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Counteraction of Adriamycin-Induced Oxidative Damage in Rat Heart by Selenium Dietary Supplementation

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Many reports indicate that dietary selenium, potentially increasing the activity of glutathione peroxidase, could offer protection against free-radical-induced damage. The effects of diets moderately enriched in selenium, as sodium selenite or as a lyophilized selenium-rich food, were studied in rats. Adriamycin, an anticancer drug causing a free-radical-mediated cardiotoxicity, was administered intraperitoneally to some rats. The onset of an oxidative damage was indicated by the increase in the plasma level of reactive oxygen metabolites coupled to a decrease in the total antioxidant activity but without modification of glutathione peroxidase activity, which were observed in all rats, independent of the dietary treatment. On the contrary, in the heart, selenium supplementation caused an increase in the total antioxidant activity, glutathione concentration, and glutathione peroxidase and catalase activities leading to a decreased generation of reactive oxygen metabolites. These results clearly indicate that a moderate Se dietary supplementation counteracts adriamycin-induced cardiotoxicity by preservation of endogenous antioxidants.

KEYWORDS: Selenium; heart; adriamycin; oxidative damage; glutathione peroxidase activity; catalase activity; reactive oxygen metabolites (ROMs); total antioxidant activity; glutathione

INTRODUCTION

Because of the presence of a less developed antioxidant defense mechanism, the heart is particularly vulnerable to injury by reactive oxygen species (ROS) (*1*). Oxidative stress associated with an increased formation of ROS has been proposed to explain the pathogenesis of ischemia-reperfusion injury (*2*) and appears also implicated in cocaine-induced cardiac dysfunction (*3*) and adriamycin-induced cardiotoxicity (*1*).

Adriamycin, an anthracycline antibiotic, is widely used in the treatment of human malignancies; like most of the anticancer drugs, it also causes various toxic effects, the commonest of which is a dose-dependent cardiotoxicity. Cellular damage induced by adriamycin is mediated by the formation of an iron−anthracycline complex that generates free radicals (*1*).

Oxidative stress modifies lipids and proteins leading to lipid peroxidation and oxidation of thiol groups, consequently altering membrane permeability and configuration and producing functional modification of various cellular proteins.

Because of the deleterious effects of oxidative stress, considerable efforts have been made upon using antioxidants to protect the heart against free radicals, and nutritional strategies designed to augment cellular defense systems have been identified as a promising approach to combat oxidative stress-associated disease conditions (*4*, *5*). In this respect, dietary supplementation with selenium (Se), potentially increasing the activity of one of the antioxidant enzymatic systems, glutathione peroxidase (GPx), could offer protection in preventing free-radical-induced cardiac injury.

GPx is one of the most active antioxidant enzymes in the myocardium (*2*), and Se, present in its the active site, is essential for its activity (*6*). One of the major role of this essential trace element within the body is to act as a cofactor of this key antioxidant enzyme in which it contributes to both catalytic activity and spatial conformation. Therefore, any significant modification of the Se status would lead to changes in the activity of the enzyme GPx and have important consequences on the susceptibility of the tissue to oxidative stress (*7*, *8*). In regard to the role of Se, many studies have been concentrated on Se deficiency (*9*, *10*), but little is known about the effect of Se supplementation, particularly with respect to cardiac cells and heart diseases.

In previous studies (*11*, *12*), we demonstrated the protective effect of Se supplementation in cultured cardiomyocytes submitted to hypoxia/reoxygenation. In the present study, using male Wistar rats, we have verified the possibility to counteract oxidative damage by Se dietary supplementation. To verify possible differences because of bioavailability, Se was supplemented to the diet by the addition of sodium selenite (SS) or a lyophilized food (LF) having a high Se content. Specifically,

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the food used was a potato commercially available in Italy, in which the Se content is increased by foliar Se supplementation during plant growth (*13*). The Se final concentration in the two experimental diets was the same (0.1 mg/kg diet) and doubled that of the control diet (0.05 mg/kg diet). To induce an oxidative stress, after 60 days of the dietary treatment, adriamycin was administered intraperitoneally to some rats [10 mg/kg of body weight (b.w.), in a single dose]. A total of 48 h later, the effects of adriamycin administration were observed in the plasma and heart of control and Se-supplemented rats.

