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Dietary oils high in oleic acid, but with different non-glyceride contents, have different effects on lipid profiles and peroxidation in rabbit hepatic mitochondria

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Abstract

The influence on the lipid profile and lipid peroxidation in rabbit-liver mitochondria exerted by different edible oils high in oleic acid but different non-glyceride phenolic fractions was studied. High-phenolic virgin olive oil from the variety "Picual", the same oil submitted to an exhaustive process of washing to eliminate the phenolic fraction without altering the lipid profile and high-oleic sunflower oil (poor in phenolic compounds) were added to rabbit diets. The results reveal the importance of the different oleic: linoleic ratio of the lipid sources on the lipid profile of mitochondrial membranes. This is highlighted by the greater proportion of saturated fatty acids and the lower content in oleic acid ($p < 0.05$) shown by the rabbits fed on high-oleic sunflower oil. The group fed on the fat rich in phenolics exhibited the highest level of antioxidants (α-tocopherol, ubiquinone 10) and the highest activity of glutathione peroxidase as well as the lowest content in hydroperoxides and TBARS. The study provides evidences *in vivo* about the considerable antioxidant capacity of the phenolic fraction of virgin olive oil in rabbit-liver mitochondria and the important role that this non-glyceride fraction can play in the overall antioxidant benefits attributed to this oil.

Keywords: Non-glyceride fraction; Phenolic compounds; Olive oil; Lipid peroxidation; Membrane lipid profile; Mitochondria; Rabbits

1. Introduction

It is known that dietary fat can modify the degree of lipid peroxidation in the cell membranes, both by the influence which the lipid profile of the fat ingested exerts on the lipid profile of the cell membranes [1,2,3], as well as by the number of structural and functional changes that occurs in the membranes, including cholesterol mobilization [4], increased coenzyme Q synthesis [5,6] and modulation of enzyme activity and levels [7,8].

In recent years, we have performed a series of studies comparing the effect on lipid peroxidation of different dietary lipid sources with varying degrees of unsaturation (virgin olive oil, olive oil, corn oil and sunflower oil). We found that ingestion of virgin olive oil offers greater protection of the mitochondrial and microsomal membranes against damage caused by endogenous peroxidative stress, as well as that caused by the administration of xenobiotics [2,4,9,10]. However, these studies, as in most similar studies, used lipid sources with a different lipid composition and their results and discussions centre on the importance that the greater oleic acid content in this oil confers in combating lipid peroxidation and preventing disease. This, in part, has given rise to the market in new vegetable oils enriched in this monounsaturated fatty acid as, for example high-oleic sunflower oil. However, although the fatty acids composition of these new oils is similar to that found in olive oil, major differences exist, especially in the non-glyceride (also called unsaponifiable) fraction [11,12,13]. It should be borne in mind that these oils require refining; a process which results in loss of the non-glyceride fraction [14,15]. This aspect impacts on the question of oxidative stress and the health benefits

Abbreviations: AAPH = 2,2′-Azobis (2-amidinopropane) dihydrochloride; GPX = Glutathione peroxidase; GR = Glutathione reductase; HOS = High-oleic sunflower oil; HPLC = High performance liquid chromatography; SOD = Superoxide Dismutase; TBARS = Thiobarbituric acid reactive substances; VO = Virgin olive oil "Picual"; WO = Washed olive oil.

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Table 1
Fatty acid and non-glyceride composition of the different fat sources used for the diet composition (VO - Virgin olive oil "Picual"; WO - Washed olive oil and HOS - High-oleic sunflower oil). Values are means ± SEM (n = 6)

	VO	WO	HOS
		Fatty acids (%)	
Palmitic acid (16:0)	9.0 ± 0.0	9.1 ± 0.0	4.2 ± 0.1
Palmitoleic acid (16:1)	0.6 ± 0.1	0.7 ± 0.0	0.1 ± 0.1
Stearic acid (18:0)	3.7 ± 0.0	3.7 ± 0.0	3.9 ± 0.0
Oleic acid (18:1n-9)	81.7 ± 0.7	81.4 ± 0.3	80.2 ± 0.3
Linoleic acid (18:2n-6)	3.6 ± 0.1	3.5 ± 0.0	10.7 ± 0.1
SFA	13.1 ± 0.1	13.2 ± 0.1	8.1 ± 0.1
PUFA	4.2 ± 0.0	4.1 ± 0.0	10.7 ± 0.9
MUFA	82.3 ± 0.0	82.1 ± 0.3	80.3 ± 0.3
Oleic/linoleic	22.4 ± 0.1	23.2 ± 0.1	7.5 ± 0.0
		Non-glyceride fraction	
Tocopherol (mg/Kg)	306.9 ± 7.4	214.2 ± 7.3	921.6 ± 10.4
Phenols (ppm. caffeic acid)	738.0 ± 5.0	39.7 ± 1.0	12.0 ± 0.1

