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UTILIZATION OF EPR DETECTION TO MONITOR NUTRITIONALLY-INDUCED OXIDATIVE STRESS IN RATS

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EPR is mostly used in biological studies to measure direct and indirect indicator of free radical damage. Experimental data from the laboratory illustrated the usefulness of spin-trapping technique using EPR to detect increased primary hydroxyl radicals production in magnesium deficient rats muscles. Moreover, spin labeling technique and EPR to monitor the physical properties of lipid bilayer membranes have been used in copper deficient rats. This allowed to draw relationships between membrane physical properties and oxidative stress-induced dysfunction in this model. Moreover, beside those well recognized utilization of EPR and because of its high specificity, we used EPR detection to monitor total antioxidant capacity (TAC) in plasma. We improved a method, defined as Lat-RPE, which is based on the principle of the original Trolox Equivalent Antioxidant Capacity, and using EPR detection of the lag time for ABTS cation radical appearance. We applied this method in nutritional models of antioxidant depletion and of oxidative stress, i.e. respectively vitamin E deficiency and high sucrose diet in rats. Altogether, allowing the evaluation of the prevention capacity of antioxidants in plasma against ROS-induced damage to biomolecular targets, our results are in favor of the use of Lat-RPE as a biomarker of oxidative stress.

INTRODUCTION

There is growing interest for nutrition and oxidative stress interrelation. However, demonstration of a role for free radicals in any physiological or pathological process is relatively complex. The choice of indicator of free radical activity is problematic essentially because of the lack of any "gold standard" assay. That is why multiple approaches are used that include: measurement of direct or indirect indicator of free radical activity and measurement of endogenous antioxidant levels (Jackson, 1999). In this context, the most direct technique available for the detection of free radicals is electron paramagnetic resonance (EPR) spectroscopy.

EPR detection of direct and indirect free radical activity (spin-trapping and spin labeling techniques)

Because it allows to detect radicals that are normally present at very low concentration or whose life-time are too short to be detected by direct EPR analysis, EPR measurement of free radical production using spin-trapping has been used in our laboratory in magnesium-deficient rats. An inflammatory syndrome occurs in this model, Mg deficiency elevates circulating levels of inflammatory cytokines (Rayssiguier, Durlach, Gueux, Rock & Mazur, 1993), and the question addressed was that inflammatory cells may generate oxidative injury that could be involved in muscle lesions from these animals. In accordance with a lower level of protein thiol groups and a higher thiobarbituric acid-reactive substances (TBARS) in magnesium deficient rats muscles, while using DMSO and PBN as the spin trap, we demonstrated the contributing role of free radicals in the formation of skeletal muscle lesions that occur in this model (Rock, Astier, Lab, Vignon, Gueux, Motta & Rayssiguier, 1995a). Moreover, while no spectrum was detected in the absence of DMSO, our results suggest that primary free radicals rather than lipid derived secondary radicals may be generated in this conditions. The hyperfine split constants being consistent with PBN/CH3°; the methyl radical detected in these condition probably originated from hydroxyl radicals because DMSO in excess is known to react preferentially with this particularly species (Burkitt & Mason, 1991). The favorable conditions used in our study thus highlighted the great advantage of EPR using spin-trapping technique as it allowed us to identify primary hydroxyl radicals which are generated in magnesium deficient rat muscles.