MATERIALS AND METHODS

Materials. Diets were prepared by Mucedola (Milano, Italy). Adriamycin was from Pharmacia (Milano, Italy). All chemicals and solvents were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the highest analytical grade.

Animals and Diets. A total of 30 male Wistar rats, aged 21 days, were used. Animals were fed for 4 days a standard diet, and then they were randomly divided into three groups, one fed ad libitum a standard diet with a content of Se corresponding to the normal Se intake for rats (0.05 mg/kg; C diet) and the others fed Se-enriched diets (0.1 mg/kg). Se was supplemented to the experimental diets as sodium selenite (SS diet) or by the addition of the lyophilized form of a common food having a high Se content (LF diet). The food used was a potato commercially available in Italy, in which the Se content is increased by foliar Se supplementation during plant growth (*13*). Food lyophilization was obtained by two 24-h cycles using a Drywinner 3 lyophilizer (Heto-Holten/Jouan Nordic, Hallerød, Denmark). The Se concentration in the lyophilized food was 0.0882 μg/g, as determined by inductively coupled plasma−atomic emission spectrometry (ICP−AES) (*14*). Both SS and LF were added in appropriate amounts to diets during their preparation to obtain a Se final concentration of 0.1 mg/kg diet. All diets were normoproteic (21 g/100 g diet), normolipidic (8 g/100 g diet), and normoglucidic (61.5 g/100 g diet) and contained appropriate amounts of minerals and vitamins.

Animals were housed in individual cages in strictly controlled conditions of temperature (20 ± 2 °C) and humidity (60−70%), with a 12-h dark−light cycle, and were weighted each week. Water and food were provided ad libitum, and food consumption was recorded daily.

After 60 days of dietary treatment, rats of each group were divided into two subgroups, one receiving intraperitoneally 10 mg/kg of b.w. adriamycin and the other a similar volume of physiological solution.

A total of 48 h later, animals were sacrificed with anaesthetic ether, blood was sampled, and the heart was quickly excised, washed in PBS, and immediately frozen at −80 °C. The Animal Care Committee of the University of Bologna approved the study.

Methods. The concentration of reactive oxygen metabolites (ROMs) in plasma was measured by applying the d-ROMs test (Diacron, Grosseto, Italy) as reported in ref *15*. This test is based on the ability of transition metals to react with peroxides by the Fenton reaction. The reaction produces free radicals that, trapped by an alchilamine, form a colored compound detectable at 505 nm. The same method was used to determine the ROM concentration in the lipid fraction of the heart, obtained from about 1 g of tissue by the method of Folch et al. (*16*).

The heart was homogenized in 50 mM potassium phosphate buffer, centrifuged at 3000*g* for 10 min at 0−4 °C, and the resulting supernatant was used for enzymatic assays and to determine the total antioxidant activity (TAA). TAA was measured in both the plasma and heart using the method of Re et al. (*17*), on the basis of the ability of the antioxidant molecules in the sample to reduce the radical cation of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), determined by the decolorization of $ABTS^{\circ+}$, and measured as the quenching of the absorbance at 734 nm. Values obtained for each sample were compared to the concentration−response curve of the standard trolox solution and expressed as micromoles of trolox equivalent (TE).

GPx activity was assayed spectrophotometrically in the plasma and heart according to the method described by Flohe et al. (*18*), which is based on the reduction of oxidized glutathione coupled to the oxidation of NADPH. The disappearance of NADPH is followed at 340 nm. One unit of GPx activity is defined as the amount of enzyme that catalyses the reduction of 1 μmol of NADPH min^{-1}.

Catalase (CAT) activity was measured in the heart according to Aebi (*19*). Briefly, 50 μL of the sample were added to a 3.0 mL cuvette containing 1.95 mL of 50 mM phosphate buffer (pH 7.0) and 1.0 mL of 30 mM hydrogen peroxide. Changes in the hydrogen peroxide absorbance were followed at 240 nm for 1 min at an interval of 15 s. One unit of CAT activity is defined as the amount of enzyme that catalyses the consumption of 1 μmol pf H_2O_2 min^{-1}.

To measure the reduced glutathione (GSH) concentration, the reaction mixture containing 0.3 M phosphate buffer (pH 8.4), 5 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and the sample was incubated for 10 min and the absorbance was measured at 412 nm (*20*). The level of GSH was determined from the standard curve.