PUFA. - Polyunsaturated fatty acids; MUFA. - Monounsaturated fatty acids; SFA; Saturated fatty acids.

of such oil and it has received little attention to date [11,13].

Virgin olive oil, a monounsaturated fat mainly traditional in the Mediterranean diet, apart from its high content in oleic acid, shows an unsaponifiable fraction rich in antioxidant compounds, including phenolic compounds. In fact, this is the only dietary oil that contains these compounds, which have been shown to contribute to the oxidative stability of this oil [16,17]. Numerous studies have shown the antioxidative capacity of phenolics, which reduce the susceptibility of LDL and erythrocyte membranes to lipid peroxidation [18,19,20,21]. However, most of these studies have been performed *in vitro* and have used individual phenolic compounds.

With reference to the phenolic components in olive oil, it should be indicated that some oils during pressing and especially refining are submitted to rather prolonged washing, producing oil with the same saponifiable composition but a highly variable non-glyceride composition [14,15], since especially the phenolic fraction is very easily lost in such processes.

The present study has a dual objective. First, to examine the influence that the ingestion of different commercial oils with a high content in oleic acid but different composition of phenolic can exert on the fatty-acid composition of rabbit-liver mitochondrial membranes. Secondly, to establish the relative importance of the saponifiable and non-glyceride fractions of these oils in reducing lipid peroxidation in these membranes, as well as the possible antioxidant effect *in vivo* of these phenolic components of the virgin olive oil. For these objectives, the following oils were used: a variety of virgin olive oil called 'Picual', which is very rich in phenolic compounds; the same oil submitted to an exhaustive process of washing, which results in the complete loss of the phenolic fraction without altering the lipid profile; and a commercial variety of sunflower rich in oleic acid.

2. Materials and methods

2.1. Materials

All chemicals used were purchased from Sigma Chemical Co., and all solvents were pure reagents of Merck. Different standard ubiquinone homologues (kindly donated by Eisai Co., Tokyo, Japan) were stored as solutions in absolute ethanol at −80°C at concentrations ranging from 1 to 10 mM, as determined spectrophotometrically at 275 nm using extinction coefficients typical of each homologue [22], and according to Mayer and Isler [23].

2.2. Animals and diets

Twenty-four male New Zealand rabbits (*Oryctolagus cuniculus*) were randomly assigned to three groups of 8 animals. The animals were individually housed under standard conditions and the room was maintained on a cycle of 12 h light/12 h darkness and at a temperature of 22°C ± 1°C. For 8 weeks each group was fed a semi-synthetic isoenergetic diet composed of 19% caseine, 23% starch, 29.6% sugar, 14% cellulose, 6% fat, 7% mineral supplement, 1% vitamin supplement, 0.3% methionine and 0.1% choline, to provide the rabbits their dietary needs [24]. Diets differed in the nature of the fat (Table 1). Experimental fats consisted of extra-virgin olive oil from the variety 'Picual' (VO); the same olive oil but submitted to continuous washings with water at room temperature until almost complete loss of the phenolic fraction, but without loss of the saponifiable fraction (WO); and a commercial high-oleic sunflower oil (HOS). The diets were stored at 4°C, and the rabbits were provided *ad libitum* access to fresh diet and water every day.

The lipid profile of the lipid sources used were determined by gas chromatography (Regulation EEC/2568/91)

and the phenolic content by spectrophotometry, the results being expressed in ppm of caffeic acid [25]. Also, α-tocopherol was evaluated by HPLC according to Ueda and Igarashi (1990) [26].

2.3. Analytic determinations

At the end of the experimental period, the rabbits were anaesthetised with a solution of sodium pentothal in ultrapure water (1/50) and then exanguinated through a carotid cannule. The rabbits were sacrificed at the same time of day (between 8:00 and 9:00) to avoid any circadian fluctuation. Livers were rapidly removed and mitochondrial fraction was isolated according to Fleischer et al. [27].