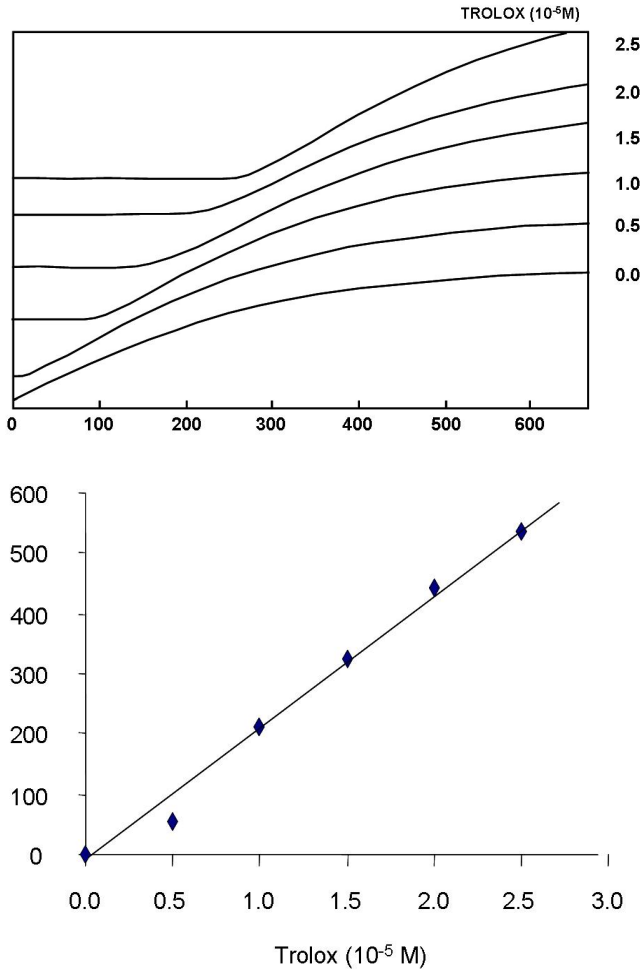

Fig. 1 Kinetic of ABTS radical apparition with increasing concentration of Trolox (0.5 to 2.5 10^{-5}M) (A). Standard curve of the dependence of lag time on antioxidant concentration of Trolox (B). The concentrations of ABTS, H_2O_2 and horseradish peroxidase (HRP) were determined by measuring their absorbance using $\varepsilon_{340\ nm}$ = 36,000 $M^{-1}cm^{-1}$ for ABTS (Childs & Bardsley, 1975), $\varepsilon_{240\ nm}$ = 43.6 $M^{-1}cm^{-1}$ for H_2O_2 (Bielski & Allen, 1977) and $\varepsilon_{403\ nm}$ = 100,000 $M^{-1}cm^{-1}$ for HRP (Nakajima & Yamazaki, 1979). HRP, ABTS, a calculated amount of PBS were premixed with either Trolox or plasma sample before the reaction was initiated with H_2O_2. To 770µl of homogenate was added 80µl of 0.1 mol/L ABTS, 25µl of 10^{-7} mol/L HRP and 100µl Trolox or plasma. 25µl of 1 mol/L H_2O_2 was added at room temperature to start the reaction and time was measured. Three capillaries of 20 µl were filled with the sample and transferred into a quartz tube. The kinetic of the ABTS radical appearance was recorded at room temperature using an EPR spectrometer (Brucker ECS 106, Karlsruhe, Germany) operating at 9.79 GHz with the following conditions: microwave power, 20mW; modulation frequency, 10KHz; modulation amplitude, 1.018 G; time constant, 81.92 ms; receiver gain, 1.10^5.

Spin labeling is also one of the most often used technique, using EPR detection, in biological studies. It allows the investigation of physical properties of lipid bilayer membranes which are highly dependent on their constitutive lipids and proteins molecules and consequently on oxidative stress damage that could appear on these molecules. Detection of the rotational motion of the 16-doxyl -stearic acid (16-DS) spin label by EPR has been used to study indirect consequences of oxidative stress in another nutritional-induced oxidative stress model. Copper deficiency in rats leads to anemia (Cohen, Keen, Hurley & Lonnerdal, 1985) involving, at least partly, decreased antioxidant defense, via decreased Cu Zn-superoxide dismutase (Cu-Zn-SOD) activity (Bartoli, Palozza & Piccioni, 1992). Results from the laboratory suggest in post weaning rat model that copper deficiency anemia is the consequence of a shorter survival of erythrocytes because of an increased destruction of these cells (Rock, Gueux, Mazur, Motta & Rayssiguier, 1995b). Impaired lipid metabolism in copper deficient rats (Nassir, Mazur, Gueux, Serougne & Rayssiguier, 1994) may also lead to alterations in the composition and the physical properties of red blood cells (RBC) membranes. The determination of the correlation time of 16-DS indicated an increased fluidity state of RBC membranes, in accordance with a decreased cholesterol to phospholipid ratio found in RBC from copper-deficient rats. We suggested that this apparently paradoxical result between shorter survival and higher fluidity state of RBC may reflect an adaptation mechanism. Hence, after in vitro H_2O_2 treatment, RBC from copper-deficient rats were found more susceptible to hemolysis. Interestingly, the correlation time of 16-DS indicated a significantly higher rigidity compared to control confirming higher susceptibility of copper deficient RBC membranes to peroxidation. (Rock *et al.*, 1995b). This result highlighted the usefulness of spin labeling technique to set up a relation between membrane physical properties and biological (dys)function.