All data are means ± SD. Statistical analysis was by the one-way analysis of variance, the Bonferroni *t* test, and Student's *t* test.

RESULTS

During the 12 weeks of the dietary treatment, food consumption was similar in all rats and no differences were detected in the body weight among the different groups (data not shown).

As reported in **Figure 1A**, in the basal condition, the plasma ROM level was similar in the different groups. After adriamycin administration, plasma ROMs increased in all rats, independent of the dietary treatment.

In basal conditions, plasma TAA was also similar in all groups (**Figure 1B**), and it was significantly decreased by the adriamycin administration, regardless of the dietary treatment.

In plasma, the GPx activity was similar in all animals in basal conditions, and it was not modified by adriamycin administration (**Figure 1C**).

In the basal condition, the cardiac ROM level was similar in all groups; after adriamycin administration, it increased in controls and, to a lesser extent, in rats fed the SS diet but not in LF fed ones (**Figure 2A**).

In both the basal condition and after adriamycin administration, cardiac TAA was significantly higher in Se-supplemented rats than in controls. Within the same dietetic group, TAA was not modified by the induction of the oxidative stress (**Figure 2B**).

In basal conditions, GSH appeared significantly more concentrated in the heart of Se-supplemented rats than in controls and adriamycin administration did not significantly affect its concentration (**Figure 2C**).

In both basal conditions and after adriamycin administration, cardiac GPx activity appeared significantly influenced by Se dietary supplementation (**Figure 3A**). As in the plasma, the enzyme activity was not influenced by adriamycin administration.

The CAT activity was similar in the heart of rats fed the different diet in basal conditions; in control rats, adriamycin administration caused a significant decrease in the enzyme activity, which kept its level constant or slightly increased in Se-supplemented rats (**Figure 3B**).

DISCUSSION

Adriamycin, an anthracycline antibiotic, is a widely used anticancer agent. Despite its high antitumor efficacy, its use in clinical chemotherapy is limited because of diverse toxicities, including cardiac toxicity. Oxidative damage to membrane lipids and other cellular components is believed to be a major factor in the adriamycin toxicity (*21*), which is mediated by the formation of an iron−anthracycline complex that generates free

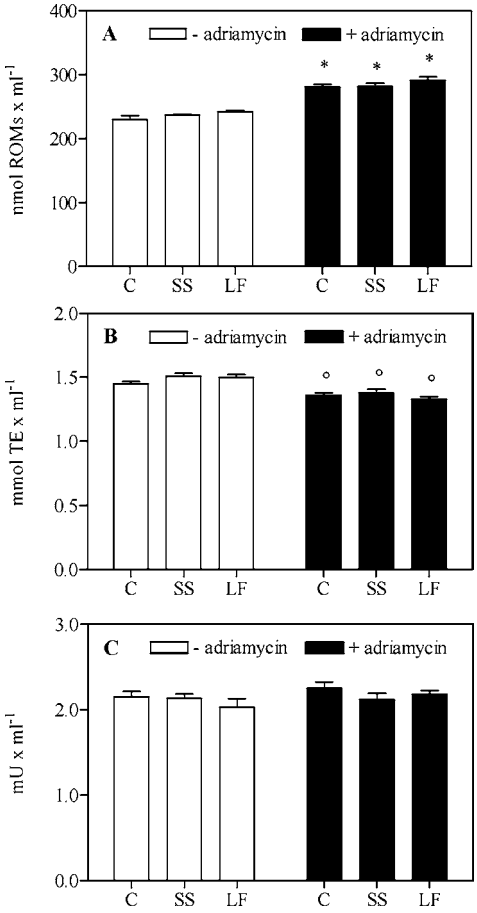

Figure 1. ROMs level, TAA, and GPx activity in the plasma of rats fed control or Se-supplemented diets, in basal conditions and after adriamycin administration. ROMs level (A), TAA (B), and GPx activity (C) were measured in the plasma as reported in the Materials and Methods. Data are means ± SD of five animals in each group. Statistical analysis was performed by the one-way analysis of variance to compare the three different diets in basal condition (not significant) and after adriamycin administration (not significant) and by Student's *t* test to compare the same dietetic group in basal conditions and after adriamycin administration [(°) $p < 0.01$; (*) $p < 0.001$].

