Vitamin E, Markers of Oxidative Stress and Nitric Oxide Levels in Senescence

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Abstract

Background: Cellular damage by reactive oxygen species including those associated with altered plasma antioxidant reserve and lipid peroxidation is now accepted to involve the aging process. It is conceivable that vitamin E supplementation reduces the age related modifications by reducing oxidative stress.

Aim: The present study was undertaken to assess the markers of oxidative stress i.e. serum paraoxonase, erythrocyte malondialdehyde, plasma uric acid, vitamin C, E and nitric oxide levels in the blood samples of different age group subjects and to investigate the effect of vitamin E supplementation in ameliorating the levels of these markers in middle aged and elderly subjects.

Methods: These parameters were estimated by using standard methods in 30 healthy younger individuals (20-30 years) who served as controls and in 60 healthy subjects categorized into two groups i.e. Group I (40-55 years) and Group II (≥ 56 years) before and after 3 months of vitamin E supplementation. The obtained values were compared statistically by using student’s t-test.

Result: Vitamin E supplementation (200 mg/day) brought about an improved antioxidant status with significantly raised plasma vitamin C, E, nitric oxide and serum paraoxonase levels (p<0.05), and simultaneously depleted levels of plasma uric acid and erythrocyte malondialdehyde (p<0.05) in middle aged and elderly subjects.

Conclusion: These findings support the protective and anti-aging role of vitamin E supplementation in reducing oxidative stress.

Key words: Aging, paraoxonase, nitric oxide, uric acid, malondialdehyde.

Introduction

Aging is a universal and inevitable normal biological phenomenon, associated with progression and generalized impairment of function resulting in the loss of adaptive response to stress and increasing risk of age related diseases. Accumulating evidences indicate that various types of reactive oxygen species (ROS) are produced in the cells and tissues contributing to aging process.1,2 Reactive oxygen species are indiscriminate and if not promptly neutralized, they can inflict major interrelated derangements of cell metabolism including DNA strand breakage, damage to membrane ion transporters, specific proteins and lipid peroxidation.3 Prime targets of these free radicals attack are polyunsaturated fatty acids (PUFA) in the membrane lipids causing lipid peroxidation. Among reactive aldehydes, malondialdehyde (MDA) is an abundant product of lipid peroxidation and various studies have been reported regarding the etiopathological role of lipid peroxidation in aging and disease development.4 These free radicals are efficiently removed by antioxidant defense system which includes antioxidant enzymes and antioxidants. In our previous study, alteration in antioxidant enzyme activities with increasing age have been well documented.4 Recently paraoxonase (PON1) a calcium-dependent A-esterase synthes-
ized primarily in the liver and secreted into the serum as HDL-associated enzyme that prevents oxidation of low density lipoprotein (LDL) and responsible for anti-atherogenic property of high density lipoprotein (HDL), has received much attention in ageing and its related diseases.\(^5\)

Oxidant scavenging role of these enzymes are well supported by cooperative action of non-enzymic antioxidants such as vitamin C & E and uric acid which may have a significant role in regulating the oxidative stress mediated cascade responsible for age related biomolecular deterioration. Their role in scavenging free radicals in inhibiting lipid peroxidation and in prevention of age related diseases have been well documented.\(^2,3,6,7\) However, their role as pro-oxidant and relation of uric acid with vascular injuries reflect the need of further investigation.\(^8,9\)

Nitric oxide (NO) is a powerful endothelium derived vasodilator, produced from the precursor L-arginine in human body cells. It plays a significant role in circulatory, digestive, neural and immunological systems. It takes part in blood pressure control, inhibits mast cells degranulation, possess antioxidant and anti-aggregant properties and regulates vascular tone. It also inhibits proliferation of smooth muscle cells and adhesion of leukocytes and platelets.\(^10\) Alteration in the levels of these antioxidants, vasodilators and increased levels of lipid peroxides with aging process further amplify the life threatening incidences.

It is conceivable that vitamin E, mainly α-tocopherol, can ameliorate the modifiable indexes via regulating the free radical production. In this context, considerable attention has been devoted to the potential use of α-tocopherol, a potent chain breaking antioxidant, in the prevention of age related alterations.\(^11\) Although in previous epidemiological and experimental animal studies, vitamin E reduces oxidative stress mediated damages and facilitates various physiological activities.\(^12,13\) The objective of present study was to investigate the therapeutic effect of vitamin E supplementation in replenishing the serum paraoxonase activity, plasma antioxidant reserve, plasma nitric oxide level and in controlling the progression of lipid peroxidation in different age groups.

