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RESEARCH ARTICLE

Anti-atherosclerotic effect of antioxidant and homocysteine lowering therapy in experimental atherosclerosis model

Mustafa Ozgul [1*]

[1] Alanya Training and Research Hospital, Department of Cardiology, Alanya, TÜRKİYE

ABSTRACT

Background: We aimed to investigate the effect of antioxidant and homocysteine (Hcy)-reducing therapy on atherosclerosis in this study. We used folic acid and B12 as Hcy-reducing therapy.

Materials and methods: A total of 32 male Wistar albino rats with an average weight of 200-300 grams were used in this study. After all the animals were sacrificed at the end of 4,5 months, blood samples were collected to analyze SOD, CAT, GLUT, GSH-PX, Hcy, TG, and total cholesterol.

Results: In treated groups, antioxidant enzymes were determined at various levels. The changes of antioxidant enzymes showed that antioxidant vitamins decreased oxidative stress, as well as partly. This finding suggests that antioxidant vitamins might have slowed or perhaps stopped atherogenesis.

Conclusion: Antioxidant vitamins are effective in preventing oxidative stress induced by hypercholesterolemia, but this beneficial effect has not been definitively proven in clinical study. Therefore, we need more primary and secondary prevention studies on this subject.

Keywords: atherosclerosis, oxidative stress, homocysteine

Correspondence:
Mustafa Ozgul, MD

Address: Department of Cardiology, Alanya Training and Research Hospital, Alanya, TÜRKİYE

Email:
drmustafaozgul@hotmail.com

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INTRODUCTION

There have been extremely important developments in the 20th century in atherosclerosis, which has been known since ancient times, and extensive research has been conducted on its pathogenesis and its treatment. However, there are currently many details we do not know about atherosclerosis, and the most common cause of death in developed countries is still cardiovascular diseases caused by atherosclerosis [1, 2].

Homocysteine (Hcy) is increasingly recognized as a risk factor for coronary artery disease [3]. Understanding its metabolism and the importance of vitamins B6 and B12, as well as folate, in regulating enzyme levels will help develop therapeutic strategies that may also reduce risk by reducing circulating concentrations [4-6]. Possible mechanisms by which high Hcy levels lead to the development and progression of vascular disease include effects on platelets, clotting factors, and endothelium [7].

One of the possible mechanisms in research to explain the development of Hcy-induced thrombosis and atherosclerosis is that Hcy may catalyze the oxidation of serum lipids in vivo and cause LDL modification [8]. With the increase in Hcy level, the antithrombotic and fibrinolytic effects of endothelium are impaired, and it acquires prothrombotic properties [9].

Hyperhomocysteinaemia (Hhcy) represents an independent risk factor for atherosclerotic cardiovascular disease, stroke, peripheral arterial occlusive disease, and venous thrombosis [10, 11]. The increased risk of coronary artery disease is not limited to patients with severe Hhcy; the risk is also increased in people with mild to moderately increased serum Hcy levels [12, 13]. The importance of Hcy in vascular function and arteriosclerosis was discovered by demonstration of arteriosclerotic plaques in children with homocystinuria [14, 15].

Table 1. TC and TG values obtained from group I and group II

	Group I (n: 6)		Group II (n: 6)	
	TC (mg/dl)	TG (mg/dl)	TC (mg/dl)	TG (mg/dl)
Mean ± standard deviation	93.00 ± 6.06	81.66 ± 10.80	117.50 ± 9.04	117.50 ± 8.52
Median	92 ♥	79 ♣	118 ♥	117 ♣
Minimum	85	69	107	107
Maximum	101	98	133	129

Note. ♥p = 0.004; ♣p = 0.004; Group I: Fed on normal food; & Group II: Fed with cholesterol-rich feed

Table 2. Antioxidant and MDA values between group I and group II

		MDA (nmol/gr Hb)	CAT (U/gr Hb)	GLUT (mg/gr Hb)	SOD (U/gr Hb)	GSH-PX (U/gr Hb)
Group I	Mean ± standard deviation	124.05 ± 5.20	22.68 ± 4.50	2.72 ± 0.38	4,129.80 ± 258.30	91.63 ± 5.30
	Median	121.78	23.38	2.60	4,071.91	91.96
	Minimum-maximum	119.36-132.96	15.90-27.76	2.35-3.41	3,898.7-4,488.8	84.50-99.16
Group II	Mean ± standard deviation	195.43 ± 9.28	30.24 ± 5.10	2.00 ± 0.25	4,948.50 ± 358.80	106.60 ± 6.40
	Median	194.39	31.06	2.04	4,924.49	105.33
	Minimum-maximum	186.25-210.87	24.16-36.68	1.64-2.37	4,518.00-5,394.10	99.60-118.70
p-value		0.004	0.025	0.006	0.004	0.004

Note. Group I: Fed on normal food & Group II: Fed with cholesterol-rich feed

In atherosclerosis, there is a correlation between the increase in plasma cholesterol and the increase in plasma Hcy level. Micronutrients that facilitate Hcy catabolism reduce plasma Hcy, cholesterol, triglyceride (TG), and LDL levels [16].

