

## Effect of Vitamin E on Apoptosis of the Endothelial Cells of the Carotid Arteries in Hypercholesterolemic Male Rabbits

Seyed Mohammad Jafar Haeri<sup>(1)</sup>, Gholamreza Dashti<sup>(2)</sup>, Mohammad Mardani<sup>(2)</sup>, Bahman Rashidi<sup>(2)</sup>, Amene Nikgoftar Fathi<sup>(3)</sup>, Narges-al-sadat Haeri<sup>(4)</sup>

### Original Article

#### Abstract

**INTRODUCTION:** Cardiovascular disease is the principal cause of mortality and morbidity in developed countries, leading to the formation of atherosclerosis plaques and thrombosis. Apoptosis of endothelial cells is one of the primary factors in vascular thrombosis. Lipids, when oxidized by endothelial cells, result in an increased thickness of the arterial wall. Iron is also recognized as an atherogenic element that induces atherosclerosis. There remains uncertainty about the antioxidative role of vitamin E in the formation of atherosclerosis. In this study, the authors evaluated the effect of iron and vitamin E on the apoptosis of endothelial cells in the carotid arteries of hypercholesterolemic male rabbits.

**METHOD:** Thirty white male rabbits were randomly divided into five groups and fed the following diet for six 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 six weeks. Finally, the animals were sacrificed with ketamine, and carotid arteries were removed. The samples were fixed in 10% formalin, and TUNEL staining was used after the tissue processing. Cell counts were carried out under a light microscope.

**RESULTS:** Vitamin E decreased Serum cholesterol and apoptotic endothelial cells in the hypercholesterolemic + vitamin E diet ( $P < 0.05$ ). However, they increased significantly in the interference groups compared 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.

**Keywords:** Apoptosis, Endothelial cell, Atherosclerosis, Iron, Vitamin E, Carotid Artery

*Date of submission: 2022-Jun-19, Date of acceptance: 2022-Nov-20*

#### Introduction

Cardiovascular disease is the leading cause of mortality in developed countries, associated with atherosclerosis and thrombosis<sup>1</sup>. Atherosclerosis plays a pivotal role in the pathogenesis of several disorders, including

cardiovascular risk factors. It is not a degenerative process, but an active one that begins with lipid deposition in the intima of the arteries<sup>2,3</sup>. Lipid retention, apoptosis of vascular smooth muscle cells (VSMCs), endothelial dysfunction, and fibrosis contribute to the progression of atherosclerosis by causing plaque destabilization

1- Department of Anatomy, Medical School, Arak University of Medical Sciences, Arak, Iran

2- Department of Anatomy, School of medicine, Isfahan University of Medical Sciences, Isfahan, Iran

3- Department of Anatomical Sciences, Medical School, Arak University of Medical Sciences, Arak, Iran

4- Department of Physiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

**Address for correspondence:** Gholamreza Dashti, Department of Anatomy, School of medicine, Isfahan University of Medical Sciences, Isfahan, Iran. E-mail address: [dashti@med.mui.ac.ir](mailto:dashti@med.mui.ac.ir)

and rupture<sup>3,4</sup>. Although the mechanisms of apoptosis induction are not yet fully understood, it has been shown that Oxidized Low-Density Lipoproteins (Ox-LDL) induce apoptosis in vascular endothelial cells that are normally resistant to the Fas-ligand-dependent mechanism<sup>5,6</sup>. Following primary endothelial cell damage, inflammation and lipid accumulation are initiated by monocytes-fibronectin adhesion, causing oxidative level alteration and accelerating apoptosis<sup>6,7</sup>. Reactive oxygen species (ROS) can initiate lipid peroxidation, leading to the activation of unmodified LDL to Ox-LDL. This reaction requires multiple enzyme systems, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, mitochondrial electron transport enzymes, cytochrome P450 enzymes, and uncoupled endothelial nitric oxide synthetase (eNOS)<sup>8</sup>. The Ox-LDL-induced pro-oxidant state leads from the beginning to acute thrombotic events by disrupting the fibrous cap via the release of MMPs and the formation of a platelet clot in narrowed arteries<sup>9</sup>.

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<sup>10,11</sup>. Hence, these functions of vitamin E explain its protective role against the progression of atherosclerosis. Many studies have reported the preventive aspects of vitamin E (α-tocopherol) supplementation against atherosclerosis, but a secure correlation is yet insufficiently explored.

