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RESEARCH ARTICLE

In Vitro Effects of Ascorbic Acid and Alpha-tocopherol on Serum Malondialdehyde formation in Diabetic Patients

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ABSTRACT

Free radicals are responsible for onset and progression of diabetes. The link between oxidative stress and secondary complications of diabetes has been also documented. In present study we have studied *In vitro* effect of ascorbic acid and alpha tocopherol in terms of Malondialdehyde (MDA) formation in blood serum from diabetic as well as non-diabetic persons. Total 60 blood samples from average age group of 48.12 $\pm$ 11.60 years were studied. Each sample is fractionated into control and experimental group for ascorbic acid and alpha tocopherol respectively. Given fractions were incubated for 1 hr, 6 hr and 24 hr duration for studying time dependent results. There was a time dependent increase in malondialdehyde (MDA) in all control as well as experimental groups. But there found highly significant ($p<0.001$) decline in rate of malondialdehyde (MDA) formation in both experimental group as compared to their respective controls. In conclusion *in vitro* supplementation of antioxidant vitamins has a potential to minimize the oxidative stress hence recommended for their use in *invitro* systems.

Key words: *Oxidative Stress, Diabetes, Lipid Peroxidation, Ascorbic acid, Alpha tocopherol.*

INTRODUCTION

Free radicals are atoms or group of atoms having unpaired electron in their outermost orbit. Reactive oxygen species (ROS) are continuously generated in physiological conditions and effectively neutralized by intracellular as well as extracellular antioxidant systems (Halliwell and Gutteridge, 1989). Oxidative stress occurs when free radical production exceeds the body's ability to neutralize them.

Diabetes is a disease condition with concomitant higher levels of glucose in blood. Hyperglycemic condition also leads increased oxidative stress and inturn complications associated with disease. (Rolo and Palmeira, 2006). In fact, excess oxidative stress is responsible for onset and

progression of diabetes and its associated complications (Oberley, 1988; Prasad and Sinha 2010).Free radicals target various biomolecules including polyunsaturated fatty acids (PUFA) making plasma membrane more vulnerable to ROS (Aitken *et al*., 1989) by the process of peroxidation of membrane lipids causing structural and thus functional abnormality of target membrane (Rao *et al*., 1989; Sharma and Agarwal, 1996).

Malondialdehyde (MDA) is an end product of lipid peroxidation and considered as a reliable marker for free radical damage. MDA also inactivates enzymes (Chen and Yu, 1994; Tanaka *et al*., 1999), acts as endogenous fixative, reacting with proteins & nucleic acids to form heterogeneous cross links (Chio and Tappel, 1969). A notable increase in free radicals has been reported in both diabetic rat aorta (Langenstroer and Pieper, 1992) and more recently endothelial cells grown under hyperglycaemic conditions (Graier *et al*., 1999). Many studies have shown that increased lipid peroxides and/ or oxidative stress are present in diabetic subjects (Keaney and Loscalzo, 1999; West, 2000).Studies also shown diabetic persons have defective metabolism of vitamin C (Sundaram *et al*., 1996.)

Non enzymatic antioxidants viz Alpha Tocopherol and Ascorbic Acid have confirmed their antioxidant potential (Theng *et al*., 2007; 2009). Therefore, the idea of using these antioxidant so as to minimize diabetes induced lipid peroxidation getting strengthen. (Ceriello *et Al*., 1997; Maxwell *et al*.,1997). Many studies have evaluated changes in level of antioxidant and MDA in blood of type 2 diabetes and healthy subjects (Mohamed *et al*., 2014)

Most of studies reported yet have compared the level of plasma/serum antioxidants with respect to MDA formed *in vivo*. It has also been reported that diabetic patients possess a lower quantity of antioxidant potential in the plasma (Abuja and Albertini, 2001). And hence may require more antioxidants compared to healthy individuals. Thus, this study is aimed to find out the *in vitro* effect of additional alpha tocopherol and ascorbic acid individually with respect to malondialdehyde in blood serum.

METHODS AND MATERIAL

The study was conducted on 30 normal and 30 prediagnosed type 1 and 2-diabetic human subjects, in the mean age group of 48.12 $\pm$ 11.60 years. The subjects with history of smoking/ alcoholism/ any chronic disease were excluded from the study. Patients were not taking any other medicines other than oral anti-diabetic.

Collection of blood samples

6 mL of blood was collected in a collecting tube. Incubated in an upright position at room temperature for 30-45 min. to allow clotting. Serum was separated by centrifuging the blood sample at 3000 rpm. for 5 min. and then carefully aspirated the supernatant (serum) in another sample tube.