In this study, we aimed to examine the effects of B12 and folic acid, which reduce Hcy levels, on oxidative stress and Hcy levels in the atherogenesis process, and therefore on the development of atherosclerosis, in an experimental model.

MATERIALS AND METHODS

A total of 32 male Wistar albino rats with an average weight of 200-300 grams were used in this study. The rats were fed with standard light (12 hours of daylight/12 hours of darkness), temperature (22 ºC), and sufficient water and feed for a total of 4.5 months. This study was carried out in Suleyman Demirel University experimental animals laboratory and biochemistry laboratory, and the study was approved by the institutional ethics committee (ethical approval number: 0953-TU-04).

Group I (placebo group, n: 6): Rats in this group were fed with standard pellet rat chow.

Group II (control group, n: 6): They were fed weekly with a cholesterol feed created by grinding the standard feed and adding 2% cholesterol [17].

Group III (n: 7): 500 microgram/kg intramuscular B12 was administered every day in addition to the cholesterol feed [18].

Group IV (n: 7): In addition to the cholesterol feed, 10 mg/kg folic acid was administered by gavage every day [18].

Group V (n: 6): In addition to the cholesterol feed, 500 microgram/kg intramuscular B12 and 10 mg/kg folic acid were administered by gavage every day [18].

At the end of 4.5 months, after the diet and treatment application, the rats were starved for 1 night and under anesthesia of 10% ketamine and 2% xylazine, they were drained of blood from the beating heart, and then the experiment was terminated by removing heart and the aorta.

Hcy, cholesterol parameters, protein, malondialdehyde (MDA) levels and glutathione (GLUT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activities were studied from all samples obtained.

The collected data were evaluated with the statistical package for social sciences 13.0 program. A non-parametric test was applied to evaluate the findings, and average values were considered. The Mann-Whitney U test was used to compare paired groups with each other. Values below $p < 0.05$ were considered significant.

RESULTS

Total cholesterol (TC) and TG levels were found to be significantly higher in group II, which was fed a cholesterol-rich diet, than in group I, which was fed a normal diet. The differences between TC and TG values obtained in group I and group II are summarized in **Table 1**.

Antioxidants and MDA levels between group II fed a cholesterol-rich diet and group I fed a normal diet are shown in **Table 2**.

Table 3. Hcy values between group I and group II

		Group I: Fed on normal food	Group II: Fed with cholesterol-rich feed	p-value
Hcy (μM/L)	Mean ± standard deviation	12.14 ± 0.93	17.11 ± 0.99	0.004
	Median	12.140	16.765	
	Minimum-maximum	11.05-13.62	15.93-18.43	

Table 4. MDA and antioxidant values and comparison between groups II-group V

		MDA (nmol/gr Hb)	CAT (U/gr Hb)	GLUT (mg/gr Hb)	SOD (U/gr Hb)	GSH-PX (U/gr Hb)
Group II	Mean ± standard deviation	195.40 ± 9.20	30.24 ± 5.10	2.00 ± 0.25	4,948.50 ± 358.80	106.60 ± 6.48
	Median	194.39	31.06	2.04	4,924.49	105.33
	Minimum-maximum	186.20-210.80	24.16-36.68	1.64-2.37	4,518.00-5,394.10	99.64-118.74
Group III	Mean ± standard deviation	155.01 ± 32.00	40.91 ± 5.70	2.61 ± 0.31	4,508.80 ± 752.90	95.92 ± 8.80
	Median	162.25	40.85	2.66	4,608.69	97.05
	Minimum-maximum	98.37-185.60	33.67-50.80	2.08-2.98	3,029.70-5,523.00	81.46-108.23
Group IV	Mean ± standard deviation	198.83 ± 32.00	48.04 ± 11.02	3.50 ± 0.48	7,195.80 ± 1,001.0	101.41 ± 9.70
	Median	198.00	50.86	3.39	6,786.80	99.75
	Minimum-maximum	163.02-260.42	35.63-63.98	3.07-4.46	6,064.50-8,619.80	6,064.50-8,619.80
Group V	Mean ± standard deviation	163.97 ± 20.80	60.11 ± 15.70	3.54 ± 0.80	5,305.20 ± 692.00	130.36 ± 9.90
	Median	162.94	56.59	3.61	5,437.14	129.92
	Minimum-maximum	131.19-189.68	45.19-81.96	2.47-4.34	4,407.60-6,008.80	120.00-144.95
$p\text{-value}_1$		0.003	0.010	0.010	0.199	0.032
$p\text{-value}_2$		0.668	0.007	0.003	0.003	0.391
$p\text{-value}_3$		0.010	0.004	0.004	0.423	0.004

