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Procyanidins from *Vitis vinifera* Seeds: In Vivo Effects on Oxidative Stress

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The purpose of this study was to evaluate the effect of supplementation with procyanidins from *Vitis vinifera* on markers of oxidative stress. Ten healthy volunteers received a daily dose of 110 mg of procyanidins for 30 days. Fasting venous blood samples were taken before and at the end of the supplementation period and after 7 days of wash-out. The total antioxidant activity and the plasma concentrations of α-tocopherol were not modified. Conversely, the levels of α-tocopherol in red blood cell membranes increased significantly from 1.8 ± 0.1 to 2.8 ± 0.2 mg/g. Similarly, the lymphocyte oxidized DNA [8-oxo-7,8-dihydro-2′-deoxyguanosine/2′-deoxyguanosine ratio] was reduced from 7.23 ± 2.47 to 2.34 ± 0.51, and the red blood cell membrane fatty acid composition shifted to a higher level of polyunsaturated fatty acids. On the basis of these results, it may be suggested that dietary procyanidins exert their antioxidant protection in vivo by sparing liposoluble vitamin E and reducing DNA oxidative damage.

KEYWORDS: Procyanidins; plasma; total antioxidant activity; 8-oxo-7,8-dihydro-2′-deoxyguanosine; human

INTRODUCTION

Proanthocyanidins (condensed tannins) are oligomers consisting of flavan-3-ol units linked by C−C and occasionally C−O−C bonds (**Figure 1**). They differ structurally according to the number of hydroxyl groups on aromatic rings A and B and the configuration of the asymmetric carbons of the flavanol heterocycle (*1*). The most common proanthocyanidins are procyanidins, deriving from (epi)catechin (3′,4′-OH) and prodelphinidins, consisting of (epi)gallocatechin units (3′,4′,5′-OH). Procyanidins are distributed in food (apples, grapes, plums, barley, cranberries, kaki) and medicinal plants (*Crataegus* spp., *Vaccinium vitis idaea*, *Salix* spp., and *Potentilla erecta*) and are also present in grape seeds and *Pinus maritima* bark (*2*).

Procyanidins have been reported to exert a widelarge range of pharmacological activities including anti-inflammatory, antibacterial, antiallergic, and vasodilatory actions (*3*−*7*). In addition, procyanidins are known to inhibit platelet aggregation and capillary permeability and to modulate the activities of different enzymes (*8*, *9*). Procyanidins have been shown to limit free radical formation by inhibiting enzymes (*10*) or chelating metals involved in their generation and to lower the level of reactive oxygen species. However, most of the work concerning the antioxidant ability of procyanidins has been done in vitro (*11*−*14*), and in vivo evidence in humans is lacking. Moreover, it is not yet clear whether procyanidins or some related antioxidant polyphenolic compounds in grape seed extract are absorbed and can contribute to in vivo antioxidant defense.

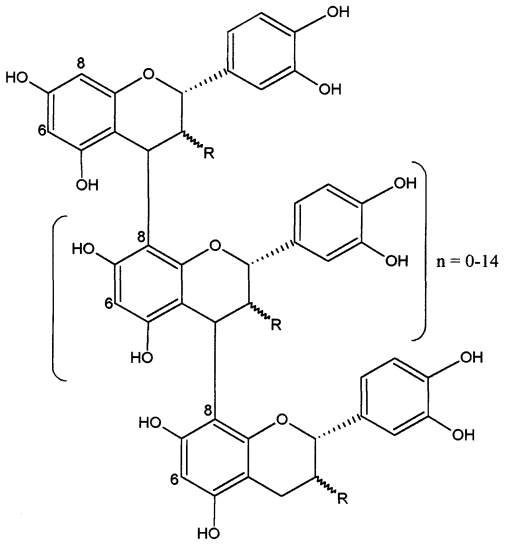

Figure 1. Grape seed procyanidins. R = 3β-OH → monomer, (+)-catechin; R = 3α-OH → monomer, (−)-epicatechin; R = 3β-*O*-gallate → monomer, (+)-catechin 3-*O*-gallate; R = 3α-*O*-gallate → monomer, (−)-epicatechin 3-*O*-gallate.