Material and Methods

**Study design:** To study the effect of vitamin E supplementation in the amelioration of markers of oxidative stress and plasma nitric oxide levels in middle age group (40-55 years) and elderly subjects (56 years onwards).

**Setting and locale of the study:** In the present study, 90 healthy subjects were included and divided into 3 groups of 30 subjects each i.e. control group (30 subjects) of age group 20 – 30 year), Group I (30 subjects of age group 40 – 55 years) and Group II (30 subjects of age group 56 years onwards). In each group, 15 male and 15 female (1:1 ratio) were included. These subjects were selected randomly from urban area of Allahabad city of Uttar Pradesh, after taking their informed consent and approval of protocol by ethics committee of college. A general information questionnaire regarding demographic information, family history and limited physical examination was completed from all the subjects. Height and weight were measured with subject barefoot and light dressed. The body mass index (B.M.I.) was calculated as \([B.M.I = \text{weight (Kg) / height (metre)}^2]\).\(^14\)

**Inclusion criteria:** Subjects having no history of disease, not under any medical treatment.

**Exclusion criteria:** Patients with diabetes mellitus, hypertension, renal insufficiency, hepatic disease or under any medicinal treatments were excluded. Pregnant and lactating women, obese (B.M.I \(>25\)), hypertensive (BP \(>120/80\) mmHg), smokers and subjects who did not follow study instructions were excluded from the study.

Fasting blood samples were collected in EDTA vials from the antecubital vein of the subjects and processed immediately. Markers of oxidative stress and nitric oxide levels were estimated in Group I A and Group II A and after 3 months of vitamin E supplementation (Group I B and Group II B) and compared it with that of younger controls. Vitamin E was supplemented orally (dose: 200 mg/day) in form of capsules.\(^15\)

Serum paraoxonase activity was estimated by Gan et al method using p-nitrophenyl acetate (5.5 mM/L) as a substrate.\(^16\) The increase in the absorbance of p-nitrophenol formed was measured spectrophotometrically (412 nm). The activity of paraoxonase (PON) was measured in Tris buffer (20 mM/L; pH 8.0) containing 1 mM CaCl\(_2\). The generated product p-nitrophenol was calculated by using molar extinction coefficient of 17000 per mole/cm at pH 8.0. Results were expressed as units/ml (1 nmol p-nitrophenol formed per minute).

Erythrocyte malondialdehyde (MDA) levels were measured as thiobarbituric acid reactive substances, after preparation of hemolysate.\(^17\) The heat induced reaction of malondialdehyde (MDA)
with thiobarbituric acid (TBA) in the acid solution forms a trimethine coloured substance which is measured spectrophotometrically at 532 nm. Plasma ascorbic acid levels were estimated by McCormick and Greene method. Ascorbic acid in plasma is oxidized by Cu^2+ to form dehydroascorbic acid which reacts with acidic 2,4–dinitrophenyl hydrazine to form a red bishydrazone, which is measured at 520 nm.

Plasma tocopherol level was estimated by Hashim and Schutttringer method. Protein in the plasma was precipitated by an equal volume of absolute ethanol. The whole mixture was subjected to extraction by an equal volume of n-heptane. α,α\textsuperscript{1}-dipyridyl was added followed by ferric chloride reagent to the system which produced light pinkish orange color.

Plasma total nitrate and nitrite levels were measured with the use of Griess reagent as described earlier. The Griess reagent consists of sulfanilamide and N-(1-napthyl) ethylenediamine. The method is based on a two-step process. The first step is the conversion of nitrate to nitrite using nitrate reductase. The second step is the addition of Griess reagent, which converts nitrite into a deep-purple azo compound; photometric measurement of the absorbance at 540 nm due to this azo chromophore accurately determines the nitrite concentration.

Plasma uric acid levels were estimated by Caraway’s method in which uric acid reacts with phosphotungstic acid in alkaline medium forming a blue color complex which is measured at 700 nm. Values were expressed as mean ± SD. The significance of mean difference between groups was compared by using Student’s ‘t'-test, linear regression analysis and Pearson correlation test.