Note. Group II: Fed with cholesterol-rich feed; Group III: Cholesteral feed + B12; Group IV: Cholesterol feed + folic acid; Group V: Cholesterol feed + folic acid + B12; $p\text{-value}_1$: p-value obtained by comparing group II and group III; $p\text{-value}_2$: p-value obtained by comparing group II and group IV; & $p\text{-value}_3$: p-value obtained by comparing group II and group V

MDA, a lipid peroxidation breakdown product, was significantly higher in the group fed with a cholesterol diet. It was determined that all of the anti-oxidant system elements evaluated in the study were significantly different in group II, which was under oxidative stress, that is, fed with a cholesterol-rich diet, compared to group I, which was fed a normal diet.

Hcy values between group II, fed with a cholesterol-rich diet, and group I, fed with a normal diet, are shown in **Table 3**. Hcy level was found to be significantly higher in group II, which was given a cholesterol-rich diet.

Antioxidant and MDA values of group II fed with a cholesterol-rich diet and B12, folic acid, and B12 + folic acid given in addition to the cholesterol-rich diet are shown in **Table 4**.

MDA levels: It was detected significantly less in group III and group V, which received treatment, than in group II. No significant change was detected between the MDA level obtained in group II and group IV MDA level.

CAT levels: It was found to be significantly higher in groups III, IV, and V than in group II.

GLUT levels: While it was lower in group II than in group I, it was found to be higher in all treatment groups compared to group II.

SOD levels: While it was higher in group II than in group I, it was found to be higher in group IV than in group II, and no difference was detected in groups III and V compared to group II.

GSH-PX levels: While they were detected to be higher in group II than in group I, they were found to be higher in group V than in group II and was found to be lower in group III. No difference was detected in GSH-PX values between group IV and group II.

Hcy values between group II-group V and the difference between them are shown in **Table 5**. Hcy levels were found to be significantly lower in groups III, IV, and V, which were given Hcy-lowering treatment, compared to group II.

TC and TG values and differences between groups II, III, IV, and V are summarized in **Table 6**. In all groups receiving treatment, TC and TG levels were found to be significantly lower than in group II.

Table 5. Hcy values between group II-group V

		Group II	Group III	Group IV	Group V
Hcy (μM/L)	Mean ± standard deviation	17. 10 ± 0.99	12.60 ± 0.95	13.98 ± 0.68	13.99 ± 2.10
	Median	16.765	12.660	14.210	13.150
	Minimum-maximum	15.93-18.43	11.46-13.85	12.73-14.59	12.00-17.41
p-value			0.003 ($p\text{-value}_1$)	0.003 ($p\text{-value}_2$)	0.025 ($p\text{-value}_3$)

Note. $p\text{-value}_1$: p-value obtained by comparing group II and group III; $p\text{-value}_2$: p-value obtained by comparing group II and group IV; & $p\text{-value}_3$: p-value obtained by comparing group II and group V

Table 6. TC and TG values and comparison between groups II-group V

		Group II	Group III	Group IV	Group V
TC	Mean ± standard deviation	117.50 ± 9.04	90.71 ± 8.40	85.14 ± 9.80	82.00 ± 10.80
	Median	118	89	89	79
	Minimum-maximum	107-133	82-105	70-97	71-99
TG	Mean ± standard deviation	117.50 ± 8.05	73.80 ± 16.50	64.00 ± 18.60	65.00 ± 24.00
	Median	117	75	59	65.5
	Minimum-maximum	107-129	46-100	42-89	34-92
p-value			0.003 ($p\text{-value}_1$)	0.003 ($p\text{-value}_2$)	0.004 ($p\text{-value}_3$)
			0.003 ($p\text{-value}_4$)	0.003 ($p\text{-value}_5$)	0.004 ($p\text{-value}_6$)

