Disruption of redox homeostasis and carcinogen metabolizing enzymes changes by administration of vitamin E to rats.

Disruption of redox homeostasis and carcinogen metabolizing enzymes changes by administration of vitamin E to rats

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A B S T R A C T

Aims: A large meta-analysis of randomized clinical trials has seriously questioned chemoprevention based on vitamins including vitamin E (VE), and an increased risk for cancer among long-term users was actually seen. However, the mechanism underlying these findings still remain unknown. To clarify the mechanism, in an *in vivo* model we studied the putative disruption of redox homeostasis and the perturbation of carcinogen metabolizing enzymes determined by VE.

Main methods: Male Sprague–Dawley rats were treated *ip* with either 100 or 200 mg/kg b.w. daily for 7 or 14 consecutive days. Controls received vehicle only. Cytochrome P450 (CYP) content, CYP-reductase, CYP-linked monooxygenases, as well as phase-II and the antioxidant enzymes catalase and NAD(P)H:quinone reductase were investigated in both liver and kidney. Free radical species in tissue subcellular preparations were measured by electronic paramagnetic resonance (EPR) spectroscopy coupled to a radical probe technique.

Key findings: No substantial changes of hepatic xenobiotic metabolism enzymes were determined by VE. Conversely, a powerful booster effect of various renal phase-I carcinogen bioactivating enzymes at both dosages and observational times was recorded. While no relevant changes of post-oxidative phase-II reactions were found in the liver, a significant inactivating effect was caused by VE in renal tissues. Antioxidant enzymes were found mainly downregulated by the treatment. In the kidney, a marked free radical over-generation linked to CYP induction was observed.

Significance: This study proved that VE acts as a co-carcinogen and pro-oxidant agent. Such epigenetic mechanisms may contribute to explain the harmful outcomes observed in humans.

1. Introduction

Vitamin E (VE) is a term that encompasses a group of potent, lipid-soluble, chain-breaking antioxidants composed of a group of eight naturally occurring tocopherols and tocotrienols of subtypes α, β, γ, and δ. Of these, α-tocopherol subunit seems to be the most abundant, biologically active, and it is preferentially absorbed and accumulated in humans [1,2]. VE is absorbed together with lipids, then transported to the liver and this process is similar for all forms of vitamin E. ω-Hydroxylation of the tocopherol phytyl side-chain by cytochrome P450 (CYP) play a major role in regulating tissue tocopherol concentrations, followed by stepwise removal carbon moieties then converted to the carboxyethyl-hydroxychromane (CEHC) metabolite that in turn is largely excreted in the urine after glucuronide conjugation [3].

Since its discovery and isolation, VE has been considered a "safe" agent [4], and its antioxidant activity has persuaded many scientists to further investigate its abilities in preventing chronic diseases, especially those believed to have oxidative components such as cardiovascular disorders, atherosclerosis, and cancer [5–9]. Although the relationship between VE supplementation and cancer risk has been investigated in many epidemiological studies and clinical trials, results are conflicting, and its putative protective role is now under debate more then ever.

The Nutritional Prevention of Cancer (NPC) study and the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study showed a

Abbreviations: APND, aminopyrine N-demethylase; b.w., body weight; CAT, catalase; CEHC, carboxyethylhydroxychromane; CYP, cytochrome P-450; CYP-red, NADPH-(CYP)-c-reductase; DCPIP, dichlorophenolindophenol; EPR, electronic paramagnetic resonance; EROD, ethoxyresorufin *O*-deethylase; ECOD, ethoxycoumarin *O*-deethylase; GST, glutathione S-transferase; *ip*, intraperitoneal; MROD, methoxyresorufin *O*-demethylase; NQO1, NAD(P)H:quinone reductase; p-NFH, p-nitrophenol hydroxylase; PROD, pentoxyresorufin *O*-dealkylase; UDPGT, UDP-glucuronosyl transferase; VE, vitamin E.

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reduction in prostate cancer risk of respectively 63% and 32% after selenized yeast and α-Tocopherol supplementation [10,11]. In addition, a randomized-controlled trial reported that VE, in combination with selenium and β-carotene, reduced the overall cancer mortality [12], and these observations were supported by epidemiologic data [13,14]. However, several subsequent trials and a meta-analysis largely rejected these results. Many of these studies pointed out that VE was neither beneficial nor harmful, not supporting the routine use of VE [15–19] while more recent studies show how VE taken at high doses (far exceeding the intake derived from dietary sources) revealed a dose-dependent relationship between VE supplementation and all-cause mortality [20] as well as representing a hazard for several cancer forms including prostate, non-melanoma skin cancer, colorectal cancer and lung adenocarcinoma [21–24]. These outcomes have once again taken the role of VE to the heart of scientific debate, particularly considering that more and more people take large amounts of VE for its alleged therapeutic or prophylactic value [21]. However, the underlying mechanism of the observed detrimental effects is still unknown.

Evidence from both *in vitro* and *in vivo* models suggested that VE might increase the hepatic production of cytochrome P450 (CYP). CYP2C11, and several CYP1 and CYP2 family members were found to be up-regulated in VE-supplemented rats [22–24]. Such induction phenomenon may increase the biotransformation of ubiquitous precarcinogens (co-carcinogenicity) and generate an over-production of oxygen centred radicals [25–32].