Iron, a significant oxidant, can play an essential role in atherosclerosis by catalyzing the formation of highly reactive radicals such as the hydroxyl radical from superoxide and hydrogen peroxide. It promotes the proliferation of smooth muscle cells, decreases levels of plasma antioxidants, and affects circulating lipid levels<sup>12</sup>. 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 authors assessed the effect of iron and vitamin E as two separate and

combined factors on endothelial cell apoptosis in the carotid arteries of male rabbits fed with a high cholesterol-vitamin E-iron diet.

## Materials and Methods

### *Animals*

In this prospective, experimental study, 30 white male rabbits were randomly divided into five 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)<sup>11,12</sup>. It is noted that atherogenesis was induced by oral administration of 1gr Cholesterol/ 4 cc oil/ 100 gr rat diet. Serum cholesterol, TG, HDL, and LDL levels were assessed after six weeks, and then the 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<sup>13</sup>. For this study, diagnostic kits from Roch Co. were used to detect apoptotic cells based on kit instructions. Sections (3 μm) of fixed and dewaxed tissues were treated with proteinase k and incubated at 37 °C for 30 min. This was followed by one wash of PBS (25 °C) and placement in Tris-HCl (0.1 M) for 30 min at room temperature, and then rinsed 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) was 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.

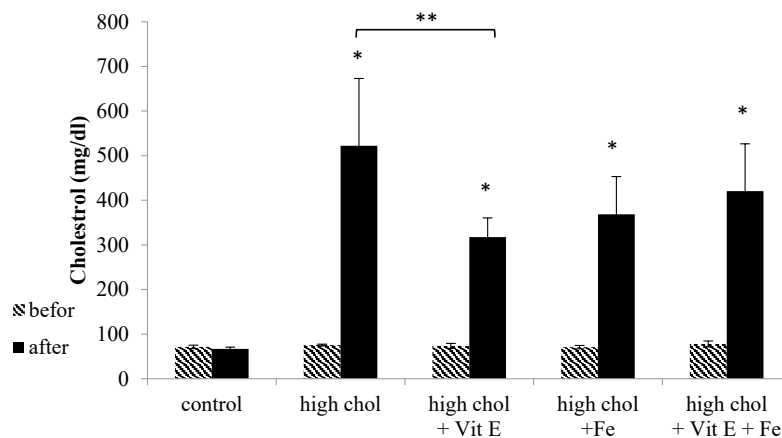
## Results

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

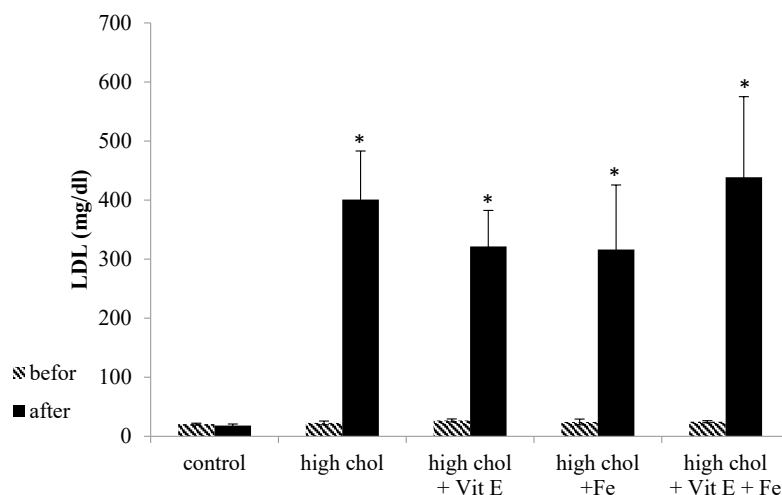
Statistical analysis of serum levels of cholesterol, TG, LDL, and HDL showed an

equal amount of these factors before diet administration. However, some changes were observed in most groups after receiving high cholesterol and vitamin E and Fe in different diets for six weeks.

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. The serum level of cholesterol in group 3 that used Vitamin E decreased compared to group 2 or HCD ( $P < 0.05$ ) (Figure 1).



