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## Accepted Manuscript

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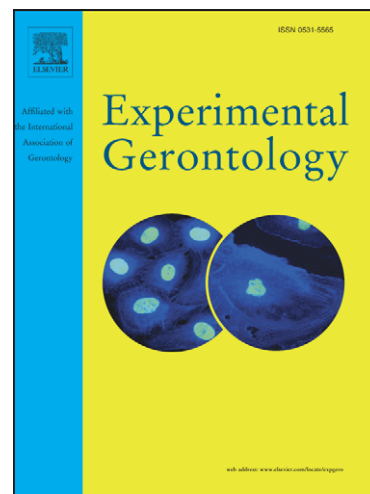
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## Effects of Zinc supplementation on antioxidant enzyme activities in healthy old subjects.

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**ABSTRACT**

A large body of experimental research indicates that oxidative stress contributes to the processes related to aging and age-related diseases. Trace elements, particularly zinc (Zn), are essential components of the endogenous enzymatic antioxidant defenses. The aim of this study was to determine the activity of three main antioxidant enzymes in plasma [i.e. superoxide dismutase (pSOD), catalase (CAT), glutathione peroxidase (GPx)] and of SOD in erythrocyte (eSOD) in a group of 1108 healthy elderly subjects from different European countries. The same enzymatic activities were evaluated in a subgroup of 108 subjects before and after Zn supplementation. We observed that eSOD activity increased with age, whereas plasma Zn decreased. Moreover, we found that women showed higher eSOD activity and lower plasma Zn compared to men. There were no age and gender-related differences in the activities of pSOD, CAT and GPx. After Zn supplementation, the activities of Zn-dependent enzymes (pSOD and eSOD), as well as plasma Zn concentration, were significantly higher than before supplementation. These results were not influenced by age, gender, plasma Zn variations ( $\Delta$  Zn) and geographic area. These data suggest the potential beneficial effects of Zn supplementation on Zn-dependent antioxidant enzymes in healthy elderly subjects.

## INTRODUCTION

The involvement of reactive oxygen species (ROS) in the ageing process has been well documented (Harman, 1992), and an enhanced oxidative stress in the elderly has been reported as being related to several pathologies such as neurodegenerative and vascular diseases (Mariani et al., 2005). Uncontrolled free radical production in ageing is considered to be the result of both increased production of ROS –mainly generated in the organism as byproducts of normal cellular metabolism, especially through the mitochondria pathway (Balaban et al., 2005)- and lowered antioxidant defenses.

Antioxidant enzymes, i.e. superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), operate in concert together with several non-enzymatic molecules to contrast the ROS actions and to avoid oxidative damage. SOD catalyzes the reduction of superoxide anion into hydrogen peroxide, which is subsequently detoxified by CAT and GPx at both intra- and extra-cellular levels. Three different isoforms of SOD have been identified: SOD1 (Copper Zinc-SOD, CuZn-SOD), localized in cytoplasm, which includes Cu and Zn at its catalytic site, SOD2 (Manganese-SOD, Mn-SOD), localized in mitochondria, which has Mn as a co-factor, and finally SOD3 (EC-SOD), an extracellular antioxidant enzyme, which, such as SOD1, presents Cu and Zn at its catalytic site. Other transition metals are important components of the enzymatic antioxidant defenses: Cu and iron (Fe) are, in fact, essential components of CAT, and selenium (Se) is a constitutive component of GPx.

Consequently, metal ions are crucial for life. In particular, Zn has several antioxidant properties other than being co-factor of SOD1 and SOD3, such as maintenance of protein sulphhydryl groups (Bray and Bettger, 1990; Powell, 2000), protection against vitamin E depletion, stabilization of membrane structure, and the maintenance of tissue concentrations of metallothioneins, powerful free radical scavengers (DiSilvestro, 2000). Therefore, Zn supplementation may be beneficial in guaranteeing powerful antioxidant defenses.

