# Differential Inhibitory Effect of alpha-, beta-, gamma-, and delta-Tocopherols on the Metal-Induced Oxidation of Cholesterol in Unilamellar Phospholipid-Cholesterol Liposomes

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**ABSTRACT: Cholesterol oxidation products (COPs) are present in biological tissues and in foods. The inhibitory effect of antioxidants, such as tocopherols, on COPs formation has been only partially investigated. The antioxidant effect of dl alpha-, dl beta-, dl gamma-, and dl-delta tocopherol on the metal-induced oxidation of phosphatidylcholine (PC): cholesterol liposomes was assayed. Formation during liposome oxidation of six different COPs was monitored by gas chromatography. dl alpha-, and dl gamma-tocopherol show good inhibitory effect against PC-fatty acid oxidation and also on COPs formation. dl delta-Tocopherol is less effective than the alpha- and gamma-homologous, beta-tocopherol being unable to prevent PC and cholesterol oxidation. dl alpha-, and dl gamma-Tocopherol are more effective to prevent the oxidation of the lateral chain of cholesterol molecule. At the highest tocopherol concentration assayed, dl alpha-tocopherol shows prooxidant effect, enhancing liposomal oxidation and COPs formation. It is concluded that the tocopherols assayed can inhibit cholesterol oxidation but to a different degree.**

**Keywords: cholesterol oxidation products, liposome oxidation, tocopherols, natural antioxidants**



## Introduction

CHOLESTEROL IS A MOLECULE WITH AN UNSATURATED BOND, THEREFORE it is prone to oxidation (Paniangvait and others 1995; Schroepfer 2000). The sterol is sensitive to free radical oxidation by diatomic molecular oxygen (Smith 1987). The autoxidation of cholesterol has been extensively studied and is well documented (Kubow 1993). Cholesterol molecules function as an integral part of the lipid bilayer of cell membranes and are closely associated with membrane phospholipids (Morel and Lin 1996). As cholesterol contains one Δ5-double bond, formation of any oxygen radical or free radical is expected to initiate cholesterol oxidation. Up to 17 different cholesterol oxidation products (COPs) can be identified from biological tissues and food samples (Brown and Jessup 1999).

Antioxidants are organic lipid- or water-soluble substances of either synthetic or natural origin that can prevent or delay the development or the progress of polyunsaturated fatty acid oxidation (Namiki 1990). The protective effect of antioxidants on triacylglycerol oxidation in a wide variety of fats and oils, and also on phospholipids in biological and artificial bilayer membranes, is widely documented (Frankel 1993). However, the effect of antioxidants on cholesterol oxidation has not been extensively studied up to date, results are diverse and sometimes contradictory. Vitamin E, among natural antioxidants, is the best known and widely used antioxidant (Bieri 1984). This substance is a mixture of tocopherols and tocotrienols, tocopherols being the only molecules exhibiting antioxidant activity (Logani and Davies 1980). Tocopherols (dl alpha-, dl beta-, dl gamma-, and dl delta-) exhibit different antioxidant behaviors, depending on the system and/or conditions where they are assayed. It is also accepted that dl alpha- and dl gamma-tocopherol are the most effective as antioxidants, dl delta-tocopherol being less active and dl beta-tocopherol almost totally inactive under some conditions (Pokorny 1991).

Liposomes are simply vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules. Phosphatidylcholine (PC): cholesterol (CHO)-containing liposomes constitute a very clean membrane model to assess the oxidative stability of the phospholipid fatty acid component and/or of the sterol fraction (Barclay and others 1985). These membrane models are also very suitable to assay the effectiveness of lipid- or water-soluble antioxidants (Sevanian and McLeod 1987).

Although tocopherols have received much attention as antioxidants in foods, as well as in biological systems, the individual effects of the different tocopherols composing vitamin E on the oxidative stability of cholesterol have not been extensively studied. The present work was designed to evaluate the effect of different concentrations of dl alpha-, dl beta-, dl gamma-, and dl delta-tocopherols on the COPs formation from PC:CHO-containing unilamellar liposomes when oxidation of liposomes is induced by $Fe^{2+}$-ascorbate.

