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Oxidative status of plasma and muscle in rabbits supplemented with dietary vitamin E

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Abstract

Thirty New Zealand white rabbits, mean weight 2 kg, were divided into three equal groups balanced for body weight and randomly assigned to a diet containing 60 (C), 150 (T1) or 375 (T2) mg/kg of all-*rac*-α-tocopheryl acetate. After 29 days, the animals were slaughtered. α-Tocopherol was assayed in muscle (*longissimus dorsi*) and plasma; triglycerides and cholesterol (total, high density lipoprotein, low density lipoprotein) were analysed in plasma; reactive oxygen metabolites (ROMs) were analysed in serum; and thiobarbituric acid-reactive substances (TBARS) were analysed in muscle. There were no body weight and food intake differences between the groups. The plasma vitamin E and vitamin E:lipid ratio were significantly higher in groups T1 and T2 than in C, but increases were not linearly related to dietary levels. Muscle α-tocopherol concentrations in the treated groups were significantly higher than in C, and linearly related ($R = .67$) to the vitamin E:lipid ratio. ROM and vitamin E levels in blood were inversely related ($R = .74$), with ROMs significantly lower in the treated groups than in C. The 60-mg/kg dose of C recommended by the National Research Council was unable to control ROM production. Lipid oxidation in muscle was significantly lower in T2 than in the other groups, and TBARS correlated significantly with muscle vitamin E ($R = .61$) and serum ROM ($R = .73$). These data suggest that vitamin E supplemented at 375 mg/kg diet can effectively control ROM production and improve muscle lipostability. ROM assay provides a useful indirect estimate of the oxidative status of muscle in vivo.

Keywords: Rabbit; ROMs; α-Tocopherol; Lipoperoxidation

1. Introduction

There is now considerable emphasis on modification of the fatty acid composition of animal tissues in order to produce "healthier" foods. However, increasing the degree of unsaturation of the muscle cell membranes by dietary manipulation results in increased oxidative deterioration [1,2], and oxidized lipids in foods may have adverse effects on health [3]. The susceptibility of muscle to lipid oxidation can be reduced by antioxidants. α-Tocopherol (α-ol) is a highly effective chain-breaking antioxidant [4,5] and its presence within muscle cell membranes reduces lipid oxidation, improving the quality characteristics of meat such as colour, flavour, texture and nutritional value, and also extending its shelf life [6]. Dietary vitamin E supplementation results in elevated concentrations of α-ol in cell membranes, especially mitochondrial and microsomal membranes, significantly lowering susceptibility to lipid oxidation [7–9].

The optimal concentration of vitamin E required to protect membrane lipids in meat from oxidative damage has not been clearly established. This study was designed to analyse the effects of increasing supranutritional dietary doses of α-ol acetate on biomarkers of oxidative status in the blood and muscle of adult rabbits.

2. Methods and materials

2.1. Diets and study design

Thirty New Zealand White rabbits of average weight (2000 g) were divided into three numerically equal groups

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by restricted randomisation, ensuring the groups were balanced for body weight. The groups were allocated to receive a pelleted diet containing 60 mg (control group, C), 150 mg (treated group, T1), or 375 mg (treated group, T2) all-*rac*-α-tocopheryl acetate/kg for 29 days prior to slaughtering. The chemical composition of the basal pellet diet (Panzoo spa, Forlì, Italy) was moisture humidity 11.50%, crude protein 17.00%, fat (ether extract) 3.50%, crude fiber 14.00%, ash 9.00% and vitamins and minerals (added to the premix by manufacturers).

Rac-α-tocopheryl acetate (Rhône-Poulenc Animal Nutrition, France) was added to the basic pellets to give the 60, 150 and 375 mg/kg levels. The vitamin E content of the control diet was recommended by the National Research Council (NRC) [10].

The animals were housed in stainless steel cages in controlled temperature and light conditions (12-h light/dark cycle) and were allowed free access to feed and water. Feed intake and weight gain were monitored weekly on an individual basis. Rabbits were slaughtered at the end of the trial, by which time the animals were 90 days old.