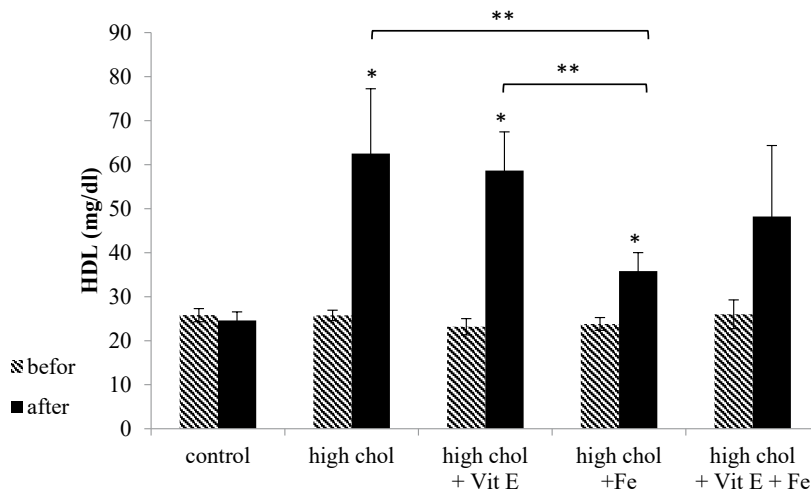
**Figure 1:** Mean  $\pm$  SE of serum cholesterol levels following high-cholesterol diet  
 \*Significant difference between before and after and also compare to control group ( $P < 0.05$ ).  
 \*\*Significant difference between the mean changes of two groups ( $P < 0.05$ ).



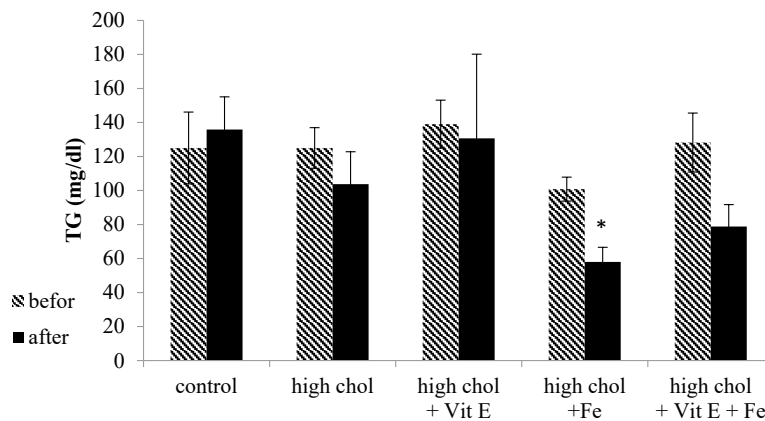
**Figure 2:** Mean  $\pm$  SE of LDL levels following high-cholesterol diet  
 \*Significant difference between before and after and also compare to control group ( $P < 0.05$ ).

The serum level of LDL increased in all interference groups after six weeks ( $P < 0.05$ ), but there was no significant difference between trial groups (Figure 2). The amount of HDL increased in groups 2, 3, and 4 after the treatment ( $P < 0.05$ ). However, the serum level of HDL in group 4 was significantly lower

than in groups 2 and 3 ( $P < 0.05$ ) (Figure 3). Serum triglyceride (TG) did not show any significant changes except in group 4, which significantly decreased after six weeks ( $P < 0.05$ ). There were no differences between trial groups (Figure 4).



**Figure 3:** Mean  $\pm$  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 ( $P < 0.05$ ).

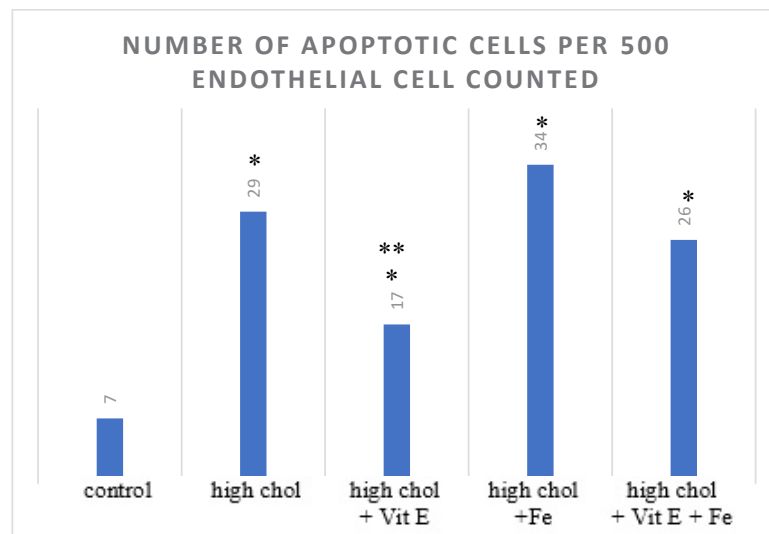


**Figure 4:** Mean  $\pm$  SE of TG levels following high-cholesterol diet  
 \*Significant difference between before and after and also compare to control group ( $P < 0.05$ ).

