

## ORIGINAL ARTICLE

### ROLE OF VITAMIN E ON OXIDATIVE STRESS IN SMOKERS

Nafeeza Mohd. Ismail, Asma Harun\*\*, Ahmad Asmadi Yusof, Zaiton Z\*, and Alini Marzuki\*\*,

Depts. of Pharmacology, \*Physiology, Faculty of Medicine, \*\*Dept. of Pharmacy,  
Faculty of Allied Health Sciences, Universiti Kebangsaan Malaysia,  
Kuala Lumpur, Malaysia.

Cigarette smoke contains numerous oxygen free radicals that when inhaled, overwhelm antioxidant defenses and produce a condition of oxidative stress. This study investigated whether or not supplementation with vitamin E can affect the state of oxidative stress in healthy smokers. In this randomised double blind trial, 32 smokers received 200 mg of vitamin E or placebo daily for 8 weeks. All smokers in the vitamin E group completed the trial whilst only nine in the placebo group completed the trial. Plasma vitamin E concentrations increased significantly [ $P < 0.02$ ] in the vitamin E group. The release of malondialdehyde [MDA] from erythrocytes was not significantly different between the two groups at baseline and was clearly reduced [ $P < 0.01$ ] after 8 weeks of vitamin E supplementation. Vitamin E increased erythrocyte superoxide dismutase activity [ $P < 0.02$ ] and decreased glutathione peroxidase activity [ $P < 0.02$ ]. No changes were detected in plasma MDA. We conclude that daily supplementation with 200 mg of vitamin E for 8 weeks improved the oxidative stress state in smokers.

*Key words : Vitamin E, smokers, oxidative stress, antioxidants*

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#### Introduction

Cigarette smoke has an abundance of oxidants that lead to a tremendous production of free radicals. There is overwhelming evidence from epidemiological and experimental studies showing the detrimental effects of free radicals on health. The body has the ability to produce endogenous antioxidants such as superoxide dismutase, catalase and glutathione peroxidase.

Under normal circumstances, there is a balance between these endogenous antioxidants and the production of free radicals in the body. In the instance where there are excessive free radicals, the available tissue antioxidants may become depleted, leading to oxidative damage as in the case of cigarette smoke. The most logical course to defeat smoking is the implementation of smoking cessation programmes, but given the poor success rates of these programmes, there is compelling evidence for a beneficial role of exogenous antioxidants to minimise smoking-related oxidative damage.

Compounds, which possess antioxidant properties, have the potential to decrease oxidative stress and thus may protect against smoking-induced pathology. Compounds that have been investigated are lipoic acid, taurine, ubiquinone, selenium, garlic, ginkgo biloba and polyphenols (1 – 6). Other compounds, which have been widely tried for their antioxidant properties, are vitamins such as alpha-tocopherol (7,8), and ascorbic acid (9, 10). Vitamin C doses of between 500 mg and 2000 mg have produced controversial results (9, 10). Despite these antioxidative and anti atherogenic effects, the clinical results of vitamin E supplementation in human subjects varied (7, 8). The Cambridge Heart Antioxidant Study using alpha-tocopherol showed that a higher dose of 800 iu daily did not have additional advantage over a dose of 400 iu daily (7).

Of the tocopherols, alpha-tocopherol exhibits the greatest antioxidant potential *in vivo*. Although there is no clear evidence that the antioxidant capacity of tocotrienols is superior to tocopherols, tocotrienols have been found to be more potent

antioxidants (11). Therefore, the objective of this study was to assess the effect of a tocotrienol-rich mixture on the status of oxidative stress in otherwise healthy smokers.

**Methods**

1) Study subjects

a) Recruitment of study subjects

Subjects were volunteers who responded to an advertisement to participate in this study. Thirty two volunteers who satisfied all the criteria for inclusion were included in the study.

b) Inclusion criteria

The criteria for inclusion were males, aged between 25 - 45 years, who are current smokers and had been smoking 20 cigarettes/day for at least 5 years.

c) Baseline investigations

All subjects underwent a clinical examination. Blood samples (10 ml) were drawn after 12 hour of fasting for lipid profile, blood sugar, liver and renal

function tests. Lung function test was performed and FEV<sub>1</sub>, FVC and FEV<sub>1</sub>/FVC were determined. Chest X-ray and an electrocardiogram [ECG] were done.

d) Health status

All volunteers did not have a history of asthma or other respiratory illnesses. All volunteers had normal biochemical parameters, lung function test, chest X-ray and ECG.

e) Drug and additional vitamin E restriction

During the study, drugs such as aspirin and allopurinol as well as additional intake of vitamin C or vitamin E were not permitted.