The aim of the present study, therefore, was to evaluate the effect of supplementation with procyanidins from *Vitis vinifera*

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seeds on selected indicators of oxidative stress, including leukocyte DNA damage, plasma total radical-trapping antioxidant parameter, plasma and red blood cell α-tocopherol, and red blood cell membrane phospholipid fatty acid composition.

MATERIALS AND METHODS

Chemicals. The grape seed extract (Leucoselect) was obtained from Indena (Milano, Italy). Gallic acid, (+)-catechin, (−)-epicatechin, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ascorbic acid, 2,6-di-*tert*-butyl-4-methylphenol (BHT), α-tocopherol, Histopaque 1077, Triton X-100, deferoxamine mesylate, sucrose, sodium dodecyl sulfate, Tris-HCl, Na_2EDTA, $MgCl_2$, NaI, $ZnSO_4$, sodium acetate, protease (EC 3.4.24.31), ribonuclease A (EC 3.1.27.5), ribonuclease T_1 (EC 3.1.27.3), nuclease P_1 (EC 3.1.30.1), acidic phosphatase (EC 3.1.3.2), 2′-deoxyguanosine, and 8-oxo-7,8-dihydro-2′-deoxyguanosine were purchased from Sigma-Aldrich (St. Louis, MO); (−)-epigallocatechin gallate and (−)-epigallocatechin were from Extrasynthese (Genay, France). 2,2′-Diazobis(2-amidinopropane) dihydrochloride was from Wako Chemicals (Richmond, VA). *R*-Phycoerythrin was from Molecular Probes (Eugene, OR). Omegawax test mix was from Supelco (Sigma-Aldrich, St. Louis, MO). All other reagents were of analytical grade (J. T. Baker, Deventer, Holland).

Subjects. Ten healthy subjects, mean age of 40 ± 12 years, body mass index of 23.1 ± 2.1 kg/m^2, were selected with a self-administered questionnaire to assess the intake of phenolics-rich foods (bran cereals, vegetables such as onions, spinach, tomatoes, and lettuce; fruits such as apples, apricot, blackberries, cranberries, grapes, grapefruit, strawberries; and beverages such as beer, coffee, fruit juices, tea, and wine). They were not taking any supplements or drugs. Informed written consent was obtained from each participant, and the protocol was approved by the Local Ethics committee.

Protocol. The subjects were given two capsules (280 mg of total amount) providing ∼110 mg of procyanidins from grape seed extract for 30 days. Blood samples were collected from fasting subjects at the beginning of the period of supplementation, after 30 days, and after 7 days of wash-out. Plasma and red blood cells were separated by centrifugation at 10000g for 1 min.

Analysis of the Grape Seed Extract. *Sample Preparation.* The grape seed extract was dissolved in 20% v/v aqueous methanol solution (C = 2 mg/mL).

MS Analysis. Electrospray mass spectrometric (ESi-MS) analyses were performed on a Hewlett-Packard (Palo Alto, CA) model 5989A equipped with an electrospray interface 59987A. Nitrogen was used as nebulizing gas at a pressure of 50 psi and a temperature of 300 °C. The samples were analyzed by direct infusion in ESI-MS by means of a syringe pump (Harvard Apparatus, Natick, MA) at a flow rate of 10 μL/min in scan mode.

Plasma Total Radical-Trapping Antioxidant Parameter (TRAP). TRAP was determined according to the method of Ghiselli et al. (*15*), modified as previously described (*16*).

Plasma and Red Blood Cell (RBC) α-Tocopherol. *Sample Preparation for Plasma α-Tocopherol Determination.* Extraction and HPLC determination of plasma α-tocopherol were performed as described by Vuilleumier et al. (*17*).