Note. $p\text{-value}_1$: p-value obtained by comparing the TC values of group II and group III; $p\text{-value}_2$: p-value obtained by comparing the TC values of group II and group IV; $p\text{-value}_3$: p-value obtained by comparing the TC values of group II and group V; $p\text{-value}_4$: p-value obtained by comparing the TG values of group II and group III; $p\text{-value}_5$: p-value obtained by comparing the TG values of group II and group IV; & $p\text{-value}_6$: p-value obtained by comparing the TG values of group II and group V

DISCUSSION AND CONCLUSION

Atherosclerosis is a multifactorial disease, and its development shows heterogeneity. Many predisposing factors such as smoking, diabetes, hyperlipidemia, mechanical stress, and inflammation play a role in the development of atherosclerosis. Many studies conducted to date have proven that free radicals have a significant effect on the development of atherosclerosis [19].

Epidemiological evidence unequivocally supports the association between moderate Hhcy and coronary, cerebral, and peripheral atherosclerosis [20]. In other words, Hhcy is associated with an increased risk of stroke, heart attack, and venous thrombosis [21]. Despite some negative prospective studies, Hhcy is an independent risk factor for coronary artery disease. In the Norwegian study, a strong relationship was found between Hcy levels above 9 μmol/L and mortality in coronary artery disease [20].

Experimental studies support Hcy can promote atherogenesis thanks to its toxic effect on the vascular endothelium via oxidative stress. Folic acid is very effective in reducing plasma Hcy levels when used alone or in combination with other B vitamins. Although there is no randomized controlled trial yet showing that folic acid treatment reduces cardiovascular risk, observational studies support that B vitamins reduce cardiovascular risk and carotid atherosclerosis [20]. In our study, we investigated the effect of antioxidant and Hcy-lowering treatment on antioxidant system elements by increasing the formation of free oxygen radicals, which have an important role in the development of atherosclerosis, with a hypercholesterolemic diet. In our study, we found that Hcy levels increased in group II, which was fed a high-cholesterol diet and was not given treatment. Increased Hcy level also contributes to oxidative stress caused by hypercholesterolemia. Also, we determined the level of thiobarbituric acid-reactive substances, which we determined as an indirect indicator of oxidation in the process of hypercholesterolemic atherosclerosis, with MDA measurements. In group II, which was fed a cholesterol-rich diet, serum MDA values were higher than in group I. These findings support that hypercholesterolemia increases the production of reactive oxygen species (ROS) and causes endothelial cell damage that initiates the atherosclerosis process, as suggested by many researchers.

The MDA findings in our study are compatible with the results of the study in [17] about inducing hypercholesterolemia. Similarly, in the study in [22], MDA level was found to be higher in the group fed with a cholesterol diet. In addition to the increase in MDA, which is an indicator of increased ROS production in hypercholesterolemia, changes in antioxidant parameters were also observed. The role of oxidative stress due to hypercholesterolemia in the pathogenesis of atherosclerosis is clear.

Compared to group I, CAT, SOD, and GSH-PX activities were found to be higher and GLUT levels were lower in group II, which was fed a high cholesterol diet. Moreover, it was found an increase in CAT and SOD activities in the rat group in which they induced hypercholesterolemia compared to the control group, and these findings are compatible with our study [22]. Again, similar to our study, the GLUT level was found to be lower than the control group. They attributed the increased SOD activity to the stimulation of SOD, which is normally found at low levels in plasma, by oxidative stress, and observed that SOD is highly inducible under oxidative stress.

In our study, we believe that the SOD, CAT, GSH-PX enzyme activities detected at higher levels in group II than in group I were to compensate for the increased oxidative stress secondary to hypercholesterolemia. The level of non-enzymatic antioxidant GLUT decreases in an environment where lipid peroxidation increases, and it also contributes to the increase of SOD by preventing the destructive effect of superoxide radical on SOD [23]. These findings are fully and partially compatible with many studies on the subject. Various studies on experimental atherosclerosis show different results regarding increases and decreases in antioxidant enzyme activities, which shows us that a hyperlipidemic diet creates different modifications in antioxidant defense mechanisms. The different results may be due to the method of inducing hypercholesterolemia, the amount of cholesterol used, and the degree and duration of oxidative stress. The fact that the level of MDA, an indicator of lipid peroxidation, in group II is significantly higher than in group I helps to explain the increase in the antioxidant enzyme system as a result of compensation against oxidative stress.