## Materials and Methods

### Chemical materials

PC with linoleic acid at the sn-1 and sn-2 positions was purchased from Nu-Chek-Prep., Inc. (Elysian, Minn., U.S.A.). CHO (cholest-5-3 beta-ol) of the highest purity available (essentially free of COPs), 7 alpha-hydroxycholesterol (cholest-5-en-3 beta, 7 alpha-diol) (7 alpha-OH), 7 beta-hydroxycholesterol (cholest-5-en-3 beta, 7 beta-diol) (7 beta-OH), 7-ketocholesterol (3 beta-hydroxycholest-5-en-7-one) (7-keto), cholestanetriol (5 alpha-cholestane-3 beta, 5, 6 beta-triol) (5 alpha-3OH), 20-hydroxy-

cholesterol (cholest 5-en-3 beta, 20 diol) (20-OH), 25-hydroxycholesterol (cholest-5-en-3 beta, 25-diol) (25-OH), and 5 alpha-cholestane, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). dl alpha-, dl beta-, dl gamma-, and dl delta-Tocopherols (95 to 98%) were a gift from Productos Roche de Chile (Santiago, Chile). Sylon BTZ was purchased from Supelco Inc. (Bellefonte, Pa., U.S.A.).

### Column chromatography of cholesterol

Commercial CHO was subjected to a silicic acid-column chromatography to separate possible COPs derived from CHO extraction and/or further processing and handling according to Kim and Nawar (1991). Purity of the preparation was tested by thin layer chromatography as evidenced by a single spot when compared to the CHO standard.

### Liposome preparation

Unilamellar liposomes of PC and CHO, 2 : 1 mole ratio, and containing 50, 100, and 150 μM of each tocopherol were prepared through a modification of the method described by Sevaninan and McLeod (1987). The liposome suspension was sonicated with 5 pulses of 30 s at 300 W (Branson Sonifier 350). Control liposomes were prepared by the same procedure but avoiding addition of tocopherols. The buffer for sample preparations was sodium citrate (pH 5.6). Liposomes were maintained at 4 °C, under $N_2$ with gentle agitation until use (always within the next 12 h). Concentration of liposomes was estimated through PC content and adjusted to 30 mg liposomes/mL (Bartlett 1959). Liposome oxidation was carried out at 42 °C, under slow stirring (100 rpm), in 100 mL sealed glass bottles containing 75 mL of liposome emulsion by adding 50 μM $Fe^{2+}$ ($FeCl_2$) and 0.5 mM ascorbate (in citrate buffer) according to Kagan and others (1990). Oxidation was monitored at 0, 2, 4, 6, 8, and 10 h by measuring the thiobarbituric acid reactive substances (TBARS) formation according Schmedes and Holmer (1989). Absorption of aliquots (1.5 mL) taken at every time during incubation were measured by UV-VIS spectrophotometry at 532 nm and recorded as TBA-nr (optical density units/aliquot, TBA-nr) according to Yin and Faustman (1993). Liposome samples having TBA-nr > 0.05 before each assay were discarded.

### Gas-chromatography assay for COPs.

COPs formation after liposome oxidation was assessed by capillary gas chromatography, according the procedure of Addis and others (1989) after some modifications. At the end of each oxidation assay, lipids were extracted by adding 100 mL chloroform-methanol (2:1 v/v) and 150 μL of 5 alpha-cholestane as internal standard, and shaken for 2 min. After centrifugation at 1000 × g for 20 min, the organic phase was evaporated under vacuum. Saponification of the recovered lipids from the organic phase was performed by adding 20 mL of 1N KOH in methanol and maintained for 20 h at 20 °C and under a steam of $N_2$. Unsaponifiables were extracted with anhydrous diethyl ether, dried over $Na_2SO_4$, filtered (Whatman nr 1 filter paper), and evaporated to 1 mL volume. Trimethylsilyl ester derivatives were formed by the addition of 100 μL pyridine and 50 μL Sylon BTZ. Gas chromatography analyses of COPs, as trimethylsilyl esters derivatives, were made with a Hewlett Packard 5890 Series II G.C., equipped with a flame ionization detector. The GC conditions were: Ultra 1 Hewlett Packard 50 m capillary column (0.2 mm id., 0.33 μm film thickness); temperature programming from 180 °C to 285 °C at 5 C/min; injector at 250 °C; and detector at 300 °C.