EPR detection to monitor antioxidant defense measurement

Oxidative stress is defined as an "imbalance between oxidants and antioxidants in favor of the oxidants" that could lead to molecular and cellular damages (Sies, 2000). Together with direct free radical production and biomolecular damage measurements, disturbances in the antioxidant system may thus be useful to indicate conditions of oxidative stress in any physiological or pathological process. Because of the difficulties in measuring each antioxidant components separately and the interactions among them in the serum or plasma, number of assays have been introduced for the measurement of total antioxidant capacity of body fluids (Cao, Alessio & Cutler, 1993; Miller, Rice-Evans, Davies, Gopinathan & Milner, 1993). We improved a method, defined as Lat-RPE and based on the principle of the original 2,2'-azino-bis(3-ethylbenzathiazoline-6-sulfonic acid (ABTS) one (Miller *et al.*, 1993). ABTS is a peroxidase substrate which, when oxidized in the presence of H_2O_2 in a typical peroxidative reaction generates a metastable radical with a high chemical stability (Arnao, Cano, Hernandez-Ruiz, Garcia-Canovas & Acosta, 1996). Several analytical strategies, namely decolorization assay, fixed-time point inhibition assay, reaction rate inhibition assay and lag phase measurement assay have been proposed by using this system (Rice-Evans & Miller, 1994). Because of its high specificity we used EPR to follow the kinetic of appearance of ABTS cation radical and determined the lag phase.

The typical course of ABTS oxidation in the presence of increasing concentration of Trolox, an hydrophilic analogue of vitamin E, retards the lag time for $ABTS^{\circ+}$ appearance in a dose dependent manner as it is shown in Fig.1 confirming the results obtained by Bartosz and Bartosz (1999) while using spectrophotometric detection. Moreover, it is well known that the antioxidant capacity of the cell is mainly attributable to the enzyme system, whereas that of plasma is mostly accounted for by low molecular weight antioxidants. These "sacrificial" compounds, rapidly consumed during the scavenging of ROS, can be regenerated or replaced by new dietary-derived compounds (Ghisella, Serafini, Natella & Scaccini, 2000). One can thus assume that the "sacrificial" antioxidant capacity can be evaluated by the lag time before ABTS cation radical appearance as it is done by Lat-RPE. To validate this hypothesis, we applied Lat-RPE on plasma obtained from rats fed a diet either adequate or deficient in vitamin E for two weeks. We observed that vitamin E deficient rats have a lower TAC as measured by Lat-RPE (Fig. 2). Moreover we observed a good relationship between Lat-RPE and plasma vitamin E level in this model. Vitamin E being one of the most important scavenging antioxidant in plasma, this result validate our method. To further improve this hypothesis, we then applied Lat-RPE to a model in which plasma vitamin E is decreased not because of a low dietary vitamin E intake but rather as a consequence of a higher consumption of this antioxidant to counteract ROS production. This was