Result

In the present study, mean blood pressure and demographic indices of the study group subjects are depicted in Table 1. On vitamin E supplementation, insignificant (p<0.1; 12.5% low) change occurred in BMI and blood pressure of middle age and elderly subjects. There were significant changes in antioxidant status and MDA levels in study group subjects before and after vitamin E supplementation, as represented in Table 2. Serum PON activity was significantly low (20.08%; p<0.05) in Group II A subjects as compared to healthy controls that was found to be increased significantly (15.19%; p<0.05) on vitamin E supplementation. However, serum PON activity was reduced insignificantly in Group I A subjects (p<0.1; 12.5% low) possibly due to low oxidant overload as compared to Group II A subjects, which increased upto certain extent on vitamin E supplementation i.e. 9.6% high.

Table 1. Demographic profile of study group. (Mean ± SD)

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Control group (n = 30)</th>
<th>Group I A (n = 30)</th>
<th>Group I B (n = 30)</th>
<th>Group II A (n = 30)</th>
<th>Group II B (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>26.0 ± 4.5</td>
<td>48.5 ± 7.0</td>
<td>--</td>
<td>62.0 ± 6.0</td>
<td>--</td>
</tr>
<tr>
<td>M:F ratio</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
</tr>
<tr>
<td>BMI (kg/m\textsuperscript{2})</td>
<td>23.38 ± 2.6</td>
<td>21.56 ± 2.4</td>
<td>21.84 ± 3.0</td>
<td>23.70 ± 2.3</td>
<td>23.96 ± 2.0</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>112 ± 8.4</td>
<td>118 ± 7.5</td>
<td>116 ± 6.8</td>
<td>110 ± 6.0</td>
<td>108 ± 5.5</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>76.0 ± 3.7</td>
<td>80.0 ± 5.5</td>
<td>78.0 ± 4.5</td>
<td>76.0 ± 3.0</td>
<td>74.5 ± 2.0</td>
</tr>
</tbody>
</table>

Plasma vitamin C, E and NO levels were found to be significantly low in both the non-supplemented groups (p<0.05; 23.5%, 26.2% and 22.0% low respectively in Group I A and p<0.05; 30.6%, 34.0% and 29.3% low respectively in Group II A). On vitamin E supplementation, marked amelioration in their levels were observed i.e. p<0.05; 20.5%, 23.7% & 20.8% high respectively in Group I B and p<0.05; 19.0%, 21.6% and 18.9% high respectively in Group II B (Table 2). Similarly, marked elevated levels of erythrocyte malondialdehyde (MDA) and plasma uric acid were observed in non-supplemented groups i.e. p<0.05; 24.43% & 20.4% high in Group I A and p<0.05; 35.9% & 27.3% high in Group II A subjects respectively as compared to healthy controls which decreased significantly (p<0.05; 23.2% & 13.3% low respectively) in Group I B and (p<0.05; 22.6% & 14.4% low respectively) in Group II B subjects.

Altered levels of oxidative stress markers, NO and their correlation with plasma vitamin E level in vitamin E supplemented middle aged and elderly subjects (Table 3) reflect the association of plasma vitamin E level with amelioration of NO and markers of oxidative stress levels in study group subjects after vitamin E supplementation.
Table 2. Effect of Vitamin E supplementation on Serum peroxyxanese (PON) activity, erythrocyte MDA, plasma nitric oxide and non-enzymic antioxidants level (Mean ± SD).

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Control group (n = 30)</th>
<th>IA group (n = 30)</th>
<th>IB group (n = 30)</th>
<th>IIA group (n = 30)</th>
<th>IIB group (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON (µmol/L)</td>
<td>228.5 ± 42.8</td>
<td>219.1* ± 12.4</td>
<td>182.6** ± 27.5</td>
<td>210.4** ± 29.3</td>
<td></td>
</tr>
<tr>
<td>NO level</td>
<td>8.34 ± 6.50</td>
<td>7.85** ± 1.47</td>
<td>5.90** ± 1.47</td>
<td>7.09* ± 1.47</td>
<td></td>
</tr>
<tr>
<td>(µ mol/L)</td>
<td>1.47 ± 2.4</td>
<td>1.34** ± 2.4</td>
<td>0.97** ± 1.47</td>
<td>1.18** ± 1.47</td>
<td></td>
</tr>
<tr>
<td>Vitamin E (mg %)</td>
<td>± 0.40 ± 0.27</td>
<td>± 0.31 ± 0.23</td>
<td>± 0.23 ± 0.29</td>
<td>± 0.29 ± 0.29</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (mg %)</td>
<td>0.82 ± 0.63**</td>
<td>0.75** ± 0.56</td>
<td>0.66** ± 0.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg %)</td>
<td>± 0.20 ± 1.5</td>
<td>± 0.17 ± 0.11</td>
<td>± 0.13 ± 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uric acid (mg %)</td>
<td>4.50 5.42** ± 1.36</td>
<td>4.77* 5.73**</td>
<td>4.90** ± 1.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malondialdehyde (µmol MDA/ml)</td>
<td>2.64 3.57**</td>
<td>2.80** 3.78**</td>
<td>2.93** ± 1.34</td>
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</table>

