# Effects of Alpha- Tocopherol on the Velocity of Low Density Lipoprotein Oxidation by Cupric Ions

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**Abstract-** We studied the effect of different concentrations of alpha-tocopherol on *in vitro* cupric ions induced oxidation of low density lipoproteins (LDL). Human native LDL (50 µg protein/ml) oxidation was induced by 10 µmol/L of $CuSO_4$. Conjugated dienes were measured spectrophotometrically for up to 440 minutes. The length of the lag phase ($T_{lag}$), maximum velocity of the reaction ($V_{max}$) and the maximum amount of generated dienes were obtained from kinetic data. Alpha-tocopherol increased $T_{lag}$ and decreased $V_{max}$ with a dependence upon concentration (0-100 µmol/L). There was no difference between the $D_{max}$ obtained with cupric ions alone or in the presence of the various concentrations of alpha-tocopherol. The results suggest that alpha-tocopherol may decrease free radicals presence in LDL and thus decrease velocity of LDL oxidation by cupric ions. This mechanism may be a reason for alpha-tocopherol effect in ameliorating atherosclerosis.



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**Key words:** Lipoprotein, LDL; cupric ions; alpha- Tocopherol; oxidation- reduction

## Introduction

Hypercholesterolemia is a major risk factor of atherosclerosis, therefore the oxidative modification of low density lipoprotein (LDL) is believed widely to be involved in the pathogenesis of human atherosclerosis, the underlying cause of coronary heart disease and strokes(1). Oxidized LDL produced in the arterial intima is intensively taken by scavenger receptors on the macrophages, which directly contributes to foam cell formation. These cells, loaded with cholesteryl esters, are characteristic of the precocious lesions of the atherosclerotic plaques (2). Moreover, oxidized LDL may affect many other aspects of arterial wall metabolism and thus contribute to the atherogenic process (3). However, this process may be inhibited by natural or synthetic antioxidants that protect LDL against oxidative modifications (4). Oxidation of LDL by cupric ions ($Cu^{+2}$) *in vitro* is a model frequently used to evaluate the protective effect of such antioxidants. This model was reported to produce oxidized LDL sharing many structural and functional properties common to LDL oxidized by cells or LDL extracted from arterial atherosclerotic plaques (5). Copper mediated LDL oxidation is a free radical mediated process based on the peroxidation of LDL polyunsaturated fatty acids (6). During the initiation phase, lipid peroxyl radicals are formed, but the propagation of the oxidation process is suppressed by endogenous antioxidants within the LDL particle (tocopherols, carotenes, etc), which results in the lag phase of oxidation. When endogenous antioxidants are depleted, a rapid propagation phase occurs leading to more peroxyl radicals that are transformed into conjugated dienes by molecular rearrangement. Finally, during the decomposition phase, the dienes fragment to low molecular mass products, particularly aldehydes such as malondialdehyde (MDA) (7).

Alpha-tocopherol is the active form of vitamin E and is contained principally in vegetable oils such as soybean, corn, cottonseed and sunflower oils. Supplementation with alpha-tocopherol has been found to have beneficial effects on the susceptibility of LDL to oxidation in healthy, diabetic and dyslipidemic subjects as well as in smoker (8,9). Alpha-tocopherol is lipid soluble and has been found to be the most abundant antioxidant in LDL particles. Alpha-tocopherol supplementation has been found to decrease the

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susceptibility of LDL particles to oxidation, which suggests that alpha-tocopherol could prevent atherosclerotic lesions through the decrease of LDL oxidation (10). Consequently, this study investigated effect of alpha-tocopherol on velocity of LDL oxidation by cupric ions ($Cu^{+2}$) in an *in vitro* model.

## Patients and Methods

### Materials

$Na_2HPo_4$, $NaH_2Po_4$, NaCl, KBr, agarose, bovine serum albumin, dimethyl sulfoxide and 2-thiobarbituric acid were obtained from Merck (Damstadt, Germany). EDTA, $CuSo_4$ and alpha-tocopherol were obtained from Sigma (St. Louis, Mo, USA). Dialysis membranes (SPECTRUM 12,000 – 14,000 Da) were purchased from Biogene (Mashhad, Iran).

