Antioxidant Role of Vitamin E in Atherogenesis induced by Hyperfibrinogenemia

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SUMMARY

With the aim of studying the effect of vitamin E over oxidative stress due to hyperfibrinogenemia (HF) in an experimental model of atherogenesis and the possible normalization of oxidative stress markers; nitric oxide (NO), L-citrulline, superoxide dismutase (SOD) and involution of histopathologic lesions in the thoracic aorta were evaluated. The study was performed in 36 rats, Wistar strain, which were divided into three groups (n = 12 each): A, control; B, HF x 90 days; C, HF x 90 days + vitamin E. HF was induced by adrenaline injections (0.1 ml/day/rat) for 90 days. The dose of vitamin E was 2 mg/day/rat for 75 days. Fibrinogen levels (mg/dl), NO (μM) and L-citrulline (mM) were measured in plasma and SOD activity (U/ml) was measured in red blood cell lysate using spectrophotometry. Sections of thoracic aorta were analyzed using optical microscopy (OM). For the statistical analysis, MANOVA and Fisher’s exact test were used; a significant level of p < 0.05 was established. A significant increase of fibrinogen in group B (407 ± 8.9 mg/dl) in comparison with groups A (203 ± 9 mg/dl) and C (191.58 ± 17.79 mg/dl) (p<0.001) was observed. NO decreased significantly in group B (13.73 ± 1.76 μM) against groups A (23.58 ± 0.08 μM) and C (26.64 ± 3.65 μM) (p<0.001). L-citrulline increased significantly in groups B (4.99 ± 0.18 mM) and C (6.60 ± 0.16 mM) in comparison with group A (3.03 ± 0.13 mM) (p<0.001). SOD increased its activity in groups B (251.67 ± 10.34 U/ml) and C (304.75 ± 10.43 U/ml) against group A (139.44 ± 4.74 U/ml) (p<0.001). Optical microscopy showed endothelial denudation, intima-media thickness and wall protrusion in group B (90%) and recovery of endothelial denudation and a 50% decreased of the intima-media thickness in group C (p<0.001). High SOD levels would be insufficient to prevent alterations in the oxidative stress pathway induced by HF. Vitamin E would stop the chain reaction started by free radicals and as a consequence, superoxide anion would decrease, stimulating an increase in NO bioavailability and normalizing the concentrations of plasma fibrinogen.

Key words > Hyperfibrinogenemia - Atherogenesis - Oxidative stress - Superoxide dismutase - Vitamin E

Abbreviations > VSMC - Vascular smooth muscle cells
eNOS - Endothelial nitric oxide synthase
RNS - Reactive nitrogen species
ROS - Reactive oxygen species
GC - Guanylate cyclase
cGMP - Cyclic guanosine monophosphate
HP - Hyperfibrinogenemia
LDL - Low-density lipoprotein
OM - Optical microscopy
mRNA - Messenger ribonucleic acid
NO - Nitric oxide
oxLDL - Oxidized low-density lipoprotein
SOD - Superoxide dimutase
TNF-α - Tumor necrosis factor-alpha
vit - Vitamin E

BACKGROUND

Atherogenesis presents a lipid deposit and an inflammatory component in the arterial wall. In previous works, we have shown the existing relationship between hyperfibrinogenemia and the functional alteration of the vascular endothelium that leads to a loss of normal hemostatic properties, changing the endothelium-dependent relaxation due to a decrease of nitric oxide (NO) synthesis and/or bioavailability which could be an earlier and important phenomenon of endothelial dysfunction. (1) Other researchers confirm that oxidative stress and endothelial dysfunction predict the risk of suffering from cardiovascular ischemic events. (2)
A reduction in NO endothelial bioavailability could promote a proinflammatory and prothrombotic phenotype at a vascular wall level, (3) since this strong vascular vasodilator with low molecular weight and lipophilic nature spreads easily through the cell membranes crossing the vascular intima until reaching the smooth muscle tissue of the arterial wall where soluble guanylate cyclase (GC) is activated and it increases the levels of cyclic guanosine monophosphate (cGMP), which is a mediator of its physiological effects, and it causes the relaxation of vascular smooth muscle cells (VSMC). (4) These events help the endothelium to restrict the uncontrolled entrance of low-density lipoproteins (LDL) and inhibit the adhesion, migration and accumulation of monocytes and T lymphocytes in the subendothelial space. All this would induce an alteration in the endothelial homeostatic equilibrium and consequently would cause endothelial dysfunction. (5, 6) However, in oxidative stress conditions, NO and L-citrulline synthesis would follow a physiopathological pathway, due to eNOS dissociation, in subclinical stages and even when there are no structural damages on the wall. That is why they could be used as oxidative stress markers in subclinical atherosclerosis. (7)