### Statistical analysis

Data were analysed statistically by Student's t-test. Mean values were obtained by averaging 4 independent measurements and expressed ± S. D. Differences between different average measurements were considered significant at $p < 0.05$.

## Results and Discussion

### Effect of tocopherols on liposome oxidation

Figure 1 (A to D) shows the individual effect of different concentrations of dl alpha-tocopherol (A), dl beta-tocopherol (B), dl gamma- tocopherol (C), and dl delta-tocopherol (D) on the kinetics of the metal-induced oxidation of PC : CHO unilamellar liposomes, and expressed as TBA-nr. It can be observed that dl alpha- and dl gamma-tocopherol show a concentration dependent inhibitory effect on liposome oxidation when assayed at the lower concentrations (50 μM and 100 μM). However, at the higher concentration assayed (150 μM) dl alpha-tocopherol showed a prooxidant effect, which was not observed for dl gamma-tocopherol. In our experimental condition, dl delta-tocopherol showed a very low antioxidant effect, while dl beta-tocopherol was unable to prevent liposome oxidation.

### Effect of tocopherols on cholesterol oxidation

Figures 2 to 5 (A to D) show the individual effect of each tocopherol when assayed at the different concentrations on the remaining CHO concentration, and on the different amounts of COPs formed from the liposomes after 10 h oxidation, and compared to the control liposomes without added tocopherols. Controls showed an appreciable amount of COPs as product of CHO oxidation (Figure 2 to 5, A). From the 6 COPs assayed, 7 alpha-OH, 7 beta-OH, 20-OH, and 25-OH are the most relevant oxidation products. 5 alpha-3OH and 7-keto were also formed, but in small amounts. dl alpha-Tocopherol, when assayed at 50 and 100 mM (Figure 2 B to C), can efficiently inhibit COPs formation. However, when the same tocopherol was assayed at 150 mM (Figure 2 D), it was not only unable to inhibit cholesterol oxidation,

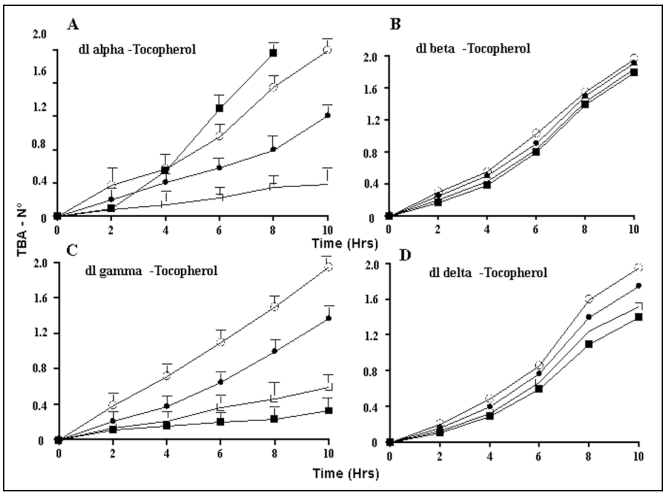


**Figure 1–Effect of dl alpha-tocopherol (A), dl beta-tocopherol (B), dl gamma-tocopherol (C), and dl delta-tocopherol (D), assayed at 50 μM (-●-), 100 μM (-■-), and 150 μM (-○-), compared to control (-m-), on the metal-induced oxidation of phospholipid : cholesterol liposomes. Results represent the average of 4 assays ± S.D. Other experimental conditions are in the text.**