### 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

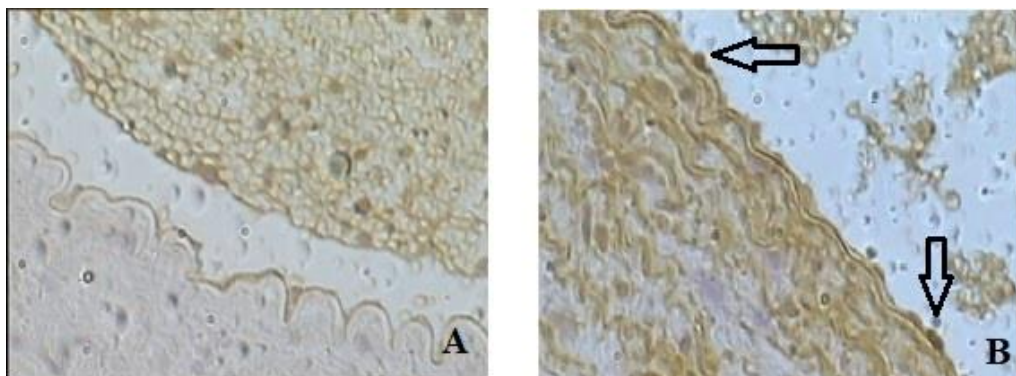
all hypercholesterolemic rabbits regardless of other supplements, while in group 3 that received Vitamin E supplement, it was significantly less than in other trial groups (Figures 5 and 6) ( $P < 0.05$ ).



**Figure 5:** Mean  $\pm$ 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 ( $P < 0.05$ ).



**Figure 6:** Light microscopy image of TUNEL staining. A: Control group (X400). B: Nucleus of apoptotic endothelial cells in group 4 (X400).

## Discussion

Previous research has demonstrated that the use of antioxidant compounds can reduce Ox-LDL, one of the risk factors for atherosclerosis<sup>14</sup>. Damage to endothelial cells can be responsible for thrombosis, atherosclerosis, and vascular lesions. On the other hand, apoptosis of endothelial cells is known as one of the main causes of thrombosis and embolism, especially in carotid arteries, which can lead to stroke<sup>15</sup>. However, several studies have shown the effect of cholesterol on apoptosis<sup>16</sup>. 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 serum cholesterol, TG, and LDL levels and prevent cardiovascular diseases like atherosclerosis<sup>12,17</sup>. In previous studies using vitamin E deficient animals, a diet with  $\alpha$ -tocopherol was found to be clearly correlated with a reduction of atherosclerosis<sup>17</sup>. Vitamin E prevents apoptosis in rat kidneys by suppressing iron peroxidation and decreasing oxidative stress<sup>18</sup>. However, clinical trials have not proven this<sup>19</sup>, but the current study provides evidence with respect to the potential efficacy of vitamin E's 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<sup>20</sup>. In vitro studies demonstrate that iron is required for LDL oxidation in endothelial cells, macrophages, and smooth muscle<sup>21</sup>. It facilitates atherosclerosis progression by smooth muscle proliferation and plasma antioxidant reduction<sup>22</sup>. On the other hand, iron reduction enhances LDL oxidation and induces atherogenesis in the arteries<sup>23</sup>. Previous studies showed that iron influences the TG/HDL ratio and provokes coronary atherosclerosis progression<sup>25</sup>. Iron dextran injection in (0.3 mg/mice) reduces cholesterol and triglyceride serum without altering liver morphology<sup>25</sup>. 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<sup>26</sup>. In this study, iron decreased TG and HDL levels, and the apoptosis rate in the 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<sup>27</sup>. The results of a study on hyperlipidemic mice revealed that the prevention of atherosclerosis by a vitamin E diet in severe vitamin E deficiency was found to have an independent effect of preventing lipid oxidation in the vessel wall<sup>28</sup>. 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 atherosclerosis. Also, using vitamin E reduced endothelial apoptosis in hypercholesterolemic rabbits compared to those using an iron supplement. 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.

### Acknowledgments

The authors would like to thank and express

their gratitude to Isfahan University of medical sciences for their financial support and help to conduct it.

### Conflict of Interest

The authors declare that they have no conflicts of interest.

### Data Availability Statement

Data are available on request from the authors.