The ZincAge project was approved by the European Commission and designed to study biochemical, genetic and lifestyle factors for healthy ageing and longevity, with specific attention to the role played by oxidative stress and nutritional Zn in the maintenance of immune efficiency both at central and peripheral levels. The project involved nine laboratories from different countries of Northern and Southern Europe, in order to evaluate also the role played by different dietary habits. The study design of the ZincAge project has been described in detail elsewhere (Mocchegiani et al., 2004).

The aim of the present study was to examine a spectrum of antioxidant enzyme activities, with particular attention to the influence of age and gender, in a large number of healthy, community-dwelling elderly subjects, enrolled from different European countries. Furthermore, in a subgroup of these subjects, the effect of Zn supplementation for seven weeks on these antioxidant enzyme activities and on zinc status was also determined.

## **SUBJECTS AND METHODS**

The study included 1108 healthy free-living subjects enrolled in the ZincAge project. Subjects were recruited by announcements at general practitioners' surgeries from five European centers located in Italy (n. 371), Greece (n. 163), Poland (n. 200), France (n. 224) and Germany (n.150). All subjects were in good health condition, and without functional impairment, according to the inclusion criteria of the ZincAge protocol. Healthy status was evaluated by a specific questionnaire on health and morbidity planned for the study and filled in by the general practitioner after medical examination and analysis of the clinical record. None of the subjects studied was taking any drug that may interfere with antioxidant status, particularly nutritional integrators or antioxidant supplements. In all the participants, clinical laboratory analyses were determined, including measurements of albumin, total cholesterol and triglycerides. All subjects gave informed consent to the study.

Subjects were classified in four groups according to age: group A, 387 “young-old” subjects aged less than 70 years (range 60-69) (204 women, 183 men, mean age  $65.4 \pm 2.7$ ); group B, 415 “old” subjects aged between 70-79 years (221 women, 194 men, mean age  $73.9 \pm 2.8$ ); group C, 208 “old-old” subjects aged between 80-89 years (131 women, 77 men, mean age  $83.4 \pm 2.7$ ); group D, 98 nonagenarians ( $\geq 90$  years) (64 women, 34 men, mean age  $92.7 \pm 2.7$ ).

From all subjects, 10 ml of blood was collected in heparinized tubes and centrifuged immediately, to separate plasma and erythrocytes, which were aliquoted and stored frozen at  $-80^{\circ}\text{C}$  in the Biological Bank of INRCA until analysis. SOD3 (pSOD) (U/ml), CAT ( $\mu\text{mol}/\text{min}/\text{mg}$  protein), and GPx (nmol NADPH/min/ml) activities in plasma were measured according to the methods of L’Abbé and Fisher (1990), Beers and Sizer (1952), and Flohé and Gunzler (1984), respectively. To determine SOD1 activity in erythrocytes (eSOD), red blood cells were hemolyzed with cold distilled water, then, after extraction with ethanol/chloroform mixture (1:1), the enzymatic activity (U/g Hb) was measured in the supernatant according to Winterbourn and coll. (1975). Plasma Zn status was established by inductively coupled plasma mass spectrometry (ICP-MS). Zn concentration was defined as low when  $<10 \mu\text{M}$  (10<sup>th</sup> percentile), marginal between 10 to 11  $\mu\text{M}$ , and normal when  $>11 \mu\text{M}$  (25<sup>th</sup> percentile) (Beattie et al., 2002).

In a subgroup of 108 subjects, randomly selected among participants, [55 (50.9%) women and 53 (49.1%) men; mean age  $72.4 \pm 7.2$  years, range 60-92] from Italy (n. 34), Greece (n. 23), Poland (n. 30), France (n. 2) and Germany (n.19), the activities of eSOD, pSOD, CAT and GPx were also determined after seven weeks ( $48 \pm 2$  days) of Zn supplementation (10 mg  $\text{Zn}^{++}/\text{day}$  as 50 mg zinc-bis-hydrogen-DL-aspartate), which correspond to the recommended dietary allowances of zinc for this study population (Commission of the European Communities, 1993).