but it also increases the formation of the oxidation products. The prooxidant effect of dl alpha-tocopherol, observed for PC oxidation (Figure 1 A), also affected CHO stability. dl alpha-Tocopherol, at the highest concentration assayed, significantly increased the formation of all COPs assayed, the most remarkable being the 20-OH and 25-OH increased. This result confirms that the prooxidant effect of dl alpha-tocopherol observed for the PC-fatty acids is also extended to CHO oxidation. dl gamma-Tocopherol (Figure 4, B to D) shows an effective protection against COPs formation at the different concentrations assayed, the tocopherol behavior showing good correlation with the inhibitory effect exhibited for the PC-fatty acid oxidation. It is also remarkable that some selectivity can be observed when the protective effect of both tocopherols against CHO oxidation was compared. dl gamma-Tocopherol and dl alpha-tocopherol (at those concentrations exhibiting antioxidant activity) were good inhibitors of the 20-OH and 25-OH formation. However, the same tocopherols were less effective in inhibiting 7 beta-OH and 7 alpha-OH formation, and were almost totally unable to prevent 5 alpha-3OH and 7-keto formation. dl beta-Tocopherol (Figure 3 B to D) and dl delta-tocopherol (Figure 5 B to D) showed the same behavior than when assayed for PC oxidation. The 2 tocopherols are devoid of activity to prevent COPs formation from the metal-induced liposome-CHO oxidation.

## About the mechanism of the antioxidant effect of tocopherols on cholesterol oxidation

Tocopherols, in addition to possessing vitamin E function, are the major natural antioxidants in food and are important for the stability of vegetable oils (Burton and Ingold 1986). This antioxidant aspect of tocopherol chemistry has been studied extensively, especially regarding the relative antioxidant activity of dl alpha-tocopherol, and less extensively for the gamma- and delta-tocopherol homologs (Pokorny 1991). However, information about the antioxidant effect of these tocopherols on CHO stability is scarce. PC:CHO-containing liposomes is a good model



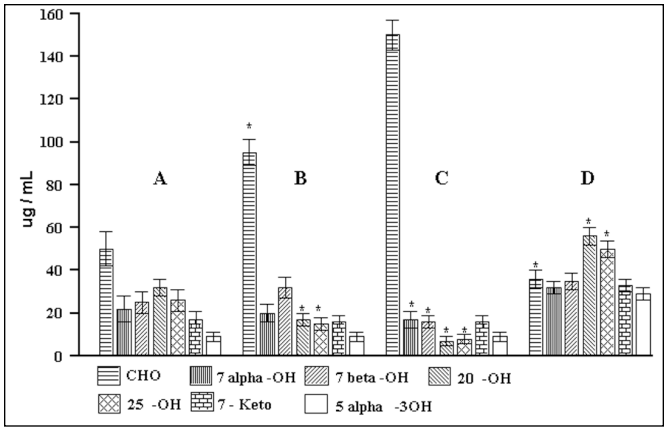


**Figure 2–Effect of dl alpha-tocopherol assayed at 50 μM (B), 100 μM (C), and 150 μM (D), compared to control (A), on the COPs formation from the metal-induced oxidation of phospholipid : cholesterol liposomes. Results represent the average of 4 assays ± S.D. Other experimental conditions are in the text.**

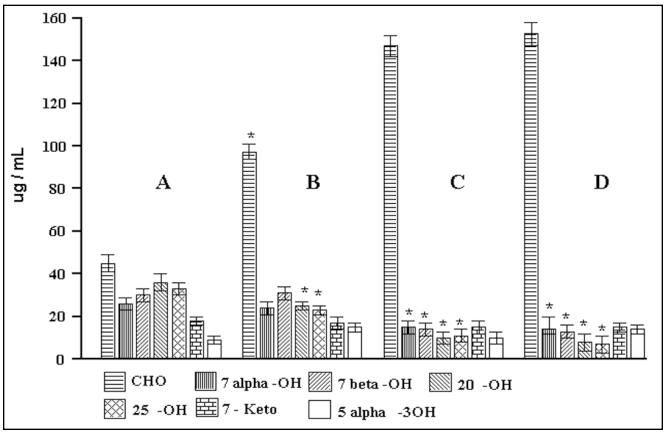


**Figure 4–Effect of dl gamma-tocopherol assayed at 50 μM (B), 100 μM (C), and 150 μM (D), compared to control (A), on the COPs formation from the metal-induced oxidation of phospholipid : cholesterol liposomes. Results represent the average of 4 assays ± S.D. Other experimental conditions are in the text.**