2) Study design

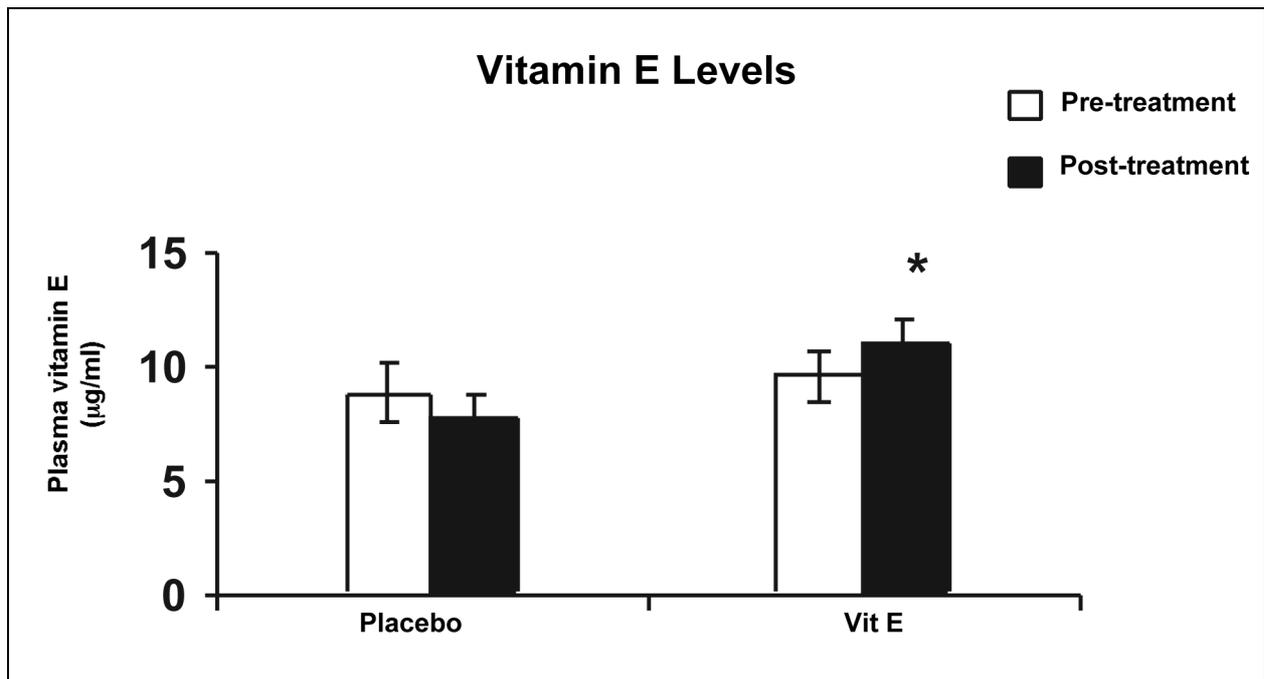
a) Ethical clearance

This study was approved by the Ethics committee of the Faculty of Medicine, Universiti Kebangsaan Malaysia.

b) Randomisation

The volunteers were randomised into two experimental groups of 16 subjects.

Figure 1 : Effect of 8 weeks of supplementation with 200 mg daily of vitamin E or placebo on plasma vitamin E concentrations \*(p<0.02). There is no difference in plasma vitamin E levels before and after treatment in the placebo group.



c) Vitamin E dose

The volunteers were given either 200 mg soft gel capsules of vitamin E containing approximately 30 % tocopherol [TF] and 70 % tocotrienol [TT] or placebo.

d) Placebo

The placebo was visually identical to the vitamin E soft gels and contained palm olein of negligible vitamin E content.

e) Treatment and duration

The period of treatment for both groups were 8 weeks. The groups consisted of all males, with a mean age of  $31.6 \pm 8.9$  years. All subjects signed an informed consent form, which was approved by the Universiti Kebangsaan Malaysia.