In an *in vivo* model we investigated whether the harmful outcomes of VE are mediated by the perturbation of carcinogen metabolizing enzymes and by disruption of the redox homeostasis.

2. Materials and methods

2.1. Chemicals

Acetic acid (PubChem CID:176), aminopyrine (PubChem CID:6009), bovine serum albumin (PubChem CID:16132389), dichlorophenolindophenol (PubChem CID:13726) (DCPIP), epinephrine (PubChem CID:5816), ethoxycoumarin (PubChem CID:35703), Folin-Ciocalteu reagent, glycerol (PubChem CID:753) from Sigma-Aldrich, glucose 6-phosphate (PubChem CID:5958) and glucose 6-phosphate dehydrogenase from Roche Diagnostic; L-glutathione oxidized (PubChem CID:71308714), L-glutathione reduced (PubChem CID:745), methanol (PubChem CID:5958), methoxyresorufin (PubChem CID:119220), Nicotinamide adenine dinucleotide phosphate in oxidized (PubChem CID:5886) and reduced form (PubChem CID:5886) (NADP+ and NADPH), p-nitrophenol (PubChem CID:980), pentoxyresorufin (PubChem CID:107683), perchloric acid (PubChem CID:24247), resorufin (PubChem CID:69462), sodium dithionite (PubChem CID:24489), trichloroacetic acid (PubChem CID:6421), Triton X-100 (PubChem CID:5590), Trizma (PubChem CID:16218782), umbelliferone (PubChem CID:4412127), 1-chloro-2,4-dinitrobenzene (PubChem CID:6), 1-naphthol (PubChem CID:7005), 7-ethoxyresorufin (PubChem CID:3294) from Sigma-Aldrich. Vitamin E in the form of DL-all-*rac* α-Tocopherol (PubChem CID: 2116).

All others chemicals were highest purity commercially available.

2.2. Animal treatment

Male Sprague–Dawley rats (Harlan Laboratories S.r.l.), weighing 150–180 g, were housed under controlled condition (12 h light–dark cycle, 22 °C, 60% humidity).

All the experimental procedures were carried out in conformity with protocols endorsed by the National Academy of Science guidelines and in accordance with EU Directive 2010/63/EU for animal experiments. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Bologna. All efforts were made to minimize suffering. Animals were fed a rodent chow and had tap water ad libitum. After 1-week acclimatization period, all animals were randomly divided into six experimental units of six animals each. VE was dissolved in corn oil and administered intraperitoneally (ip) at doses of 100 mg/kg or 200 mg/kg b.w. daily for 7 or 14 consecutive days. Animals assigned to control groups received ip an equal volume of vehicle (corn oil).

2.3. Tissue collection and subcellular fraction preparation

Rats were fasted 16 h prior sacrifice, which occurred 24 h after the last treatment. They were sacrificed by decapitation, in accordance with approved Ministerial procedures appropriate to the species. Liver and kidney were rapidly removed and processed separately. After extensive mincing with a pair of scissors, the tissue was homogenized in sucrose with IKA Ultra-Turrax homogenizer. The S9 fraction (9000 $\times g$) from liver was then prepared [33]. The post-mitochondrial supernatant so obtained was then centrifuged for 60 min at 105,000 $\times g$, after which the cytosolic fraction (supernatant) was collected and immediately frozen in liquid nitrogen and stored at −80 °C. The pellet resuspended in 0.1 M $K_2P_2O_7$, 1 mM EDTA buffer (pH 7.4) was centrifuged again for 60 min at 105,000 $\times g$ to give the final fraction. Washed microsomes were then resuspended with an hand-driven Potter Elvehjem homogenizer in a 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA and 20% (*v*/*v*) glycerol; fractions were immediately frozen in liquid nitrogen and stored at −80 °C prior to use.

2.4. Haematological and serum biochemical analyses

Blood samples were collected from each animal in both heparinized and non-heparinized tubes. Using a table top centrifuge to obtain plasma, samples collected in heparinised tubes were centrifuged for 15 min at 2000 rpm, while samples saved in non-heparinised tubes were centrifuged at 1500 rpm for 10 min after complete coagulation, to obtain serum. Biochemistry and haematology were assessed by Department of Veterinary Medical Science, School of Agriculture and Veterinary Medicine, Alma Mater Studiorum University of Bologna.

2.5. Protein concentration

Protein concentration was determined according to the method described by Lowry et al. [34] as revised by Bailey [35], using bovine serum albumin as standard and diluting microsomes 200 times and cytosol 1000 times to provide a suitable protein concentration.

2.6. Phase I enzymes

2.6.1. Cytochrome P450 (CYP) content

CYP content was determined by a differential spectrophotometric assay using the Omura and Sato method [36]. CYP (0.5 ml of microsomes, 4.5 ml of 0.05 M Tris–HCl buffer pH 7.6 and 10 mM EDTA) was measured by examining the absorbance difference (between 450 and 490 nm) of differential spectra, obtained between CYP that is reduced and linked to CO, with respect to the free reduced form ($\varepsilon = 91\ mM^{-1}\ cm^{-1}$).