Statistical analysis was performed with the program Statistical Package for the Social Sciences (SPSS version 12.0, Chicago, IL) for Windows. All data are presented as mean  $\pm$  SD. Non parametric tests were applied: Kruskal-Wallis test was used for comparisons among age groups, and gender differences were determined by Mann-Whitney test. Wilcoxon test for paired samples was

used to compare enzymatic activities and plasma Zn concentration before (baseline) and after Zn supplementation. Variation of each enzyme activities ( $\Delta$  = enzyme activity after – enzyme activity before Zn supplementation) were not associated with age, gender, geographic area and variations in Zn concentration ( $\Delta$  Zn = [Zn] after – [Zn] before supplementation) in a general linear model.  $\Delta$  enzyme activities and concentration of Zn in plasma, according to basal plasma Zn levels, were analyzed by Kruskal-Wallis test. All tests were two-tailed and considered significant at  $p < 0.05$ .

## RESULTS

At baseline, eSOD activity was higher in nonagenarians (group D) compared to the other age groups (A, B and C), and also in octogenarians (group C) compared to subjects aged less than 80 years (group A and group B), while there were no differences in the activities of pSOD, CAT and GPx among groups. Plasma Zn showed an inverse trend since it progressively decreased with age (Table 1).

Consistently, the prevalence of a condition of Zn deficiency (defined as plasma concentration lower than 10  $\mu$ M) increased with age: it occurred in about 10% of both young-old and old groups, in about 18% of the old-old subjects and reached 49 % prevalence in nonagenarians. On the other hand, only 27 % of the nonagenarians, compared with about 70 % of the other groups, showed normal values ( $>11$   $\mu$ M) of plasma Zn (Figure 1).

Analyzing the antioxidant enzyme activities according to gender, it was found that women showed a higher eSOD activity compared with men ( $p < 0.0001$ ), whereas there were no gender differences in the activities of pSOD, CAT and GPx, as well as in plasma Zn level (Table 2). eSOD activity was always higher in women than men in all age groups but independent of geographic origin (data not shown).

At the beginning of the study, more than 50% of the 108 subjects who were enrolled for supplementation exhibited plasma Zn levels below the normal value (34.4% with Zn deficiency and 19.6% with marginally reduced Zn), whereas after supplementation with Zn for seven weeks, the



prevalence was reduced to 41% (27.5% with Zn deficiency and 13.7% with marginal deficiency) ( $p < 0.001$ ) (Figure 2).

After supplementation, the activities of Zn dependent enzymes (eSOD and pSOD) were significantly higher than baseline, as well as plasma Zn concentration, whereas CAT and GPx activities decreased (Table 3).  $\Delta$  enzyme activities were not influenced by age, gender,  $\Delta$  Zn concentration and geographic area (data not shown).

There were no statistically significant differences in  $\Delta$  antioxidant enzyme activities after Zn supplementation in subjects grouped according to basal plasma Zn levels, whereas an increase of plasma Zn concentration was observed only in subjects with baseline Zn deficiency or marginal Zn deficiency, but not in subjects with normal concentration of plasma Zn at baseline (Table 4).

## DISCUSSION

One of the biological characteristics of aging is that the level of non-enzymatic antioxidant components declines during senescence (Short et al., 1997). On the contrary, the activities of several enzymatic antioxidant enzymes seem to increase with aging, which may be induced by a parallel increase of free radicals (Mecocci et al., 2000). Results of this study partially agree with these findings; we found, in fact, that in this large healthy elderly population, eSOD activity significantly and progressively increased with age, whereas there were no age-related modifications in the activities of pSOD, CAT and GPx.

These results corroborate our previous data on a smaller population of the ZincAge project (Mariani et al., 2006). However, earlier reports, which analyzed modification of the activities of these enzymes with age, showed contrasting results. Some studies found an age-related increased activity of eSOD (Ho et al., 2005; Mecocci et al., 2000; Saraymen et al., 2003), CAT (Ho et al., 2005), and GPx (Bolzan et al., 1997; Ceballos-Picot et al., 1992), whereas other authors reported a decrease in the activity of eSOD (Andersen et al., 1997; Ceballos-Picot et al., 1992; Guemouri et al., 1991), pSOD (Adachi et al., 2000; Guemouri et al., 1991), CAT and GPx (Guemouri et al.,

1991) with age. Moreover, consistent with our results, the activities of CAT (Andersen et al., 1997; Bolzan et al., 1997), and GPx (Andersen et al., 1997; Ho et al., 2005; Mecocci et al., 2000) were unchanged with increasing age.