Protein concentration of the samples was determined by the technique of Lowry et al. [28], using bovine albumin as a standard. The phosphorus content in the phospholipids of the mitochondrial membrane was determined as previously described [29].

Hydroperoxides content was determined using the method of Jiang et al. [30]. This technique is based on the rapid oxidation of Fe^{2+} to Fe^{3+} by hydroperoxides under acid conditions. We performed two separate determinations, the first one, referred to as baseline levels of hydroperoxides, were made using the mitochondrial membranes with no further inductions. The second determination was developed after the induction of the sample with 2.5 mM of AAPH (a potent generator of free radicals). This measure allows determination of the maximal level of peroxides that a mitochondrial membrane is able to produce. Additionally, the difference in the induced levels of hydroperoxides minus the baseline level was used as an index of oxidation susceptibility of the membranes to lipid peroxidation.

The thiobarbituric acid reactive substances (TBARS) in mitochondrial membranes were analysed following the method of Orrenius [31], through the spectrophotometric measurement of substances that react with thiobarbituric acid. Both parameters were expressed in nmol/μmol of inorganic phosphorus (Pi).

Fatty-acid profiles in mitochondrial membranes were determined according to the method of Lepage and Roy [32], using a Hewlett Packard HP 5890 Series II chromatograph (Palo Alto, CA) and a Supelco column (Palo Alto, CA) PHASE: SP™ 2330 FS capillary column, 60 m long, 32 mm id and a thickness of 20 mm. The data are expressed as a percentage of the total fatty acids determined.

Coenzyme Q and α-tocopherol concentrations were assayed by high-performance liquid chromatography (HPLC). Coenzyme Q and α-tocopherol were extracted by a mixture of ethanol: petroleum (60:40) using the method of Kroger [33], after centrifugation at 2500× for 5 min, the upper layer was collected by aspiration and the residue was re-extracted twice with 1 ml of petroleum ether. The dry residue of combined extracts was diluted in the HPLC mobile phase (ethanol: water, 97:3). The HPLC system consisted of an apparatus equipped with a Diode Array Detector, model 168 (Beckman Instruments, Inc., Fullerton, CA, USA) and the column was a reverse-phase C18 Spherisorb ODS 1 of 25 × 0.46 cm reverse phase C18 column with a guard column containing the same material as the main column.

Catalase activity was determined following the method described by Aebi [34], based on monitoring the H_2O_2 decomposition by spectrophotometric measures at 240 nm, as a consequence of the catalytic activity of catalase. Superoxide dismutase (SOD) was determined by the method of Fridovich et al. [35], based on the inhibition by SOD on the reduction of the cytochrome C and spectrophotometric measurement at 550 nm. For classic glutathione peroxidase (cGPX), we used the technique of Flohé et al. [36], a method based on the instantaneous formation of glutathione oxidized during the reaction catalysed by glutathione peroxidase, which is continually reduced by an excess of active glutathione reductase and NADPH present in the cuvette. The subsequent oxidation of NADPH to $NADP^+$ was monitored spectrophotometrically at 340 nm. Finally, glutathione reductase activity (GR) was measured according to Carlberg et al. [37]. This method is based in the reduction of oxidized glutathione (added in excess) during the reaction catalyzed by glutathione reductase. The subsequent oxidation of NADPH was monitored spectrophotometrically at 340 nm.

2.4. Statistical analysis

Results are presented as means ± S.E.M. of 8 animals. All the results were submitted to one-way ANOVA analysis. Duncan's test was performed *a posteriori* to evaluate differences between groups. All p values of 0.05 or less were considered significant. The statistical treatments were carried out using the SPSS package (SPSS for Windows, 6.1, 1994, SPSS Inc., IL, USA).

3. Results

The data corresponding to the composition of the oils used in the study are shown in Table 1. Concerning the fatty acid composition, all the oils had a similarly high percentage of oleic acid, the HOS being the lipid source with the highest percentage of linoleic acid and in turn the one lowest in saturated fatty acids. With respect to the oleic: linoleic acid ratio, the HOS presented the lowest value. The non-glyceride fraction showed greater differences, and thus the HOS presented a high tocopherol content but almost a null content in phenolic compounds, with the VO showing the highest content in these compounds.