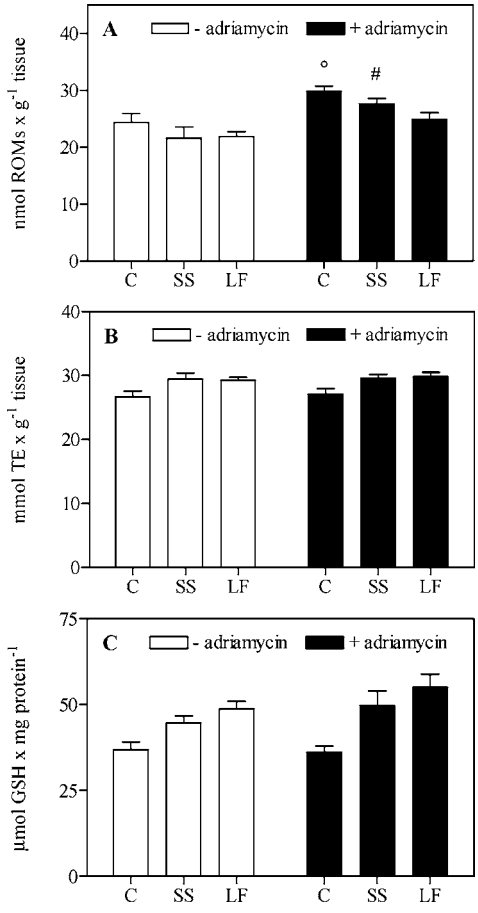

Figure 2. ROMs level, TAA, and glutathione concentration in the heart of rats fed control or Se-supplemented diets, in basal conditions and after adriamycin administration. ROMs level (A), TAA (B), and glutathione concentration (C) were measured in the heart as reported in the Materials and Methods. Data are means ± SD of five animals in each group. Statistical analysis was performed by the one-way analysis of variance and the Bonferroni *t* test to compare the three different diets in both basal conditions [ROMs level, not significant; TAA, $p < 0.05$; GSH concentration, $p < 0.01$ (C versus LF fed $p < 0.05$)] and after adriamycin administration [ROMs level, $p < 0.05$ (C versus LF fed $p < 0.05$); TAA, $p < 0.05$; GSH concentration, $p < 0.01$ (C versus SS fed and C versus LF fed $p < 0.05$)] and by Student's *t* test to compare the same dietetic group in basal conditions and after adriamycin administration [(#) $p < 0.05$; (°) $p < 0.01$].

radicals (*1*). In our study, the onset and the extent of an oxidative condition following the administration of adriamycin was clearly indicated by the increase in the plasma ROM level, which is reported as a valid indicator for oxidative damage measurements by different authors (*22*−*24*).

Adriamycin administration significantly decreased plasma TAA, which can be considered as the summation of the interactions among various antioxidants. As already observed in cultured cardiomyocytes (*11*, *12*), this reduction in TAA reflects a consumption of antioxidant molecules because of the adriamycin-induced increase in ROS production. No modifications in the plasma GPx activity were detected after the induction of the oxidative stress; similar results were obtained by Fadillioglu and Erdogan (*25*) who, using a higher dose (20 mg/kg of b.w.) of adriamycin, observed an increase in plasma thiobarbituric acid reactive substances (TBARS) without modification in the plasma GPx activity.

Results obtained in the plasma in basal conditions and after the oxidative stress were independent of the dietary treatment; Se supplementation did not influenced either plasma ROM level and TAA and did not cause an increase in the plasma GPx activity. Plasma GPx (GPx 3) is a tetrameric glycoprotein, immunochemically distinct from erythrocyte GPx, synthesized in a range of tissues, with the major source being the kidney (*26*). The GPx 3 activity does not appear strictly related to the Se status; patients with renal diseases have very low GPx 3 activity not associated with Se deficiency (*27*). The insensitiveness of plasma GPx activity to dietary Se supplementation observed in this study could partially explain the lack of protection against oxidative stress observed in the plasma of Se-supplemented rats. Se is an essential constituent of a number of enzymes other than GPx, some of which have antioxidant functions. Among them, selenoprotein P is an abundant extracellular glycoprotein that is rich in selenocysteine. Four isoforms of selenoprotein P are present in rat plasma, and evidence supports the functions of the protein in Se homeostasis and oxidant defense; however, the mechanisms of these apparent functions remain speculative, and much work on the mechanism of the selenoprotein P function lies ahead (*28*). The measurement of selenoprotein P in human plasma has shown that it is depressed by Se deficiency and by cirrhosis (*28*), but little is

