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The effects of folic acid on vascular reactivity in a hyperhomocysteinemic rat model

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ABSTRACT

Objective: This study aimed to investigate the antioxidant effects of folic acid and its effects on contraction and relaxation responses in rat aorta in hyperhomocysteinemic rats.

Material and Method: Thirty-four male Wistar Albino rats were allocated into four groups. Rats in the hyperhomocysteinemia group (Group 1, n=9) received 1 g/kg/day methionine via orogastric gavage for 30 days and then injected with intraperitoneal saline for the next 7 days. In the hyperhomocysteinemia+folic acid group (Group 2, n=9), following the administration of methionine for 30 days, folic acid (4 mg/kg/day) was delivered intraperitoneally for 7 days. Sham group rats (Group 3, n=8) received orogastric saline for 30 days, which was followed by an IP injection of saline for another 7 days. Animals allocated into the folic acid group (Group 4, n= 7) had orogastric saline for 30 days and intraperitoneal folic acid for 7 days. After 5 weeks of treatment, blood samples were obtained, all animals were sacrificed, and hearts were harvested. Thoracic aortic segments were suspended on individual organ baths, and acetylcholine-induced (endothelium-dependent) relaxation responses of isolated aortic rings were evaluated.

Results: Relaxation responses in Group 1 through 4 were 73.88±9.96, 76.15±9.28, 76.61±8.83, and 69.26±15.68, respectively. There was no significant difference in the organ bath in terms of relaxation response to acetylcholine at a dose of 10-9 mM between the groups (p=0.550).

Conclusion: Folic acid therapy failed to produce a significant improvement in vascular reactivity.

Keywords: Hyperhomocysteinemia, folic acid, vascular reactivity

INTRODUCTION

Background/Rationale

Atherosclerotic cardiovascular diseases are among the leading causes of death in the world, as well as in Turkey (1,2). Hence, the effects of atherosclerosis at the cell level and their prevention continue to attract researchers.

Folic acid helps to make nucleic acids and convert some amino acids (such as the conversion of serine, glycine, and homocysteine to methionine, catabolism of histidine to glutamic acid) (3). In folic acid deficiency, especially in pregnant women, megaloblastic (macrocytic) anemia, cancers (such as colon, stomach, and uterus), and other pathologies (such as anencephaly, spina bifida, and cardiovascular disease) may occur together with elevated serum homocysteine level (4). It is stated that one of the most essential factors causing hyperhomocysteinemia (HHcy) is folic acid deficiency (5). In recent years, the link between folate homeostasis and homocysteine metabolism has been shown to play a crucial role in many vascular diseases (6).

HHcy is an independent risk factor for atherosclerotic vascular disease, stroke, and arterial as well as venous thromboembolism (5). Some mechanisms proposed for the formation of HHcy-related atherosclerosis can be listed as endothelial dysfunction, vasodilatation with an impaired flow, increased proliferation in vascular smooth muscle cells, and increased coagulation (7). Another undesirable effect of HHcy on vascular endothelium is reducing the production of nitric oxide (NO) and its bioavailability. NO is a critical molecule that plays an essential role in maintaining vital balance in all

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systems of the body (such as vascular reactivity, platelet aggregation, immune system, nerve conduction, and the production of various hormones) (8). We hypothesized that the demonstration of favorable effects of folic acid on contraction and relaxation responses can contribute to studies on the use of folic acid in vascular diseases.

Objectives

This study aimed to investigate the antioxidant features of folic acid and its effects on contraction and relaxation responses in rat aorta in hyperhomocysteinemic rats.

MATERIAL AND METHOD

Study Design

A randomized and controlled experimental animal study was designed. The study protocol was approved by Adnan Menderes University Animal Experiments Local Ethics Committee (IRB number: 2011-001, Date: 09.02.2011). The study was conducted in 2014 .The research institution is a third-level health center that provides specialized diagnosis, treatment, and service by using modern knowledge and technology.

Participants

Thirty-four Wistar albino male rats were obtained from the Adnan Menderes University Experimental Animal Production Laboratory. Rats were kept in their cages and were fed ad libitum with standard rat food and tap water until about 16 weeks old. A 12-hour day and night rhythm was applied. The animals were kept in a controlled room with a temperature of 20-25 degrees Celsius. Weights of the rats were measured weekly, starting from the first day of the study. The drug doses were adjusted accordingly (9). The animals were randomly allocated into four groups. Rats were kept in 4 different wire cages throughout the study. At the end of the ten-day adaptation period, the rats were weighed as 350-400 gr. Hyperhomocysteinemia was induced with a dose of 1 g/kg/day L-methionine (Sigma-Aldrich (M9625, USA) dissolved in 150 mM phosphate buffer (pH 7.4) given by oral gavage (9). Folic acid was given at a dose of 4 mg/kg/day dissolved in 1% sodium carbonate (10). Antioxidant levels were examined biochemically in tissue and blood samples in all groups.

