to the specific competition of each sports modality (triathlon, n = 10; running, n = 15; judo, n = 10 and swimming, n = 15). The athletes kept their eating habits until the 22 hours of the competition day which were always held in the morning. After this time, fasting would start for blood collection which occurred in the Center of Nutritional Orientation for Athletes (Nutrition Institute – UERJ) at 8:00 hours by venous punction in tests tubes containing heparin (30 U/tubo). All precautions were taken to avoid contamination by minerals during the blood collection and processing. The blood was separated for hemogram performance. The remaining was centri-

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fuged at 1.800 *g* for 10 minutes for plasma separation. The buffy coat was removed and the remaining erythrocytes were three times washed with cold NaCl at 0.9%. The washed cells were straightened and completed to the initial volume with cold deionized water. The plasma shares and straightened erythrocytes were stored at –20^{0}C until the moment of the analyses.

Laboratory analyses

Hematocrit, hemoglobin and leukocytes counting were determined by an automatic hematologic analyzer (Cell-Dyn/Cobas Vega, Florida/USA). Serum zinc and copper were measured by spectrometry of atomic absorption (Perkin Elmer Plasma model AA1475, Massachusetts/USA). The used heparin had low amount of zinc (0.005 µg Zn/1000 U) which did not affect the final amount of serum zinc. Cerulloplasmin was determined in serum through the method based on its oxidase activity over the *p*-phenilenediamine[15]. The superoxide dismutase activity in the erythrocytes was determined by the pyrogallol method[16] and the results were expressed by hemoglobin gram. The metallothionein in the erythrocytes was measured by adaptation of the affinity method of ^{109}Cd to hemoglobin[17] as described by Zapata *et al*.[18]. It was also expressed by hemoglobin gram. The intra-essay variation coefficient for all measurements was lower than 5%. All analyses were twice or thrice performed.

Cutting point determination for serum copper

In order to evaluate the influence of the serum copper concentration over the copper-dependent indicators, the obtained outcomes were subdivided according to the serum copper concentration (< 11 µmol/L; 11-13 µmol/L and > 13 µmol/L) according to the DRI/02[19] which states that serum values lower than 11 µmol/L should be considered as an indication of copper deficiency.

Statistical analyses

The Kolmogorov-Smirnov test was applied in order to verify whether the data were within a normal distribution. The statistical comparison among the biochemical indices was performed by one-way on ranks ANOVA, followed by the test by Dunn. The association between the indicators was determined by the Spearman correlation coefficient. The *p* level considered as significant was of < 0.05. The SCG Plus Version 7.0 (Statistical Graphics Corporation Plusware, USA) software was used for statistics calculation.

RESULTS

The athletes presented mean age of 27 ± 6 years, the hemoglobin mean values (155 ± 15 g/L) and hematocrit (45 ± 3%) were suitable and similar, not demonstrating to influence other indicators by hemodilution or hemoconcentration. All athletes presented serum zinc mean value above 10 µmol/L, not demonstrating signs of deficiency of this mineral. The leukocytes counting (5.5 ± 2.0 x 10^3 cells/µL) did not demonstrate presence of infectious processes.

In table 1, the results of the copper-dependent indicators are grouped according to the serum copper concentration. About 32% (n = 16) of the athletes had serum copper concentration lower than 11 µmol/L, classified as deficient by the DRI 2002[19]; 38% (n = 19) with concentration between 11-13 µmol/L and 30% (n = 15) with concentration > 13 µmol/L. The plasma values presented significant difference (p < 0.05) among all groups. That result guaranteed that the association of different concentrations of serum copper with copper-dependent metalloproteins in elite athletes could be verified.

Considering the different levels of serum copper applied in this study, the serum cerulloplasmin concentration, the Cu-Zn SOD activity and of the erythrocyte metallothioneins presented opposite behavior. While the Cu-Zn SOD (p = 0.05) and metallothionein (p = 0.06) reduced their activity, the serum cerulloplasmin concentration increased (p = 0.03) concerning the high values of serum copper (> 13 µmol/L).

TABLE 1
Distribution of the copper-dependant biochemical indicators in the athletes evaluated according to serum copper concentration

Variables	Serum copper levels, µmol/L		
	< 11	11-13	> 13
n	16	19	15
Serum copper (µmol/L)	9.8 ± 1.0	11.9 ± 0.5	14.1 ± 0.8*
Metallothionein (nmol/g hemoglobin)	3.1 ± 1.0	3.1 ± 0.9	2.8 ± 0.6
Serum Ceruloplasmin (mg/L)	248 ± 49	295 ± 93	306 ± 71**
Cu-Zn superoxide dismutase (U/g hemoglobin)	900 ± 200	873 ± 209	764 ± 142***

Results described as mean ± standard deviation. Comparison by one-way on ranks ANOVA, followed by the test by Dunn.
* Significant difference among all groups (p < 0.005).
** Significant difference (p < 0.05) between group > 13 µmol/L *vs* group < 11 µmol/L
*** Significant difference (p < 0.05) between group > 13 µmol/L *vs* groups 11-13 µmol/L and < 11 µmol/L.

The associations observed between the indicators, when the athletes groups were considered, are shown in table 2.

TABLE 2
Associations between the copper-dependent biochemical indicators in the evaluated athletes (n = 50)

Indicators	Degree of association	
	r	p
Serum copper *x* Cu-Zn SOD	–0.32	0.02
Serum copper *x* Ceruloplasmin	0.31	0.04
Metallotionein *x* Cu-Zn SOD	0.73	0.00
Metallotionein *x* Ceruloplasmin	–0.40	0.006
Cu-Zn SOD *x* Ceruloplasmin	0.37	< 0.0001

Degree of association determined by the Spearman correlation coefficient.