Regarding gender, we found that eSOD activity was higher in women than in men in all age groups. On the contrary, the activity of CAT, that appeared to be higher in women compared to men in our previous report on a smaller population (Mariani et al., 2006), showed no difference between women and men, as well as pSOD and GPx. These results were observed also in all age groups and independent of geographic area, consistent with several studies (Ceballos-Picot et al., 1992; Guemouri et al., 1991; Ho et al., 2005), but not with others (Bolzan et al., 1997).

The different results observed may be justified by several factors. In most reports, with the exception of Guemouri et al. (1991), populations analyzed were quite small (from 100 to 200 subjects), random samples were not used, and selection bias may have a strong influence. Moreover, it must be considered that genetic background, dietary habits and micronutrient intake (especially between women and men) play an important role, all factors that are difficult to disentangle.

Our results are in agreement with the evidence that normal ageing is associated with a consistent loss of plasma Zn, that probably reflects a progressive decline of Zn content in the body (Andriollo-Sanchez et al., 2005). We found that plasma Zn concentration progressively decreased among age groups, from “young old” to nonagenarians, who also presented more frequently a condition of Zn deficiency. It was suggested that Zn deficiency in elderly subjects may be related to multiple causes, such as reduced intake with diet, inadequate mastication due to loss of teeth and intestinal malabsorption (Oteiza and Mackenzie, 2004).

The main objective of the present study was to evaluate the effect of short-term Zn supplementation on antioxidant enzyme activities. We found that Zn supplementation caused a significant increase in the activity of eSOD and pSOD, the two Zn-dependent enzymes, independent of age, gender, baseline Zn status and geographic area. On the contrary, CAT and GPx showed

decreased activities after supplementation. Plasma Zn concentration also increased after supplementation, but only in subjects with baseline level below the normal values.

Several mechanisms have been proposed to explain the potential antioxidant effects of Zn. A part its structural role as co-factor of SOD1 and SOD3, Zn protects sulfhydryl groups from oxidative damage, binding directly to them and thus reducing their reactivity and preventing intramolecular disulfide formation. Zn contributes to the inhibition of ROS production competing with transition metals, especially with Fe, for chelation by the organic ligand cysteine, preventing that cysteine-bound Fe transfers electrons to oxygen and produce  $\text{OH}^\bullet$  (Bray and Bettger, 1990). Other antioxidant actions of Zn have been suggested. For example protection against vitamin E depletion, since a zinc-adequate diet is important for good intestinal absorption of vitamin E in rats (Kim et al., 1998). Finally, Zn may be implicated in the maintenance of tissue concentration of metallothioneins, scavengers of free radicals, since they are inducible by Zn administration (Cousins, 1985).

To our knowledge, few studies have evaluated the effect of Zn supplementation on antioxidant enzyme activities, with inconclusive results. Zinc supplements have been shown to decrease eSOD activity (Abdallah and Samman, 1993; Fischer et al., 1984; Yadrick et al., 1989) but not in elderly subjects (Galan et al., 1997; Girodon et al., 1997) or in diabetic patients (Anderson et al., 2001; Roussel et al., 2003). There are also a small number of studies examining the effect of dietary Zn intake on extracellular SOD activity. Increased Zn intake seems to increase (Davis et al., 2000), whereas Zn deficiency decrease pSOD (Ruz et al., 1992). Conversely, a cross section study did not find any association between dietary Zn intake and pSOD activity (Paik et al., 1999). Regarding plasma GPx, an increased activity was shown in elderly subjects supplemented with Zn and Se (Girodon et al., 1997), and in adults after supplementation with Zn and Se plus ascorbic acid, beta-carotene and alpha tocopherol, although it is probable that the effect was mostly due to the Se supplementation, being GPx a Se-dependent enzyme (Preziosi et al., 1998). To our knowledge, there are no studies that investigated the relationship between Zn supplementation and CAT

activity. It was recently found that SOD activity was significantly enhanced in gallium-stressed cultures of *Pseudomonas fluorescens*, mimicking an oxidative environment. In this condition CAT activity was markedly lowered (Beriault et al., 2007). Authors suggested that, since gallium is known to antagonize biochemical reactions necessitating Fe and it may inhibit the genesis of enzymes or proteins requiring Fe, the perturbation in Fe-metabolism related to gallium toxicity might determine the decreased CAT activity. Similarly, the competition between Zn and Fe could explain the decrease activity of CAT we observed after Zn supplementation.