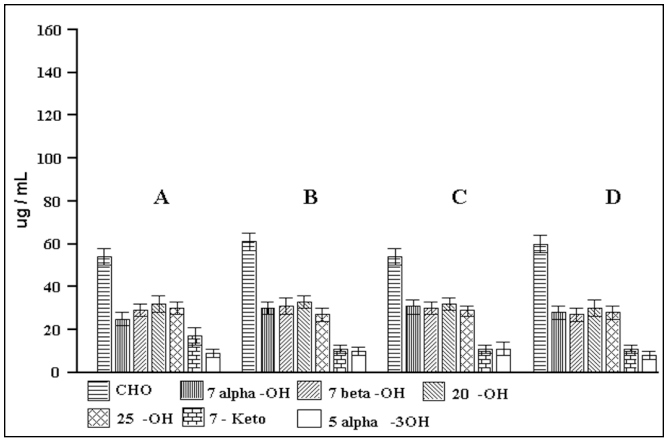


**Figure 3–Effect of dl beta-tocopherol assayed at 50 μM (B), 100 μM (C), and 150 μM (D), compared to control (A), on the COPs formation from the metal-induced oxidation of phospholipid : cholesterol liposomes. Results represent the average of 4 assays ± S.D. Other experimental conditions are in the text.**

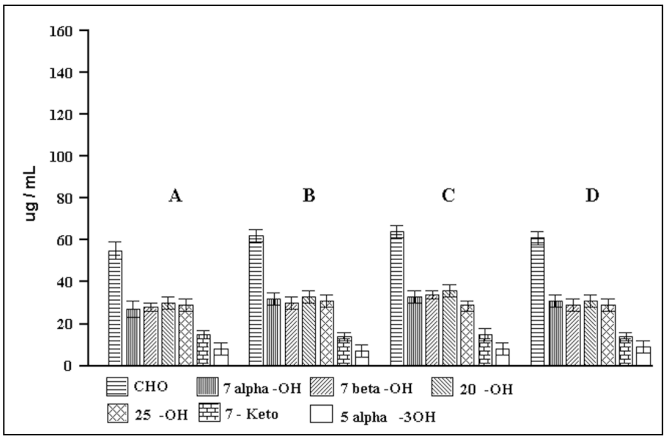


**Figure 5–Effect of dl delta-tocopherol assayed at 50 μM (B), 100 μM (C), and 150 μM (D), compared to control (A), on the COPs formation from the metal-induced oxidation of phospholipid : cholesterol liposomes. Results represent the average of 4 assays ± S.D. Other experimental conditions are in the text.**