DISCUSSION

The present study was the first one to have as aim to determine the association between different serum copper concentrations and copper-dependent metalloproteins with antioxidant action concentrations. The limitation of this study was not to have determined the daily copper intake of the athletes; however, this limitation did not harm the results' interpretation.

The serum copper concentration in athletes may act high[8] or unchanged[14]. Some authors believe that the magnitude of the copper loss through sweat justifies these alterations, although it does not lead to copper deficiency[14,20-21]. The several serum concentrations found by different studies[8,14] may be also partially explained by the chronic physical training which apparently stimulates antioxidant defense mechanisms, triggering the serum copper for cerulloplasmin and Cu-Zn SOD synthesis[22].

In the present study, the distribution of athletes according to the serum copper concentration made it possible to observe that 32% of the athletes presented levels lower than the minimum threshold of suitability recommended by the DRI/02 (< 11 µmol/L[19]). Despite the low serum copper concentration, no athlete presented cerulloplasmin values below the recommendation (180 mg/dL[19]). Nevertheless, we cannot nutritionally classify them as copper-deficient, neither as suitable, once the serum copper and cerulloplasmin may be affected by intense physical activity.

Cerulloplasmin is a copper-dependent glycoprotein present in the serum, which is synthesized by the liver[9]. Suzuki *et al*.[23], have found in rats deficient in copper, low amounts of cerulloplasmin due to the preference of mobilization of hepatic copper available for the chaperones (CCS) responsible for the Cu-Zn superoxide dis-

mutase synthesis, and not for the chaperones (ATOX 1) involved with the cerulloplasmin synthesis, avoiding thus, the copper return for the blood flow as cerulloplasmin.

The antioxidant function of the cerulloplasmin consists not only in its capacity of keeping the copper and iron ions joined to the their specific proteins, avoiding that they participate in the Fenton reaction, but also in its screening effect over the superoxide and other reactive oxygen species[24].

During intense physical exercise there is release of reactive oxygen species[7] and stimulus to the inflammatory process through the release of cytokines[25], which may result in increase of the cerulloplasmin concentration[26], once both the cerulloplasmin synthesis and release in the serum are stimulated by the interleukins 1 and 6[27]. Nevertheless, it is possible that the increase in cerulloplasmin in response to the interleukins in the physical exercise is also associated with its role as antioxidant[22,26].

The cerulloplasmin concentration in the present study was sensitive to the serum copper concentration. The athletes did not present leukocytes counting relevant to the presence of inflammatory processes**.** Therefore, we may suggest that, in this case, the cerulloplasmin would play a role of plasma antioxidant, protecting the body from the highest copper amounts available. The intracellular metalloproteins play a role similar to the cerulloplasmin, being important in the antioxidant protection as well as maintenance of copper homeostasis[28-29].

Since the erythrocytes are constantly exposed to the oxygen, they require very active antioxidant defense mechanisms in order to prevent or reduce oxidative injuries. The erythrocyte Cu-Zn superoxide dismutase protects these cells from the action of the superoxide ions which are harmful radicals[30]. Since intense physical exercise promotes free radicals, some authors found higher amounts of metalloproteins after physical activity[14,22,31], while others have not found similar results[30]. The reported discrepancy can be justified by the different essays applied for the determination of the superoxide dismutase activity. Moreover, other factors influence the activity of this enzyme, such as the moment of collection[7].

Metallothionein is a protein rich in cysteine traces which is able to link zinc, cadmium and copper, being important in the intracellular maintenance of suitable levels of these metals[11,13]. It presents antioxidant properties, inhibiting propagation reactions of free radicals through the selective connection of pro-oxidant metal ions such as iron and copper, and potentially toxic ones such as cadmium and mercury. Moreover, it is a powerful screener of reactive oxygen species[10]. Only two studies determined the erytrocite metallothionein concentration in athletes and concluded that the metallothionein is sensitive to intense and high-impact physical exercise[22], but not to the post-exercise resting time[32].

In the present study, the Cu-Zn SOD and the erythrocyte metallothionein presented lower values when the serum copper was in higher concentration, possibly due to copper greater mobilization by the liver for the cerulloplasmin synthesis and maintenance of the plasma antioxidant system[10]. Such premise may be observed by the associations of the serum copper with the Cu-Zn SOD (negative) as well as with the cerulloplasmin (positive), when all athletes have been considered. The positive associations observed between the erythrocyte metalloprotein and the Cu-Zn SOD and of these with the cerulloplasmin, demonstrated the sensitive balance between the intra and extra cellular antioxidant systems in elite athletes.

In conclusion, the copper-dependent metalloproteins studied keep association with different serum copper concentrations, being the intracellar (Cu-Zn SOD and metallothionein) sensitive to low concentration, and the extracellular one (cerulloplasmin) sensitive to higher concentration. Possibly, due to the copper mobilization for the antioxidant systems, suggesting that there is a homeostatic imbalance between the intra and extracellular antioxidant due to the protection to the different copper levels.

Nutritional supplementation with micronutrients is growing in the sports environment but its real need has been questioned[33]. Although the present study has not evaluated the eating habit of the athletes, we call attention to the fact that dietetic manipulations (indiscriminate use of supplements with zinc or iron) may affect the metalloprotein levels studied harming the copper homeostasis as well as the fulfillment of its antioxidant function, directly affecting the cellular functionality and integrity.

All the authors declared there is not any potential conflict of interests regarding this article.

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