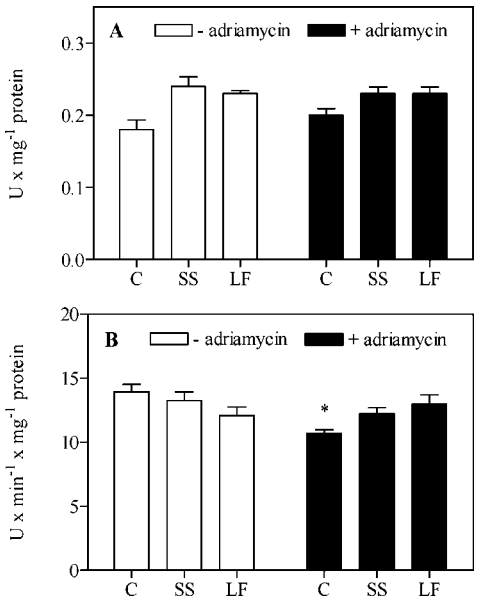

Figure 3. GPx and CAT activities in the heart of rats fed control or Se-supplemented diets, in basal conditions and after adriamycin administration. GPx (A) and CAT activities (B) were measured in the heart as reported in the Materials and Methods. Data are means ± SD of five animals in each group. Statistical analysis was performed by the one-way analysis of variance and the Bonferroni *t* test to compare the three different diets in both basal conditions [GPx activity, $p < 0.01$ (C versus SS fed and C versus LF fed $p < 0.05$); CAT activity, not significant] and after adriamycin administration [GPx activity, $p < 0.05$; CAT activity, $p < 0.05$ (C versus LF fed $p < 0.05$)] and by Student's *t* test to compare the same dietetic group in basal conditions and after adriamycin administration [(*) $p < 0.001$].

known about the influence of Se supplementation. Although, in this work, we did not measure selenoprotein P, the invariance of plasma TAA could indicate an invariance of this antioxidant protein. This is in agreement with the work of Persson-Moschos et al. (*29*), who reported that, at a low Se status, selenoprotein P levels increased in a similar fashion after supplementation with different forms of Se but, at a normal Se status, no significant effects of supplementation with the same amount of Se were observed.

Multiple effects of Se supplementation were observed in the heart. First of all, the onset of an adriamycin-induced oxidative damage, indicated by the increase in the ROM level, was clearly observed in control rats, and it was partially prevented by the SS-supplemented diet and completely prevented by the LF-supplemented one. Therefore, the extent of the adriamycin-induced oxidative stress appears modulated, in the heart, not only by the amount of Se in the diet but also by the form in which Se is supplemented to the diet. In SS- and LF-fed rats, the reduction in ROM production was coupled to an increase in cardiac TAA. In previous studies, using cultured cardiomyocytes (*11*, *12*), we demonstrated that GPx represents one of the main determinants of cardiac TAA. In this study, the intake of exogenous antioxidants was the same in all rats, because they received similar diets, which differed only for the Se content; therefore, we focused our attention on endogenous antioxidants as determinants for TAA modifications.

The GSH concentration was higher in the heart of Se-supplemented rats, in both basal conditions and after the oxidative stress. An improvement in GSH levels in the liver and brain of diabetic mice after selenite treatment has been recently reported also by Sheng et al. (*30*). Because glutathione is one of the essential compounds for maintaining cell integrity because of its reducing properties and participation in the cell metabolism (*31*) and the glutathione redox cycle is one of the most important intracellular antioxidant system, the GSH increase could be one of the mechanisms for cardiac protection by Se supplementation.