Sample Preparation for Red Blood Cell α-Tocopherol Determination. Extraction and HPLC determination of RBC α-tocopherol were performed as described by Hatam and Kayden (*18*), with the following adjustments: 0.3 g of red blood cells, separated from whole blood by centrifugation at 10000*g* for 1 min and washed two times with an equal volume of a physiologic solution (0.9% NaCl, w/v), was weighed in 10 mL plastic tubes. Five hundred microliters of Triton X-100 (1% w/v in water), 500 μL of distilled water, and 1 mL of ascorbic acid (1% w/v in ethyl alcohol) were added. After 10 s of vortexing, an ultrasonic bath was employed for 5 min to perform cells lyses. Four milliliters of hexane was added, and the tubes were vortexed for 10 min. After centrifugation at 800*g* for 10 min, 3.5 mL of the supernatant was transferred into 10 mL clean tubes containing 50 μL of BHT (0.1% w/v in hexane). Hexane (2.5 mL) was added to the tubes containing RBC to perform a second extraction by vortexing for 10 min, and after centrifugation at 800*g* for 10 min, 2 mL of the supernatant was transferred in the corresponding tube. The whole extraction solvent was evaporated under vacuum and nitrogen flow, and then 500 μL of hexane was added, the tubes were vortexed for 10 s, and the contents were transferred into a glass vial.

HPLC Analysis. A model 9001 (Varian, Cary, Australia) pump connected to an Ultra Wisp 715 (Waters, Milford, MA) autosampler and to a LC 240 fluorescence detector (Perkin-Elmer, Beaconsfield, U.K.) was used. Chromatogram integration was obtained with a Millennium workstation (Waters). Separations were performed on a 250 × 4.6 mm i.d. LiChrosorb Si60 column (Merck, Darmstadt, Germany), using hexane/ethyl acetate (1000:75, v/v) as mobile phase. The flow rate was 2 mL/min. The fluorescence detector was set at 290 nm in excitation and at 330 nm in emission. Calibration curves were obtained by injecting standard α-tocopherol, and linearity was assessed in the range of 0.8−8 μg/mL.

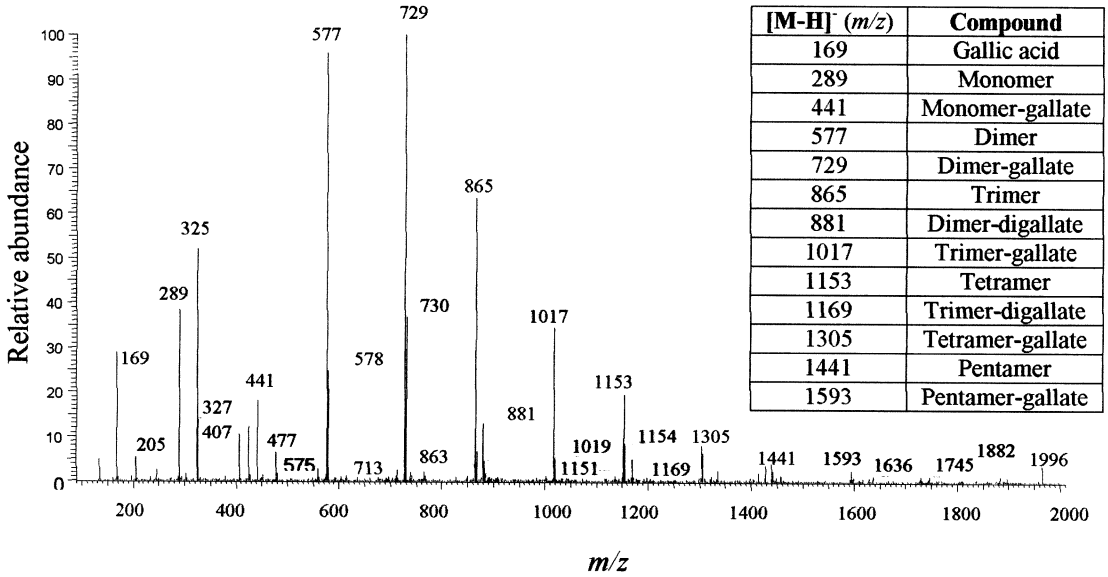
Analysis of Fatty Acid Composition of Phospholipids of Red Blood Cell Membrane. *Sample Preparation.* One milliliter of distilled water, 300 mg of RBC, 2.5 mL of BHT (110 μg/mL in methanol), and 5 mL of chloroform were transferred in 10 mL plastic tubes. Samples were vortexed for 3 min and centrifuged at 1200*g* for 10 min. The chloroform phase was transferred into 10 mL plastic tubes. 2.5 mL of chloroform was added to the tubes containing RBC to perform a second extraction by vortexing for 3 min, and, after centrifugation at 1200*g* for 10 min, the supernatant was transferred into the corresponding tube. The chloroform phase was eluted on a silica cartridge (Sep-Pack Plus Silica, Waters), which was then evaporated with nitrogen. The silica phase was transferred into Pyrex glass tubes, and 2.5 mL of a toluene/methanol (1:4 v/v) mixture and 200 μL of acetyl chloride were added (*19*). After 1 h in an oven at 100 °C, 5 mL of K_2CO_3 (6% w/v in water) was added to the silica. After centrifugation at 1200*g* for 10 min, the supernatant was transferred into amber glass vials and analyzed by gas chromatography.