### References

1. Kasper DL, Fauci AS, Hauser SL, Longo DL, Jameson JL, Loscalzo J. Harrison's Principles of Internal Medicine. 19<sup>th</sup> ed. New York: McGraw Hill Professional; 2022.
2. Hajhashemi V, Dashti GR, Saberi S, Malekjamshidi P. The effect of hydroalcoholic extract and essential oil of *Heracleum persicum* on lipid profile in cholesterol-fed rabbits. *Avicenna J Phytomed* 2014; 4(3): 144-150.
3. Dashti G, Behdadipour Z, Vahedi P, Pestechian N. Effect of ascorbic acid on the right and left coronary arteries of male rabbits fed with high cholesterol diet. *Arya Atherosclerosis J* 2007; 2(4): 180-182.
4. Unal O, Ulukan MO, Bakuy V, Kaytaz B, Artan S, Aral E, et al., Comparison of the apoptotic effects of topically applied papaverine, diltiazem, and nitroprusside to internal thoracic artery. *Braz J Cardiovasc Surg* 2020; 35: 626-633. <https://doi.org/10.21470/1678-9741-2019-0251>
5. Zibaenejad F, Mohammadi SS, Sayadi M, Safari F, Zibaenezhad MJ. Ten-year atherosclerosis cardiovascular disease (ASCVD) risk score and its components among an Iranian population: a cohort-based cross-sectional study. *BMC Cardiovasc Disord* 2022; 22(1): 1-8. <https://doi.org/10.1186/s12872-022-02601-0>
6. Melaku LS, Debela N. Molecular description of fibroblast growth factor and mechanisms by which fibroblast growth factor-21 mediating biological actions and acting as a biologic biomarker of cardiovascular diseases. *Indian J Health Sci Biomed Res (KLEU)* 2020; 13(2): 68. [https://doi.org/10.4103/kleuhsj.kleuhsj\\_92\\_20](https://doi.org/10.4103/kleuhsj.kleuhsj_92_20)
7. Begum J, Weaver SRC, Iqbal AJ, Rainger GE, McGettrick HM. Cell migration in cardiovascular