2.2. Blood and muscle samples

At slaughtering, plasma samples were drawn into 7-ml vacutainers (protected from the light) containing lithium heparin for all 30 rabbits; in 6 rabbits per each group, serum samples were also drawn into 6 ml vacutainers containing a gel and clot activator (Becton and Dickinson, Milan, Italy). Within 30 min of collection, the samples were centrifuged at 4°C and 1000 *g* for 15 min; the plasma, protected from the light, and serum samples were stored at −35°C pending analyses for α-ol, total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), triglycerides (TRI; in plasma) and reactive oxygen metabolites (ROMs; in serum). A sample of *longissimus dorsi* muscle was taken from each carcass and stored at −35°C pending determination of α-ol and thiobarbituric acid-reactive substances (TBARS). The latter is a measure of the resistance of the muscle lipids to oxidation.

2.3. Determination of α-ol

Vitamin E was determined in plasma as described by Vuilleumier et al. [11] and in muscle as described by Zapel and Csallany [12]. Plasma was always protected from the light. Briefly, the plasma was thawed and vortexed to disperse any precipitate; 200 μl was pipetted into a 4-ml centrifuge tube and diluted with 200 μl of water. Ethanol (400 μl) was added, followed by 800 μl of n-hexane with continuous vortexing. The tube was sealed, vortexed a further 5 min, and centrifuged at 2000 *g* for 5 min. An aliquot from the hexane phase was transferred into conical vials. Eighty microlitres of sample extract in hexane phase was introduced into an isocratic straight-phase high-performance liquid chromatography (HPLC) equipped with a Phenomenex column (250 × 4.6 mm) filled with sphereclone 5 μm Si, a Kontron model 422 pump, an automatic injector (Kontron model 465) and a Kontron fluorescence spectrophotometer (SFM25). Mobile phase flow rate was 1.7 ml/min. Retention time was about 3.5 min. The effluent passed through the fluorimeter set at excitation 298 nm, emission 328 nm. The resulting peak areas calculated by integrator were compared with the peak areas of standard solutions of α-ol treated exactly like the plasma samples (external calibration).

Muscle samples (100 mg) were thawed and homogenised in a Tissumizer with 20 volumes of acetone. The homogenate was centrifuged (1300 *g*, 10 min.), the supernatant filtered through a 0.2-μm filter, evaporated under a stream of nitrogen and resuspended in ethanol (100 μl) containing α-tocopheryl acetate as internal standard. This mixture was evaporated under a stream of nitrogen, resuspended in diethyl ether (30 μl) and methanol (80 μl) and 50 μl was injected into the Kontron HPLC now equipped with a C18 reverse-phase symmetry column (150 × 4.6 mm, 3.5 μm, Waters, Milford, MA, USA) and diode array detector model 440. The mobile phase was 100% methanol with flow rate 1.5 ml/min. Retention times were α-ol, 4.1 min; α-tocopheryl acetate, 5.0 min. Tocopherols were monitored at 292 nm. α-ol concentrations in the samples were calculated from peak area responses using a standard curve established by chromatography of known amounts of pure α-ol. Standard stock solutions of α-ol and α-tocopheryl acetate (100 and 200 μmol/l ethanol, respectively) were used to establish the calibration curves.

2.4. Plasma lipid analysis

TC, HDL-C, LDL-C and TRI were determined using dry chemistry kits and an automatic Ektachem DT 60 II system (Kodak SpA, Milan, Italy). The vitamin E:lipid ratio (mg/g) was calculated as reported by Thurnham et al. [13], dividing the plasma α-ol concentration by the sum of TC plus TRI concentrations in plasma.

2.5. Measurement of oxidative stability of muscle

The oxidative stability of rabbit muscle was determined using a modification of the method described by Monahan et al. [1], itself a combination of the Kornbrust and Mavis [14] method for the induction of lipid peroxidation and the Beuge and Aust [15] method for determination of extent of lipid peroxidation by assaying two TBARS. One gram of tissue homogenate with 9 ml of 1.15% KCl was incubated at 37°C in 40 mmol Tris-maleate buffer (pH 7.4) with 1 mmol $FeSO_4$ (to catalyse lipid peroxidation). After 300 min, an aliquot was removed for measurement of TBARS, expressed as nmol malondialdehyde (MDA)/g of muscle tissue per min.