### Plasma samples

Blood samples were obtained by veinpuncture from healthy volunteers fasted overnight, i.e. 20 men 20 – 30 years old, and collected in glass tubes containing 1 g/L of EDTA. After 10 minutes at room temperature, plasma was separated by centrifugation at 3000 g for 20 minutes at 20°C. The plasma immediately treated for LDL isolation.

### LDL isolation

LDL (d = 1.019 to 1.063) were isolated by ultracentrifugation using a single step discontinuous gradiant in a Damond B-60 ultracentrifuge using potassium bromide as density adjustment reagent (11 – 12). LDL containing fraction was dialyzed in dark for 24 hours at 4°C against 0.01 mol/L phosphate buffers, pH 7.4, containing 0.15 mol/L NaCl and 0.01% EDTA. The protein concentration of LDL was measured by the method of Lowry using bovine serum albumin as a standard (13). Purity of LDL preparations was checked by agarose gel electrophoresis on 0.8% gel (14).

### LDL oxidation and conjugated diene measurement

Just before experiments, LDL preparations were further dialyzed twice for 16 hours against the phosphate buffer saline, described above but without EDTA, to remove EDTA. At the end of dialysis, LDL (50 µg protein/ml) was incubated with 10 µmol/L copper without or with alpha-tocopherol (10, 50 and/or 100 µmol/L) at 37°C in 0.01 mol/L phosphate buffer saline, pH 7.4. The kinetics of LDL oxidation was determined by monitoring the change in absorbance at 234 nm against the buffer with a Perkin-Elmer, Lambda 2, UV spectrophotometer. Absorbance was recorded every 5 minutes for 440 minutes (15). The changes in absorbance per minutes as a function of time, i.e. the oxidation velocities, were also calculated. Three LDL oxidation parameters were considered: the length of the lag phase ($T_{lag}$), which was determined graphically by the time intercept of the tangents to the slow and rapid increase of the kinetic profile, the maximum velocity of diene production ($V_{max}$) and the maximum amount of generated dienes ($D_{max}$). The conversion of absorbance into concentration was based on a diene molar absorptivity of 29,500 $mol^{-1}.L.cm^{-1}$ (16).

### Statistical analysis

Results are presented as mean ± SD. $p<0.05$ was considered significant. Statistical analyses were performed by student's t-test.

## Results

LDL isolation from plasma was confirmed by agarose gel electrophoresis (Figure 1). Figure 1 shows that band seen for fraction separated from plasma (lane 1) is corresponding to LDL band in plasma as control (lanes 2 and 3). Figure 2 shows examples of conjugated diene absorbance kinetics obtained with LDL alone (no added cupric ions), LDL plus cupric ions ($Cu^{+2}$ alone) or LDL plus cupric ions in the presence of different alpha-tocopherol concentrations. In the absence of added cupric ions, absorbance slightly increased to reach a value of approximately 0.07. This time course of conjugated diene formation was similar to that reported when low concentration of copper (<0.1 µmol/L) were added to LDL (17), indicating that some cupric ions was present in buffers and/or LDL preparations. In the presence of cupric ions alone, absorbance kinetics revealed the three oxidation phase, i.e. lag phase, propagation phase and decomposition phase and maximum absorbance approximately was 0.5.