Table 3. Correlation coefficient (r) between plasma Vitamin E level and various other parameters (NO and markers of oxidative stress levels) in supplemented middle age and elderly group.

<table>
<thead>
<tr>
<th>Particulars</th>
<th>PON NO level</th>
<th>Vitamin C level</th>
<th>Uric acid level</th>
<th>MDA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middle age group</td>
<td>+0.406</td>
<td>+0.295</td>
<td>+0.318</td>
<td>-0.293</td>
</tr>
<tr>
<td>Elderly group</td>
<td>+0.363</td>
<td>+0.251</td>
<td>+0.276</td>
<td>-0.246</td>
</tr>
</tbody>
</table>

Discussion

Several studies have documented enhanced production of ROS as well as altered levels of antioxidant reserves in plasma and tissues with advancing age.1-4 Theoretically, it is conceivable that exogenous administration of non-enzymic antioxidants such as vitamin E may prevent or reduce oxidative stress mediated biocellular deterioration which is responsible for disease development with advancing age although contradictory evidences have been documented.22,23 In this context, an attempt was made to control the oxidative stress by exogenous vitamin E supplementation in different age group subjects.

In particular, lipid peroxidation considered to be a major phenomenon, initiates a complex cascade that promotes aging effects such as atherosclerotic plaque formation, inhibition of NO and prostacyclin synthesis, enhancement of cytosolic free calcium and peripheral vascular resistance leading to hypertension and leakage of lysosomal hydrolases via breakdown of lysosomal membranes which cause dystrophic changes in muscle fibers leading to weakness of muscles with growing age.24,26 In the present study, erythrocyte MDA levels were high in non-supplemented groups, which reduced significantly (p<0.05) in vitamin E supplemented middle age and elderly subjects. These findings clarify the chain breaking antioxidant property of vitamin E by which it protects the membrane bound lipids and nascent LDL against free radical mediated lipid peroxidation and thus plays a significant role in the reductions of aging process.

Oxidation of lipids is well controlled by antioxidant enzymes including serum paraoxonase, an enzyme found in association with HDL contributing it to antiatherogenic and antioxidant capability by hydrolyzing specific oxidized phospholipids and cholesterol linoleate hydroperoxides and by neutralizing hydrogen peroxide.5,27 Alteration in the paraoxonase activity may have significant effect in aging process possibly due to increased production of reactive aldehydes. In the present study, serum paraoxonase activity was found to be decreased continuously in middle aged followed by elderly. It could be explained on the basis of inactivation of enzyme itself due to interaction of oxidized lipids with the paraoxonase free sulphhydryl group. Similar findings have been reported by Sarkar et al in patients with coronary artery disease in age group 40-70 years.28

On vitamin E supplementation, serum paraoxonase activity was found to be increased significantly in supplemented elderly subjects and to certain extent in middle age subjects as compared to non supplemented group. It reflects directly to the antioxidant enzyme replenishing property of vitamin E by quenching free radical to inhibit lipid peroxidation and thereby inhibiting the resultant inactivation of paraoxonase enzyme to combat oxidant overload with advancing age. Jarvik et al also reported a direct relationship between vitamin C and E intake and serum paraoxonase 1 activity.29 On the contrary, Kleemola et al observed an inverse relationship between serum paraoxonase 1 activity and the intake of antioxidant vitamin.23