Zinc supplementation had a positive effect on plasma Zn concentration. Investigations on diabetic subjects found that, after daily supplementation with 30 mg of Zn gluconate for three months, all subjects display plasma Zn concentration above the level considered at risk of Zn deficiency (Anderson et al., 2001; Roussel et al., 2003). Supplementation with two moderate doses of zinc (15 or 30 mg of Zn gluconate) for six months significantly increased serum Zn concentration in healthy subjects aged 55-70 years, participating at the Zenith study, but did not modify Cu, Fe or vitamin E status, neither had a significant effect on LDL oxidation, that represented the principal aim of the investigation (Feillet-Coudray et al., 2006). The effect of zinc supplementation was recently confirmed in the total sample of the Zenith study, including also subjects aged 70-85 years: serum zinc concentration, in fact, increased significantly over the 6 months of supplementation, in both groups receiving 15 and 30 mg/day of zinc, with no change in the placebo group. Nevertheless, erythrocyte zinc concentration was not different between supplemented and un-supplemented subjects (Intorre et al., 2007).

Consistently, in our study we found a significant increase of plasma Zn after supplementation, especially in elderly subjects who presented Zn deficiency at baseline. However, after supplementation, we observed subjects who still presented marginally reduced Zn or Zn deficiency. These findings may be related to the short term of supplementation in this study, only seven weeks instead of three to six months of the other studies reported above. Short-term Zn supplementation (30 mg/d as amino acid chelate for three weeks) in diabetic women, in fact, was found to improve

plasma zinc, but not 5'-nucleotidase activities, a sensitive indicator of zinc status, and did not showed a significant effect on vulnerability of LDL and VLDL to oxidation (Blostein-Fujii et al., 1997).

The present study demonstrates the efficacy of Zn supplementation, given at nutritional dose, on biochemical indicators of zinc status, such as plasma Zn concentration and Zn-dependent antioxidant enzymes activities, but it was not sufficient to restore a normal zinc status in all treated subjects. Further researches are required to address the nature of these observations by taking into consideration a larger panel of biomarkers of oxidative stress (antioxidant/oxidized molecules), multiple supplementation with other trace elements and multiple antioxidant administration, for a longer period and at higher doses, in order to better understand the role of Zn in delaying and/or preventing aging and age-related diseases also in maintaining an optimal redox status in the organism.

