Effect of vitamin E and iron on apoptosis of the endothelial cells of the carotid arteries in hypercholesterolemic male rabbits

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**Abstract:**

**Background:**

Cardiovascular disease is the main cause of mortality and morbidity in developed countries causing atherosclerosis plaques and thrombosis. Endothelial cell apoptosis is one of the main factors in vascular thrombosis. Lipids oxidized by endothelial cells lead to an increase thickness of the arterial wall. Iron is also known as an atherogenic element that causes atherosclerosis. As there is still doubt about the antioxidative role of vitamin E in atherosclerosis formation. In this study we evaluated the effect of iron and vitamin E on endothelial cell apoptosis of the carotid arteries in hyhypercholesterolemic male rabbits.

**Material and Methods:**

Thirty white male rabbits were randomly divided into 5 groups, and fed the following diet for 6 weeks: Group 1: control, Group 2: cholesterol (1%), group 3: cholesterol (1%) + vitamin E (50 mg/kg), group 4: cholesterol (1%) + Iron (50 mg/kg) and group 5: cholesterol (1%) + vitamin E (50 mg/kg) + Iron (50 mg/kg). Serum cholesterol, TG, HDL, and LDL levels were assessed after 6 weeks. Finally, the animals were sacrificed with ketamine and carotid arteries removed. The samples were fixed in 10% formalin and TUNEL staining was used after the tissue processing. Cell count were carried out under a light microscope.

**Results:**

Vitamin E decreased Serum cholesterol and apoptotic endothelial cells in hypercholesterolemic+ vitamin E diet (P< 0/05). However, they increased significantly in the interference groups in comparison to the control group (P< 0/05).

**Conclusion:**

According to our findings, vitamin E showed to have a beneficial effect on preventing cardiovascular diseases and may play a positive role in the prevention of atherosclerosis.

**Key Words**: Apoptosis, Endothelial cell, Atherosclerosis, Iron, Vitamin E, Carotid Artery.

**Introduction**

Cardiovascular disease is the leading cause of death in developed countries associated with atherosclerosis and thrombosis (1). Atherosclerosis plays a key role in the pathogenesis of several disorders including cardiovascular risk factors. It is not a degenerative process; but it is an active process that begins as lipids deposition in the intima of the arteries(2,3).Lipid retention, apoptosis of vascular smooth muscle cells (VSMCs), endothelial dysfunction, and fibrosis are involved in atherosclerosis progression by causing plaque destabilization and rupture (3,4). However, the mechanisms of apoptosis induction are not yet fully understood but it has been shown Oxidized Low Density lipoproteins (Ox-LDL) induce apoptosis in vascular endothelial cells that are normally resistant to the Fas-ligand-dependent mechanism (5,6). Following primary endothelial cell damage, inflammation and lipid accumulation initiates by monocytes-fibronectin adhesion cause oxidative level alteration and accelerate apoptosis( 6,7). Reactive oxygen species (ROS) can commence lipid peroxidation leading to activation of unmodified LDL to ox-LDL. This reaction requires multiple enzyme systems like nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, mitochondrial electron transport enzymes, cytochrome P450 enzymes and uncoupled endothelial nitric oxide synthetase (eNOS)( 8).The Ox-LDL induced pro-oxidant state from beginning to acute thrombotic events by disruption of fibrous cap via release of MMPs and formation of platelet clot in narrowed arteries(9).

Vitamin E, a lipid-soluble and chain-breaking antioxidant can decelerate LDL oxidation, prevent platelet adhesion and aggregation, attenuate the synthesis of leukotrienes and increase the release of prostacyclin by regulating the expression of cyclooxygenase and cytosolic phospholipase A2 ( 10,11). Hence, these functions of vitamin E explain its protection against the progress of atherosclerosis. Many studies have reported the preventive aspects of vitamin E (a-tocopherol) supplementation against atherosclerosis, but a secure correlation is yet insufficiently explored.

Iron, a major oxidant can play an important role in atherosclerosis by catalyzing the formation of highly reactive radicals such as hydroxyl radical from superoxide and hydrogen peroxide. It promotes the proliferation of smooth muscle cells, decreased levels of plasma antioxidants and effect on circulating lipid levels (12).Although these studies suggest the role of oxidized activated iron in atherosclerosis pathogenesis, the mechanism of such involvement has not yet been elucidated.