to assay the effectiveness of the different tocopherols to prevent CHO oxidation, because these membrane-like structures can incorporate the tocopherols into the lipid matrix due the lipid-solubility of these substances. $Fe^{2+}$, which is capable of generating free radicals by Fenton-like reactions (Schaich 1992), is also a good catalyst for PC and for CHO oxidation, and no harsh conditions (for example, temperature) are needed to induce liposome oxidation. In addition, the unilamellar structure of liposomes allows a good interaction of the $Fe^{2+}$ ion at the lipid/water interphase of the liposome macromolecule, allowing the PC-fatty acid and the sterol oxidation to proceed (Sevanian and McLeod 1987). According to our results, dl alpha- and dl gamma-tocopherols show antioxidant effect for PC oxidation and also for CHO oxidation. However, the comparative effectiveness of these 2 tocopherols is controversial. While some researchers claim that gamma-tocopherol is a better antioxidant than alpha-tocopherol (Pokorny 1991; Lampi and others 1999), other researchers reported a better effectiveness of alpha-tocopherol as antioxidant (Yoshida and others 1993). Differences are probably due to the different oxidation models used to assess the antioxidant activity of these tocopherols (free fatty acids, highly refined oils, different phospholipids, liposomes, and so on). Although it is generally accepted that alpha-tocopherol has a higher ability for hydrogen donation than its gamma- homologous, the latter was often found to be a better antioxidant in pure lipid-phase systems, especially when comparison was made using high concentrations (Huang and others 1994). The prooxidant effect of alpha-tocopherol on fats and oils at high concentrations, which was also observed for our liposomal model, was described long ago (Labuza 1971). This work confirmed the above observation not only for PC oxidation, but also for CHO oxidation. However, the antioxidant-prooxidant behavior of alpha-tocopherol remains controversial. Recently Mäkinen and others (2000) demonstrated that alpha-tocopherol at high concentrations (up to 1000 ppm) was not prooxidant when assayed in methyl linoleate as oxidation model. As discussed above, the experimental model appears to be determinant for explaining the results. The antioxidant effect of beta- and delta-tocopherols, also remains to be a matter of controversy. Some researchers report that beta-tocopherol shows the same antioxidant effectiveness as gamma-tocopherol, both tocopherols being better antioxidants than delta-tocopherol for fatty acids oxidation (Yoshida and others 1993). However, others claim that beta-tocopherol is devoid of any antioxidant activity, and that delta-tocopherol is less effective than the alpha- and gamma-tocopherol homologs (Behrens and Madére 1985). Our results support the antioxidant behavior attributed to alpha- and gamma-tocopherol on fatty acid oxidation, but also confirm that these tocopherols can prevent CHO oxidation. Considering the prooxidant effect observed for dl alpha-tocopherol at the higher concentration assayed, our ranking of effectiveness to prevent COPs formation in the liposome-oxidation model must be: dl gamma-tocopherol > dl alpha-tocopherol > > dl beta- tocopherol ≅ dl delta-tocopherol.

The fact that dl alpha- and dl gamma-tocopherols are more efficient inhibitors of the formation of 20-OH and 25-OH than of the other COPs assayed, suggests that these tocopherols may elicit a better interaction with those free radicals formed at the lateral chain of the CHO molecule than with free radicals formed at the sterol nuclear structure. The formation of 7 alpha-OH, 7 beta-OH, 7-ketosterol, and 5 alpha-3OH, requires previous hydration or dehydration stages of the CHO molecule. Therefore, intermediary products such as 7-hydroperoxide cholesterol or 5,6-epoxycholesterol must be formed before the formation of the end products (Paniangvait and others 1995). Probably formation of 20-OH and 25-OH derivatives, which occurs after oxygen free-radical attack to the lateral chain of the CHO molecule, does not lead to the formation of intermediary products. dl alpha- and dl gamma-Tocopherols may be counteracting most directly free radicals involved in the oxidation of the lateral chain than those involved in the formation of the intermediary and/or end oxidation products at the nuclear structure of the CHO molecule. Also, stereochemical problems between the aromatic structure of tocopherols and the CHO steroidal structure may be impeding the free radical scavenging action of the tocopherols. The antioxidant effect of dl alpha-tocopherol and of some synthetic antioxidants against COPs formation has been studied in models of diverse complexity, such as spray-dried egg yolk (Huber and others 1995), fish oil, sunflower oil, and palm oil (Li and others 1996), or dehydrated and powdered anchovy (Shozen and others 1997). For all of these models, dl alpha-tocopherol has been identified as the most efficient antioxidant, with a better activity than other well known antioxidants, such as the butylated hydroxyanisole (BHA). To our knowledge, no other tocopherols have been assayed as antioxidant against COPs formation, therefore, the present results may open possible future applications for the gamma-tocopherol structure, which is now commercially available, to prevent cholesterol oxidation in food systems.

## Conclusions

SOME TOCOPHEROLS SUCH AS DL ALPHA- AND DL GAMMA-TOCOpherols can inhibit, but with different efficiency, the formation of COPs from PC:CHO liposomes when oxidation is induced by a metal. These tocopherols are more effective in inhibiting the oxidation of the lateral chain than the oxidation of the nuclear structure of the cholesterol molecule. At the highest tocopherol concentration assayed, dl alpha-tocopherol showed prooxidant effect, enhancing liposomal PC-fatty acid oxidation and also COPs formation.

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