Se dietary supplementation increased the cardiac GPx activity in both basal conditions and after adriamycin administration, confirming the possibility to improve the enzyme activity by a moderate Se dietary supplementation. Within the same dietary group, cardiac GPx activity was not modified by adriamycin administration. Mukherjee et al. (*1*) observed a decrease in the enzyme activity after adriamycin treatment, but this discrepancy could be explain by the lower dose of the drug used in our study (10 mg/kg of b.w. versus 30 mg/kg of b.w.). According to Mukherjee et al., the CAT activity decreased in control rats after adriamycin administration even at the dose used in this study. CAT appeared therefore more sensitive than GPx to adriamycin treatment. These two enzymes have complementary intracellular localization, with CAT being located almost exclusively in peroxisomes, while GPx is found in the cytosol and mitochondrial matrix, as well as complementary catalytic activities. In the present work, the CAT activity was not decreased by adriamycin administration in Se-supplemented rats. Preservation of the CAT activity, improvement of GPx activity, and increase in GSH concentration all contributed to the enhancement of TAA, leading to a decrease in adriamycin-induced peroxide formation. Furthermore, an increased GSH concentration and GPx activity could enhance TAA, also influencing the regeneration of tocopheryl radicals, therefore maintaining tocopherol levels.

Dietary Se may also influence other endogenous antioxidants, e.g., the enzyme thioredoxin reductase (TRxR). Both GPx and TRxR are produced by cardiac tissue (*32*, *33*) and catalyze reactions essential to the protection of cellular components against oxidative and free-radical damage. TRxR displays a broad specificity and plays an important antioxidant role, not only by supplying reducing equivalents to the thioredoxin/thioredoxin peroxidase systems but also in directly reducing H_2O_2 and lipid peroxides (*34*). Furthermore, TRxR is involved in recycling the antioxidant vitamins C and E (*35*). Both GPx and TRxR endogenous activities are dependent upon an adequate supply of the micronutrient Se. Recently, a significant decrease in the TRxR activity was observed in the heart of rats receiving a Se-free diet when compared to those receiving a normal diet, and a dose-dependent increase in the TRxR activity with an increasing Se content in the diet was observed in both the liver and heart (*36*).

Although further studies are needed to completely elucidate the contribution of all endogenous antioxidants to TAA and the mechanisms by which they are influenced by Se supplementation, our results clearly indicate that, in the heart, a moderate Se dietary supplementation counteracts oxidative damage.

Oxidative damage is the final result of an unbalance between free-radical production and the entity of antioxidant defenses, and the onset of the damage may be accounted to free-radical overproduction, antioxidant deficiency, or both. It is well-documented that adriamycin causes oxidative damage by increasing free-radical production (*1*); in this study, this is indicated by the increase in the ROM level observed in the plasma of all rats and in the heart of control rats. In the heart, Se supplementation increased the GSH concentration and CAT activity and counteracted the adriamycin-induced decrease in the GPx activity; these increased endogenous antioxidant defenses balanced by the adriamycin-induced overproduction

of free radicals, therefore re-equilibrating the ratio of oxidants/antioxidants and limiting the oxidative damage.

The protective effect of Se appears therefore ascribable to the increased cardiac concentration/activity of endogenous antioxidant observed in SS- and LF-fed rats. This protective effect has not been observed in the plasma, where Se supplementation did not increase the TAA and GPx activity.

The protection against oxidative damage appeared most significant when Se was supplemented as LF; in fact, although the GSH concentration and antioxidant enzyme activities were only slightly higher in the LF diet fed rats than in SS diet fed rats, the ROM level was significantly lower in the first group than in the second one. This could be accounted for by a higher availability of Se contained in LF, other antioxidant components of LF, or both and could underline the importance of food as a source of nutrients in dietary supplementation.

Prospective epidemiological studies have shown that the incidence of numerous cardiovascular pathologies is correlated with the body Se status, and it has been demonstrated that the preischemic body Se status is one of the major determinants of the outcome of myocardial ischemia *in vivo* in rats, probably because it influences the cellular redox status (*37*). Further studies are needed before drawing conclusions on the effects of Se supplementation in humans, but in individuals at risk of cardiac oxidative damage, dietary Se supplementation, particularly via consumption of Se-rich foods, may be taken into account as a safe and convenient way of increasing antioxidant protection.

ABBREVIATION USED

ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); b.w., body weight; CAT, catalase; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); GPx, glutathione peroxidase; GSH, reduced glutathione; ICP−AES, inductively coupled plasma−atomic emission spectrometry; LF, lyophilized food; ROS, reactive oxygen species; ROM, reactive oxygen metabolite; Se, selenium; SS, sodium selenite; TAA, total antioxidant activity; TBARS, thiobarbituric acid reactive substances; TRxR, thioredoxin reductase; TE, trolox equivalent.

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