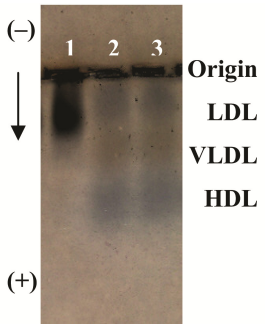


**Figure 1.** Electrophoresis analysis of LDL fraction (1) and plasma (2, 3) on 0.8% agarose gel.
*LDL=Low density lipoprotein, VLDL=Very low density lipoprotein, HDL=High density lipoprotein*

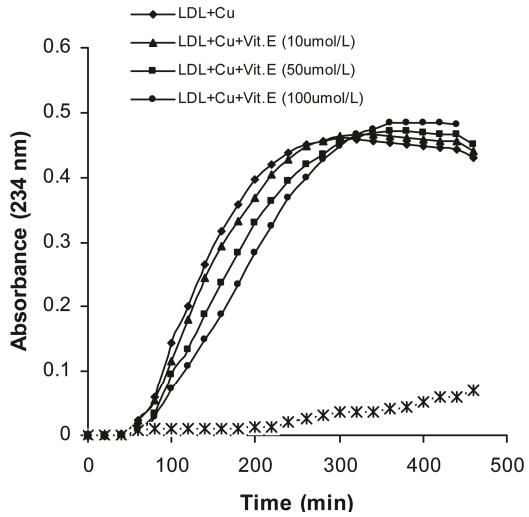


**Figure 2.** Conjugated diene absorbance kinetics measured during oxidation of LDL (50 µg protein/ml) in the air (LDL, no added cupric ions) or induced by cupric ions (10 µmol/L) in the absence or presence of different alpha-tocopherol concentrations. Each curve is one example of measures obtained during one LDL oxidation test

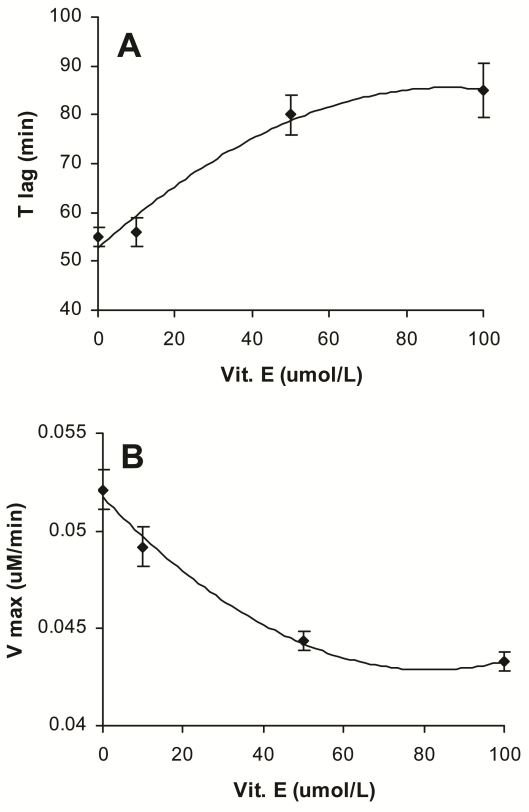


**Figure 3. A)** Length of lag phase ($T_{lag}$) and **B)** maximum velocity of diene production ($V_{max}$) during oxidation of LDL (50 ug protein/ml) induced by cupric ions (10 µmol/L) in the absence or presence of different alpha-tocopherol concentrations. Each point is the mean ± SD of three separate experiments.

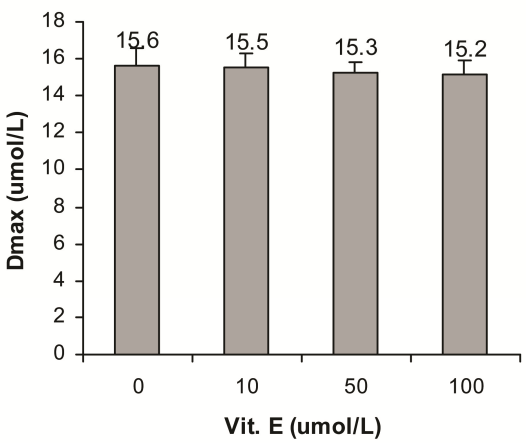


**Figure 4.** Maximum amount of generated dienes ($D_{max}$) during LDL oxidation by cupric ions (10 µmol/L) in the absence or presence of 10, 50 and 100 µmol/L of alpha-tocopherol concentrations. Each point is the mean ± SD of three separate experiments.

