Research Paper

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Are Antioxidants Always Helpful in Thalassemic Patients?

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ABSTRACT

Repeated blood transfusion in beta thalassemia major patients may lead to peroxidative tissue injury by secondary iron overload. so antioxidants are usually advised .But several clinical antioxidant trials on various diseases have shown that exogenously administered antioxidants may not be as beneficial as predicted from the earlier works. This may be due to the lesser dosage of the antioxidants administered or the stage of the disease at which such antioxidants were administered may not have been responsive to the therapy.We therefore hypothesized and hence ventured to investigate whether or not exogenously administered antioxidants are really beneficial or whether they could act as pro-oxidants in a particular situation of a cell. In the present study, 150 children with beta thalassemia major were included. Neutrophils were isolated and the cells were treated with Vitamin E, Vitamin C and combination of both the vitamins E and C , to check there protective role in liberartion of biochemical parameters like ONOO-, O2-, cytosolic Ca and Cathepsin D release and Cu, Zn SOD during Oxidative stress. Where it had been proved that vitamin E might sometimes acts as a pro oxidant while Vitamin C have certain antioxidant effects.

INTRODUCTION

Beta thalassemia major is the most prevalent type of thalassemia as it is common in certain populations. In India,every year more than 10,000 children are born with thalassemia major. It produces severe anemia in its homozygous state (1). About 190 million people throughout the world have genetic mutations associated with different hemoglobinopathies and more than 90 million of them carry defective genes leading to thalassemia (2,3).The disease is associated with profound anemia, jaundice, splenomegaly, expanded bone marrow space, siderosis and cardiomegaly. These symptoms appear after about 2-4 months of age. Impaired erythropoeisis, hemolysis in the peripheral circulation and deposition of excess iron in the tissues, are some of the causes of clinical manifestations(2). Earlier studies have shown that, in thalassemia there is excess production of reactive oxygen intermediates, such as superoxide anion (O2–), hydroxyl radical (OH·), singlet oxygen and hydrogen peroxide (H2O2) within the erythrocytes, all these events lead to oxidative stress. This oxidative stress and a possible consequential accelerated apoptosis may contribute to shortened life span of erythrocytes. Malondialdehyde (MDA), a product of lipid peroxidation is generated in excess amounts in supporting the fact that large amount of membrane bound iron is present in thalassemic erythrocytes (2,4). Trace metals, especially Iron are implicated as causative agents in excessive generation of free radical which are capable of causing oxidative damage to erythrocytes (1). Antioxidants are complex and diverse group of molecules that protect key biological sites from oxidative damage. They scavenge free radicals and other reactive oxygen species (ROS) (5-15). The present study was initiated to evaluate the role of oxidants, antioxidants and trace elements in beta thalassemia major .

Methods

MATERIALS AND METHODS

Fifty Thalassemic major patients were the subject of the investigation, who were undergoing through iron chelating therapy. Normal Neutrophils were collected from the healthy individuals. PMN were isolated through lynch at al. CHEMICAL METHODS Protein: estimated by the method of Lowry at (16).Measurement of $NO2^-$: Nitrite was estimated as per the method of Green et. al. (17).Measurement of Peroxynitrite: Peroxynitrite ($ONOO^-$) was measured as per the method of Beckman et al (18).Estimation of cytosolic calcium using Autozyme Cacium Kit.

Biochemical Methods:

Ca^{2+}/Mg^{2+} ATPase of the plasma membrane: The activity of Ca^{2+}/Mg^{2+} ATPase was assayed as per the method of Lynch et al (19). Copper –Zinc SOD,Pre treated neutrophils were subjected to Triton HBSS & centrifuged at 6000RPM for 10 minutes. The supernatant was used for this assay. The supernatant was directly read at 258 nm as per the method of Beyer et al. (11).Superoxide The amount of superoxide was measured by method described by Johnston et al. (21).Cathepsin D activity:-Cathepsin D activity was measured in the supernatant using the method of Sapolsky (22).

Pre-Treatments

Neutrophils were pre-incubated at 37°C for 45 mins as per the following:
Normal Neutrophils
Thal affected Neutrophils
Thal affected Neutrophils + Vit C
Thal affected Neutrophils + Vit E
Thal affected Neutrophils + Vit C + E.