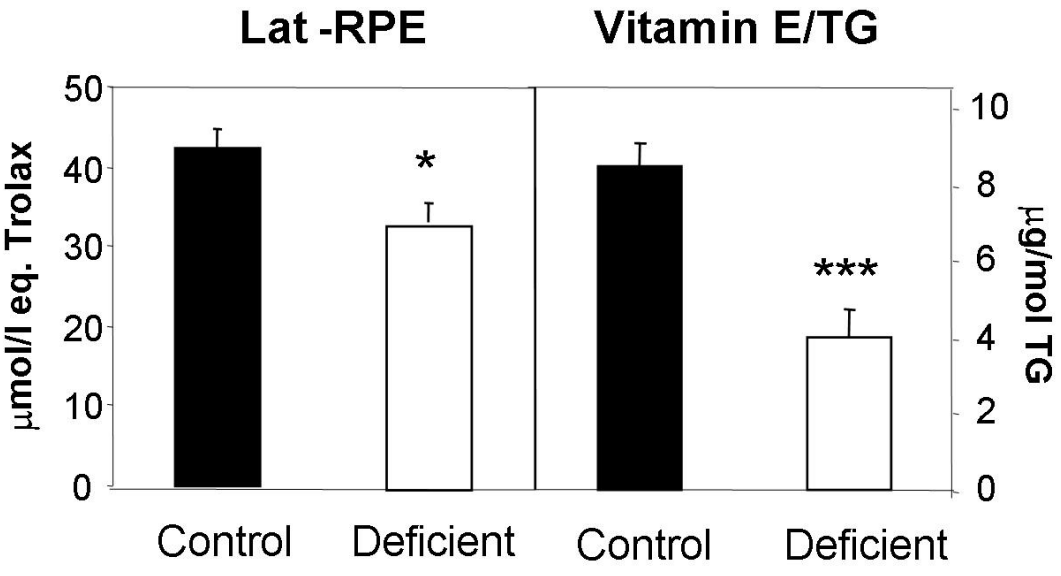

Fig. 2. Plasma Lat-RPE and vitamin E levels of control and dietary vitamin E deficient rats. Triacylglycerol levels (Biotrol, Paris, France) were determined in plasma by enzymatic procedures. Plasma vitamin E was assayed by reversed-phase high phase liquid chromatography (HPLC) using an hexane extract as previously described (Gueux, Azaïs-Braesco, Bussière, Grolier, Mazur & Rayssiguier, 1995). Values are expressed as means ± SEM, n=8 * $P< 0.05$. *** $P< 0.001$.

done with another model of nutritional-induced oxidative stress by feeding rats with diets containing high level of sucrose since sucrose feeding is known to induce oxidative damages (McDonald, 1995). That oxidative stress should occur in this model is supported by previous findings showing that fructose has deleterious effect when antioxidant defenses are decreased, in copper deficient rats (Rayssiguier, Gueux, Bussière & Mazur, 1993).

Furthermore, we recently showed prooxidant effect of sucrose even in case of normal vitamin and mineral supply (Busserolles, Rock, Gueux, Mazur, Grolier & Rayssiguier, 2002). We observed that after two weeks of experiment, whereas plasma vitamin E was effectively lower in the sucrose group, Lat-RPE was not different between the two groups (Fig. 3). We know that many antioxidants probably react rapidly with both free radical generated by the reaction and ABTS radical (Yu & Ong, 1999), thus ABTS radical should be detected only after all these antioxidants in the medium are depleted. Consequently, other molecules than vitamin E in this model with such antioxidant power may act. Moreover, in this model susceptibility of erythrocyte to hemolysis is not different between the two groups. The hypothesis we made was that TAC being not decreased and susceptibility of erythrocytes to hemolysis not increased in the sucrose group, Lat-RPE should represent a biomarker to evaluate risk of oxidative stress. To confirm this hypothesis, we decided to