In the presence of alpha-tocopherol, the higher the concentrations were, the more the curves tended to the right, indicating that protection against oxidation was concentration dependent. Figure 3 shows relationships between $T_{lag}$ or $V_{max}$ and alpha-tocopherol concentration obtained during LDL oxidation tests. $T_{lag}$ increased and $V_{max}$ decreased with alpha-tocopherol concentrations in a quadratic manner ($P$<0.01). This means that a curve (second degree equation) more accurately describes the phenomenon than a straight line. In other words, there would be saturation in antioxidant potency at concentrations greater than 100 µmol/L of alpha-tocopherol. The 50 µmol/L of alpha-tocopherol concentration increased $T_{lag}$ and decreased $V_{max}$ to approximately 50%. The values of $D_{max}$ (the maximum amount of generated dienes) during LDL oxidation test without or with different concentrations of alpha-tocopherol are presented in Figure 4. As expected from kinetics (Fig. 2), was not there any difference significant in $D_{max}$ between all the alpha- tocopherol concentrations and cupric ions alone. Figure 5 shows $V_{max}$ as a function of $T_{lag}$ for the alpha-tocopherol at 10 to 100 µmol/L concentrations. Each ellipsoid is the mean ± 95% confidence interval for the mean of $V_{max}$ (vertical diameter) and $T_{lag}$ (horizontal diameter). This graphical representation permits a rapid assessment of the antioxidant potency of the compounds, i.e. their capacity to increase $T_{lag}$ and/or decrease $V_{max}$. Alpha-tocopherol at 100 µmol/L decreased Vmax and increased $T_{lag}$ to a similar extant as 50 µmol/L, whereas decreased $V_{max}$ and increased $T_{lag}$ to a greater extent than 10 µmol/L alpha-tocopherol ($P$<0.01).

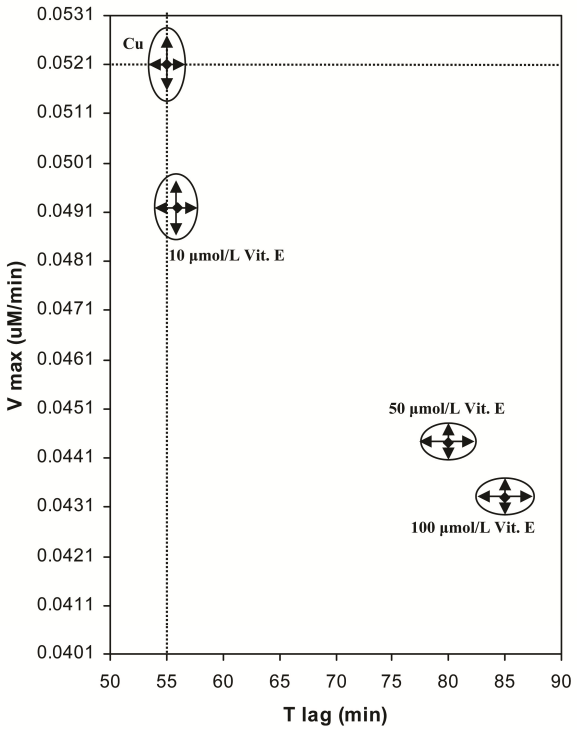


**Figure 5.** Maximum velocity of diene production ($V_{max}$) as a function of the length of the lag phase ($T_{lag}$) during oxidation of LDL (50 ug protein/ml) induced by cupric ions (10 µmol/L) in the absence (Cu ellipsoid) or presence of different alpha-tocopherol concentrations (Vit. E ellipsoid). The center of each ellipsoid is the mean of three LDL oxidation tests; vertical and horizontal diameters of each ellipsoid are 95% confidence interval for the means of $V_{max}$ and $T_{lag}$, respectively.