Quantification of serum MDA level

From the 2 ml separated blood serum, four aliquots of 0.5 ml each were made into pre-sterilized glass vials. Each fraction of semen sample was treated with 1.0 ml of Ringer-Tyrode solution (RT) as follows.

1. Control for Ascorbic acid (0.5 ml blood serum + 1.0 ml Ringer-Tyrode) = 1.5 ml
2. Ascorbic acid treated group (0.5 ml blood serum + 1.0 ml Ringer-Tyrode containing 1mM ascorbic acid)
3. Control for alpha tocopherol (0.5 ml blood serum + 1.0 ml Ringer-Tyrode containing 0.0025 % ethanol)
4. Alpha tocopherol treated group (0.5 ml blood serum + 1.0 ml Ringer-Tyrode containing 1mM alpha tocopherol)

After addition of RT each fraction is having 1.5 ml of solution, which again split into three similar fractions of 0.5 ml each. Now each sub- fraction is designated for differential incubation of 1 hr,6 hr and 24 hr respectively.

After desired incubation period (i.e 1 hr) 0.5 ml from this suspension was transferred to pre-cleaned and pre-chilled glass mortar. It was allowed to freeze for 1 hr in deep freezer.

Immediately after freezing the samples were homogenized thoroughly and uniformly in 2.0 ml reaction mixture containing Potassium phosphate buffer pH 7.04 (75mM), $FeCl_3$ (1 mM) and Ascorbic acid (1 mM)

Aliquots of 0.2 ml of this homogenate were added to 1.8 ml of distilled water and 1ml of 20% TCA. Immediately 2ml of 0.67% TBA was added and the tubes were placed in boiling water bath for 10 min. All tubes were cooled and centrifuged. Absorbance was read at 532nm. (Wills, 1966)

Control was prepared by adding 2ml distilled water, 1ml TCA 20%, 2 ml TBA 0.67%. Zero absorbance was set by control.

$$\text{nM of MDA/ml of sample} = \frac{\text{O.D. of sample}}{0.156 \times 0.0133}$$

0.156 = the absorbance for 1 nM of MDA in 1 cm thick cell at 532 nM.

0.0133 is the serum dilution factor (0.5 ml serum was supplemented with 1.0 ml of Ringer-Tyrode(1.5ml) from which 0.5 ml sample was frozen and homogenized with 2.0 ml reaction mixture (2.5ml) from which 0.2 ml was used for the assay.

Similarly same protocol is followed with 6 hr and 24 hr incubation respectively for estimation of malondialdehyde.

A set of 30 samples from normal group is also tested for baseline values in normal individuals i.e. without diabetics

***Statistical analysis*:**

Data were processed by computing the arithmetic mean and standard deviation. Since the same serum sample was used for various interval treatments, the level of significance of difference between the two means was carried out by paired't' test followed by ANOVA.

RESULTS AND DISCUSSION

In this study, we found time dependant increase in lipid peroxidation in the form of nM of malondialdehyde (MDA). An increased level of MDA which is one of the end products of lipid peroxidation is an indicator of increased oxidative stress (Pryor and Stanley, 1975).

When compared the diabetic MDA with non diabetic ones, there is highly significant ($p<0.001$) increase in the rate of MDA formation .When results of ascorbic acid and alpha tocopherol treated groups were compared, there was a highly significant protective value ($p < 0.001$)of alpha tocopherol was found ,which is more superior over ascorbic acid .

Table 1: Effect of ascorbic acid and alpha-tocopherol separately on lipid peroxidation measured in the form of nM of malondialdehyde/ml of the serum incubated at various time intervals from diabetic blood.

Hrs of incubation	Control for Ascorbic acid	Ascorbic acid treated	Control for Alpha tocopherol	Alpha tocopherol treated
1ST	156.29 ± 2.05	123.26±5.84***	157.96 + 3.10	113.5± 8.19***$
6th	161.24 ± 7.51	135.25 ± 5.71***	150.80 ± 7.95	119.60 ± 5.33***$
24TH	178.46 ± 15.22	168.37±13.54 ***	181.69 ± 14.12	154.78± 12.59***$

**** indicates P< 0.001 i.e. highly significant experimental group compared with its control .*
$ indicates p< 0.001 highly significant alpha tocopherol treated group compared withA.Aorbic acid treated group.
ANOVA : calculated F ratio >>> tabular F value at p = 0.0001

Table 2: Effect of ascorbic acid and alpha-tocopherol separately on lipid peroxidation measured in the form of nM of malondialdehyde/ml of the serum incubated at various time interval from non-diabetic blood.