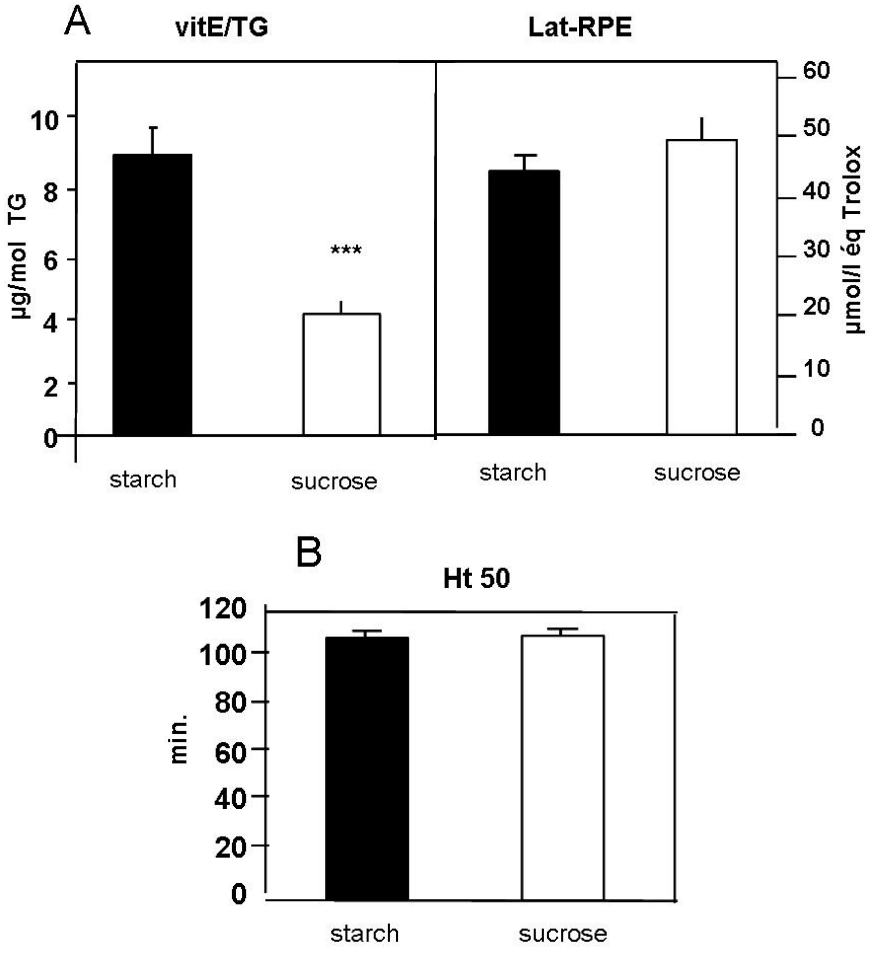

Fig. 3. Plasma Lat-RPE and vitamin E levels of rats fed a starch or sucrose rich diet for 2 weeks (A). Susceptibility of erythrocytes from rats fed a starch or sucrose rich diet for 2 weeks (B). 150 µl of a 4% suspension of erythrocytes in buffered saline was mixed with 100 µl of 6 mmol/L AAPH. Hemolysis of isolated red blood cells was performed with 4% of washed erythrocytes into 2.5 ml of PBS. The mixture was incubated at 37°C under continuous stirring and the absorbance decreases at 623nm were recorded as a function of time. The hemolysis (%) was calculated on the basis of the maximum absorbance (100%) in the aliquots of erythrocytes completely hemolyzed in distilled water. The time required for 50% hemolysis was calculated. Values are expressed as means ± SEM, n=8. *** $P < 0.001$.