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ACCEPTED MANUSCRIPT

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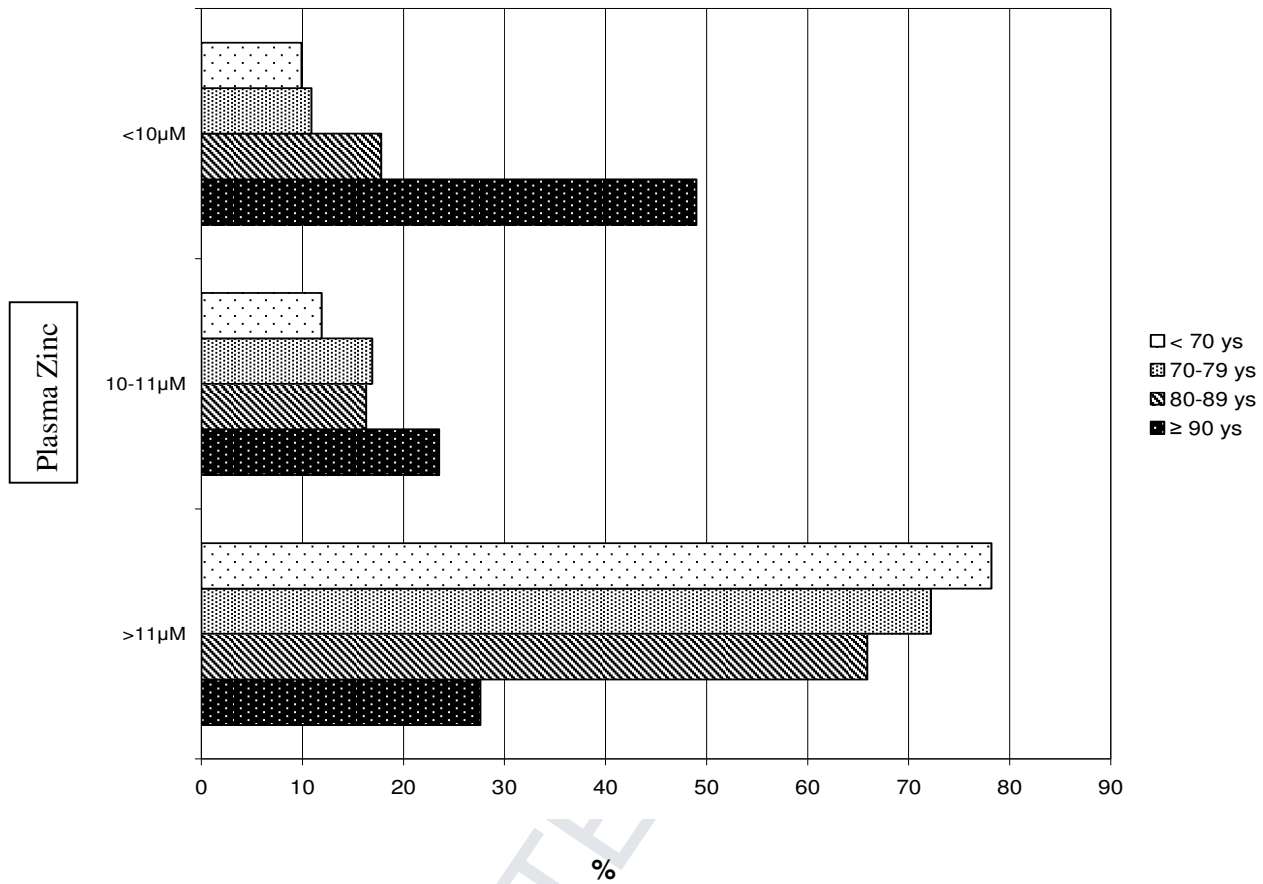
**Table 1.** Mean values  $\pm$  SD of antioxidant enzyme activities and concentration of Zn in plasma at baseline

	<b>&lt;70 ys (A)</b> <b>(n. 387)</b>	<b>70-79 ys (B)</b> <b>(n. 415)</b>	<b>80-89 ys (C)</b> <b>(n. 208)</b>	<b><math>\geq</math> 90 ys (D)</b> <b>(n. 98)</b>
<b>eSOD (U/g Hb)</b>	3124.1 $\pm$ 593.3	3104.6 $\pm$ 641.8	3325.8 $\pm$ 711.5 * <sup>^</sup>	3431.7 $\pm$ 784.1 * <sup>^</sup> <sup>°</sup>
<b>pSOD (U/ml)</b>	20.75 $\pm$ 3.12	21.09 $\pm$ 2.90	20.68 $\pm$ 2.99	20.69 $\pm$ 3.04
<b>CAT</b> <i>(<math>\mu</math>mol/min/mg prot)</i>	20.88 $\pm$ 2.72	20.97 $\pm$ 2.73	20.94 $\pm$ 2.65	20.55 $\pm$ 2.36
<b>GPx</b> <i>(nmol NADPH/min/ml)</i>	0.100 $\pm$ 0.009	0.099 $\pm$ 0.010	0.099 $\pm$ 0.010	0.099 $\pm$ 0.006
<b>plasma Zn (<math>\mu</math>M)</b>	12.77 $\pm$ 2.62	12.33 $\pm$ 2.27 *	11.87 $\pm$ 2.01 * <sup>^</sup>	9.96 $\pm$ 2.13 * <sup>^</sup> <sup>°</sup>