2.6.2. NADPH-(CYP)-c-reductase (CYP-red)

The analytical method is based on the determination of the reduction rate of cytochrome c at 550 nm ($\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$), according to previously defined procedures [37]. Incubation mixture contained 1.6 ml of 0.05 M Tris–HCl buffer (pH 7.7) with 0.1 mM EDTA + 0.5 mg cytochrome c + 0.2 ml of microsomes. Reaction starts with the addiction of 0.2 ml NADPH. The specific reaction was read at 550 nm against buffer plus cytochrome c [38].

Table 1
Expression of cyp-linked monooxygenase in hepatic subcellular fraction from VE treated rats.

Parameters	Controls	Treatment (seven days)		Treatment (fourteen days)	
		100 mg/kg b.w.	200 mg/kg b.w.	100 mg/kg b.w.	200 mg/kg b.w.
Cytochrome P450 (nmol × mg^{-1})	0.39 ± 0.03	0.37 ± 0.02*	0.44 ± 0.03**	0.35 ± 0.01**	0.39 ± 0.02
NADPH-cytochrome (P450) reductase (nmol × mg^{-1} × min^{-1})	21.05 ± 1.56	21.90 ± 1.38	19.10 ± 1.67	17.04 ± 1.67**	21.29 ± 1.57
Aminopyrine *N*-demethylase (nmol × mg^{-1} × min^{-1}) (CYP3A1/2)	27.01 ± 1.88	30.15 ± 3.56	30.00 ± 3.51	30.14 ± 1.93*	24.14 ± 1.22**
p-Nitrophenol hydroxylase (nmol × mg^{-1} × min^{-1}) (CYP2E1)	0.39 ± 0.01	0.39 ± 0.01	0.42 ± 0.02**	0.37 ± 0.01**	0.41 ± 0.01**
Ethoxycoumarin *O*-deethylase (pmol × mg^{-1} × min^{-1})	0.54 ± 0.01	0.56 ± 0.01*	0.50 ± 0.01**	0.53 ± 0.01	0.56 ± 0.01*
Pentoxyresorufin *O*-dealkylase (pmol × mg^{-1} × min^{-1}) (CYP2B1/2)	10.48 ± 0.48	12.50 ± 0.43*	14.35 ± 1.12**	10.62 ± 1.26	11.48 ± 0.60
Ethoxyresorufin *O*-deethylase (pmol × mg^{-1} × min^{-1}) (CYP1A1)	28.13 ± 2.45	19.77 ± 0.70**	22.23 ± 1.81**	19.58 ± 0.84**	19.04 ± 1.30**
Methoxyresorufin *O*-demethylase (pmol × mg^{-1} × min^{-1}) (CYP1A2)	24.64 ± 1.12	22.48 ± 1.19	21.64 ± 1.86*	20.24 ± 0.82**	17.11 ± 1.20**

Each value represents the mean ± S.D. of six independent experiments on six rats. See Section 2 for details and experimental procedures.
Mean values were significantly different compared with the control groups (Wilcoxon's rank method): *$p < 0.05$, **$p < 0.01$.

2.6.3. Aminopyrine N-demethylase (APND)

Activity was determined by quantification of CH_2O release, according to Mazel [39]. The total incubation volume was 3 ml, composed of 0.5 ml of water solution of 50 mM aminopyrine and 25 mM $MgCl_2$, 1.48 ml of 0.60 mM NADP+, 3.33 mM G6P in 50 mM Tris–HCl buffer (pH 7.4), 0.02 ml G6PDH (0.93 U/ml) and 0.125 ml of sample. After 5 min of incubation at 37 °C, the yellow colour developed by the reaction of the released of CH_2O with the Nash reagent was read at 412 nm, and the molar absorptivity of 8000 used for calculation [40].

2.6.4. p-Nitrophenol hydroxylase (p-NFI)

Activity was determined in a final volume of 2 ml: 2 mM p-nitrophenol in 50 mM Tris–HCl buffer (pH 7.4), 5 mM $MgCl_2$, and a NADPH-generating system consisting of 0.4 mM NADP+, 30 mM isocytrate, 0.2 U of isocytrate dehydrogenase and 1.5 mg of proteins. After 10 min of incubation at 37 °C, the reaction was terminated by addition of 0.5 ml of 0.6 N perchloric acid. Precipitated proteins were removed by centrifugation and 1 ml of the resultant supernatant was mixed with 1 ml of 10 N NaOH. Absorbance at 546 nm was immediately recorded and 4-nitrocathecol determined ($\varepsilon = 10.28\ mM^{-1}\ cm^{-1}$) [41].

2.6.5. Pentoxyresorufin O-dealkylase (PROD), ethoxyresorufin O-deethylase, (EROD) and methoxyresorufin O-demethylase (MROD)

Reaction mixture (PROD) consisted of 0.025 mM $MgCl_2$, 200 mM pentoxyresorufin, 0.32 mg of proteins and 130 mM NADPH in 2.0 ml 0.05 M Tris–HCl buffer (pH 7.4). Resorufin formation at 37 °C was calculated by comparing the rate of increase in relative fluorescence to the fluorescence of known amounts of resorufin (excitation 563 nm, emission 586 nm) [42]. EROD and MROD activities were measured exactly in the same manner as described for the pentoxyresorufin assay, except that substrate concentration was 1.7 mM for ethoxyresorufin and 5 mM for methoxyresorufin [43].