GC Analysis. Before injection, samples were evaporated to dryness with nitrogen and an appropriate volume of hexane was added. The GC analysis was performed as described by Ackman (*20*), partly modified. Separations were performed with a 30 m × 0.32 mm i.d. Omegawax 320 capillary column, under these conditions: initial isotherm, 140 °C for 5 min; temperature gradient, 2 °C/min to 210 °C; final isotherm, 210 °C for 20 min.

The injector temperature was 250 °C. Injection volume was 1 μL with a split ratio of 1/100, and the FID temperature was 250 °C. Carrier and makeup gas were hydrogen and nitrogen, respectively. Fatty acid retention times were obtained by injecting the Omegawax test mix as standard.

Determination of DNA Oxidative Damage. *Sample Preparation.* Lymphocytes were isolated by density gradient sedimentation using Histopaque according to the manufacturer's procedure. DNA isolation, purification, and hydrolysis were performed using the method described by Helbock et al. (*21*). Briefly, lymphocytes isolated from 10 mL of whole blood were pelletted at 240*g* for 4 min. The resulting pellets (∼2 × 10^7 cells/sample) were resuspended twice in 2 mL of lysis buffer A (10 mM Tris-HCl, 320 mM sucrose, 5 mM $MgCl_2$, 0.1 mM deferoxamine, and 1% Triton X-100; pH 7.5) and centrifuged at 1500*g* for 10 min. The pellets were then resuspended in 0.6 mL of lysis buffer B (10 mM Tris-HCl, 5 mM Na_2EDTA, and 0.15 mM deferoxamine; pH 8). SDS (35 μL, 10% w/v in water) was added, and the mixture was vortexed before the addition of 30 units of ribonuclease A (1 mg/mL) and 8 units of ribonuclease T_1. The resulting samples were incubated at 50 °C for 15 min. Then 30 μL of protease (20 mg/mL) was added, and the samples were incubated for 1 h at 37 °C.

DNA was precipitated by the addition of 0.8 mL of NaI solution (7.6 M NaI, 20 mM Na_2EDTA, 40 mM Tris HCl, and 0.3 mM deferoxamine; pH 8.0) and 2 mL of 100% propan-2-ol followed by gentle inversion of the tube. The mixture was centrifuged at 5000*g* for 10 min, the supernatant discarded, and the nucleic acid pellet washed with 1 mL of 40% propan-2-ol and 1 mL of 70% ethanol. DNA was then dissolved in 100 μL of 0.1 mM deferoxamine and hydrolyzed by the addition of 10 units of nuclease P_1 and 0.5 unit of acidic phosphatase, both dissolved in 10 μL of nuclease P_1 buffer (300 mM sodium acetate and 1 mM $ZnSO_4$; pH 5.3). Samples were incubated for 90 min at 37 °C. Fifty microliters of chloroform was added, and



[M-H]⁻ (*m/z*)	Compound
169	Gallic acid
289	Monomer
441	Monomer-gallate
577	Dimer
729	Dimer-gallate
865	Trimer
881	Dimer-digallate
1017	Trimer-gallate
1153	Tetramer
1169	Trimer-digallate
1305	Tetramer-gallate
1441	Pentamer
1593	Pentamer-gallate

Figure 2. Typical negative mass spectrum of grape seed extract.

the sample was vortexed for 10 s and centrifuged at 5000*g* for 5 min. The supernatant was collected and analyzed by HPLC-UV-EC within 24 h.