3) Chemical analyses

The release of MDA from erythrocytes *in vitro* was used as a functional measure of vitamin E status in the subjects. The plasma vitamin E levels were determined using HPLC. The indicators measured to reflect the status of oxidative stress were plasma MDA, antioxidant enzymes such as superoxide dismutase and glutathione peroxidase. All

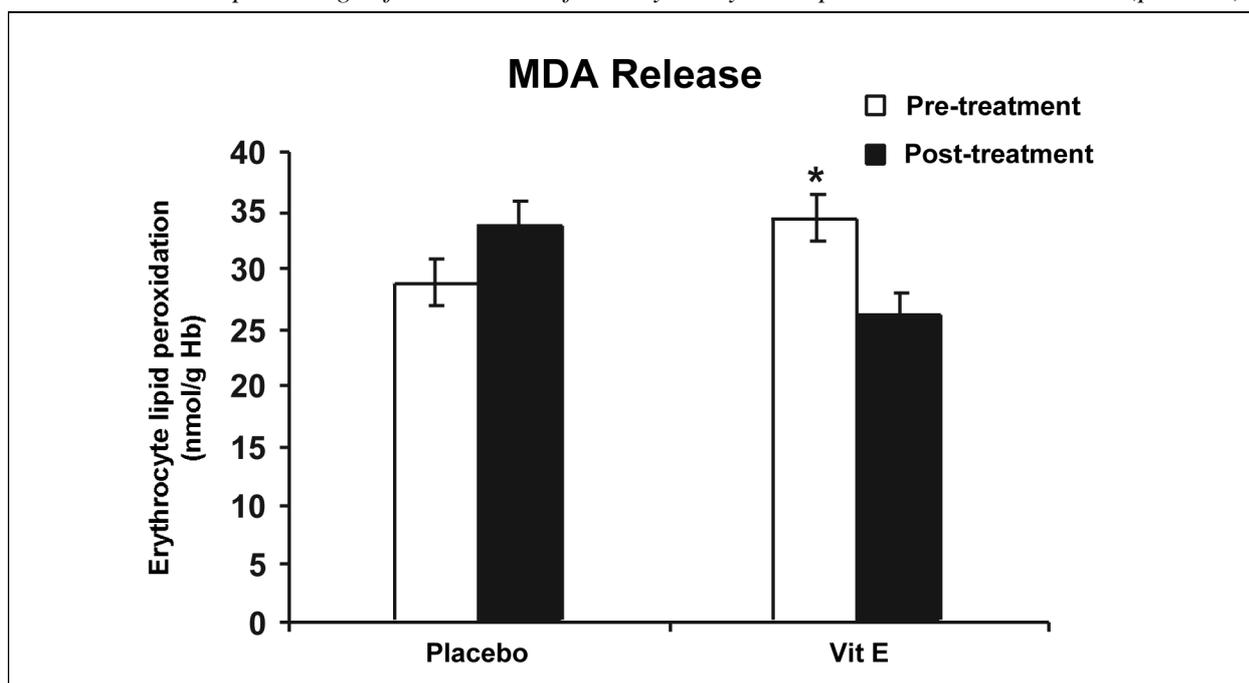
parameters were measured at the beginning and at the end of the treatment period.

a) Measurement of MDA release from erythrocyte

The determination of MDA release from erythrocytes *in vitro* was done according to a method described previously (12). Isolated erythrocytes were washed twice with phosphate buffered saline at a pH of 7.4. Two aliquots of 0.2 ml erythrocytes were placed in two separate test tubes. One aliquot was placed in a test tube (test tube 1) containing 3.8 ml phosphate buffered saline and the second aliquot of packed RBCs was suspended in another test tube (test tube 2) containing 3.8 ml phosphate buffered saline to which sodium azide had been added. Both suspensions were vortexed for 15 seconds.

One ml of the erythrocyte suspension was taken from test tubes 1 and 2 and mixed with 1.0 ml of 3 % hydrogen peroxide and 0.75 % hydrogen peroxide, respectively. These samples were prepared for duplicate incubation. All test tubes were vortexed for 10 seconds prior to incubation at 37°C in a shaking water bath. After an hour, 1 ml of trichloroacetic acid (TCA) in sodium arsenite was added to all tubes. Thiobarbituric acid (1 ml) was added to 2 ml of supernatant that was removed from each tube following centrifugation (3000 x g for 10

Figure 2 : Effect of 8 weeks of supplementation with 200 mg daily of vitamin E or placebo on erythrocyte lipid peroxidation. The group supplemented with 200mg vitamin E for 8 weeks had significantly lower percentage of MDA release from erythrocyte compared to baseline values  $(p < 0.01)$ .