Table 2 lists the percentages of the various fatty acids as well as some of the indicators of the lipid profile in the mitochondrial membranes of the rabbit liver in the different experimental groups. All these point to oleic acid as the major fatty acid, the HOS group presenting the lowest

Table 2
Fatty acid profile in liver mitochondrial membranes of rabbits fed for eight weeks on different fat sources (VO - Virgin olive oil "Picual"; WO - Washed olive oil and HOS - High-oleic sunflower oil). Values are means ± SEM (n = 8). The appearance of non-coinciding letters for the same parameter indicates significant statistical differences ($p < 0.05$)

	VO	WO	HOS
Palmitic acid (16:0)	16.5 ± 0.3^{a}	16.3 ± 0.3^{a}	17.9 ± 0.4^{b}
Stearic acid (18:0)	14.3 ± 0.3^{a}	14.6 ± 0.6^{a}	15.4 ± 0.3^{a}
Oleic acid (18:1n-9)	33.7 ± 0.7^{b}	33.3 ± 1.1^{b}	29.0 ± 0.8^{a}
Linoleic acid (18:2n-6)	20.8 ± 0.8^{a}	20.4 ± 1.0^{a}	21.2 ± 0.7^{a}
Linolenic acid (18:3n-3)	0.8 ± 0.0^{b}	0.6 ± 0.1^{ab}	0.5 ± 0.0^{a}
Arachidonic acid (20:4n-6)	5.7 ± 0.3^{a}	5.6 ± 0.5^{a}	5.9 ± 0.2^{a}
Docosahexaenoic acid (22:6n-3)	0.5 ± 0.2^{a}	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}
SFA	32.0 ± 0.4^{a}	32.0 ± 0.7^{a}	34.8 ± 0.5^{b}
PUFA	30.9 ± 1.0^{a}	30.0 ± 1.0^{a}	32.2 ± 0.8^{a}
PUFA n-6	28.5 ± 1.1^{a}	28.3 ± 1.2^{a}	29.5 ± 0.8^{a}
PUFA n-3	2.0 ± 0.2^{a}	2.1 ± 0.2^{a}	1.9 ± 0.1^{a}
SFA/PUFA	1.0 ± 0.0^{a}	1.1 ± 0.0^{a}	1.0 ± 0.1^{a}
Oleic/linoleic	1.6 ± 0.1^{a}	1.6 ± 0.1^{a}	1.4 ± 0.1^{a}

PUFA - Polyunsaturated fatty acids; MUFA - Monounsaturated fatty acids; SFA - Saturated fatty acids.

percentage of this fatty acid, and in turn registering the highest percentage of total saturated fatty acids, with substantial statistical differences with respect to the other two groups ($p < 0.05$). With respect to the percentage of polyunsaturated fatty acids, as well as linoleic acid, no significant differences were found between any of the three groups.

The content in TBARS (Fig. 1) shows the group of rabbits fed the VO oil to have the lowest values, with statistically significant differences ($p < 0.05$). The hydroperoxide content (Fig. 2) presented a pattern similar to that of the TBARS, both for the basal determination as well the induced one, group VO again registering the lowest value for susceptibility.

With respect to the antioxidants α-tocopherol and coenzyme Q (Fig. 3), the WO and HOS groups presented the lowest content in these antioxidants, with tocopherol being significantly lower than in the group of rabbits fed the VO oil.

Finally, no significant differences were detected among any of the groups for catalase, SOD or GR (Table 3). Nevertheless, the rabbits fed VO had higher cGPX (Fig. 4).

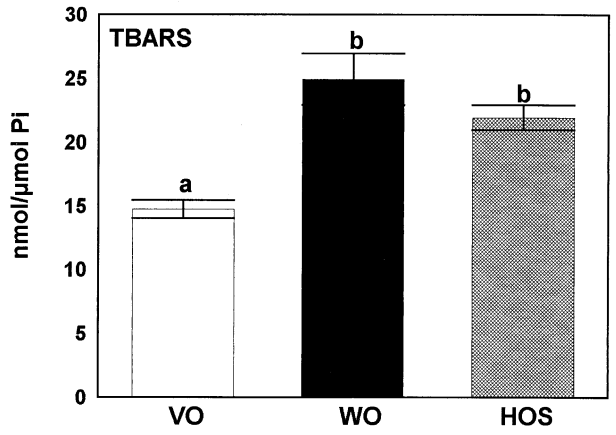

Fig. 1. Concentration of TBARS in liver mitochondrial membranes of rabbits fed for eight weeks with different fat sources. The abbreviations used are: VO = Virgin olive oil "Picual"; WO = Washed olive oil; HOS = High-oleic sunflower oil. Values represented are means ± SE (n = 8). Columns with different superscript letters are significantly different ($p < 0.05$).