HPLC Analysis. An instrument, equipped with a model 510 (Waters) pump connected to a model 996 (Waters) photodiode array detector coupled with an electrochemical detector, model Coulochem II (ESA, Chelmsford, MA), was used. Separations were performed on a 250 × 4.6 mm i.d. Waters Symmetry Shield C18 column, using 50 mM phosphate buffer (pH 5.5)/methanol (90:10, v/v) as mobile phase. The flow rate was 0.8 mL/min, and UV detection was set at 245 nm, whereas electrochemical detection was achieved by setting the guard cell (ESA model 5020) at +500 mV and the analytical cell (ESA model 5011) at +50 and +350 mV.

Statistical Analysis. All values are reported as mean ± SE. Statistical analysis was performed on a personal computer using the Statistica RM-ANOVA module (StatSoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

The composition of grape seed extract, according to the manufacturer's data sheet, was as follows: (+)-catechin, (−)-epicatechin, and gallic acid (15%); (−)-epicatechin gallate, dimers, trimers, tetramers, and their gallates (80%); pentamers, hexamers, heptamers, and their gallates (5%).

Total flavanols in each capsule are ∼39.6%. The other components present in the capsules are commonly used excipients.

A negative mass spectrum of grape seed extract is shown in **Figure 2**. Due to the limited availability of procyanidin standards, which are needed for cochromatography in HLPC, the extract was evaluated by mass spectrometry. This allowed several components, including gallic acid (*m/z* 169), (epi)-catechin (*m/z* 289), and their gallates (*m/z* 441) and different procyanidins, that is, dimer to pentamer in free and gallate form (**Figure 2**), to be identified.

The data from the self-administered questionnaire showed that the basal daily intake of procyanidins was ∼100 mg. Therefore, supplementation with 110 mg/day of procyanidins from grape seed was considered appropriate, as it doubled the basal intake.

Supplementation with grape seed extract for 30 days had a marginal effect on the plasma antioxidant potential, as measured by TRAP (**Table 1**). This result is in agreement with other studies indicating that midterm intake of antioxidant-containing foods or beverages has little influence on the blood antioxidant status, possibly due to homeostatic mechanisms (*23*). Similarly, the level of plasma α-tocopherol was not affected by the supplementation (**Table 2**).

Table 1. Time Course (Micromolar Trolox Equivalents) of Plasma Total Radical-Trapping Antioxidant Parameter

subject	*t* = 0 days	*t* = 30 days	*t* = 37 days
LC	582	707	583
LO	963	1339	1085
PA	990	842	922
SA	969	788	1136
ST	748	920	1077
ZE	897	880	717
AN	1074	1227	1209
LG	957	864	950
MR	927	953	992
VA	900	867	611
mean	**901**	**939**	**928**
SE	**47**	**65**	**74**
	$P_{(0-30)} = 0.497$	$P_{(30-37)} = 0.869$	$P_{(0-37)} = 0.634$

Table 2. Time Course (Micrograms per Milliliter) of Plasma α-Tocopherol

subject	*t* = 0 days	*t* = 30 days	*t* = 37 days
LC	11.8	10.3	10.1
LO	10.3	9.1	9.0
PA	9.5	9.2	8.4
SA	11.2	9.8	9.6
ST	11.4	12.7	11.7
ZE	7.3	8.3	9.4
AN	10.6	12.0	8.5
LG	6.6	9.1	11.2
MR	12.9	14.8	15.5
VA	12.2	10.4	12.3
mean	**10.4**	**10.6**	**10.6**
SE	**0.7**	**0.7**	**0.7**
	$P_{(0-30)} = 0.749$	$P_{(0-37)} = 0.824$	$P_{(30-37)} = 0.993$