In this study, the effect of iron and vitamin E as two separate and combined factors on endothelial cells apoptosis was assessed in the carotid arteries of male rabbits fed with high cholesterol-vitamin E- iron diet.

**Material and method**

**Animals**

In this prospective, experimental study 30 white male rabbits randomly divided in 5 following groups (n=6): Group 1: normal diet, Group 2: High Cholesterol Diet (HCD,1%), group 3: HCD+ vitamin E (50 mg/kg) , group 4: HCD+ Iron (50 mg/kg) and group 5: HCD + vitamin E (50 mg/kg) + Iron (50 mg/kg)(11, 12). It is noticed that atherogenesis induced by oral administration of 1gr Cholesterol/ 4 cc oil/ 100 gr rat diet. Serum cholesterol, TG, HDL and LDL levels were assessed after 6 weeks then animals were sacrificed with ketamine (75 mg / kg). Carotid arteries were removed and prepared for tissue sectioning.

**TUNEL Staining**

Recognition of genomic DNA Segmentation using terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling (TUNEL assay) is common during apoptosis (13). For this study, diagnostic kits of Roch Co. were used to detect apoptotic cells based on kit instructions. Sections (3 μm) of fixed and dewaxed tissues after treatment with proteinase k incubated at 37 ° C for 30 min. Then followed by one wash of PBS (25 ° C) and placed in Tris-HCl (0.1 M) for 30 min at room temperature and rinse with PBS again. The reaction compound of TUNEL was added and then incubated in a wet chamber for 60 min at 37°C. Sections were rinsed and diaminobenzidine (DAB) added for 15-30 min at room temperature. Finally, they were rinsed with water and stained using hematoxylin prepared to be studied by an optic microscope. Images were taken using Motic Image software and used for cell counting.

**Statistical Analysis**

Statistical evaluation was conducted with SPSS version 23 and results were expressed as mean ± S.E. The paired t-test was used to analyze and compare the mean of biochemical and histological factors before and after the test in the groups. The one-way analysis of variance (ANOVA) and Tukey test was used for the analysis of mean change of variables between the groups. The *P*<0.05 was considered a statistically significant difference.

**Results**

**Serum Levels of Cholesterol, LDL, HDL and TG**

Statistical analysis of serum level of cholesterol, TG, LDL, and HDL showed an equal amount of these factors before diet administration. However, some changes after receiving high cholesterol and vitamin E and Fe in different diets for 6 weeks were observed in most groups.

There was a significant increase in serum levels of cholesterol in all interference groups. A significant difference in serum level of cholesterol between groups 2 and 3 was seen after treatment. Serum level of cholesterol in group 3 that used Vitamin E decreased compared to group 2 or HCD(P<0.05) (**Figure** 1).

Serum level of LDL increased in all interference groups after 6 weeks (P <0.05) but there was no significant difference between trial groups (Figure2). The amount of HDL increased in groups 2, 3 and 4 after the treatment (P <0.05). While the serum level of HDL in group 4 was significantly lower than in groups 2 and 3 (P <0.05) (Figure3).

Serum triglyceride (TG) didn’t show any significant changes except in group 4, which decreased significantly after 6 weeks (P <0.05), but were no significant differences was observed between trial groups (Figure4).

**Apoptotic endothelial cells**

After TUNEL staining the number of apoptotic cells was counted in sections per five hundred endothelial cells. The number of apoptotic cells increased significantly in all hypercholesterolemic rabbits regardless of other supplements, while in group 3 that received Vitamin E supplement it was significantly less than in other trial groups (Figures5-&6) (P <0.05).

**Discussion**

Previous research has shown that use of the antioxidant compounds can reduce Ox-LDL one of atherosclerosis risk factors (14). Endothelial cell damage can be responsible for thrombosis, atherosclerosis, and vascular lesions. On the other hand, endothelial cell apoptosis is known as one of the main causes of thrombosis and embolism, especially in carotid arteries, which causes stroke (15).However, several studies have shown the effect of cholesterol on apoptosis (16). According to the present results, apoptosis was significantly increased in hypercholesterolemic groups.