minutes). These specimens were then boiled for 10 minutes in a water bath, cooled to room temperature and absorbance at 535 determined using a spectrophotometer. The concentration of MDA in the samples was obtained from a standard absorption curve for MDA.

b) Determination of plasma vitamin E levels

The sample preparation and the analysis of vitamin E by HPLC according were performed to a method described by Lang et al. (13). The Gilson HPLC system was used with a fluorescent detector. Vitamin E was detected at an excitation wavelength of 294 nm and an emission wavelength of 330 nm. The concentration of vitamin E was determined using a standard curve.

c) Determination of plasma MDA

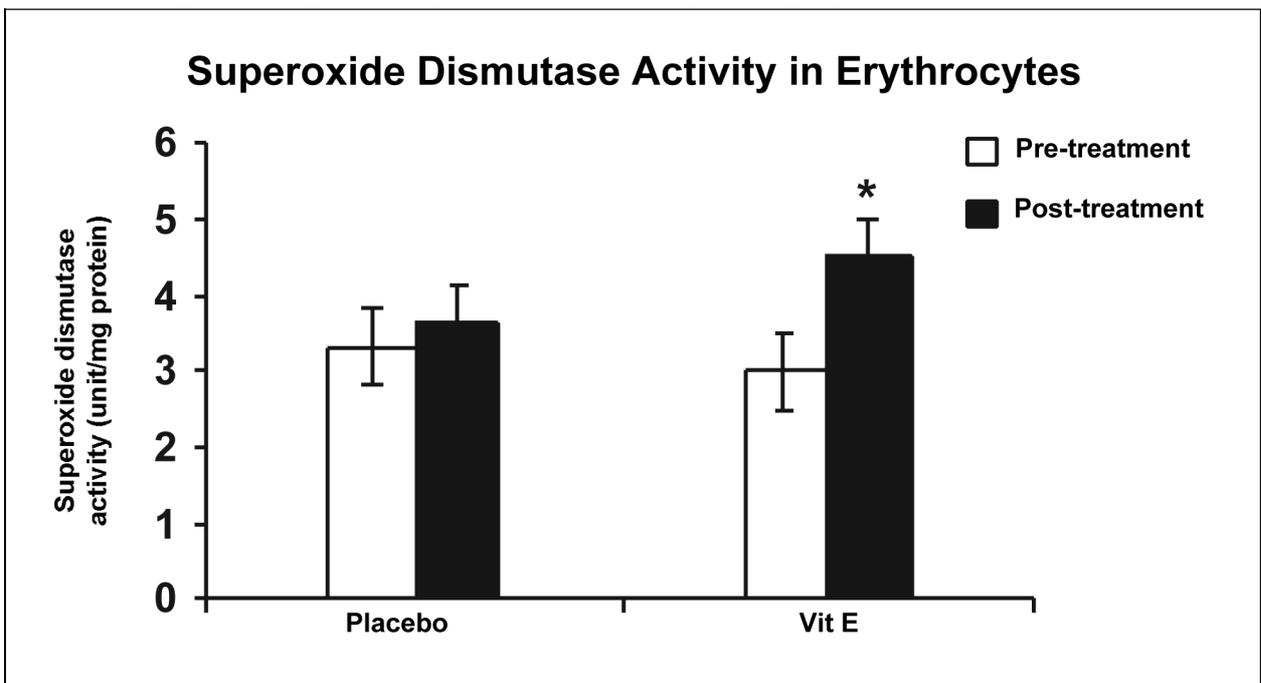
The plasma content of MDA was determined using a method described by Ledwozyw *et al.* (14) with some modifications. A plasma sample of 0.5 ml was acidified with 2.5 ml of 1.22 M trichloroacetic acid /0.6 M HCl and left to stand at room temperature for 15 min after which 1.5 ml of 0.67% thiobarbituric acid/0.05 M NaOH was added. The samples were incubated in a 100°C water bath

for 30 min. Subsequently it was left to cool at room temperature before the addition of 4 ml of n-butanol. After thorough mixing, the mixture was centrifuged for 10 min. at 1500 x g. The absorbency of the upper phase was read at 535 nm. Various amounts of MDA standard, freshly prepared by acidification of 1,1,3,3-tetraethoxypropane were subjected to the identical test procedure as the basis for constructing a standard curve for thiobarbituric acid reactivity as MDA equivalent.