Hrs of incubation	Control for Ascorbic acid	Ascorbic acid treated	Control for Alpha tocopherol	Alpha tocopherol treated
1ST	59.29 ± 3.15	50.26±2.9***	64.96 ± 3.0	45.5± 4.8***$
6 th	71.55 ± 1.36	57.96 ± 3.68 ***	82.26 ± 4.28	54.93 ± 5.32***$
24th	110.5± 2.9	100± 3.3***	120 ±2.65	94.4 ± 5.3***$

*** indicates P< 0.001 i.e. highly significant experimental group compared with its control .
$ indicates p< 0.001 highly significant Alpha tocopherol treated group compared with.Asorbic acid treated group.

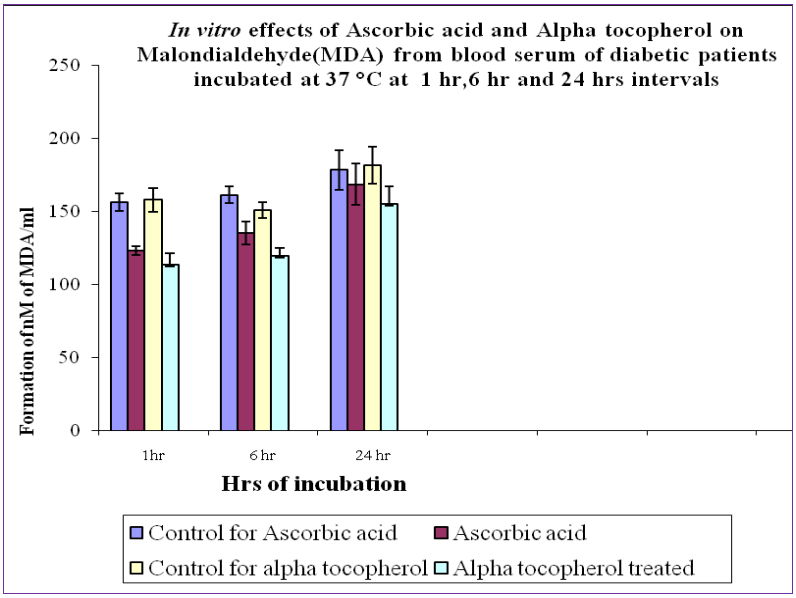

Fig. 1: showing formation of MDA in different treatment group at different time interval viz 1 hr, 6 hr and 24 hrs respectively.

Free radicals react with polyunsaturated fatty acids of the membrane and cause peroxidative damage (Sharma and Agarwal, 1996). In the present study there was time dependant increase in lipid peroxidation in all the groups, indicating time dependant increase in oxidative stress. Ascorbic acid and alpha tocopherol separately treated groups exhibited highly significant decrease ($p<0.001$) in MDA concentration as compared to their respective controls indicating protective role of these antioxidants against lipid peroxidation. Increased production of ROS causes defects in cellular function through lipid peroxidation. Lipid peroxidation mediated membrane perturbation results in loss of structural and functional integrity of RBCs and WBCs, ultimately decreasing viability (Preffer and Suisloki, 1982).

On contrary, without co-oxidant, vitamin E found to enhance peroxidative damage (Bowry *et al*.1995), while in some cases (Michael and Fowler, 2008) do not shown promising protection against diabetic complications. Pro-oxidant nature of vitamin C has been reported by earlier studies (Halliwell B 1996). Elina Porkkala-Sarataho *et al*.2000 also reported inadequacy of vitamin C alone against lipid peroxidation.

Results of this study also supports our previous studies which confirmed antioxidant efficiency of these antioxidants when used separately (Theng *et al*., 2007; 2009)., since the treatment of ascorbic acid and alpha tocopherol decreased the MDA levels therefore membrane perturbation or damage is significantly ($p<0.001$) protected.

CONCLUSION

Oxidative stress induced due to diabetic condition results in higher values of MDA formation in diabetic serum. Although it is not possible for ascorbic acid or alpha tocopherol group to minimize the MDA formation in diabetic group like that in normal group, but when compared experimental group for Ascorbic acid and Alpha-tocopherol with their respective controls, they have shown highly significant($p<0.001$) decrease in MDA formation indicating their *in vitro* efficiency in protection against oxidative damage. This prospective also can be used in places like blood banks where blood and their components encounters storage time dependent oxidative stress.

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