In contrast, red blood cell α-tocopherol levels rose significantly ($p < 0.001$), reaching a 59.5 ± 8.5% increase after 30 days of supplementation, with an almost complete recovery after 7 days of wash-out (**Figure 3**). This result indicates that α-tocopherol is preserved in important cell structures, and it represents a further confirmation of the sparing effect exerted by dietary polyphenols on α-tocopherol (*10*, *11*, *24*, *25*).

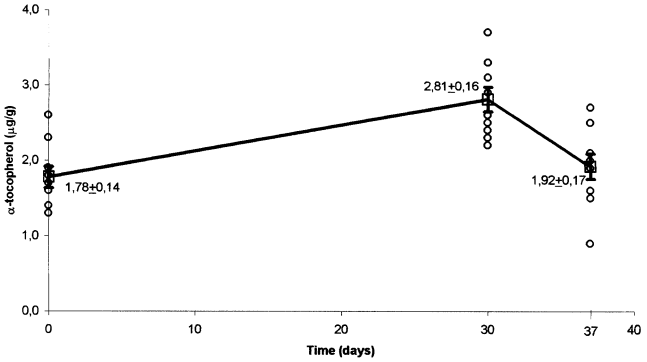


Figure 3. Time course (days) of red blood cell α-tocopherol levels. Value are means ± SE, $n = 10$.

Table 3. Polyunsaturated Fatty Acid (Percent) Content of Red Blood Cell Membrane Phospholipids[a]

fatty acid	$t = 0$ days	$t = 30$ days	$t = 37$ days	$P_{(0-30)}$	$P_{(30-37)}$	$P_{(0-37)}$
*n*9	18.59 ± 0.37	18.04 ± 0.25	17.79 ± 0.44	0.198	0.538	0.078
*n*6	23.43 ± 0.37	24.12 ± 0.54	24.31 ± 0.61	0.085	0.734	0.113
*n*3	1.44 ± 0.10	1.52 ± 0.10	1.58 ± 0.10	0.378	0.408	0.235
SFA	54.93 ± 0.32	54.78 ± 0.56	54.83 ± 0.37	0.791	0.904	0.650
MUFA	20.19 ± 0.35	19.50 ± 0.24	19.27 ± 0.41	0.160	0.442	0.042
PUFA	24.87 ± 0.38	25.63 ± 0.51	25.90 ± 0.63	0.089	0.682	0.122

[a] Values are expressed as mean ± SE, $n = 10$.