In our study, we used folic acid and B12 as Hcy-lowering treatments against the oxidative stress we created. Folic acid can be considered a free radical scavenger. It can protect biological structures against free radical damage at physiological concentrations. Co-administration of folic acid and B12 creates a synergistic effect against oxidative stress [24]. In our study, we aimed to benefit from both the Hcy-lowering effect and antioxidant effect of B12 and folic acid.

In our study, MDA levels in group III, which was given B12 against the oxidative stress caused by hypercholesterolemia and increased Hcy level, and in group V, which was given folic acid along with B12, were found to be significantly lower at the end of the treatment than in group II. However, the MDA level in group IV, which was given only folic acid, was not statistically different from group II. Hcy levels of groups III, IV, and V were found to be significantly lower than group II. Similar to the result we obtained in our study, in the clinical study in [20]. The MDA level did not change compared to the beginning in the group receiving folic acid treatment alone; however, similar to our study, the Hcy level decreased with folic acid treatment compared to the beginning. The fact that no MDA decrease was observed in group IV suggests that although folic acid can suppress lipid peroxidation due to Hcy increase, it cannot suppress lipid peroxidation due to hypercholesterolemia. In our study, CAT levels were higher in groups III, IV, and V compared to group II. While the SOD level did not show any significant change in groups III and V compared to group II, it was found to be higher in group IV. While the GSH-PX level was lower in group III than in II and higher in group V, no significant change was detected in group IV. GLUT levels were significantly higher in groups III, IV, and V than in group II. It was observed that folic acid and B12 treatments affected antioxidant enzymes and GLUT levels differently in different groups. Hcy level was found to be significantly lower in groups III, IV, and V compared to group II.

There are differences between the results of clinical studies on B vitamins. It was investigated the effect of folic acid on endothelial dysfunction in patients with type II diabetes and Hhcy; no significant improvement was found in endothelial dysfunction indicators despite a decrease in Hcy level [25]. The NORVIT (the Norwegian vitamin) study investigated the effect of Hcy-lowering treatment with B vitamins in secondary protection after acute heart attack. There was no decrease in cardiovascular deaths and complications in the groups receiving B vitamin combination or monotherapy. Contrary to expectations, an increase in clinical endpoints was detected in the group receiving combined treatment. Folic acid treatment with or without B6 after a heart attack did not reduce recurrent cardiovascular events or death, therefore, such treatment is not recommended for secondary prevention after a heart attack and coronary stent procedure [26]. In our study, folic acid alone did not reduce the level of MDA, which is an indicator of lipid peroxidation. This result may be the reason for the insufficiency of folic acid in secondary protection.

Briefly, we investigated the effects of folic acid and B12, which are known to have Hcy-lowering and antioxidant effects, on oxidative stress, which is very important in the pathogenesis of atherosclerosis. Studies show that the effects of these vitamins on the antioxidant system vary. So much so that if the given vitamin causes a decrease in the antioxidant system, this finding can be interpreted as the given vitamin combating oxidative stress. Therefore, the reflex increase of the biological antioxidant system is not observed. Again, if the given vitamin causes an increase in the antioxidant system, this can be interpreted as the given vitamin combating oxidative stress and preventing the reflexively increased biological antioxidant system from being consumed by oxidative stress. In our opinion, both interpretations are correct. Many studies conducted on these subjects support this finding. What is important is how these changes in the plasma antioxidant system are reflected in the clinic. Positive and negative results have been obtained in primary and secondary protection studies on the

cardiovascular clinical benefits of vitamins. Since the groups in clinical studies are not homogeneous with each other, it would not be correct to say that vitamins do not have beneficial effects on cardiovascular disease as a result of these studies.

More randomized controlled clinical studies are needed on the effects of vitamins on real-life data on whether they prevent atherosclerosis. Although this experimentally planned study can provide an idea about real-life data, the small number of rats in the groups is an important limitation of the study.

In conclusion, Vitamins given to support biologically existing antioxidant systems against oxidative stress, which is an important step in atherosclerosis, can partially suppress oxidative stress by acting together with antioxidant systems. Our study data suggest that vitamins may have the potential to prevent or slow down the development of atherosclerosis. Therefore, in line with the results we obtained, it would be a correct approach to recommend a vitamin-rich diet to strengthen our existing defense system against oxidative stress.

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Declaration of interest: No conflict of interest is declared by the author.

Data sharing statement: Data supporting the findings and conclusions are available upon request from the author.

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