d) Analysis of superoxide dismutase

The measurement of this enzyme was done in erythrocytes according to previously described methods (15). This method was based on the ability of superoxide dismutase (SOD) to inhibit the reduction of nitro blue tetrazolium whereby 1 unit of SOD was taken as a 50% inhibition of reduction of nitro blue tetrazolium. A mixture that consisted of a substrate solution (L-methionine, Triton X-100 and nitro blue tetrazolium) and 20 ml sample of lysed erythrocytes or PBS (control) were vortexed. A riboflavine mixture (riboflavine, 0.1 ml of EDTA in PBS, pH 7.8) was then added. The tubes were then placed in a box lined with aluminium paper and lighted with a fluorescent lamp (20 W). After 7 minutes, the absorbance of the mixture at 560 nm

Figure 3 : Effect of 8 weeks of supplementation with 200 mg daily of vitamin E or placebo groups on erythrocyte superoxide dismutase activity. The vitamin E group had a significant increase in the erythrocyte SOD activity \*( $p < 0.02$ ) at the end of the supplementation period. There was no difference before and after treatment in the placebo group.



was determined using a spectrophotometer (Shimadzu UV-160 A) against a blank without the sample.

e) Analysis of glutathione peroxidase

The method used was that of Lawrence & Burk (16). A 0.1 ml sample of lysed erythrocytes and a buffered solution 1 mM NaN<sub>3</sub>, 1mM EDTA, 0.2 mM NADPH, 50 units of glutathione reductase (GSSG) and 1mM GSH, 50mM PBS, (pH 7) was incubated at room temperature for 5 minutes, then 0.1 ml 0.25 mM hydrogen peroxide was added. The absorbance of the mixture at 340 nm was determined using a spectrophotometer (Shimadzu UV-160 A) against a blank without the sample.

4) Determination of lung function

Spirometric measurements were made using a Cosmed Pony Graphic 3.5 spirometer with subjects in a sitting position. After practice blows, recordings were repeated until three satisfactory tracings were obtained. Analysis was based on the average of these three readings. The measurement that was considered in this study was the percent ratio of FEV<sub>1</sub> and FVC.

5) Statistical Analysis

All values are expressed as mean ± S.E.M. Wilcoxon's nonparametric signed rank test for paired observations was used for comparison of all parameters before and after supplementation with vitamin E. A p value <0.05 was considered significant.