- diseases. In: *Cell Movement in Health and Disease*. 1st ed. Academic Press (Elsevier); 2022. p. 159-175. <https://doi.org/10.1016/B978-0-323-90195-6.00008-5>
8. Cho KH. Impairment of HDL by Pollutants. In: *High-Density Lipoproteins as Biomarkers and Therapeutic Tools*. Springer, Singapore; 2019. p. 213-285. [https://doi.org/10.1007/978-981-13-7387-9\\_4](https://doi.org/10.1007/978-981-13-7387-9_4)
  9. Obama T, Ohinata H, Takaki T, Iwamoto S, Sawada N, Aiuchi T, et al., Cooperative action of oxidized low-density lipoproteins and neutrophils on endothelial inflammatory responses through neutrophil extracellular trap formation. *Front Immunol* 2019; 10: 1899. <https://doi.org/10.3389/fimmu.2019.01899>
  10. Saqib U, Khan MA, Alagumuthu M, Parihar SP, Baig MS. Natural compounds as antiatherogenic agents. *Cell Mol Biol* 2021; 67(1): 177-188. <https://doi.org/10.14715/cmb/2021.67.1.27>
  11. Bachschmid MM, Schildknecht S, Matsui R, Zee R, Haeussler D, Cohen RA, et al. Vascular aging: chronic oxidative stress and impairment of redox signaling-consequences for vascular homeostasis and disease. *Ann Med* 2013; 45(1): 17-36. <https://doi.org/10.3109/07853890.2011.645498>
  12. Dashti G, Poormoghadas M, Sanei M, Hoseini M, Ghayoomi S. Effect of Ferrous and Vitamin E on Rabbit Serum Lipoproteins. *J Isfahan Med Sch* 2011; 28(118): 1275-1280.
  13. Reisi P, Dashti GR, Shabrang M, Rashidi B. The effect of vitamin E on neuronal apoptosis in hippocampal dentate gyrus in rabbits fed with high-cholesterol diets. *Adv Biomed Res* 2014; 3: 42. <https://doi.org/10.4103/2277-9175.125731>
  14. Rafiee L, Keshvari M, Movahedian Atar A, Hamidzadeh Z, Dashti GR, Rafieian-Kopaei M, et al. Effect of *Gundelia tournefortii* L. on some cardiovascular risk factors in an animal model. *J Herbmed Pharmacol* 2017; 6(4): 191-195.
  15. Zille M, Ikhsan M, Jiang Y, Lampe J, Wenzel J, Schwaninger M. The impact of endothelial cell death in the brain and its role after stroke: a systematic review. *Cell Stress* 2019; 3(11): 330. <https://doi.org/10.15698/cst2019.11.203>
  16. Zmyslowski A, Sztark A. Current knowledge on the mechanism of atherosclerosis and pro-atherosclerotic properties of oxysterols. *Lipids Health Dis* 2017; 16(1): 1-19. <https://doi.org/10.1186/s12944-017-0579-2>
  17. Dutta A, Dutta SK. Vitamin E and its role in the prevention of atherosclerosis and carcinogenesis: a review. *J Am Coll Nutr* 2003; 22(4): 258-68. <https://doi.org/10.1080/07315724.2003.10719302>
  18. Bergin P, Leggett A, Cardwell CR, Woodside JV, Thakkinstian A, Maxwell AP, et al., The effects of vitamin E supplementation on malondialdehyde as a biomarker of oxidative stress in haemodialysis patients: a systematic review and meta-analysis. *BMC Nephrol* 2021; 22(1): 1-10. <https://doi.org/10.1186/s12882-021-02328-8>
  19. Goszcz K, Deakin SJ, Duthie GG, Stewart D, Leslie SJ, Megson IL. Antioxidants in cardiovascular therapy: panacea or false hope?. *Front Cardiovasc Med* 2015; 2: 29. <https://doi.org/10.3389/fcvm.2015.00029>
  20. Heinecke JW, Rosen H, Chait A. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J Clin Invest* 1984; 74(5): 1890-4. <https://doi.org/10.1172/JCI111609>
  21. Chistiakov DA, Melnichenko AA, Orekhov AN, Bobryshev YV. How do macrophages sense modified low-density lipoproteins?. *Int J Cardiol* 2017; 230: 232-40. <https://doi.org/10.1016/j.ijcard.2016.12.164>
  22. Pari L, Karthikeyan A, Karthika P, Rathinam A. Protective effects of hesperidin on oxidative stress, dyslipidaemia and histological changes in iron-induced hepatic and renal toxicity in rats. *Toxicol Rep* 2015; 2: 46-55. <https://doi.org/10.1016/j.toxrep.2014.11.003>
  23. Dashti GR, Mardani M, Rashidi B, Haeri MJ. Effects of Iron and Vitamin E on Thickness of Carotid Artery Wall of Hyper Cholesterolemic Rabbits. *Qom Univ Med Sci J* 2012; 5(4): 73-8.
  24. Zohrabi T, Movahedian A, Paknahad Z, Hashemi Jazi SM, Maracy MR. Biochemical Parameters of Iron Metabolism and TG/HDL-C Ratio in Patients with Coronary Artery Disease. *J Isfahan Med Sch* 2011; 29(159): 1680-9.
  25. Setiawati DA, Syamsunarno MRA, Fianza PI, Atik N, Anggraeni N, Ghozali M, et al. Iron Overload Reduces Cholesterol and Triglyceride Serum of Mice. *KnE Life Sci* 2017; 369-77. <https://doi.org/10.18502/cls.v3i6.1145>
  26. He L, Zhang Y, Ru D, Xue B, Wen S, Zhou H. Serum iron levels are negatively correlated with serum triglycerides levels in female university students. *Ann Palliat Med* 2020; 9(2): 414-9. <https://doi.org/10.21037/apm.2020.03.02>
  27. Saboori S, Shab-Bidar S, Speakman J, Rad EY, Djafarian K. Effect of vitamin E supplementation

on serum C-reactive protein level: a meta-analysis of randomized controlled trials. *Eur J Clin Nutr* 2015; 69(8): 867-73. <https://doi.org/10.1038/ejcn.2014.296>  
28. Suarna C, Wu BJ, Choy K, Mori T, Croft K, Cynshi O, et al. Protective effect of vitamin E supplements on

experimental atherosclerosis is modest and depends on preexisting vitamin E deficiency. *Free Radic Biol Med* 2006; 41(5): 722-30. <https://doi.org/10.1016/j.freeradbiomed.2006.05.013>

**How to cite this article:** Jafar Haeri SM, Dashti G, Mardani M, Rashidi B, Nikgoftar Fathi A, Haeri N. **Effect of Vitamin E on Apoptosis of the Endothelial Cells of the Carotid Arteries in Hypercholesteremic Male Rabbits.** *ARYA Atheroscler* 2023; 19(3): 10-17.