The hyperhomocysteinemia (HHcy) group (Group 1, n=9): Animals in this group were given orogastric 1 g/kg/day L-methionine for 30 days. Then, intraperitoneal (IP) saline was given for a week, and a model of hyperhomocysteinemia was created in rats. The dose-response curves were assessed in the organ bath.

The hyperhomocysteinemia + folic acid group (Group 2, n=9): Rats in this group were given orogastric 1 g/kg/day L-methionine for 30 days. Then, 4 mg/kg/day IP folic acid was given for a week. Hyperhomocysteinemia was induced, and the effect of folic acid was assessed using dose-response curves in the organ bath.

The sham group (Group 3, n=8): Rats in this group were not medicated; orogastric saline was given for 30 days. Then, IP sterile saline was administered for a week, and dose-response curves were investigated in the organ baths.

The folic acid group (Group 4, n=8): Rats in this group were given IP folic acid for a week after feeding orogastric saline for 30 days. Dose-response curves were investigated in the organ bath.

One of the rats was lost before analysis. Accordingly, the number of rats used in the experiment was as follows: Group 1: 9, Group 2: 9, Group 3: 8, and Group 4: 8 (**Figure**).

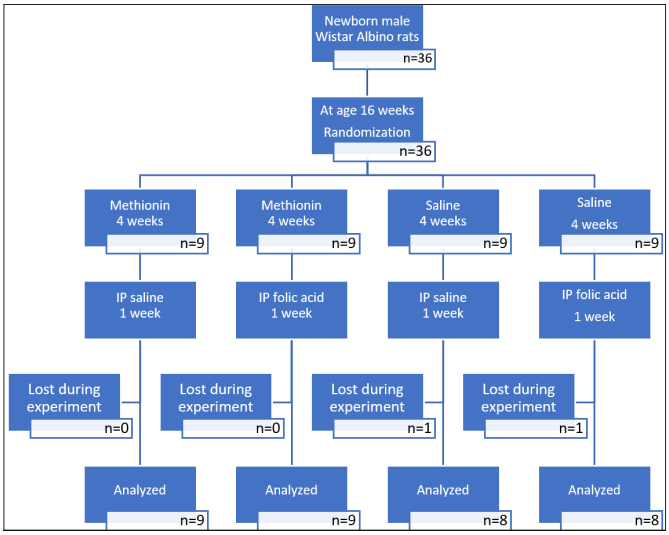

Figure. Participant flow diagram
IP: Intraperitoneal

Variables

Methionine is metabolized to form homocysteine (Hcy) over S-adenosine methionine and S-adenosine homocysteine. Additionally, methionine and its intermediate S-adenosyl homocysteine inhibits Hcy metabolism and causes an increase in plasma total Hcy levels (11). The commercial form of methionine was in the form of a sterile powder. The solution was prepared in weekly doses of 150 µM, pH 7.4, 0.25 mg/ml phosphate-buffered saline (PBS) mixture.

Folic acid was prepared at a dose of 4 mg/kg/day. Weekly doses were prepared by dissolving in 10 mg/ml PBS, and the solution was administered intraperitoneally with an insulin injector (26 G 0.45x10 mm).

The rats were enumerated by marking with picric acid. The experiment was started with four rats, and another four were added to the research every day. Thus, at the end of five weeks, the first four rats, which ended the experimental protocol for the organ bath, were sacrificed, allowing sufficient time for the organ bath.

At the end of five weeks, the rats were anesthetized with ketamine (100 mg/kg, IP) + xylazine (10 mg/kg, IP), and cervical dislocation was performed after intracardiac blood collection. Intracardiac 2-3 ml blood was taken and placed in tubes with EDTA. Later, the aorta was removed and prepared for the organ bath. The withdrawn blood was cooled in ice, centrifuged (3000 g) for 10 minutes, placed in plasma Eppendorf tubes (at least 2 separate tubes), and stored in -80°C until analysis. Heart tissue was also taken and immediately stored at minus eighty degrees for biochemical study. Organ bath experiments were carried out in the laboratory of the research institute.