\* p < 0.01 vs group A;      ^ p < 0.05 vs group B      ° p < 0.001 vs group C

eSOD = erythrocyte superoxide dismutase; pSOD = plasma superoxide dismutase; CAT = catalase; GPx = glutathione peroxidase; Zn = zinc

**Figure 1.** Percentage of subjects with reduced ( $<10 \mu\text{M}$ ), marginally reduced ( $10\text{-}11 \mu\text{M}$ ) and normal ( $> 11 \mu\text{M}$ ) concentration of plasma Zn according to age

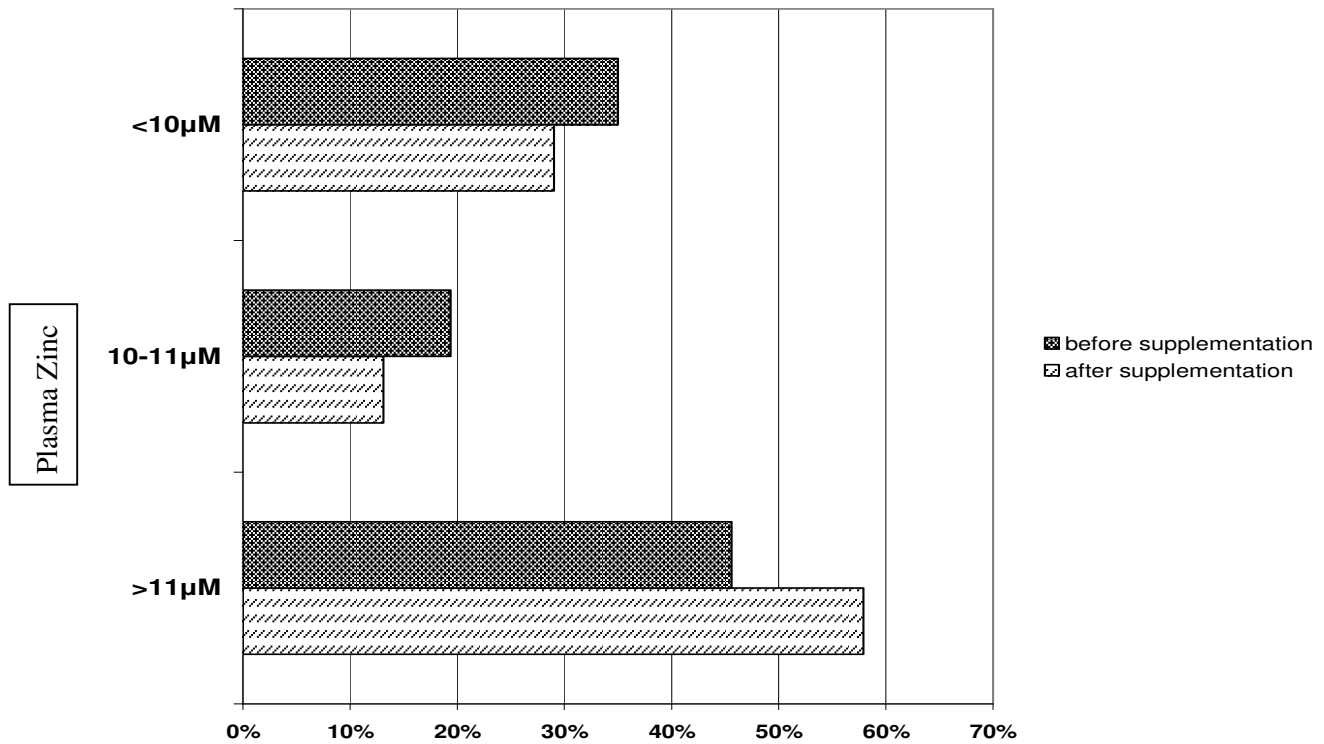


**Table 2.** Gender differences in antioxidant enzyme activities and concentration of Zn in plasma at baseline

	<b>Women</b> <b>(n. 620)</b>	<b>Men</b> <b>(n. 488)</b>	<b>p-value</b>
<b>eSOD (U/g Hb)</b>	3305.68 ± 693.93	3025.20 ± 582.66	<0.0001
<b>pSOD (U/ml)</b>	20.88 ± 2.96	20.84 ± 3.06	0.772
<b>CAT (<math>\mu\text{mol}/\text{min}/\text{mg prot}</math>)</b>	20.98 ± 2.73	20.79 ± 2.61	0.196
<b>GPx</b> <i>(nmol NADPH/min/ml)</i>	0.099 ± 0.01	0.099 ± 0.01	0.454
<b>plasma Zn (<math>\mu\text{M}</math>)</b>	12.06 ± 2.27	12.35 ± 2.68	0.083

eSOD = erythrocyte superoxide dismutase; pSOD = plasma superoxide dismutase; CAT = catalase; GPx = glutathione peroxidase; Zn = zinc

**Figure 2.** Percentage of subjects with reduced ( $<10 \mu\text{M}$ ), marginally reduced ( $10\text{-}11 \mu\text{M}$ ) and normal ( $> 11 \mu\text{M}$ ) concentration of plasma Zn before and after seven weeks of Zn supplementation



**Table 3.** Antioxidant enzyme activities and concentration of Zn in plasma before and after seven weeks of Zn supplementation

	<b>Before Zn supplementation</b>	<b>After Zn supplementation</b>	<b>p-value</b>
<b>eSOD (U/g Hb)</b>	3309.82 ± 607.21	3706.64 ± 591.91	<0.0001