2.6.6. Ethoxycoumarin O-deethylase (ECOD)

ECOD was determined by quantification of umbelliferone formation, according to Aitio A. [44]. Incubation mixture was consisted of 2.6 ml, composed of 1 mM ethoxycoumarin, 5 mM $MgCl_2$, NADPH-generating system (see aminopyrine assay) and 0.25 ml of sample. After 5 min of incubation at 37 °C reaction was stopped by addiction of 0.85 ml of trichloroacetic acid (TCA) 0.31 M. The pH of the mixture was brought to about 10 by adding 0.65 ml of 1.6 M NaOH-glycine buffer (pH 10.3); amount of umbelliferone was measured fluorimetrically (excitation 390 nm; emission 440 nm).

2.7. Phase II enzymes

2.7.1. Glutathione S-transferase (GST)

The incubation mixture for measuring overall GST activity contained 1 mM glutathione + 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in methanol + 0.025 ml of sample in a final volume of 2.5 ml 0.1 M phosphate Na+/K+ buffer (pH 6.5). The product of the reaction of the thiol group of glutathione with the electrophilic group of CDNB was read at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$) [45,46].

2.7.2. UDP-glucuronosyl transferase (UDP-GT)

UDP-GT was determined kinetically using 1-naphtol as substrate (final concentration, 50 mM) by the continuous fluorimetric (excitation 390 nm; emission 440 nm) monitoring of 1-naphtholglucuronide production in the presence of 1 mM uridine-5′-diphosphoglucuronic acid [47]. Experiments were performed in the presence or absence of Triton X-100 (0.2%) as a detergent, in order to improve the assay sensitivity [48].

2.8. Antioxidant enzymes

2.8.1. Catalase (CAT)

The reaction was started in a quartz cuvette, containing 50 mM-potassium phosphate buffer and cytosol sample, by adding 30 mM H_2O_2. The decomposition of the substrate was measured at 240 nm and catalase activity was expressed as moles of H_2O_2 consumed per minute per mg protein using a molar extinction coefficient of $43.6\ mM^{-1}\ cm^{-1}$ [49].

2.8.2. NAD(P)H:quinone reductase (NQO1)

NQO1 activity was assayed spectrophotometrically at 600 nm by monitoring the reduction of the blue redox dye of 2–6

Table 2
Expression of cyp-linked monooxygenase in renal subcellular fractions from VE treated rats.

Parameters	Controls	Treatment (seven days)		Treatment (fourteen days)	
		100 mg/kg b.w.	200 mg/kg b.w.	100 mg/kg b.w.	200 mg/kg b.w.
p-Nitrophenol hydroxylase (nmol × mg^{-1} × min^{-1}) (CYP2E1)	0.36 ± 0.08	0.38 ± 0.04*	0.70 ± 0.04**	0.72 ± 0.08**	0.93 ± 0.07**
Ethoxycoumarin *O*-deethylase (pmol × mg^{-1} × min^{-1})	0.66 ± 0.01	0.51 ± 0.01**	0.53 ± 0.02**	0.55 ± 0.01**	0.51 ± 0.01**
Pentoxyresorufin *O*-dealkylase (pmol × mg^{-1} × min^{-1}) (CYP2B1/2)	0.34 ± 0.08	0.47 ± 0.03**	0.32 ± 0.02*	0.45 ± 0.05**	0.68 ± 0.08**
Ethoxyresorufin *O*-deethylase (pmol × mg^{-1} × min^{-1}) (CYP1A1)	3.61 ± 0.28	3.78 ± 0.12**	5.87 ± 0.43**	3.93 ± 0.28	4.60 ± 0.15**
Methoxyresorufin *O*-demethylase (pmol × mg^{-1} × min^{-1}) (CYP1A2)	1.59 ± 0.14	2.67 ± 0.24**	3.37 ± 0.33**	1.75 ± 0.15	2.50 ± 0.18**

Each value represents the mean ± S.D. of six independent experiments on six rats. See Section 2 for details and experimental procedures.
Mean values were significantly different compared with the control groups (Wilcoxon's rank method): *$p < 0.05$, **$p < 0.01$.

Table 3
Phase-II enzymes in hepatic subcellular fractions from VE treated rats.

Parameters	Controls	Treatment (seven days)		Treatment (fourteen days)	
		100 mg/kg b.w.	200 mg/kg b.w.	100 mg/kg b.w.	200 mg/kg b.w.
Glutathione S-transferase (GST) (nmol × mg^{-1} × min^{-1})	11.01 ± 0.18	10.73 ± 0.73	9.71 ± 0.18**	12.53 ± 0.27**	11.76 ± 0.19*
UDPglucuronosyl-transferase (UDPGT) (nmol × mg^{-1} × min^{-1})	4.46 ± 0.32	4.03 ± 0.24	4.94 ± 0.13*	4.92 ± 0.57	3.90 ± 0.50

Each value represents the mean ± S.D. of six independent experiments on six rats. See Section 2 for details and experimental procedures.
Mean values were significantly different compared with the control groups (Wilcoxon's rank method): *$p < 0.05$, **$p < 0.01$.

dichlorophenolindophenol (DCPIP) ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$), and expressed as moles of DCPIP reduced per minute per mg protein [50].