On the other hand, there are cellular antioxidant defense mechanisms that are capable of neutralizing the levels of reactive oxigen species (ROS) and reactive nitrogen species (RNS) which preserve NO bioavailability and keep the normal vascular tone; superoxide dimutase (SOD) is a protective antioxidant, whose function consists in the elimination of superoxide ion by dismutation in H2O2 and O2 before reacting with sensitive biological molecules or originating other toxic agents. (8, 9)

Epidemiologic studies have shown a decrease in the incidence of ischemic episodes in individuals treated with exogenous antioxidants as vitamin E which belong to a non-enzymatic group of liposoluble vitamins whose greater biological activity is ideal under the isomeric expression known as α-Tocopherol. (10-12) Vitamin E works as a biological antioxidant which prevents the oxidation of polyunsaturated fatty acids in cell membranes; due to its molecular structure, vitamin E works as a liposoluble molecule capable of fixing free radicals (O2- and OH) and as it has a phenolic hydroxyl in the chroman ring responsible for the antioxidant reduction, it interrupts the chain reactions with free radicals and as a consequence oxidative stress decreases. (13-15)

In this way, vitamin E could be effective in the inhibition of initial phases in subclinical atherosclerosis. Taking into account that the natural history of atherosclerosis begins with the first long asymptomatic phase followed by a clinical phase, there is no doubt that if the early development of lesions is prevented, the incidence of clinical events would be significantly reduced.

The antioxidant effect of vitamin E, over oxidative stress due to hyperfibrinogenemia in an experimental model of atherogenesis and the possible normalization of oxidative stress markers to avoid the establishment of the first atherogenic lesions, was studied; the possible partial or total reversion of vascular wall pathology was evaluated.

**MATERIAL AND METHODS**

**Animals**
36 male inbred rats, Wistar strain, were used at the Physiology Institute of the Faculty of Medical Sciences of the NUC, with an average weight of about 280 ± 20 g, they were fed with a balanced diet for rats with a minimum of proteins (17%). The research was carried out according to the guide for care and use of laboratory animals of the National Institutes of Health, publication NIH (n° 85-23, revised 1996).

**Study groups**
Three groups made up of 12 rats each were studied sequentially and they were classified into different experimental situations.
- **Group A**: control (with no hyperfibrinogenemia induction).
- **Group B**: induced hyperfibrinogenemia for 90 days (HF x 90 days).
- **Group C**: induced hyperfibrinogenemia for 90 days + treatment with vitamin E for 75 days (HF x 90 days + vit. E).

**Induction of the inflammatory process**
Deaths or animal waste were not reported in none of the studied batches. Taking into account previous works of our laboratory, (6, 16) hyperfibrinogenemia induction was carried out with a daily subcutaneous injection of adrenaline (0.1 ml per rat) for 90 days.

**Pharmacological treatment**
The pharmacological treatment was performed through the administration of vitamin E, diluted in distilled water, in a daily dose of 2 mg per rat; this dose was equivalent to the one in human beings of 400 mg of vitamin E per day. This dose was recommended for supplementation therapies. (11, 17, 18). The treatment began the day 15 from the first HF induction and for a period of 75 consecutive days. The administration was carried out orally with a syringe of 1 ml adapted with a catheter in its end which allowed us to put the right dose of vitamin E at the esophageal level to avoid regurgitation.

**Preparation of experimental material**

**Plasma procurement**
Animals were sacrificed and 72 hrs after the last HF induction, which coincided with the day 90 of induction, blood was obtained. This blood was collected in Petri dishes with a mixture of anticoagulant constituted by ammonium and potassium oxalate in a proportion of 2:1. For the determination of SOD activity, EDTA was used as an anticoagulant; subsequently the blood was centrifuged at 3000 rpm for 15 minutes to obtain plasma and red blood cell lysate, respectively.

**Material procurement for anatomical pathology through optical microscopy**
In all the studied groups, sections of thoracic aorta from its origin to its last portion were selected. Due to that, in experimental models in rats, lesions appear preferably in this part of the aorta, in contrast with human beings in which lesions appear in abdominal aorta. 30 sections of 4 µm each section, per animal selected by single-blind experiment were carried out. The processed material for anatomical pathology was preserved in buffered formal (10%) and it was stained with hematoxylin & eosin (HE) and it was analyzed...
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by optical microscopy (OM).

**Processing of biological material**

I. **Plasma fibrinogen**: its concentration was determined by spectrophotometry according to Ratnoff and Menzie method (19) and the results are expressed in mg/dl.

II. **Nitric oxide (NO)**: it was determined by Griess test through spectrophotometry and the results are expressed in uM.