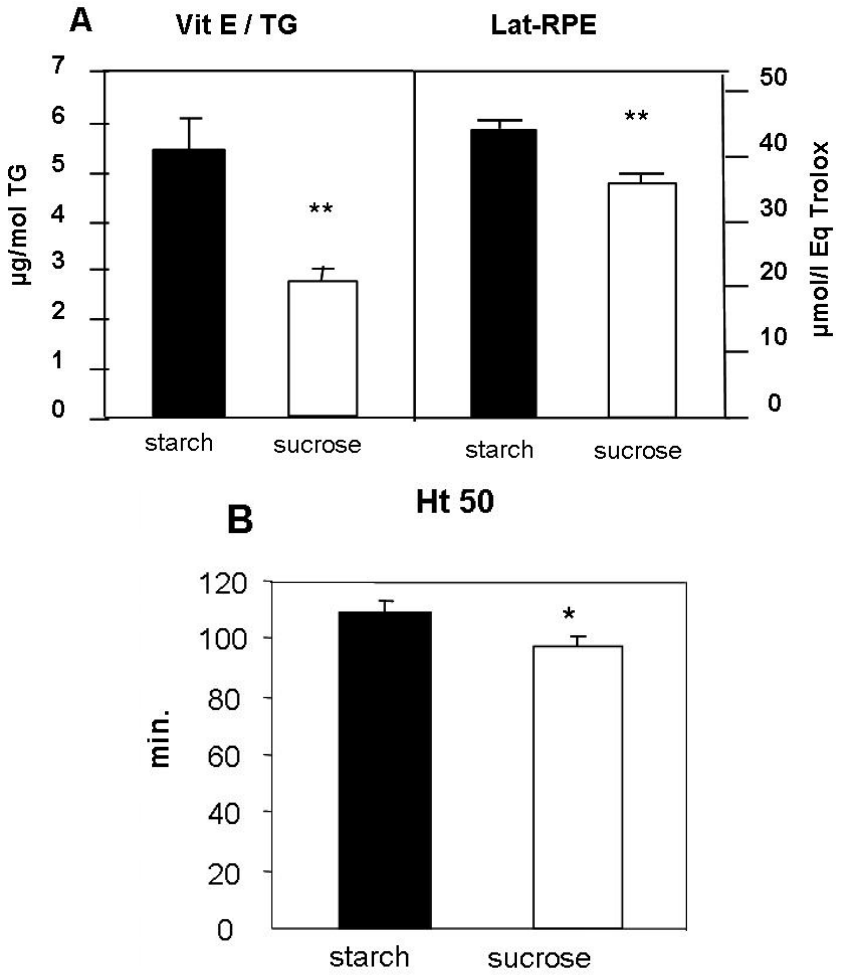

Fig. 4. Plasma Lat-RPE and vitamin E levels of rats fed a starch or sucrose rich diet for 6 weeks (A). Susceptibility of erythrocytes from rats fed a starch or sucrose rich diet for 6 weeks (B). Values are expressed as means ± SEM, n=8 * P< 0.05. ** P< 0.01.

see if after longer exposure to high sucrose diet, rats have a different Lat-RPE level than the control group. Rats fed for six weeks a high sucrose diet indeed have a lower TAC compared to starch fed rats (Fig. 4) suggesting that the total scavenging antioxidant capacity of the plasma is exceeded. Furthermore, erythrocytes susceptibility of this rats to hemolysis being increased, this highlights the importance of measuring the global sacrificial antioxidant pool in plasma.

In conclusion, beside the "classical" utilization of EPR to measure direct and indirect indicator of free radical damage, we described an improved method to evaluate TAC in plasma. Because of the high specificity of EPR, and while measuring the lag time before ABTS cation radical appearance, we show that Lat-RPE allows to measure the "sacrificial" antioxidant capacity in plasma. Finally, our results from both dietary model of oxidative stress in rats are consistent with the idea that TAC measured by this way could be of important usefulness in nutritional and more generally biological and medical studies because it evaluates the prevention capacity of antioxidants in plasma against ROS-induced damage to biomolecular targets.

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REFERENCES

Arnao M. B., Cano A., Hernandez-Ruiz J., Garcia-Canovas F. & Acosta M. (1996). Inhibition by L-ascorbic acid and other antioxidants of the 2,2'-azino-bis(3-ethylbenzathiazoline-6-sulfonic acid) oxidation catalyzed by peroxidase: a new approach for determining total antioxidant status of foods. *Anal. Biochem.*, **236**, 255-261.

Bartoli G. M., Palozza P. & Piccioni E. (1992). Enhanced sensitivity top oxidative stress in Cu,ZnSOD depleted rat erythrocytes. *Biochem. Biophys. Acta*, **1123**, 291-295.

Bartosz G. & Bartosz M. (1999). Antioxidant activity: what do we measure? *Acta Biochimica Polonica*, **46**, 23-29.