2.9. Electronic paramagnetic resonance (EPR) spectroscopy

Briefly, a small weighted portion of renal tissue from VE treated groups (mean weight = 2.7 ± 1.3 mg) and controls (mean weight = 2.4 ± 1.2 mg) was cut from each animal and immediately treated with 1 ml of 1 mM hydroxylamine probe. The hydroxylamine employed in this work was bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decandioate, capable of efficiently trapping the majority of oxygen-, carbon- and nitrogen-centred radicals, including superoxide and peroxynitrite [51]. This reaction yields a very persistent nitroxide that can be readily detected and accurately quantified by EPR spectroscopy [51–54]. Samples were prepared by treating the tissue with 1 ml of standard physiological solution containing the hydroxylamine probe (1 mM) and EDTA (1 mM) as metal chelating agent. After 5 min incubation at 37 °C, the sample was snap frozen in liquid nitrogen to stop any reaction and stored at −80 °C until EPR measurement was performed. The optimal incubation time and the most appropriate experimental conditions were determined in previous investigations [51–53]. Immediately before measurement, the sample was warmed to room temperature, and about 50 μl of the solution was transferred and sealed in a calibrated capillary glass tube, which was placed inside the cavity of a Bruker ESP 300 EPR spectrometer (Bruker Biospin S.r.l., Rheinstetten, Germany) equipped with an nuclear magnetic resonance gaussmeter for field calibration, a Bruker ER 033 M FF-lock (Bruker Biospin S.r.l.) and a Hewlett-Packard 5350B microwave frequency counter (Hewlett Packard, Houston, TX, USA). The actual amount of solution analyzed was chosen so as to cover the entire sensitive area of the instrument cavity. The spectra of the nitroxide radical, generated by the reaction of the probe with the radicals produced in the tissue, were then recorded using the following instrumental settings: modulation amplitude = 1 · 0 G; conversion time = 163 · 84 ms; time constant = 163 · 84 ms; modulation frequency 100 kHz; microwave power = 6.4 mW. The intensity of the first spectral line of the nitroxide (aN = 16.60 G; g = 2 · 0056) was used to obtain the absolute amount of nitroxide per ml of sample, after calibration of the spectrometer response with known solutions of TEMPO-coline in water, using an ER 4119HS Bruker Marker Accessory as internal standard. For simplicity, results were expressed as μmol of ROS in g of tissue. The hydroxylamine probe (CAS no. 314726-62-0) was prepared as previously described [52,53].

2.10. Statistical analysis

Statistical evaluation was performed using the Wilcoxon's rank test to assess significant differences in the considered parameters between the groups of treated animals compared with controls [55].

3. Results

3.1. Body and organ weights, blood parameters

There were no differences in body or organ weights between VE treated groups and their relative controls (data not shown). VE did not significantly affect haematological or biochemical parameters (data not shown).

3.2. Effects of VE treatment on hepatic phase-I enzymes, single probes

Table 1 shows hepatic CYP content, CYP-reductase activity and various mixed function monooxygenases measured both in controls and VE treated animals using the following selected substrates as probes of different CYP isoenzymes: aminopyrine (preferential to CYP3A4), p-nitrophenol (CYP2E1), ethoxycoumarin (mixed), penthoxyresorufin (CYP2B1/2), methoxyresorufin (CYP1A2), ethoxyresorufin (CYP1A1).

A general down regulation for many of the tested CYP isoforms, especially after fourteen-day treatment, was recorded. Ethoxyresorufin *O*-deethylase (EROD) was strongly inactivated to different extents in all situations (ranging from 21% to 32% loss; $p < 0.01$). NADPH-cytochrome (P450) c-reductase was down regulated up to 19% after fourteen-day administration at the lowest dose tested ($p < 0.01$). The longest supplementation exerted a weak but significant ($p < 0.01$) reduction N-demethylation of aminopyrine (≅11% at 200 mg/kg b.w.) and methoxyresorufin *O*-demethylase (MROD) (almost 18% at 100 mg/kg b.w. and 31% at 200 mg/kg b.w., $p < 0.01$). On the contrary, at 200 mg/kg b.w. dose, CYP content and pentoxyresorufin *O*-dealkylase (CYP2B1/2) were slightly increased up to, respectively, 13% and 37% after seven-day treatment ($p < 0.01$). The CYP2E1 associated activity showed a modest increment both after seven- and fourteen-day treatments at the highest dose, while the lowest dose caused a weak inactivation after fourteen-day treatment (about 5% loss; $p < 0.01$).

3.3. Effects of VE treatment on renal phase-I enzymes, single probes

Table 2 reports the expression of CYP-linked monooxygenases in renal subcellular preparations. At both dosages employed and

Table 4
Phase-II enzymes in renal subcellular fraction from VE treated rats.

Parameters	Controls	Treatment (seven days)		Treatment (fourteen days)	
		100 mg/kg b.w.	200 mg/kg b.w.	100 mg/kg b.w.	200 mg/kg b.w.
Glutathione S-transferase (GST) (nmol × mg^{-1} × min^{-1})	0.32 ± 0.01	0.23 ± 0.01**	0.27 ± 0.01*	0.26 ± 0.01**	0.25 ± 0.01**
UDPglucuronosyl-transferase (UDPGT) (nmol × mg^{-1} × min^{-1})	1.19 ± 0.06	0.51 ± 0.06**	0.54 ± 0.07**	0.63 ± 0.11**	0.63 ± 0.02**

Each value represents the mean ± S.D. of six independent experiments on six rats. See Section 2 for details and experimental procedures.
Mean values were significantly different compared with the control groups (Wilcoxon's rank method): *$p < 0.05$, **$p < 0.01$.