In addition to antioxidant enzymes, free radicals are efficiently removed by co-operative action of non enzymatic antioxidants such as vitamin C, vitamin E and uric acid. These may confer health benefits and may prevent or postpone the onset of degenerative diseases. Vitamin C, an exogenous water soluble antioxidant functions as...
primary defense against free radicals in plasma and disappeared more quickly. Heitzer et al observed that vitamin C alone can afford protection against the oxidant mediated damage to LDL even though it is not lipid soluble. Alteration in ascorbate levels have significant effects on collagen synthesis and thereby affect the strength of bones, tendon, teeth, cartilage and blood vessels with age. Another possible mechanism through which ascorbate plays a significant role in reducing age related deterioration include its protective effect on Na⁺-K⁺-ATPase against peroxidative damage and thereby in maintaining electrolyte balance, synergistic action to regenerate α-tocopherol, urate radical repairing action and by enhancing the availability of NO, a potent vasodilator that reduces the risk of stroke and cardiovascular disease in older people.

Vitamin E, a universal lipophilic chain breaking antioxidant and a stabilizer of biological membranes, prevents accumulation of free radicals, decreases lipid peroxidation, exerts cardioprotective effects and increases cell mediated immunity in elderly. The association of vitamin E deficiency and age related disorders such as neurological dysfunction, memory problems, cataract formation, maculopathy, myopathies and diminished erythrocyte life span in elderly has been universally accepted.

In the present study, plasma levels of these antioxidant vitamins (C & E) were significantly low in non supplemented groups as compared to control. Decreased levels of these vitamins could not be only due to their role in limiting lipid peroxidation by scavenging free radicals but also due to their utilization in maintaining the body antioxidant reserve, membrane stability and integrity and in normalization of superoxide formation. Similar findings have been documented in recent studies suggesting that aging and its related complications can be regulated by improving these antioxidant vitamins level in blood. Despite data related to antioxidant role of these vitamins, pro-oxidant properties of these vitamins also play a controversial role. On vitamin E supplementation, levels of vitamin E and C were increased significantly. Our findings are in concordance with the studies of Prasad et al and Garg et al. However, Keith et al found no significant effect of vitamin E supplementation on other markers of oxidative stress. The increased concentration of vitamin C could be explained due to sufficient amount of vitamin E after supplementation which reduces consumption of vitamin C against oxidative stress and facilitates its re-synthesis from semi dehydroascorbate via glutathione—semidehydro ascorbate reductase enzyme in neutrophils and erythrocytes.

Uric acid is an endogenous, preventive and chain breaking antioxidant which contributes about 65% of free radical scavenging action, stabilizes ascorbate, protects DNA and erythrocytes from oxidative damage. Nitric oxide is known to be implicated in a number of crucial physiological functions which declines with senescence e.g. vasodilation, penile erection, cerebral blood flow, microbicidal and tumoricidal activities of macrophages and neutrophils. Superoxide anion, produced in excess during oxidative stress reacts with nitric oxide to form toxic product peroxynitrite anion (ONOO⁻) and thereby reduces nitric oxide bioavailability i.e. an important event in progression of age related disorders. Plasma uric acid interacts with peroxynitrite anion to form a stable nitric oxide donor, thus facilitating various physiological functions of human body.

In the present study, plasma uric acid levels were found to be significantly high (p<0.05) in non-supplemented subjects. Olivieri et al also observed elevated levels of uric acid in elderly. According to Maxwell and Bruinsma, elevation of uric acid production is a secondary event and occur due to removal of xanthine oxidase inhibition via reduction in vascular nitric oxide. Vitamin E supplementation brought about significant reduction in plasma uric acid level in Group I B & II B subjects (p<0.05) which may be explained on the basis of increase in NO bioavailability by vitamin E (p<0.05) via inhibition of vascular superoxide formation and inhibition of xanthine oxidase activity leading to contro-lled production of uric acid. However, further studies are warranted to shed more light on the hidden facts related to therapeutic use of anti-oxidants.

On the basis of present study and findings of previous studies, it can be inferred that oxidative stress plays a crucial role in aging process and vitamin E supplementation provides protection against oxidative stress not only by their free radical scavenging action but also by ameliorating antioxidant reserve and by preserving endothelium derived relaxing factor (NO). In addition, present study also authenticates the fact that daily consumption of diet rich in vitamin E should be increased with advancing age in order to sustain or postpone age related modifications and its consequent sequelae.
References