III. **L-citrulline**: it was quantified by spectrophotometry and the results are expressed in Mm. (21)

IV. **SOD**: its activity was determined by spectrophotometry in red blood cell lysate, a Randox kit was used and the results are expressed in U/ml. (22)

**Statistical analysis**

For the analysis of the results of independent variables (groups of studied animals) as regards covariates (fibrinogen, NO, L-citrulline and superoxide dimutase) Infostat program was used; normality and homogeneity tests were performed with Shapiro-Wilk test and then they were studied with MANOVA. Hotelling was the post hoc test used. For the quantification of anatomopathological lesions, Axiovision 4.8 program was used; fields of pictures with a magnification 400< obtained from different studied sections in all groups were analyzed with Fisher’s exact test for qualitative variables. A significance level of p < 0.05 was established for all cases.

**RESULTS**

The results of the variations of plasma fibrinogen, NO and L-citrulline and the SOD enzymatic activity are shown in table 1.

A significant increase of plasma fibrinogen in group B (induced atherogenesis for 90 days) when it was compared to group A (control) (p < 0.001) was observed. Group C (HF x 90 days + vit E) showed a decrease in hyperfibrinogenemia with regard to group B ((HF x 90 days) (p < 0.001) and it had a similar concentration as in group A (control), in that way, the administration of the vitamin stabilized fibrinogen concentrations.

In group B (HF x 90 days) a significant decrease of NO regarding group A (control) (p < 0.001) was observed. In group C (HF x 90 days + vit E), the level of plasma NO was increased more than in group A (control) with regard to the one that showed a significant difference (p < 0.001) and also the difference with the group of non treated animals (B) (p < 0.001) was statistically significant. As far as L-citrulline plasma variations, this was increased in group B (HF x 90 days) with regard to group A (control) (p < 0.001). Group C (HF x 90 days + vit E) showed an increased in L-citrulline plasma concentrations with regard to group A (control) (p < 0.001) and even against group B (HF x 90 days) (p < 0.001).

SOD activity increased in the group with atherogenesis induced by HF for 90 days (B) with regard to non treated healthy animals (A) (p < 0.001). There was a similar behavior in the group treated with vitamin E which showed a significant increased of SOD activity against group A (control) and even in animals with atherogenesis induced by HF x 90 days (p < 0.001).

In histopathological studies, there were no changes in the control group (Figure 1); 100% of the sections showed indemnity in different layers of the aortic wall. 300 anatomopathological sections in group B, with hyperfibrinogenemia for 90 days were observed; extensive areas of endothelial denudation, protrusions in the endothelial layer and subendothelial myxoid changes in 295 of 300 studied sections were observed (Figure 2) which represents 98.33% of the total lesions (p < 0.001). The administration of vitamin E in group C (HF x 90 days + vit E) (Figure 3) showed an evolution of anatomopathological changes towards the normality of different layers in 219 (73%) of the 300 studied sections, with a significant difference (p < 0.001) against group B and with no differences with regard to group A.

**DISCUSSION**

In our results, we observed that in this atherogenic model hyperfibrinogenemia reflects and maintains the inflammatory process triggering an increase of the oxidative stress in the vascular microenvironment, situation that decreases the concentrations of NO which changes the vasodilatory reaction as a consequence of endothelial dysfunction and with a probable repercussion on vascular flow. (23) Likewise, in previous works of our laboratory, we observed that in conditions of HF, the levels of TNF-α are increased, indicating the existence of endothelial activation. (6, 24) Considering that the inflammatory component is present since the beginning of the atherogenic process and the resulting oxidative stress is spread and it deteriorates the lesion at vascular level, it is probable that antioxidants as alpha-tocopherol ease the first stages of atherosclerosis.

Vitamin E and its antioxidant effect would stabilize the membranes due to its hydrophobicity, balancing the permeability and decreasing the expression of adhesion molecules in endothelial cells that would be stimulated by proatherogenic triggers as hyperfibrinogenemia; as a result, it would reduce the contact and the inclusion of inflammatory cells, decreasing the inflammatory reactivity and consequently, hyperfibrinogenemia. This could be due to the activation of endothelial cells by proinflammatory cytokines, as TNF-α, is inhibited by alpha-tocopherol. (25) Several experimental studies suggest that therapies with alpha-tocopherol, especially in high doses, would lead to the reduction of the liberation of proinflammatory cytokines, including IL-1β, IL-6, IL-8 and TNF-α, by monocytes through the inhibition of 5-lipoxygenase activity. (11, 26, 27) Other researchers have proposed that vitamin E could control the expression of several genes involved in the inflammatory response and atherogenesis progression, as TNF-α, proteins and adhesion molecules, causing an anti-inflammatory effect. (28)