4. Discussion

With reference to the influence on the lipid composition in the mitochondrial membranes, our data indicate that although all the groups showed oleic acid to be the major fatty acid, the group of rabbits fed high-oleic sunflower oil (HOS) presented a lower content of this fatty acid, and also registered the highest percentage of saturated fatty acids, despite this fat source having the lowest saturated content. These data are opposed to those of other authors [13,38] indicating that HOS oil, despite showing an oleic acid content resembling that of olive oil, has a different influence on the lipid profile of the mitochondrial membranes of the liver. Thus, if we take into account the fact that polyunsaturated lipid sources raise the percentage of saturated fatty acids in the plasma and membranes [3,10,15], perhaps a consequence of a maintenance mechanism of membrane fluidity [39] or due to interactions of linoleic acid with delta 9-desaturase [40], which could boost palmitic and stearic acid levels, the sunflower oil rich in oleic acid appears to show a behaviour more similar to that of a polyunsaturated source than a monounsaturated source, in terms of its influence over the lipid profile.

From the standpoint of lipid peroxidation, we first should remember that the rabbits studied were not subjected to any type of additional oxidative stress beyond the simple ingestion of different lipid sources used. Secondly, the polyunsaturated fatty-acid content was quite homogeneous among all the groups, this theoretically leading to membranes with

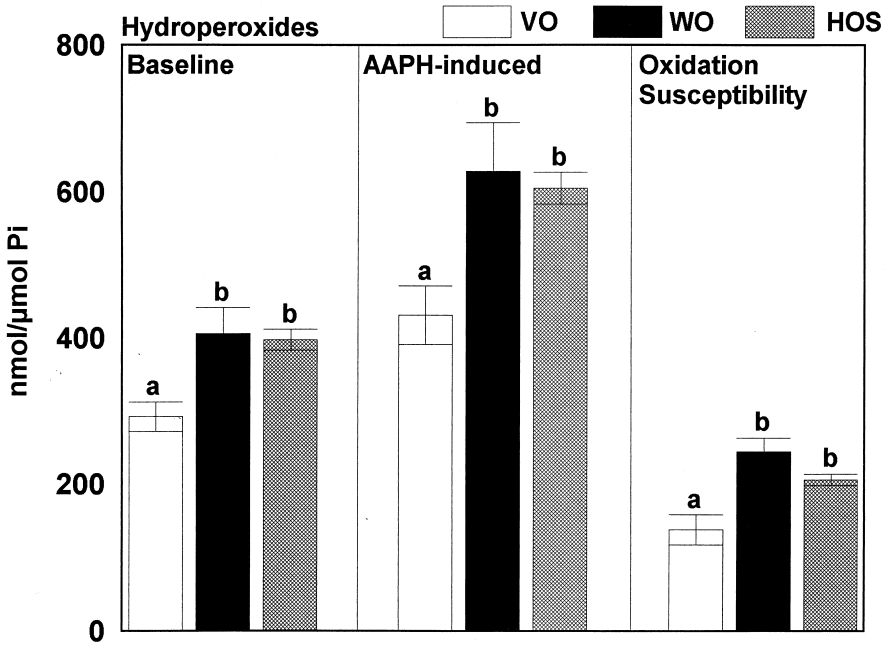

Fig. 2. Concentration of hydroperoxides at baseline levels, after the induction with AAPH and the oxidation susceptibility index (induced minus baseline levels) in liver mitochondrial membranes of rabbits fed for eight weeks with different fat sources. The abbreviations used are: VO = Virgin olive oil "Picual"; WO = Washed olive oil; HOS = High-oleic sunflower oil. Values represented are means ± SE (n = 8). For each treatment, columns with different superscript letters are significantly different ($p < 0.05$).

a similar susceptibility to lipid peroxidation, given that these fatty acids are the main ones involved in reactions of propagation and multiplication of this process [41].