Table 1
Plasma concentration of α-ol, TC, HDL-TC, and LDL-TC, TRI, serum concentration of ROMs and muscle concentration of α-ol observed in the three groups under study

	C	T1	T2	S.E.M.
Plasma α-ol (μmol/L)	6.83[a]	9.80[b]	12.12[b]	1.11
Plasma α-ol/lipid (mg/g)	1.94[a]	3.07[b]	4.12[c]	0.36
Plasma TC (mmol/L)	1.78	1.69	1.63	0.24
Plasma HDL-TC (mmol/L)	0.65	0.72	0.66	0.07
Plasma LDL-TC (mmol/L)	0.63	0.64	0.60	0.22
Plasma TRI (mmol/L)	2.56	2.06	1.79	0.58
ROMs (Carr units)	237.00[a]	107.75[b]	37.68[b]	40.73
Muscle α-ol (μg/g)	1.0[a]	4.21[b]	5.66[b]	0.99

[a,b,c] Means in the same row with different superscripts differ $P < .05$.

2.6. Determination of ROMs in serum

We used a reagent kit (DIACRON Srl., Grosseto, Italy) and spectrophotometer to determine ROMs in serum. The method depends on the production of a stable coloured organic radical cation in acidic pH (4.8) with absorption maximum at 505 nm. This chromophore is produced by quantitative reaction of serum ROM and Fe^{3+}, Fe^{2+}, and so forth, with the chromogenic compound. The ROM reagent kit measures not only ROM species existing in the matrix, but also the species developing during Fenton reaction following the lowering of pH and the release of ferryl ions from serum proteins [16,17]. In a few words, the ROOH hydroperoxide, in presence of Fe, can generate $RO^{\cdot}$ (alkoxyl radical) or $ROO^{\cdot}$ (peroxyl radical) that are titrate from N,N-dimethyl-p-phenylendiamine added as chromogen to the solution. The measurement done by the kit cannot be considered specific, as reactive oxygen species (ROS) are not; however, the kit estimates mostly alkoxyl and peroxyl radicals. The results are expressed in Carr units (1 Carr unit corresponds to 0.024 mmol/l of H_2O_2).

2.7. Statistical analysis

Growth and carcass characteristics, vitamin E (muscle and plasma levels), plasma lipids, ROMs and TBARS at 300 min were analysed by one-way analysis of variance; significant differences ($P < .05$) between treatment means were determined using Scheffé multiple comparison test [18]. The relationship between muscle vitamin E levels and the ratio of plasma α-ol to TC plus TRI was analysed by linear regression [18]. A nonlinear regression model was used to determine the relationship between ROMs and plasma vitamin E, between induced TBARS formation at 300 min and muscle α-ol and induced TBARS formation at 300 min and ROMs.

3. Results

With regard to initial body weight (C = 2025.5 g; T1 = 2015.5 g; T2 = 2008.9 g; pooled S.E.M. = 21.40) and final body weight (C = 2945.5 g; T1 = 2946.5 g; T2 = 2880.5 g; pooled S.E.M. = 43.39), no significant differences were observed among groups; therefore, dietary vitamin E supplementation did not affect growth. The mean daily feed intake was not significantly different in the three groups (C = 157.0 g/day; T1 = 157.1 g/day; T2 = 158.9 g/day), with no influence of feeding on the vitamin E intake.

Vitamin E supplementation had no effect on the lipid concentrations in plasma (Table 1). The plasma α-ol concentration and the vitamin E:lipid ratio in plasma were significantly higher in the T1 and T2 groups than in Group C (Table 1). However, the increase in plasma vitamin E was not linear: The T1 and T2 groups were supplemented with 2.5 and 6.3 times the control level, respectively, whereas plasma α-ol levels were only 1.4 and 1.8 times higher, respectively, than controls. The increase in plasma vitamin E:lipid ratio also showed a saturation effect, being 1.6 times and 2.1 times higher in T1 and T2, respectively, than in C (Table 1).