The incubation mixture was then spun at 800-900 RPM and the supernatant was discarded. To the pellet obtained was added triton-HBSS and kept for 15 mins in cold. At the end of incubation the suspension was centrifuged at 5000 RPM for 10mins and the supernatant obtained was used as a crude cytosolic preparation. The pellet constituting of the cell debris was suspended in HBSS and was used for protein and Ca-pump estimation.

RESULTS AND DISCUSSION

In general we have found that vitamin E was partially protective against free radical protection and did not protect against lysosomal degranulation induced during oxidative stress due to iron overload in Thalassemic patients undergoing through blood transfusion. Vitamin C was a better protective agent against the aforementioned parameters. However, the combination of the two vitamins was found to exert a better protection against free radical damage and lysosomal degranulation.

Generation of NO (measured herein as NO_2) is intimately connected with lysosomal degranulation and events encompassing antigenic assault. NO can mediate lysosomal degranulation in view of its ability to form ONOO- radical on one hand and on the other can activate the soluble guanylate cyclase to release c GMP, which is known to trigger lysosomal enzyme release dur-

ing various cellular conditions such as, inflammation, microbial digestion etc (23). In our current observation we did not record any protective effect against the generation of NO by vitamin E (Fig 1), which is interestingly contradictory to earlier reports (24). However, vitamin C was more effective against the generation of NO. Furthermore the combination of the two vitamins was not as effective as compared to vitamin C alone. This is an important finding in view of the tremendous significance attached to the antioxidant function of vitamin E. In this study it appears that vitamin E might as well act as pro-oxidant under certain conditions.

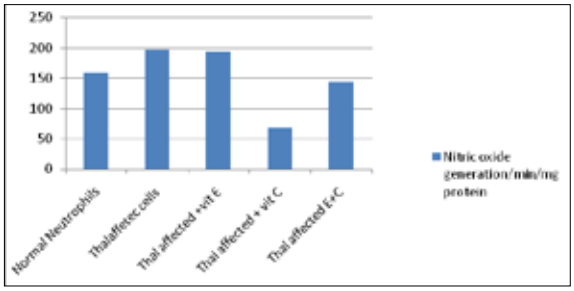

Fig 1. Effect of vitamin C and E on nitric oxide synthase (nOS) activity on Thalassemic affected child.

A more toxic form of NO e.g. ONOO- may be formed as result of combination of NO with O_2^- radicals. ONOO- is potently active and is known to oxidise sulfhydryl groups of proteins, induce protein- tyrosine nitration and can induce lysosomal degranulation as a result of direct lysosomal membrane damage (23. From figure 2 it is evident that vitamin C rendered mreprotection against ONOO- generation as compared to vitamin E alone. It is to be noted that a combination of the two vitamins further exacerbated the generation of this radical and appears to be augmented in the presence of vitamin E, as was seen in the case of NO_2^- generation.

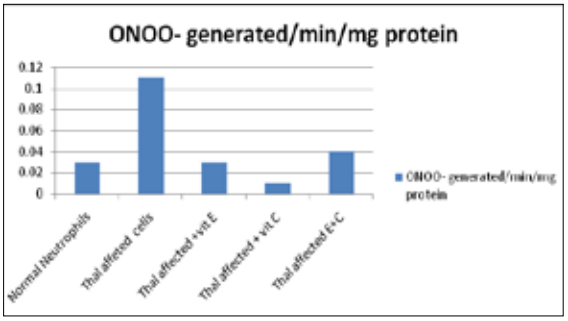

Fig 2. Peroxynitrite (ONOO-) generation in Presence of vitamins C and E in Thalassemic affected child.

Formation of ONOO- recorded in the present study augurs well with the finding of a similar pattern of generation of superoxide formation in the Thal affected neutrophils (Fig 3). Both vitamin C and E inhibited the formation of O_2^- radical in a manner similar to that observed for ONOO-. Interestingly, although vitamin C alone could diminish the generation of his radical, vitamin was ineffective in this sense.