Bielski B. H. J. & Allen A. O. (1977). Mechanism of disproportionation of superoxide. *J. Phys. Chem.*, **8**, 1048-1050.

Burkitt M. J. & Mason R. P. (1991). Direct evidence for in vivo hydroxyl-radical generation in experimental iron overload: an ESR spin-rapping investigation. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 8440-8444.

Busserolles J., Rock E., Gueux E., Mazur A., Grolier P. & Rayssiguier Y. (2002). Short-term consumption of a high sucrose diet has a pro-oxidant effect in rats. *Br. J. Nutr,* **87**, 337-342..

Cao G. Alessio H. M. & Cutler R. G. (1993).Oxygen-radical absorbance capacity assay for antioxidants. *Free Rad. Biol. Med.*, **14**, 303-311.

Childs R. E. & Bardsley W. G. (1975). The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. *Biochem. J.*, **145**, 93-103.

Cohen N. L., Keen C. L., Hurley L. S. & Lonnerdal B. (1985). Determinants of copper-deficiency anemia in rats. *J. Nutr.*, **115**, 710-725.

Ghisella A., Serafini M., Natella F. & Scaccini C. (2000). Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. *Free Rad. Biol. Med.*, **29**, 1106-1114.

Gueux E., Azaïs-Braesco V., Bussière L., Grolier P., Mazur A., Rayssiguier Y. (1995). Effect of magnesium deficiency on triacylglycerol-rich lipoprotein and tissue susceptibility to peroxidation in relation to vitamin E content. *Br. J. Nutr.*, **74**, 849-856.

Jackson M. J. (1999). An overview of methods for assessment of free radical activity in biology. *Proc. Nutr. Soc.*, **58**, 1001-1006.

McDonald R. B. (1995). Influence of dietary sucrose on biological aging. *Am. J. Clin. Nutr.*, **62**, 284S-293S.

Miller N. J., Rice-Evans C., Davies M. J., Gopinathan V. & Milner A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.*, **84**, 407-412.

Nakajima R. & Yamazaki I. (1979). The mechanism of indole-3-acetic acid oxidation by horseradish peroxidases. *J. Biol. Chem.*, **254**, 872-878.

Nassir F., Mazur A., Gueux E., Serougne C. & Rayssiguier Y. (1994). Apolipoprotein A-I, A-IV and E synthesis in the liver of copper-deficient rats. *Lipids*, **29**, 727-729.

Rayssiguier Y., Durlach J., Gueux E., Rock E. & Mazur A. (1993). Magnesium and ageing. I. Experimental data: importance of oxidative damage. *Magnesium Res.*, **6**, 369-378.

Rayssiguier Y., Gueux E., Bussière L. & Mazur A. (1993). Copper deficiency increases the susceptibility of lipoproteins and tissues to peroxidation in rats. *J. Nutr.*, **123**, 1343-1348.

Rice-Evans C. A. & Miller N. J. (1994). Total antioxidant status in plasma and body fluids. *Methods Enzymol.*, **234**, 279-293.

Rock E., Astier C., Lab C., Vignon X., Gueux E., Motta C. & Rayssiguier Y. (1995a) Dietary magnesium deficiency in rats enhances free radical production in skeletal muscle. *J. Nutr.*, **125**, 1205-1210.

Rock E., Gueux E., Mazur A., Motta C. & Rayssiguier Y. (1995b). Anemia in copper-deficient rats: role of alterations in erythrocyte membrane fluidity and oxidative damage. *Am. J. Physiol.*, **269**, C1245-C1249.

Sies H. (2000). What is oxidative stress? [In:] Keany J. F. Jr. (Eds.), *Oxidative stress and vascular disease* (pp. 1-8). Boston: Kluwer Academic Publishers.

Yu T. W. & Ong C. N. (1999). Lag-time measurement of antioxidant capacity 2,2'-azino-bis(3-ethylbenzathiazoline-6-sulfonic acid): rationale, application, and limitation. *Anal. Biochem.*, **275**, 217-223.