Mean α-ol levels in the muscle from both treated groups were significantly higher than in controls, with a saturation effect again evident: Levels were 4.2 and 5.7 times higher, respectively, in the T1 and T2 groups than Group C, but there was no significant difference between the treated groups' values. We found a good linear correlation ($R = .67$; $P < .001$) between muscle vitamin E content and the α-ol to lipid ratio (Fig. 1). The ROM data are shown in Table 1. The levels of ROS were significantly lower in the T1 and T2 groups (−55% and −84%, respectively) compared with Group C. ROM concentrations were related to plasma vitamin E levels by an equation of the form $y = ax^{-b}$, where y is ROM concentration and x is plasma vitamin E concentration (see Fig. 2). R for the correlation was .74, with $P < .001$. The curve shows that above about 7 μmol/l of α-ol, a large increase in the concentration results in only a slight improvement of oxidative status of rabbit serum. Conversely, below this level, a small decrease in vitamin E results in a sharp increase in ROM levels.

The production of TBARS in muscle in the presence of Fe^{2+} was significantly affected by the level of vitamin E supplementation (Fig. 3). Basal means of TBARS values (nmol MDA/g tissue ± S.E.) were C = 42.31 ± 2.0; T1 =

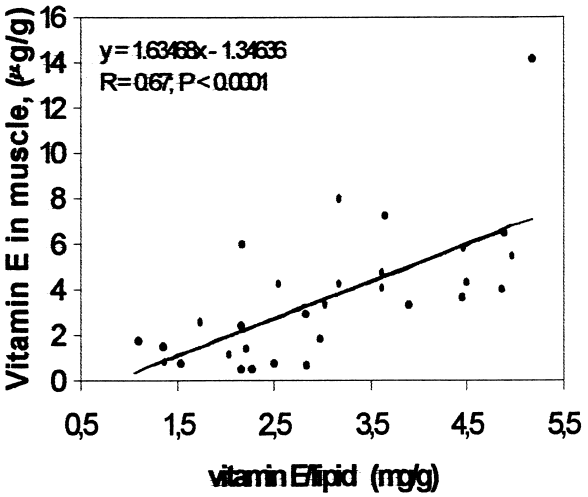

Fig. 1. Relation between muscle vitamin E (μg/g) concentration and plasma vitamin E:lipid (mg/g) ratio.

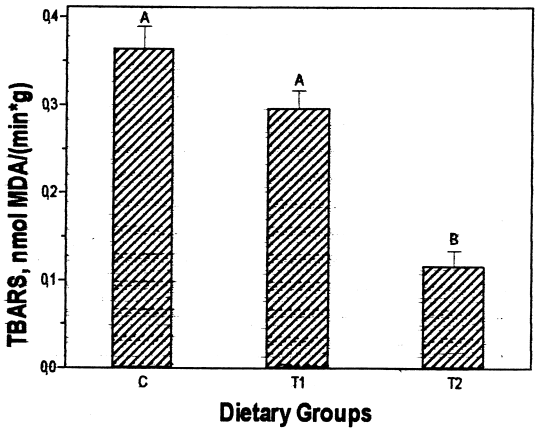

Fig. 3. Effect of dietary vitamin E (mg all-*rac*-α-tocopheryl acetate/kg diet) on TBARS production (nmol MDA/min $\times$ g) minutes following Fe^{2+} catalysed oxidation. The bars show the mean $\pm$ S.E., $n = 10$. The groups are described in the experimental design. Bars not sharing a common superscript are significantly different ($P < .01$).

40.17 $\pm$ 2.0; T2 = 26.92 $\pm$ 1.9 and means of values at the 300th min were C = 151.18 $\pm$ 9, 3; T1 = 129.50 $\pm$ 5.6; T2 = 61.72 $\pm$ 6.5, with the increments C = 256.5%, T1 = 228.5% and T2 = 128.4% between time 0 and 300th min.

The rate of TBARS production (and hence, lipid peroxidation) decreased significantly ($P < .01$) as dietary vitamin E increased, from 0.36 $\pm$ 0.03 nmol MDA per min per g tissue in Group C to 0.12 $\pm$ 0.02 nmol MDA per min in T2. However, only the TBARS level in Group T2 was significantly ($P < .01$) lower than control. The rate of TBARS production showed a significant ($R = .61$, $P < .001$) inverse correlation with the concentration of vitamin E in muscle (Fig. 4) and a significant direct correlation ($R = .73$, $P < .001$) with serum ROM levels (Fig. 5).