Table 5
Antioxidant enzymes in hepatic cytosol from VE treated male rats.

Parameters	Controls	Treatment (seven days)		Treatment (fourteen days)	
		100 mg/kg b.w.	200 mg/kg b.w.	100 mg/kg b.w.	200 mg/kg b.w.
Catalase (μmol × mg^{-1} × min^{-1})	3.47 ± 0.33	6.00 ± 0.56**	4.18 ± 0.44*	5.41 ± 0.65**	2.49 ± 0.21**
NAD(P)H:quinone reductase (μmol × mg^{-1} × min^{-1})	8.73 ± 0.50	8.37 ± 0.90	13.31 ± 1.50**	9.51 ± 1.13**	10.72 ± 1.19**

Each value represents the mean ± S.D. of six independent experiments on six rats. See Section 2 for details and experimental procedures.
Mean values were significantly different compared with the control groups (Wilcoxon's rank method): *$p < 0.05$, **$p < 0.01$.

observational times, a prevalent up regulation was noted for the majority of the tested CYP isoforms. CYP2E1 associated activity presented a significant increase at all doses tested; in particular, a 2-fold increase was seen after seven days at 200 mg/kg b.w. and after fourteen-day treatment at 100 mg/kg b.w. ($p < 0.01$). These increments became greater (up to 2.6-fold) after fourteen days at the highest dose tested ($p < 0.01$). VE significantly increased ($p < 0.01$) PROD up to 32% after seven or fourteen lowest doses; the phenomenon reached 2-fold induction ($p < 0.01$) after highest dose administration (both treatment periods). EROD activity was up regulated after seven- or fourteen-day highest dose treatment (almost up to 63% and 27% respectively, $p < 0.01$). CYP1A2-linked activity was more than doubled ($p < 0.01$) by seven-day treatment at 200 mg/kg b.w.; the same timing at the lowest dose, as well as fourteen-day treatment at the highest dose, were able to increase MROD ($p < 0.01$), even if the alteration was more modest (up to 68% and 57%, respectively). In contrast, VE significantly decreased ($p < 0.01$) ECOD (almost 30% loss after seven days at 100 mg/kg b.w., 20% and 17% loss, after seven-day highest dose, or fourteen-day lowest dose treatments, respectively).

3.4. Effects of VE on hepatic phase-II enzymes (GST and UDPGT)

Table 3 shows how VE was not able to provoke relevant changes in hepatic phase-II enzymes. GST reported mild fluctuations, as a 12% loss occurred after seven-day highest dose administration ($p < 0.01$). Contextually, fourteen-day treatment, at both dosages, caused up-regulation up to almost 14% at 100 mg/kg b.w., $p < 0.01$, and up to 7% at 200 mg/kg b.w. $p < 0.05$. UDPGT was not affected by VE treatment, with the exception of 200 mg/kg b.w. that showed a nearly 11% increase ($p < 0.05$) after seven-day treatment.

3.5. Effects of VE on kidney on renal phase-II enzymes (GST and UDPGT)

Contrary to what was observed in the liver, Table 4 reports a relevant inactivating effect attributable to VE.

GST activity, in terms of CDNB metabolism, was inactivated in all cases, ranging from 16% loss ($p < 0.05$) to 28% loss ($p < 0.01$) after seven-day treatment for either dose. This phenomenon was further exacerbated for UDPGT activity, which recorded important decreases (ranging from 47% to 57% loss; $p < 0.01$) in each group.

3.6. Effects of VE on hepatic antioxidant enzymes

Seven- or fourteen-day lowest dose treatments exerted a modest increment of CAT (up to 73% and 56%, respectively), while highest dose treatments caused a 20% ($p < 0.05$) increase after seven-day administration, and a 28% ($p < 0.01$) decrease after fourteen-day treatment (Table 5).

NAD(P)H-quinone reductase was significantly induced (59%, $p < 0.01$), in seven- or fourteen-day 200 mg/kg b.w. groups.

3.7. Effects of VE on renal antioxidant enzymes

VE decreased NAD(P)H: quinone reductase in all cases (ranging from 20% to 44% loss). Catalase recorded a 20% ($p < 0.01$) increase at the lowest dose after seven-day supplementation, while the highest dose administered for the longest treatment period induced an 11% loss ($p < 0.01$) (Table 6).

3.8. EPR radical probe measurements in renal tissue

EPR spectroscopy coupled to a radical probe technique was used to assess the level of reactive (oxidizing) radical species, in renal tissue. As shown in Table 7 and Fig. 1, we found a significant over-generation of free radicals in the samples collected from VE treated animal groups. The rate of production of the stable nitroxide radical representing the rate of reactive oxygen species in the tissue was more than 2-fold greater in each experimental treated unit compared to controls ($p < 0.05$).