Epidemiological and experimental studies indicate that antioxidants like vitamin E can cause a significant decrease in the serum cholesterol, TG, and LDL levels and prevention of cardiovascular disease like atherosclerosis(12,17).In previous studies, using vitamin E deficient animals, a-tocopherol diet was found to be clearly correlated with reduction of atherosclerosis(17).Vitamin E prevents apoptosis in rat kidneys by suppressing iron peroxidation and decreasing oxidative stress (18). However, clinical trials have not proven this (19) but the current study, provide evidence with respect to the potential efficacy of vitamin E effect on apoptosis.

The exact mechanism of LDL oxidation is also unknown, but metal ions such as iron promote lipid peroxidation by producing highly toxic hydroxyl radicals (20). In vitro studies demonstrate that iron is required for LDL oxidation in endothelial cells, macrophages and smooth muscle(21). It facilitates atherosclerosis progression by smooth muscle proliferation and plasma antioxidant reduction(22)on the other hand, iron reduction enhances LDL oxidation and induces atherogenesis in the arteries (23). Previous studies, showed that iron influence TG/HDL ratio and provoke coronary atherosclerosis progression (24). Iron dextran injection in (0.3 mg/mice) reduces cholesterol and triglyceride serum without altering liver morphology (25). These findings suggest that lower levels of iron may be a risk factor in female students with high TG serum. Supplementation of iron may be a strategy for the prevention of high TG serum in female students (26). In this study, iron decreased TG and HDL levels and the apoptosis rate in hypercholesterolemic - iron diet group was higher than in other groups, which is in agreement with previous studies.

A study observed that treatment with vitamin E can protect humans against coronary heart disease by reducing plasma lipid peroxide and the thickness of the aortic intima(27).The results of a study on hyperlipidemic mice, revealed that prevention of atherosclerosis by vitamin E diet in severe vitamin E deficiency was found to have independent effect of preventing lipid oxidation in the vessel wall (28). The results of the present findings suggest that vitamin E can inhibit and prevent an increase in serum cholesterol levels in a high-cholesterol diet, as the first step of reducing the arterial wall lipid deposition and consequently preventing the atherosclerosis. Also using vitamin E reduced endothelial apoptosis in hypercholesterolemic rabbits compared to those using iron supplement and may play a positive role in the prevention of atherosclerosis. With regard to the result of the current study, future clinical investigations can be considered to substantiate these effects and explore possible mechanisms of action.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest.

**DATA AVAILABILITY STATEMENT**

Data are available on request from the authors.

**Authors’ contributions**

GRD and SMJH designed the experiments. SMJH performed all the experiments. MM and BR helped to complete the experiments. ANF and NSH analyzed the data. SMJH, ANF and GRD wrote the manuscript. All authors read and approved the final manuscript.

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**Figure1:** Mean± SE of serum cholesterol levels following high-cholesterol diet

\*Significant difference between before and after and also compare to control group (P <0.05).

\*\*Significant difference between the mean changes of two groups (P <0.05).

**Figure2:** Mean± SE of LDL levels following high-cholesterol diet

\*Significant difference between before and after and also compare to control group (P <0.05).

**Figure3:** Mean ±SE of HDL levels following high-cholesterol diet

\*Significant difference between before and after and also compare to control group (P <0.05).

\*\*Significant difference between the mean changes of two groups (P <0.05).

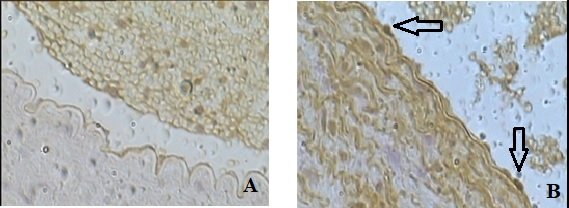
**Figure4:** Mean± SE of TG levels following high-cholesterol diet

\*Significant difference between before and after and also compare to control group (P <0.05).

**Figure5:** Mean ±SE of number of apoptotic endothelial cells following high-cholesterol diet

\*Significant difference in compare to control group (P <0.05).

\*\*Significant difference in comparison to other trial groups (P <0.05).



**Figure6:** Light microscopy image of TUNEL staining. A: Control group (X400). B: Nucleus of apoptotic endothelial cells in group 4 (X400).