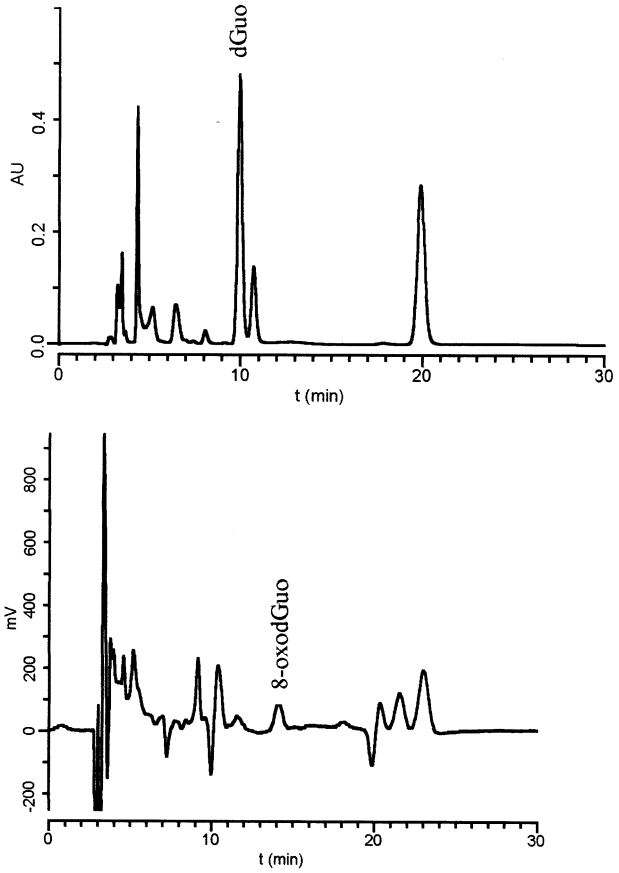


Figure 4. Typical HPLC profile of DNA hydrolysate: (top) UV trace at 245 nm; (bottom) EC trace at +350 mV. dGuo = 2-deoxyguanosine, 8-oxo-7,8-dihydro-2′-deoxyguanosine.

The increase of this vitamin in RBC membrane was accompanied by a slight and not significant rise of polyunsaturated fatty acid of RBC membrane phospholipids (**Table 3**).

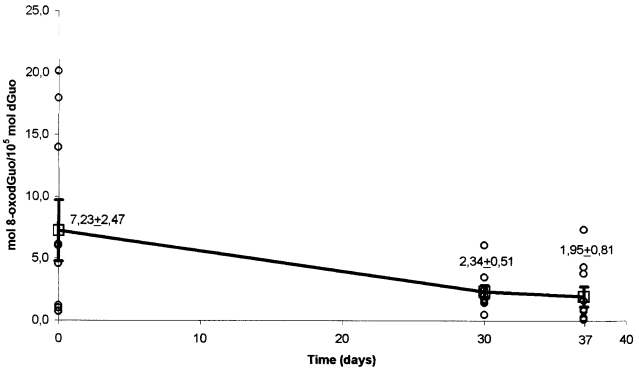


Figure 5. Time course (days) of [8-oxo-7,8-dihydro-2′-deoxyguanosine/10^5 2-deoxyguanosine] ratio in DNA isolated from lymphocytes. Values are means ± SE, $n = 10$.

To evaluate the oxidative DNA damage, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) was selected as an indicator of such damage (*26*). Therefore, 8-oxodGuo and the corresponding nucleoside 2-deoxyguanosine (dGuo) were measured in DNA isolated from peripheral lymphocytes and subjected to enzymatic digestion. A simple isocratic HPLC allowed both nucleosides to be separated (**Figure 4**), and their identities were confirmed by comparing their retention times and UV spectra with those of standards. Owing to its low levels, 8-oxodGuo was assayed by electrochemical detection. This was not the case for dGuo, the concentration of which was much higher, thereby permitting UV detection. Calibration curves were obtained by injecting standard dGuo and 8-oxodGuo, and linearity was assessed in the ranges of 10−100 μg/mL and 1−40 ng/mL for dGuo and 8-oxodGuo, respectively. The detection limits were 1 μg/mL and 1 ng/mL for dGuo and 8-oxodGuo, respectively.

The subjects showed a remarkable difference in the initial values of the ratios between 8-oxodGuo and dGuo (7.23 ± 2.47 mol of 8-oxodGuo/10^5 mol of dGuo) (**Figure 5**). This may be ascribed to the different dietary and environmental habits of the subjects and to their metabolic status, as has been demonstrated also by other authors (*27*, *28*). Despite this interindividual variation, the initial ratios decreased in all subjects, reaching a level of 2.34 ± 0.51 mol of 8-oxodGuo/10^5 mol of dGuo ($p = 0.049$, t_0 vs t_{30}) at the end of the supplementation, and remained at this value for the wash-out period (1.95 ± 0.81) (**Figure 5**). This result suggests that supplementation of healthy subjects with grape seed extract may protect DNA from oxidative damage.

This study suggests that grape seed extract intake may result in a significant sparing of α-tocopherol in red blood cell membranes, a considerable reduction of oxidative DNA damage, and a slight increase of polyunsaturated fatty acid in the phospolipids of RBC membranes.

ABBREVIATIONS USED

RBC, red blood cells; TRAP, plasma total radical-trapping antioxidant parameter; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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