4. Discussion

Our survey has outlined a complex picture in which VE exerts its effects in a completely organ-specific way. The almost neutral behaviour shown by VE on hepatic phase I and II enzyme activities, and at the same time the appreciable increase of the antioxidant machinery considered in this study (Catalase and NAD(P)H:quinone reductase) would once again stick the label of "protective agent" on VE. On the contrary, if we take into account data from the kidneys, the picture appears very different from what was recorded in the liver.

In the animal model that we proposed here, VE administration produced a booster effect on renal phase I carcinogen bioactivating enzymes.

If extrapolated to humans, the recorded CYPs inductions might have serious toxicological relevance.

In both experimental animals and humans it is well established that xenobiotic metabolizing enzymes can be induced by a wide range of drugs, pesticides, food additives, industrial chemicals, natural products and environmental pollutants [56].

On one hand, CYP changes may alter both endogenous metabolism (e.g., Leukotrienes, Vitamin D, arachidonic acid derivatives, nitric oxide, aldosterone and cholesterol metabolism) and crucial physiological functions such as growth, differentiation, apoptosis and neuroendocrine

Table 6
Antioxidant enzymes in renal cytosol from VE treated rats.

Parameters	Controls	Treatment (seven days)		Treatment (fourteen days)	
		100 mg/kg b.w.	200 mg/kg b.w.	100 mg/kg b.w.	200 mg/kg b.w.
Catalase (μmol × mg-^{1} × min^{-1})	6.36 ± 0.18	7.60 ± 0.87**	6.96 ± 0.37*	6.37 ± 0.19	5.64 ± 0.17**
NAD(P)H:quinone reductase (μmol × mg^{-1} × min^{-1})	3.40 ± 0.14	2.52 ± 0.30**	2.54 ± 0.24**	2.72 ± 0.18*	1.92 ± 0.11**

Each value represents the mean ± S.D. of six independent experiments on six rats. See Section 2 for details and experimental procedures.
Mean values were significantly different compared with the control groups (Wilcoxon's rank method): *$p < 0.05$, **$p < 0.01$.

functions. On the other hand, the increased bioactivation of ubiquitous pro-mutagens/pro-carcinogens, saturating the enzymes involved in "error-free repair", can seriously place DNA at structural damage risk.

Furthermore, the existence of NADPH-dependent production of reactive oxygen species (ROS) (O_2^-, H_2O_2, and HO•) by microsomes has been known for several decades and has been linked to CYP induction [57]; more recently, it was discovered that the regulation of various CYP isoforms including CYP1A, CYP2B, CYP3A, CYP4A, and virtually any CYP can generate very large amounts of ROS [58]. In other terms, the induction of any of these CYPs leads to increased ROS production, and this phenomenon has also been observed in studies reporting NADPH-stimulated release of ROS by subcellular preparations enriched in specific human CYPs [30]. The phenomenon could intensify further in genetically predisposed individuals who inherit certain 'high-risk' polymorphisms affecting carcinogen-metabolizing enzymes [59,60], as well as individuals who lack adequate detoxication or DNA repair capacity [61–63].

For example, the 2.6-fold increment of CYP2E1-linked activity that we recorded could rise in a putative alcohol consumer. Alcohol, which is itself an inducer of CYP2E1, can thus act with VE in boosting phase-I reactions; irrespective of the fact that genetic polymorphisms lead to the occurrence of high or poor-metabolizer phenotypes in the population [64], that further complicate the issue.

Moreover, it has been shown how CYP2E1 is one of the most active CYP isoforms in generating ROS associated with cancer progression and metastasis [65]; and recent evidence by León-Buitimea et al. supports the hypothesis that ethanol enhances oxidative stress in MCF-10A cells overexpressing CYP2E1, increasing human mammary cell activation, *via* an EGFR-dependent signalling mechanism associated with oxidative stress [66]. Translated to humans, the doubling in CYP1A1 and CYP1A2 linked activity, which activates polycyclic aromatic hydrocarbons (PAHs), would be of particular concern for smokers who are exposed to a wide range of procarcinogens that are bioactivated by the CYP apparatus, irrespective of the fact that many PAHs are themselves recognized CYP inducers [67].

Discussing the link between the CYP induction and its role on cancer, it should be also kept in mind that most of CYP forms induction takes place through the involvement of nuclear receptors such as the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), the pregnane X receptor (PXR) and the peroxisome proliferator-activated receptor alpha (PPARα). The pleiotropic response triggered by the activation of nuclear receptors can affect not only genes involved in the xenobiotic metabolism, but also those associated to physiological processes including cell proliferation [68]. For this reason, CYP induction cannot be considered only a metabolism concern, but it can be also a marker of transcription factor activation and a potential tumorigenesis sentinel. Recently, the chronic exposure of rodents to non-genotoxic CYP inducers was associated to the formation of tumours in several tissues [56].

Furthermore, the remarkable decrement observed in kidney of post oxidative enzyme activities (GST and UDPGT), and the subtler, but still important one of the enzyme-dependant antioxidant machinery highlights a lack in detoxifying capacity and an increased vulnerability to further ROS insults. Under these conditions, it appears that the fine balance between the generation of oxygen centred species and the cellular defences may be tilted in favour of oxidative stress, leading to putative macromolecular damage that can finally point toward cancer.