<b>pSOD (U/ml)</b>	21.03 ± 2.98	23.93 ± 3.52	<0.0001
<b>CAT (μmol/min/mg prot)</b>	20.85 ± 2.58	19.17 ± 2.71	<0.0001
<b>GPx (nmol NADPH/min/ml)</b>	0.0992 ± 0.006	0.0989 ± 0.006	<0.05
<b>plasma Zn (μM)</b>	10.76 ± 2.37	11.37 ± 2.29	<0.01

eSOD = erythrocyte superoxide dismutase; pSOD = plasma superoxide dismutase; CAT = catalase; GPx = glutathione peroxidase; Zn = zinc

**Table 4.** Differences in antioxidant enzyme activities and concentration of Zn in plasma after seven weeks of Zn supplementation according to basal plasma Zn levels

	Zn <10 $\mu$ M (a) (n. 38)	Zn 10-11 $\mu$ M (b) (n. 22)	Zn > 11 $\mu$ M (c) (n. 48)
$\Delta$ eSOD (U/g Hb)	404.11 $\pm$ 280.79	358.91 $\pm$ 170.00	400.06 $\pm$ 381.46
$\Delta$ pSOD (U/ml)	3.20 $\pm$ 2.18	2.88 $\pm$ 1.76	2.84 $\pm$ 1.81
$\Delta$ CAT ( $\mu$ mol/min/mg prot)	-1.57 $\pm$ 1.58	-1.92 $\pm$ 1.74	-1.79 $\pm$ 2.50
$\Delta$ GPx (nmol NADPH/min/ml)	-0.0007 $\pm$ 0.002	0.0004 $\pm$ 0.003	-0.0006 $\pm$ 0.002
$\Delta$ plasma Zn ( $\mu$ M)	1.57 $\pm$ 1.87	1.36 $\pm$ 2.34	-0.43 $\pm$ 1.91 * <sup>^</sup>

\* p &lt; 0.0001 vs. group a;

<sup>^</sup> p < 0.01 vs. group b

$\Delta$  eSOD= erythrocyte superoxide dismutase activity after – erythrocyte superoxide dismutase activity before zinc supplementation;  $\Delta$  pSOD= plasma superoxide dismutase activity after – plasma superoxide dismutase activity before zinc supplementation;  $\Delta$  CAT= catalase activity after – catalase activity before zinc supplementation;  $\Delta$  GPx = glutathione peroxidase activity after – glutathione peroxidase activity before zinc supplementation;  $\Delta$  Zn= plasma [Zinc] after – plasma [Zinc] before zinc supplementation