4. Discussion

Our results show that dietary supplementation with increasing doses of α-tocopheryl acetate increases the α-ol concentration and the α-ol:lipid ratio in plasma, but that the relationship is nonlinear. Wiseman et al. [19] found a linear-log relation between dose in diet and plasma vitamin E concentrations in the range 49–775 tocopherol equivalents per kg of diet. Burton et al. [20] also found that the vitamin E:lipid ratio was greater in human adults supplemented with vitamin E than in nonsupplemented individuals, but that there was no significant difference in this ratio between those supplemented with 100 IU/week and those given 2800 IU/week.

This saturation effect could be due to factors limiting the absorption or transport of the vitamin. Thus, it has been reported [21] that in experimental animals, the efficiency of α-ol absorption decreases as the quantity given increases. On the other hand, the plasma LDLs have a good vitamin E transport capacity; in fact, it has been reported that in 90% of humans, LDLs are not saturated with vitamin E [22].

By contrast, we found a greater increase in the vitamin E content of muscle than of plasma. At the highest dietary

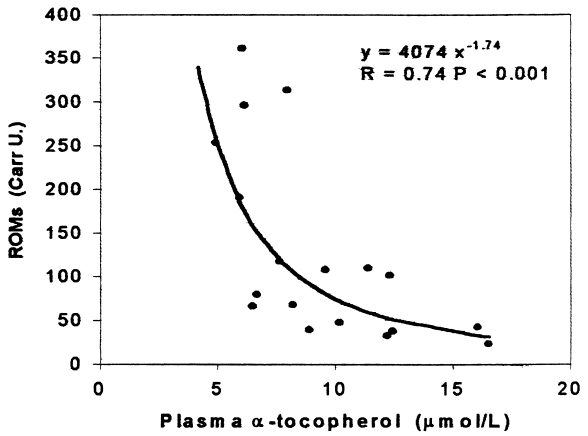

Fig. 2. Correlation between ROM level in serum (Carr units) and vitamin E (μmol/l) levels in plasma.

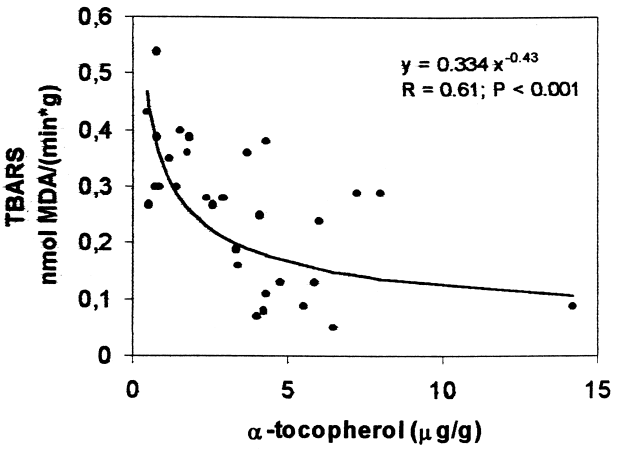

Fig. 4. Relation between TBARS production (nmol MDA/min $\times$ g) minutes following Fe^{2+} catalysed oxidation and vitamin E content in muscle.

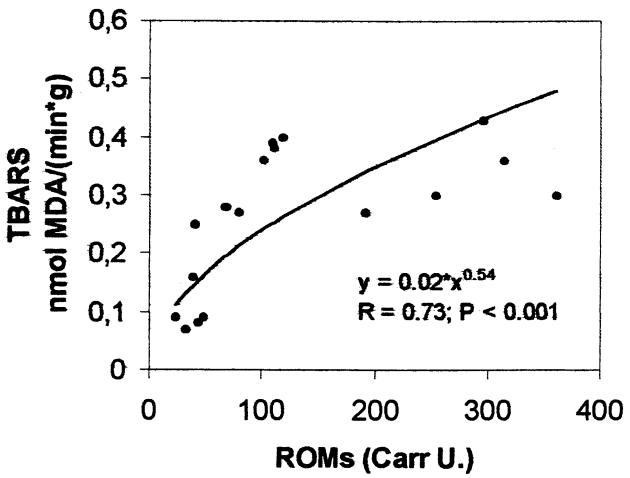

Fig. 5. Relation between TBARS production (nmol MDA/min × g) minutes following Fe^{2+} catalysed oxidation in muscle and ROM levels in serum.

dose, the α-ol content of muscle was 5–7 times higher than in controls. Other data also suggest that skeletal muscle is able to accumulate α-ol [23]. Thus, De Winne and Dirinck [24] supplemented chickens with α-tocopheryl acetate at 200 ppm for three weeks before slaughter, and found that the α-ol content of leg and breast muscle was 6–7 times greater than in the corresponding muscles of chickens on control diet.