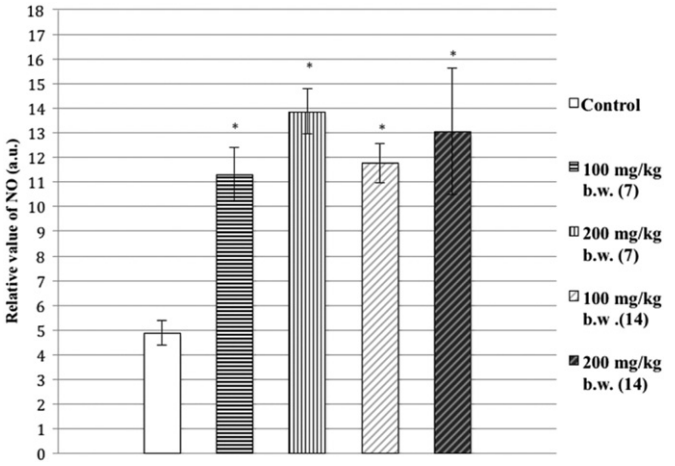

Fig. 1. Oxygen free radical species in kidney. Bars represent the amount of ROS. Measures were performed by the use of EPR spectrometry and they are expressed as the intensity of nitroxide EPR signal. Mean values were significantly different compared with the control groups (Wilcoxon's rank method): $^{*}p < 0.05$, $^{**}p < 0.01$.

Due to the widely recognized role of ROS as a factor that may increase the incidence of cancer in humans, we used EPR spectroscopy coupled to a radical probe technique, in order to evaluate the hypothesized contribution of CYPs observed induction on free radical generation in renal tissue.

Our data showed a significant increase of ROS in renal tissue samples collected from VE treated rats compared with controls: more than doubled values were recorded throughout each treatment. These findings lead to consider the hypothesis that VE could potentially increase cancer risk rather than preventing it, by inducing carcinogen-activating enzymes and enhancing oxidative stress status, that can play a role in the promotion and in virtually all steps of the carcinogenesis process [69,70].

Bearing in mind the limitation of a rat model, it is conceivable that the results of the present study support the observed cancer-promoting effect reported by Wu and co-workers [24], exemplified in a co-carcinogenic potential through different mechanisms; and, more in general, our findings may help explain previous contradictory evidence regarding VE supplementation and cancer incidence.

These findings and the emerging conclusions should not be a surprise, if we look at "unexpected" results of cancer chemoprevention trials testing the effects of β-carotene on the risk of chronic diseases such as cancer. β-carotene administered alone or in combination with vitamins for prevention of lung and other cancers in heavy smokers or asbestos workers failed to reduce cancer risk, and, in some cases, actually increased it [71–73]. Even in that case, it was documented that the harmful effects of β-carotene could be linked to its ability to stimulate metabolizing machinery, increasing bioactivation and enhancing ROS levels [74].

The term "antioxidant paradox" has been used for several years to refer to the observations that giving large doses of antioxidants to

Table 7
ROS levels in kidney tissue from VE treated rats.

Tissue	Controls	Treatment (seven days)		Treatment (fourteen days)	
		100 mg/kg b.w.	200 mg/kg b.w.	100 mg/kg b.w.	200 mg/kg b.w.
Kidney (a.u.)	4.88 ± 0.24	11.30 ± 1.06*	13.84 ± 0.91*	11.75 ± 0.79*	13.03 ± 2.56*

Each value represents the mean ± the standard error of the mean (S.E.M.) of six independent measurements on six animals. The amount of oxygen free radical species are expressed as the intensity of nitroxide EPR signal.
Mean values were significantly different compared with the control groups (Wilcoxon's rank method): $^{*}p < 0.05$, $^{**}p < 0.01$.

humans, in most studies, had no preventive or therapeutic effects [75, 76]; despite this, the market of vitamins is rising, worldwide, and many tons of vitamin E have been consumed by the population with no beneficial effects to their health and even a suggestion of harm [77].

The even more relevant fact is that, out of all the people who usually consume multivitamin cocktails, most of them take supplements not to treat a diagnosed deficiency, but to improve their health condition [77,78]. Our data bring to light a dark side of VE that is astonishingly similar to that of β-carotene and point out, once again, how harmful the well-embedded concept that "antioxidants are good, more antioxidants are better" could be [79].

We agree with Wenner Moyer M. in replying to the question "are supplements useful?" that the state of the art offers only an equivocal half-answer: "maybe yes" for some individuals, nutrients and doses, and "maybe no" for others [78].

5. Conclusions

Our study provides a further example of the potentially harmful effects of recommending supplementations with micronutrients on a large scale.

Furthermore, we hypothesize that the increased cancer risk observed in long-term clinical trials and the "paradoxical" ability of VE to generate oxidative stress which we have shown herein may be related. In the light of recent evidence, assuming that daily VE supplementation could, at worst, be ineffective should be carefully reconsidered. On the other hand, if cancer chemotherapy as often as possible involves multiple agents in order to prevent clonal selection, why would cancer chemoprevention be based on a single agent [80]?

Author contribution to study

FV, DC, AS and MP designed the study. DC, FV, AS, AV, CMDC, VL conducted the study *in vivo* and data analysis. PF and ML performed EPR measurements and relative data analysis. FV, DC and MP wrote the manuscript. All authors discussed, edited and approved the final version.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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