Tissue homogenization was performed in a Braun Potter-S tissue homogenizer (B. Braun Biotech Co., Melsungen, Germany) with tissue homogenization buffer. The tissue homogenization buffer (1 mM, pH 7.4) was prepared using phenylmethylsulphonyl fluoride (C7H7FO25, SIGMA, catalog number P-7626), di-Natriumhydrogen-phosphate-Dihydrate (Na2HPO4.2H2O, MERCK, catalog number K25979680), potassium dihydrogen phosphate (H2KPO4, MERCK, catalog number A986373), and ethylenediaminetetraacetic acid disodium (EDTA, C10H14N2O8Na2.2H2O, SIGMA, catalog number E1644).

The primary variable of our study was defined as relaxation response to acetylcholine (%). Other variables were, substrates reacting with thiobarbituric acid (TBARS) in heart tissue and serum samples, nitric oxide (NO) (μM), superoxide dismutase (SOD) (ng/mL), glutathione peroxidase (GSH-Px) (U/L), glutathione reductase (GR) (mmol/min/ml), catalase (CAT) (mmol/min) in the heart tissue and hemolysate samples.

Glutathione determination was performed according to the method used by Tietz (12). By determining the level of nitrate, which is one of the cleavage products of NO, an indirect measurement of NO was done according to the method used by Cortas et al. (13). SOD activity determination was performed by the process of Yi Sun et al. (14). Glutathione peroxidase (GPx) activity was measured according to Paglia and Valentin's method (15). The technique developed by Carlberg and Mannervik and later by Kazim Husain was used for the determination of glutathione reductase (GR) activity (16). Catalase (CAT) activity analysis was done according to the Hugo Aebi method (17). The determination of homocysteine in the blood was made using an ELISA kit (Cusabio, Catalog No: CSB-E13376r).

Organ Bath: At the end of the five-week trial, after ketamine (100 mg/kg IP) and xylazine (10 mg/kg IP) anesthesia, the rib cage was opened with a median sternal approach, and cervical dislocations were performed after drawing intracardiac blood. Thoracic aorta, which stands at the same level as the thoracic vertebra, was carefully and rapidly removed along with the arcus aorta. Using a binocular magnifier (3.5 magnification), the aorta was cleared of the adipose and connective tissues on it and then cut through an appropriate incision to obtain isolated length aortic rings. During this process, an effort was given to preserving the endothelium.

Each aortic segment was cut into approximately 3 mm-wide rings. These rings were suspended in the carbonated (95% oxygen, 5% carbon dioxide) Krebs solution (118.3 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.22 mM KH2PO4, 2.5 mM CaCl2, 25.0 mM NaHCO3 and 11.1 mM glucose, Sigma-Aldrich), which was heated and circulated at 37 °C (Heater and Circulatory system - May WBC 3044V3), without delay (18). Each aorta ring was passed through two stainless steel hooks in a 25 ml organ bath filled with Krebs solution (May 99 IOBS Ankara, Turkey), and suspended to the organ bath.

Upperparts of the two hooks were attached to a transducer (May GTA0303, Biopac Systems Inc. Model MP 100), and their contractions in mg were measured (19). The Acq Knowledge 3.8.2 (Commat Ltd, Ankara, Turkey) was used as a computer program. The standard equilibrium stage was then performed for each aortic ring. Aortic rings initially were stretched with 1 g, waited for 10 min., then the tension was increased to 2 g, waited for 10 min., and then reached 3 g and waited for 10 minutes again.

Later, 0.1 ml of norepinephrine at 10-4 M concentration was added to the bath, and contraction responses were recorded. When the contraction curves were flat, the bath was washed twice with a Krebs solution. The bath was stretched up to 4 g and waited until it was stable. First, the contraction curve was taken with 0.1 ml of norepinephrine 10-4 M concentration, and then relaxation responses were obtained with 0.1 ml of Acetylcholine 10-4 M concentration. Finally, the bath was washed twice again with the Krebs solution, 45 minutes before starting the experiment. Baths without contraction and relaxation responses at these stages were not taken to the experimental phase (20). Then, the experiment phase was started. Norepinephrine 10-4 M was added 0.1 ml to each bath. Contraction responses in the baths were observed. Acetylcholine concentrations prepared when the plateau level formed and started to be stable in the contraction curve formed were given to the baths. First, 0.1 ml of

10-9 M concentration of acetylcholine was given, and a relaxation response was observed, acetylcholine was given 3.16 x10-9 M when non-relaxation was observed. When the relaxation response ended, another concentration of 10-8 M acetylcholine was given. Thus, 3.16x10-8 M, 10-7 M, 3.16x10-7 M, 10-6 M, 3.16x10-6 M, 10-5 M, 3.16x10-5 M, 10-4 M acetylcholine were given each 0.1 ml in an order. Relaxation responses at each dose were observed and recorded. Aortic rings were studied for each of the four groups in the organ baths.