We also found that vitamin E levels in muscle were linearly related to the plasma α-ol:lipid ratio. Thus, the ratio of vitamin E in plasma lipids to that in muscle cell lipids was approximately constant as vitamin E intake increased [20]. This provides further evidence that the plasma α-ol: lipid ratio is a good biomarker of tissue levels of the vitamin.

In the dose range we used, vitamin E had no effect on the composition of plasma lipids. Two studies on New Zealand White rabbits also reported that vitamin E supplementation had no effect on plasma lipoproteins [25,26]. However, at a higher level of vitamin E supplementation (775 mg/kg), Wiseman et al. [19] found lowered LDL levels in plasma. Furthermore, other investigators reported hypocholesterolemic effects of vitamin E supplementation [27,28]. It was proposed that vitamin E stimulated cholesterol-7 α-hydroxylase activity, thereby increasing the conversion of cholesterol to bile acids [27].

It is likely that the discordant findings in this area are to be attributed to differences in the vitamin E dosages employed. More recently, it has been suggested that high doses of vitamin E may decrease hepatic very low density lipoprotein secretion due to effects on tocopherol binding protein [21].

ROMs are defined as oxygen-centred free radicals and their metabolites [29]. We determined the concentration of ROMs in serum, mostly as levels of alkoxyl and peroxyl radicals that originated from hydroperoxides in presence of ferryl ions. ROMs are generated during normal metabolism and, in particular, during the activation of lipid metabolism involving both the phospholipid and arachidonic acid pathways in the cell membranes [30,31]. ROM can enter into reactions that, if uncontrolled, can impair animal performance [32]. TBARS are the final products of oxidative processes generated starting from ROM; therefore, the ROM evaluation is an earlier marker of the oxidative process that in our study, correlates with levels of the final products estimated as TBARS. In other words, ROM determination in serum provides an indirect estimate of the postmortem oxidative stability of muscle lipids.

Antioxidants can control lipid peroxidation [33]. In particular, vitamin E, which is lipid-soluble, is thought to act as a chain breaker, reacting with the lipid peroxyl radical LOO· to produce LOOH and the tocopherone [34]. There is also evidence that vitamin E interferes with the production of free radicals in leukocytes and possibly other cells [35].

It is noteworthy that ROM levels were influenced by vitamin E supplementation in our study; however, the effect was only evident at plasma α-ol concentrations above 3 μg/ml. Hence, the average plasma α-ol concentrations obtained with NRC-recommended levels of vitamin E supplementation are not sufficient to control the oxidative state of plasma.

The induced TBARS value has been shown to be an effective measure of the oxidative stability of muscle tissue [36]. As was the case with ROMs in serum, TBARS values in muscle were only lowered at high dietary vitamin E levels, and the reduction was significant only at dietary doses more than 6 times those recommended by the NRC. This is consistent with the results of Sheldon et al. [37], who found that dietary supplements had to be more than 5 times greater than NRC-recommended levels to significantly reduce TBARS.

Both ROMs and TBARS were related to vitamin E concentrations by nonlinear equations (Figs. 2 and 4). A similar relation between oxidative stability and dietary vitamin E was found in cooked leg meat from broilers [38]. This type of relationship implies that below a threshold vitamin E concentration, lipoperoxidation occurs very readily.

A similar concept was suggested for LDL oxidation by Karlsson et al. [22]. Here, lipid peroxidation seems to occur in an all-or-nothing manner: Only when the LDL particle is almost totally depleted of antioxidant does peroxidation occur significantly, but at this point, it explodes in the typical manner of a free radical chain reaction [39].

Our data indicate that the in vivo threshold of vitamin E efficacy differs from that in vitro. We found that in vivo, ROM levels were kept under control when plasma vitamin E was above 3 ppm, whereas muscle lipostability in vitro only seemed to be significantly enhanced by vitamin E levels above 5 ppm. This is probably related to nonavailability in vitro of the additional protection provided by plasma antioxidants [19].

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