However, as can be showed in the results, despite this similarity in polyunsaturated fatty acids, the groups differed strongly in peroxidative stress, indicating that the differences with respect to the hydroperoxide content and TBARS, as well as the diverse content and activity of the antioxidants studied, are not only the result of the lipid profile of the fatty acids used, but mainly the consequence of the non-glyceride fractions present in these oils.

Thus, HOS oil showed an extremely high α-tocopherol content, approximately three-fold that of VO olive oil and four-fold that of the WO oil. Nevertheless, this is not reflected by the α-tocopherol content in the mitochondrial membrane, as this was similar to that found for the group of rabbits fed WO but lower than that of the group fed VO. This indicates that, first, a possible greater expenditure of this antioxidant molecule in the HOS and WO groups as a consequence of the greater oxidative stress shown by these groups, and secondly that the unsaponifiable fraction of the HOS oil proved less effective than the corresponding fraction of the VO oil in maintaining low oxidative stress in the membranes under study. Apparently, that response in liver mitochondria to α-tocopherol content of virgin olive oil and sunflower oil may be common in different organisms since we have previously described a similar situation in rats [10]. In addition, the plasma and the LDL of the rabbits in the present study showed a similar pattern (data not showed).

Thus, the group of rabbits that ingested the VO showed a lower content in TBARS and hydroperoxides (both under baseline conditions and after an induction *in vitro*) and less susceptibility to oxidation, higher tocopherol and ubiquinone content. In addition, this group showed the highest activity of glutathione peroxidase, as opposed to results reported by other authors [13]. This discrepancy in the glutathione peroxidase activity is perhaps due to the use of different varieties of virgin olive oil, which in other studies may have been less rich in phenolic compounds than are the oils used in the present study.

There are some interesting reflections when we compare VO oil with the rest of the lipid sources in our study. Firstly, all of them are similar from the point of view of the composition of their mitochondrial membranes in polyunsaturated fatty acids. Moreover, HOS and WO have similar levels of α-tocopherol but less than VO in these membranes. The most important factor from the point of view of the peroxidation found in the animals seem to be the difference in phenolics found in virgin olive oil (Table 1). In fact, the unwashed virgin olive oil (VO) contain approximately 18-fold more phenolics than the washed olive oil (WO), and nearly 60-fold that of the sunflower oil rich in oleic acid (HOS).

To date, there is very little information about absorption and the metabolic fate of olive oil phenolics. Recently, Visioli et al. [42] have reported that tyrosol and hydroxytyrosol are dose-dependently absorbed in humans after ingestion of 50 ml of olive oil over four days. However, these results should be taken with caution since the normal intake of olive oil, even in Mediterranean countries is, on average,

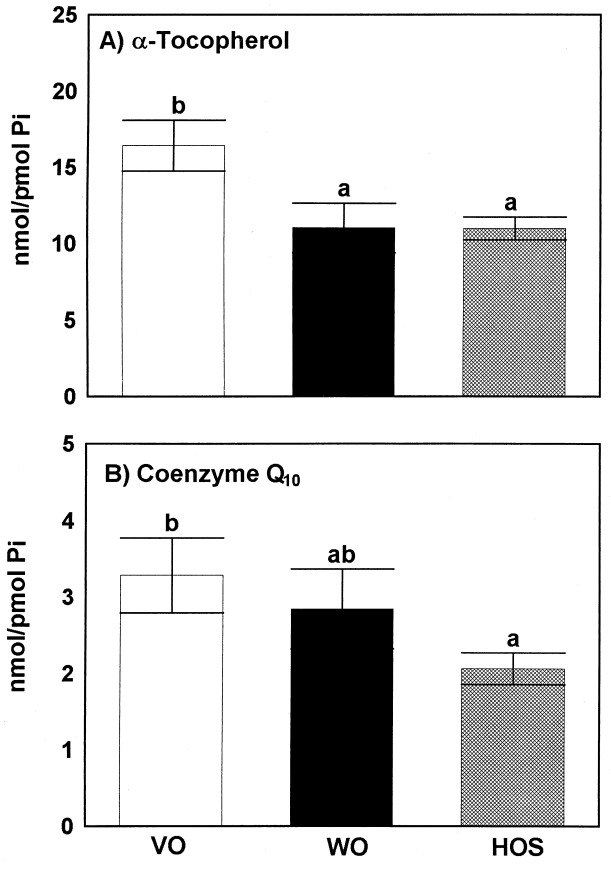

Fig. 3. Concentration of Coenzyme Q (A) and α-tocopherol (B) in liver mitochondrial membranes of rabbits fed for eight weeks with different fat sources. The abbreviations used are: VO = Virgin olive oil "Picual"; WO = Washed olive oil; HOS = High-oleic sunflower oil. Values represented are means ± SE (n = 8). Columns with different superscript letters are significantly different ($p < 0.05$).