**Results**

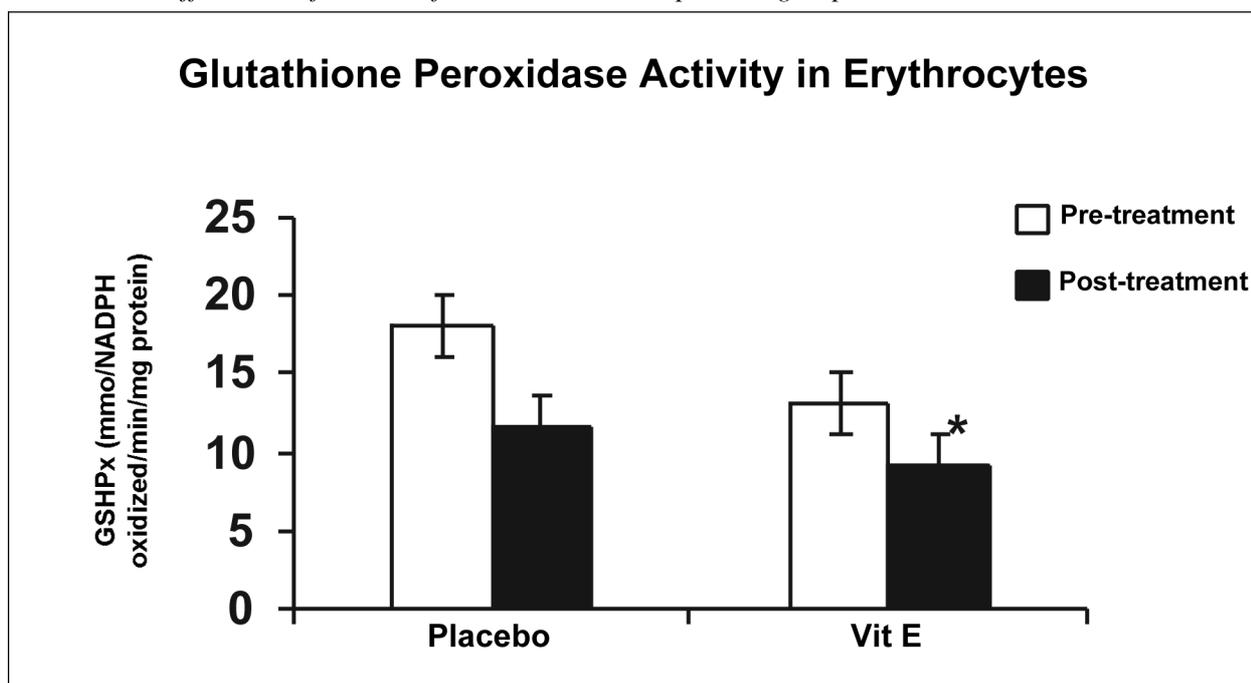
Plasma Vitamin E levels

There was no significant difference in total plasma vitamin E levels between the two groups at baseline (fig. 1). The plasma levels increased significantly (p<0.02) fig. 1 or fig 2 between the two groups after 8 weeks of supplementation with vitamin E. There was no difference in plasma vitamin E levels in the placebo group.

Effects of Vitamin E Supplementation on Erythrocyte Antioxidant Defence

There was no significant difference in the extent of erythrocyte susceptibility to peroxidation between the two groups at baseline (fig. 2). Vitamin

Figure 4 : Effect of 8 weeks of supplementation with 200 mg daily of vitamin E or placebo groups on erythrocyte GSHPx oxidised activity. The vitamin E group had significant decrease in the erythrocyte GSHPx activity\*(p< 0.02) at the end of the supplementation period. There was no difference before and after treatment in the placebo group.



E supplementation reduced erythrocyte release of MDA compared to the respective baseline levels ( $p < 0.01$ ). Erythrocytes from the placebo group showed a greater susceptibility to hydrogen-induced lipid peroxidation than those from the vitamin E supplemented group ( $p < 0.030$ ). Before supplementation, erythrocyte GSHPx and SOD activities were comparable. After supplementation with vitamin E, the erythrocyte SOD activity was increased ( $p < 0.02$ ) and the erythrocyte GSHPx activity decreased ( $p < 0.02$ ) compared to the respective baseline levels.

Plasma MDA Levels

There was no difference in baseline levels of plasma MDA levels between the two groups. Vitamin E supplementation did not affect the plasma MDA levels.

Lung Function Test

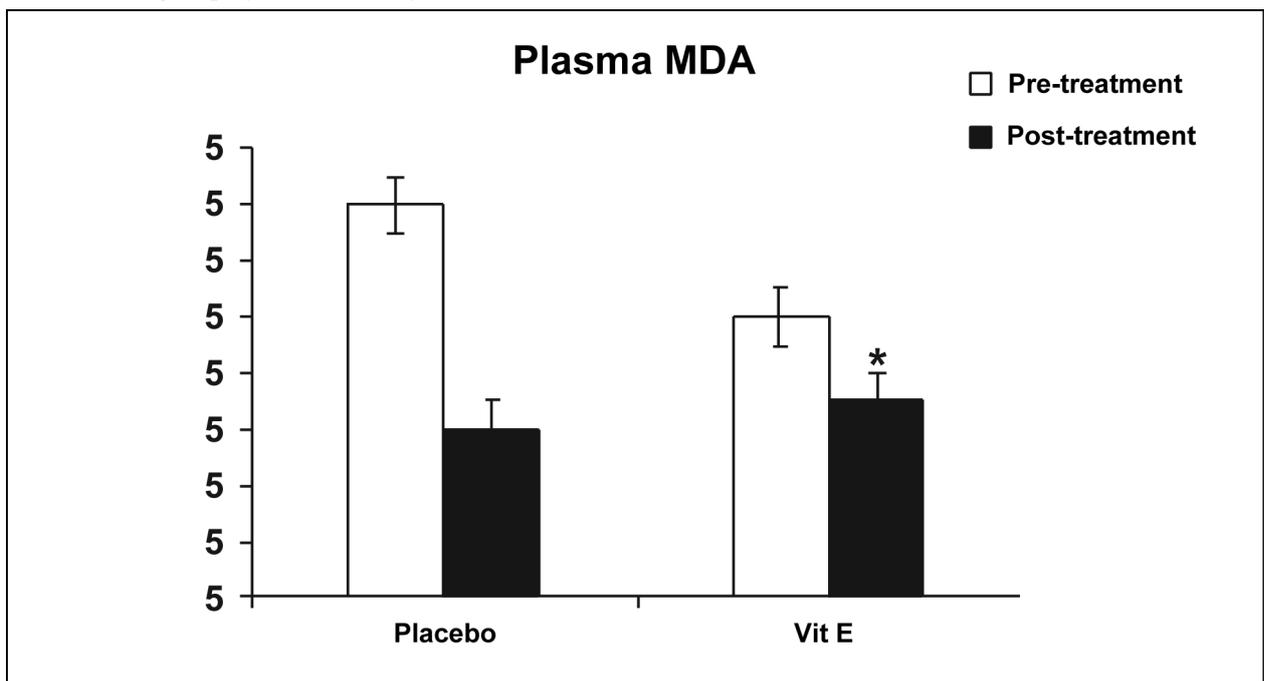
There was no difference in FVC (fig. 6), FEV<sub>1</sub> (fig.7) and the percent ratio of FEV<sub>1</sub> and FVC (fig. 8) before or after supplementation with vitamin E between the two groups. There was also no difference in the FVC, FEV<sub>1</sub> and the percent ratio of FEV<sub>1</sub> and FVC after vitamin E supplementation compared to the baseline values.