In this way, the first action target of vitamin E would be the endothelium as a consequence of the selective uptake of HDL by scavenger receptors, which takes part of vitamin E delivery. This one combined with HDL and LDL are carried in plasma through the layer of endothelial cells; then, vitamin E goes into the subendothelial space where it would prevent protein and lipid oxidation caused by oxidative stress, since vitamin E stops the chain reaction which was begun by...
free radicals. Due to that, it is considered an “electron collector” and as a consequence it would decrease the superoxide anion, stimulating an increase in NO bioavailability and, therefore, it would decrease lipid peroxidation which is reflected in the involution of histopathological lesions and in the inhibition of foam cell formation.

Consequently, vitamin E improves the vasomotor response that depends on the endothelium since it is capable of modulating the production of NO which in atherogenesis contributes in the maintenance of normal vascular homeostasis reducing the clinical incidence.

In the same way, L-citrulline behavior was similar to that one of NO, since the treatment with vitamin E for 75 days increased L-citrulline values which indicates that NO is not suffering a secondary inactivation in its physiopathological pathway, but, on the contrary, it would be available in the vascular environment to carry out its physiological and protective functions.

The increase of SOD enzymatic activity over the normal values in animals treated with vitamin E would indicate, apart from its own antioxidant effect, the accentuation of the endogenous antioxidant effect practiced by the SOD.

Probably, in the first instance, SOD activity is increased due to the rise of the substratum as a consequence of the oxidative imbalance and this could be the cause of an increase in the SOD activity in the group with atherogenesis lesions, while the greater increase of its activity in animals treated with vitamin E could be due to, on the one hand, an increase in the synthesis of superoxide dismutase and, on the other hand, to an increase in the enzyme activation.

In accordance with the results in this model, other researchers say that vitamin E would increase SOD activity and mRNA level of SOD in vascular smooth
Papel antioxidante de la vitamina E en la aterogénesis induced by hiperfibrinogenemia

Con el propósito de estudiar el efecto de la vitamina E sobre el estrés oxidativo desencadeado por hiperfibrinogenemia (HF) en un modelo experimental de aterogénesis y la posible normalización de los indicadores de estrés oxidativo, se evaluaron: óxido nítrico (NO), L-citrulina, superóxido dismutasa (SOD) e involución de lesiones histopatológicas en la aorta torácica. El estudio se realizó en 36 ratas, cepa Wistar, que se dividieron en tres grupos (n = 12 cada uno): A, control; B, HF × 90 días; C, HF × 90 días + vitamina E. La HF se indujo mediante inyecciones de adrenalin (0,1 ml/día/rata) por 90 días. La dosis de vitamina E fue de 2 mg/día/rata durante 75 días. Se dosaron en plasma los niveles de fibrinógeno (mg/dl), NO (μM) y L-citrulina (mM) y en lisado de glóbulos rojos, por espectrofotometría, se determinó la actividad de la SOD (U/ml). Se analizaron cortes de la aorta torácica por microscopía óptica (MO). Para el análisis estadístico se emplearon MANOVA y la prueba de Fisher; se estableció un nivel de significación de p < 0,05. Se observó un aumento significativo de fibrinógeno en el grupo B (407 ± 8,9 mg/dl) en comparación con los grupos A (203 ± 9 mg/dl) y C (191,58 ± 17,79 mg/dl) (p < 0,001). NO disminuyó significativamente en el grupo B (13,73 ± 1,76 U/ml) frente a los grupos A (23,58 ± 0,08 U/ml) y C (26,64 ± 3,65 U/ml) (p < 0,001). La L-citrulina aumentó en forma significativa en los grupos B (4,99 ± 0,18 mM) y C (6,60 ± 0,16 mM) en comparación con el grupo A (3,03 ± 0,13 mM) (p < 0,001). La SOD incrementó su actividad en los grupos B (251,67 ± 10,34 U/ml) y C (304,75 ± 10,43 U/ml) frente al grupo A (198,44 ± 4,74 U/ml) (p < 0,001).

La microscopia óptica mostró denudación endotelial, engrosamiento intimal y protrusión de la pared en el grupo B (90%) y recuperación de la denudación endotelial y el engrosamiento intimal en el grupo C (p < 0,001). Niveles aumentados de SOD serían insuficientes para impedir alteraciones en la vía del estrés oxidativo inducido por la HF. La vitamina E actuaría deteniendo la reacción en cadena iniciada por los radicales libres y en consecuencia disminuiría el anión superóxido, estimulando de esta manera un incremento en la biodisponibilidad del NO y normalizando las concentraciones de fibrinógeno plasmático.