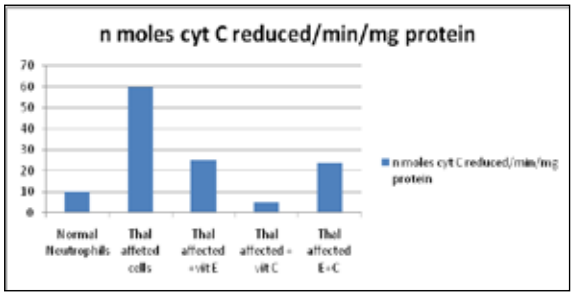

Fig 3. Pre- incubation of Vitamin C and E on Superoxide generation in thalassemic affected child.

A combination of the two vitamins, in fact, proved more deleterious rather than protective against the generation of O_2^-. However, the increased generation of O_2^- was found to be in parallel agreement with the formation of ONOO-. Furthermore, the increased generation of O_2^- could be explained by our another observation of a decreased activity of the Cu,Zn SOD enzyme (Fig 4), which is responsible for the removal of any O_2^- formed in the cells. Both the vitamins were ineffective in general to activate and increase the level of this antioxidant enzyme.

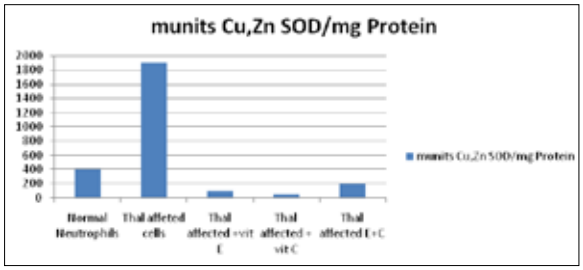

Fig 4. Cu, Zn SOD levels in response to vitamin C and E treatment to thalassemic affected cells.

Therefore from the above, it appears that the increased generation of ONOO- may be due to an increased formation of O_2^- and or decreased scavenging of this radical by Cu,Zn SOD (Fig 5) and a corresponding increase in the NOS activity as suggested by increased NO_2^- formation. Increased NO and O_2^- formation may thus lead to an increased formation of toxic and extremely reactive ONOO- leading to cell damage.

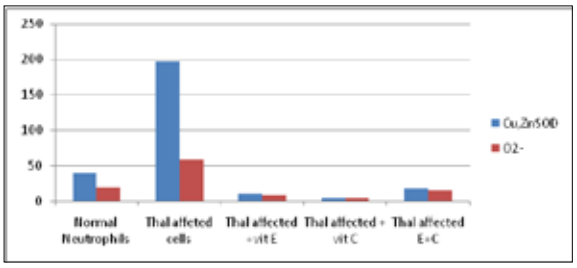

Fig 5 . Relation of cu, Zn SOD and Superoxide generation in Thalassemic affectd child.

As mentioned earlier, ONOO- can induce direct degranulation of lysosomes in view of its ability to cause lysosomal membrane damage (23). This report is corroborated by our present observation of an increased cathepsin D activity in cells treated with opsonised E coli, and stimulated cells along with vitamin E (Fig 6). Vitamin C alone was found to be protective against lysosomal degranulation (as represented by Cathepsin D release). However, in combination with vitamin E, the protective effect of vitamin C was lost, thus suggesting that vitamin E might be acting as a pro-oxidant under certain physiological status.

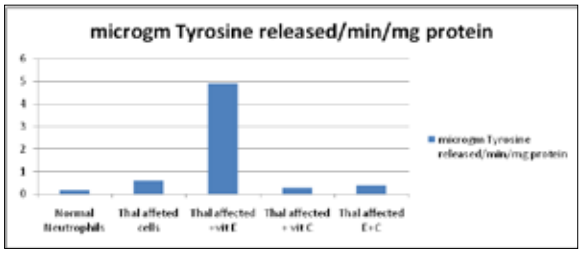

Fig 6. Effect of vitamin C and E on lysosomal degranulation on thalassemia affected child

The pro-oxidant effect of vitamin E observed by us may be due to the depletion in glutathione (GSH) in cells undergoing iron cheletion therepy . GSH, vitamin C and vitamin E are known to be in redox recycling interaction and maintain each other in a reduced state (REF). Vitamin E is the primary acceptor of reducing equivalents from vit C, which in turn takes electrons from

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GSH. In the present context it appears that depletion of GSH may break this reduction cycle and therefore keep vit E in an oxidized state which might act as a pro-oxidant.