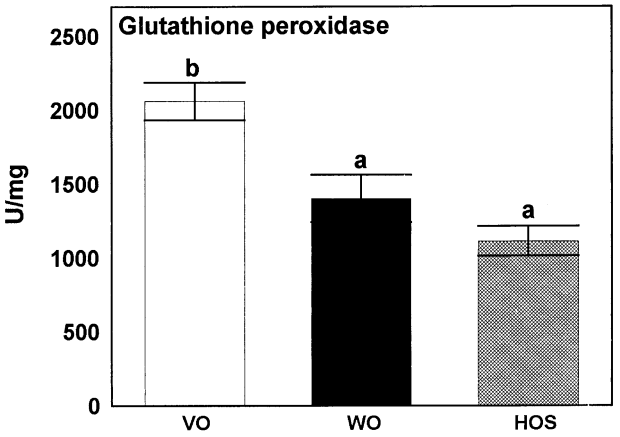

Fig. 4. Activity of classic glutathione peroxidase (cGPX) in liver mitochondrial membranes of rabbits fed for eight weeks with different fat sources. The abbreviations used are: VO = Virgin olive oil "Picual"; WO = Washed olive oil; HOS = High-oleic sunflower oil. Values represented are means ± SE (n = 8). Columns with different superscript letters are significantly different ($p < 0.05$).

lower than 50 ml/day and the oils used in the mentioned study were very rich in phenolics. Despite we did not test the presence of olive oil phenolics in the rabbit-liver mitochondria; it could be possible that a continual exposure to olive oil phenols results in a long-term accumulation of these molecules [42].

There are several mechanisms by which the phenolic compounds of virgin olive oil can act as antioxidants. The compounds of this phenolic fraction reportedly have a capacity for metal chelation or direct interaction with various free radicals [20,21,43]. Although, in view of the results of the present study, this action could be due, at least in part, to maintaining the pool of reduced tocopherol, an antioxidative mechanism similar to that described for ubiquinone 10. This, in turn, leads to a lower expenditure of this molecule, as observed in the case of the group of rabbits fed the VO. This mechanism has been also described for other phenolics [43]. Similarly, increases have been observed, after the use of phenolic compounds present in tea, in the activity of cytosolic enzymes and phase II enzymes [44]. This, together with the increased activity of the enzyme glutathione peroxidase shown by the VO group, leads us to assume a possible role of this phenolic fraction in this redox enzyme activity. However, there is no literature available on this aspect, and therefore we need further research to confirm this effect.

In conclusion, the present study shows the importance of the oleic: linoleic ratio in the lipid sources used, especially from the point of view of the lipid profile of the mitochondrial membranes in rabbit liver. This also shows the antioxidant capacity of the phenolic compounds present in virgin olive oil and the important role played by this fraction on the antioxidant benefits of this oil, at least under our experimental conditions. It is therefore necessary to have both an adequate oleic: linoleic ratio as well as an appropriate non-glyceride fraction, since when any of these fac-

Table 3
Activity of cytosolic superoxide dismutase (SOD), catalase and glutathione reductase (GR) in liver of rabbits fed for eight weeks on different fat sources (VO - Virgin olive oil "Picual"; WO - Washed olive oil and HOS - High-oleic sunflower oil). Values are means ± SEM (n = 8). The appearance of non-coinciding letters for the same parameter indicates significant statistical differences ($p < 0.05$)

	VO	WO	HOS
SOD (U/mg)	5.7 ± 0.3^a	5.6 ± 0.1^a	5.4 ± 0.3^a
Catalase (seg^{-1} *mg^{-1})	0.1 ± 0.0^a	0.1 ± 0.0^a	0.1 ± 0.0^a
GR (U/mg)	12.5 ± 0.7^a	12.4 ± 0.6^a	10.9 ± 0.8^a

tors is altered, the effect of the lipid sources on health could be negative.

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