## Discussion

Continuous measurement of conjugated dienes allowed us to estimate the effect of alpha-tocopherol on different phase of LDL oxidation process. $T_{lag}$ represents the length of the initiation phase during which LDL resist oxidation, $V_{max}$ is the maximum velocity of the propagation phase during which conjugated dienes are intensively produced and $D_{max}$ is the maximum quantity of generated diene (18). In this study, alpha-tocopherol at 10, 50 and 100 µmol/L decreased $V_{max}$ approximately 5, 16 and 17%, respectively, in compare with $V_{max}$ of cupric ions alone. Effect of this vitamin on $T_{lag}$ shows at 10, 50 and 100 µmol/L concentrations increased $T_{lag}$ approximately 10, 32 and 38%, respectively. Thus different concentrations of alpha-tocopherol had approximately the same effect on $V_{max}$, whereas they had greater effects on lag phase ($T_{lag}$). It may be noted that $T_{lag}$ is somewhat related to the slope of the rapid increase of the kinetic profile, i.e. to the oxidation velocity, because it is graphically determined using a tangent to this slope. A decreased oxidation velocity gives a low slope, which tends to produce a low $T_{lag}$. Thus the interpretation of $T_{lag}$ should be concomitant to that of $V_{max}$. This is why we suggest that $V_{max}$ be presented as a function of $T_{lag}$, using an ellipsoid whose position represents the antioxidant capacity of alpha-tocopherol compared with the ellipsoid obtained in the presence of cupric ions alone. Any ellipsoid under and/or on the right of cupric ions may represent a potentially LDL antioxidant alpha-tocopherol. The antioxidant activity was proportional to the alpha-tocopherol concentration in the LDL containing mixture, because $T_{lag}$ increased and $V_{max}$ decreased as the alpha-tocopherol concentration increased. However, the relations between oxidation parameters and alpha-tocopherol concentrations tended towards a plateau, i.e. the lag phase could not be indefinitely increased and diene production could not be completely stopped even by a high alpha-tocopherol concentration. This indicates that alpha-tocopherol impair some, but not all, of the mechanisms responsible for peroxidation. Furthermore, we found that $D_{max}$ was similar in LDL oxidized by cupric ions alone or by cupric ions associated with alpha-tocopherol, whatever their concentration. Thus alpha-tocopherol slow down, but do not completely stop, LDL oxidation. Time for the achievement of $D_{max}$ with cupric ions was less than 5 hours and maximum time for

$D_{max}$ was approximately 7 hours with alpha-tocopherol at 100 µmol/L. Thus, it is possible that alpha-tocopherol impair conjugated diene decomposition, resulting in low thiobarbitoric acid reactive substances (TBARS) production. Consistent with this hypothesis is the fact alpha-tocopherol has been reported to inhibit radical chain propagation by scavenging highly reactive lipid peroxyl and alkoxyl radicals, which promote the propagation of the chain reaction of lipid peroxidation (19,20). Several study showed that LDL oxidation was presumably initiated by the LDL bound cupric ions (12, 21). It is currently admitted that copper (II) reduction to copper (I) is required for triggering lipid peroxidation in LDL (22). After hydrogen abstraction from polyunsaturated fatty acid structures in LDL by $LOO^{\cdot}$ or $LO^{\cdot}$ radicals and the chemical rearrangement of $L^{\cdot}$ radicals leads to conjugated diene radicals which further react with $O_2$ and finally yield hydroperoxides and cyclic endoperoxides containing the conjugated diene structure (23). We have previously reported that alpha-tocopherol may with reduce of copper (II) to copper (I), decreased the affinity of copper to LDL and thus preventing copper induced oxidative (12). Thus, our findings in this study suggest that alpha-tocopherol could inhibit cupric ions induced LDL oxidation in the initial stage kinetically, as characterized by prolonged lag time, reduced maximal velocity of conjugated diene accumulation. This study demonstrates that alpha-tocopherol can slow down *in vitro* copper induced LDL oxidation and this mechanism may be a reason for alpha-tocopherol effect in atherosclerosis preventation.

## Acknowledgments

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