Intracellular calcium (Ca) is an important mediator of a wide variety of signaling processes in the cell. Ca homeostasis is tightly regulated in a cell due to its toxicity. However, the role of calcium in lysosomal degranulation is of pivotal importance in addition to the role of NO and ONOO-. In the recent study we report that there was an increase in the cytosolic Ca levels in neutrophils challenged with opsonised E coli and the levels were significantly diminished in the presence of both vitamins C and E (Fig 7).

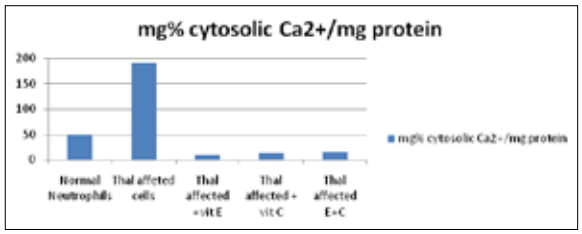

Fig7. Cytosolic free Ca2+ levels in response to vitamin C and Vitamin E in Thalassemic affected child.

To further test the reason behind the decrement in the Ca levels we examined the activity of Ca-pump (Ca^{2+} Mg^{2+} ATPase) which is responsible for the extrusion of Ca out of the cytosol. Our findings reveal an increased Ca pump activity during the phagocytic process and when the cells were treated with vit E (Fig 8).

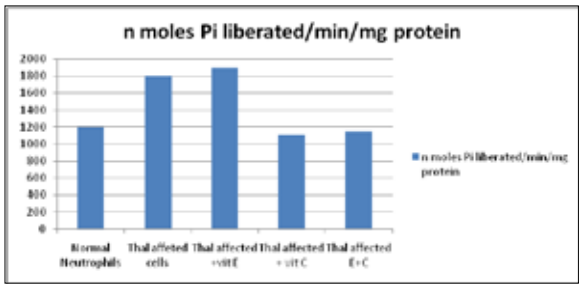

Fig 8.Effect of vitamin C and E on the plasma membrane calcium pump activity in Thalassemic affected cells.

On the other hand vit C alone and in combination with vit E did not alter the status of the pump activity. The increase in the pump activity in the vit E treated cells, akin to that found in the Thal group , indicates a possible pro-oxidant effect of vit E at least in the resent experimental conditions. However, no direct correlation ship could be established between the cytosolic ca and the Ca pump in the present work. This could be due to an experimental artifact or due to some cellular components which may scavenge free Ca. this aspect therefore requires further investigations.

Conclusions

The overall results obtained from the present work are summarized in Figure 9.

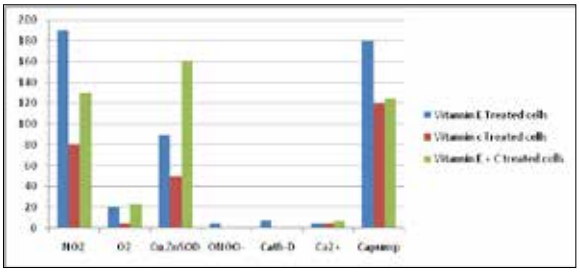

Figure 9.Comparative analysis of Vitamin E ,Vitamin C, and Vitamin E + C on various biochemical parameters as shown in the figure

Overall it could be surmised that neutrophils challenged with E coli exhibited increased generation of ONOO-, $O_2^{\cdot-}$, cytosolic Ca and Cathepsin D release and decreased Cu, Zn SOD. A similar pattern was observed for Vit E treated cells and was thus found to act as a pro-oxidant. In contrast vit C was the most protective for the Thal affected cells . However, both vitamins E and C together showed certain degree of pro-inflammatory properties akin to the actions of a pro-oxidant. A brief summary of the results are depicted in Figure 9. It therefore appears that usage of exogenous antioxidants may not be always beneficial to the cells and that more investigations are warranted to exactly determine and define the therapeutic efficacies of exogenous antioxidants.

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