Discussion

Presently, it is widely known and accepted that free radical mediated lipid peroxidation has a crucial role in the pathogenesis of many disease processes such as atherosclerosis, diabetes mellitus, carcinogenesis, inflammation and many other conditions. Hence, the use of various antioxidant supplements to prevent or reduce damage to biological tissues are currently extensively investigated in various disease conditions. The amount of exogenous antioxidants required by individuals will be influenced by the oxidative stress status of the individual as this will affect the endogenous cellular antioxidant defence system. Antioxidant defences in the lung are provided by endogenous enzyme systems and by dietary antioxidants, particularly vitamin C and E (16 – 18). Smokers have a higher index of oxidative stress resulting from inhalation of cigarette smoke that is known to contain oxidizing substances. Consequently, smokers have been shown to have a lower antioxidant status (19 – 24) that predisposes them to developing free radical mediated lung injury.

In this study, we assessed the antioxidant defences [in individuals with high oxidant stress] in response to supplementation with vitamin E as an antioxidant and compared this response to a placebo intake. It has previously been shown that

Figure 5 : Effect of 8 weeks of supplementation with 200 mg daily of vitamin E or placebo on plasma MDA concentration. There was no difference in the plasma MDA concentration in both groups. Fig. 6 There is no difference in FEV<sub>1</sub> /FVC in the vitamin E supplemented group and the placebo group after 8 weeks of treatment.



erythrocytes from smokers are more susceptible to lipid peroxidation (25). This study demonstrated that vitamin E reduces the susceptibility of erythrocytes to lipid peroxidation. The end product of lipid peroxidation, MDA was measured by fluorometric determination. Although this assay is not specific only for MDA and measures other substance that can react with thiobarbituric acid and give rise to the colored end points detected, the modifying effect of vitamin E on the generation of TBA-reacting species implies that it is a valid indicator of peroxidation *in vitro*.

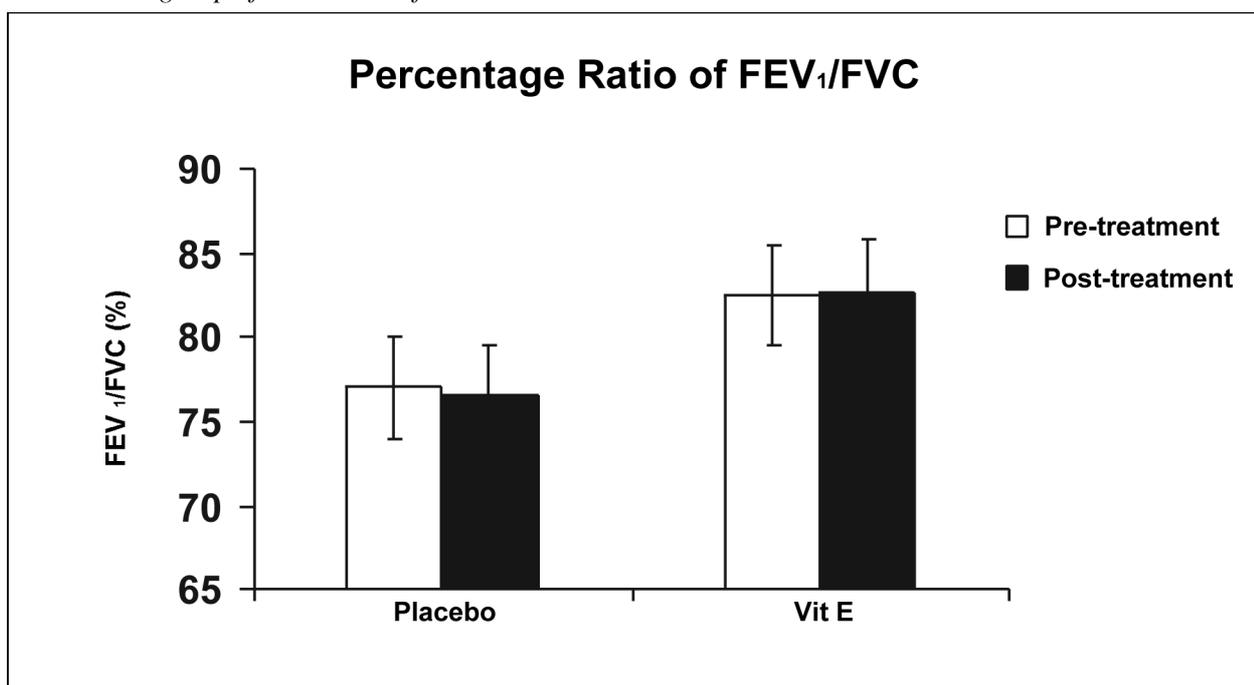
It has also been shown that erythrocyte activity is increased in smokers and this may reflect an increased spontaneous regeneration of superoxide in the cells (26). We found that vitamin E supplementation can further increase the SOD. The function of SOD is to metabolise the oxygen radical ( $O_2^-$ ). Vitamin E is also a scavenger for superoxide. Therefore, in the presence of vitamin E, SOD is utilized less thus the pool of SOD is increased. On the other hand, the constant intake of vitamin E ensures its continued presence and increases the process of scavenging  $O_2^-$ , thereby generating hydrogen peroxide, the substrate for GSHPx. With increased production of its substrate, the utilization of GSHPx is more. There is a consequent fall in the reserves of GSHPx as the cellular utilization of GSHPx exceeds the production of this enzyme. Other studies have shown that there is a decline in

glutathione (GSH) concentration with increasing erythrocyte vitamin E concentration and this may indicate an increased utilization of GSH in the reductive regeneration of the vitamin E its peroxy radical (25). Glutathione depletion studies in mice demonstrate that the rate of decline of total GSH in tissues and blood reflects its rate of utilization (27).

There have been various reports on the intake of antioxidants and lung function (28–30). Although this study demonstrates that vitamin E can improve the antioxidant status, there was no improvement of lung function. Britton *et al.* showed that a combined intake of vitamin C and E correlated positively with FEV<sub>1</sub> and FVC. It is possible that a higher dose of vitamin E is necessary to achieve an improvement in lung function, hence the improvement seen when vitamin E intake is combined with vitamin C (30). The fat-soluble vitamin E and water-soluble vitamin C are thought to act cooperatively in a system whereby vitamin E, which is mainly sequestered in cell membranes and other lipid structures, is maintained in a reduced state by interacting with water-soluble vitamin C.

The endogenous and exogenous factors influencing the cellular protection against free radicals are many and varied. Our study suggests that one such factor is the constant availability of vitamin E in the body. Although vitamin E improved the integrity of erythrocytes membrane shown by improved protection against lipid peroxidation *in*

Figure 6 : There is no difference in FEV<sub>1</sub>/FVC in the vitamin E supplemented group and the placebo group after 8 weeks of treatment.



*vitro*, it also affected the regulation of the endogenous antioxidant enzymes. Until the biological relevance of this is made clear, the recommendation of vitamin E supplements against smoking induced lipid peroxidation should be done cautiously.

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### Correspondence:

Assoc. Prof. Dr. Nafeeza Mohd. Ismail, M.B.B.S., Ph.D.

Dept. of Pharmacology, Faculty of Medicine,  
Universiti Kebangsaan Malaysia,  
Jalan Raja Muda Abdul Aziz,  
50300 Kuala Lumpur, Malaysia.  
e-mail: [nafeeza@medic.ukm.my](mailto:nafeeza@medic.ukm.my)

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