Study Size

The sample size calculation was performed based on the primary outcome relaxation response to acetylcholine at a dose of 3.16 x10-9 M with the G Power program (21). Taking the effect size as 0.70 (large), α error as 0.05, power as 80%, number of groups as 4, a total of 28 subjects are required to compare the mean values with the one-way ANOVA.

Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) (SPSS for Windows, Version 25.0, Chicago, IC, USA). Results were presented as mean and standard deviation. The normal distribution of the continuous variables was examined with the Shapiro-Wilk test. The Kruskal-Wallis test was used to check for significant differences between groups. The Mann-Whitney U test was used for bivariate comparisons. The threshold for statistical significance was taken as $p<0.05$.

RESULTS

There was a substantial difference in the blood catalase levels between Group 1 and 2. Also, there were significant differences in blood homocysteine, blood catalase, tissue glutathione peroxidase, tissue glutathione reductase, and tissue glutathione levels between Group 1 and 3. There were significant differences in blood homocysteine, blood catalase, tissue glutathione peroxidase, tissue glutathione reductase, and tissue glutathione levels between Groups 1 and 4. Besides, there were significant differences in blood homocysteine, blood glutathione reductase, blood catalase, tissue glutathione peroxidase, tissue glutathione, tissue glutathione reductase, and tissue catalase levels between Group 2 and 3. Additionally, there were significant differences in blood homocysteine, blood catalase, blood TBARS, tissue glutathione, and tissue glutathione reductase levels between Group 2 and 4. However, there was no significant difference in the comparison of the biochemical results between Group 3 and 4 (**Table 1** and **Table 2**).

There was no significant difference in the organ bath analysis concerning relaxation responses between the groups. ($p>0.005$) (**Table 3**).

Table 1. Comparisons of the blood and tissue measurements between the groups

Variable	Group	Mean	SD	H	p
Blood homocysteine	1	18.20	4.91	24.558	<0.001
	2	17.33	1.84		
	3	8.32	1.52		
	4	9.75	2.12		
Blood glutathione reductase	1	61.09	5.35	478.887	<0.001
	2	22.39	1.97		
	3	8.25	1.35		
	4	15.10	2.13		
Blood glutathione	1	5.45	3.70	0.132	0.939
	2	5.83	3.18		
	3	6.22	2.15		
	4	5.40	2.61		
Blood catalase	1	417.44	171.66	17.907	<0.001
	2	665.40	137.31		
	3	1567.62	582.32		
	4	1530.84	560.81		
Blood nitric oxide	1	45.84	1.20	8.696	<0.001
	2	41.64	5.79		
	3	40.08	1.02		
	4	46.77	1.26		
Blood TBARS	1	8.73	4.15	14.963	<0.001
	2	11.82	1.05		
	3	4.05	1.51		
	4	4.42	3.23		
Tissue glutathione peroxidase	1	0.79	0.50	7.783	<0.001
	2	0.69	0.38		
	3	1.53	0.33		
	4	1.49	0.39		
Tissue glutathione reductase	1	1.25	0.28	4.613	0.009
	2	1.15	0.49		
	3	2.03	0.72		
	4	2.34	1.34		
Tissue glutathione	1	12.79	1.47	95.330	<0.001
	2	10.53	6.16		
	3	40.26	6.84		
	4	37.45	1.44		
Tissue catalase	1	0.57	0.14	6.210	0.002
	2	0.70	0.19		
	3	1.26	0.44		
	4	1.37	0.82		
Tissue nitric oxide	1	64.04	4.23	1.388	0.266
	2	71.43	5.85		
	3	70.92	4.17		
	4	66.90	5.43		
Tissue TBARS	1	3.57	1.85	15.484	<0.001
	2	4.99	2.10		
	3	1.03	0.28		
	4	0.92	0.44		

SD: Standard deviation. TBARS: tissue substrates that react with thiobarbituric acid H: Kruskal-Wallis test value. Group1: Hyperhomocysteinemia, Group 2: Hyperhomocysteinaemia+Folic acid, Group 3: Sham, Group 4: Folic acid.

Table 2. Post hoc comparison of the groups concerning the measured variables with significant differences

Variable	Group	Group	Z	p
Blood homocysteine	1	3	2.882	0.002
		4	2.558	0.010
	2	3	3.000	0.001
		4	2.646	0.006
Blood glutathione reductase	2	3	2.143	0.035
Blood catalase	1	2	2.286	0.022
		3	2.722	0.006
		4	2.558	0.011
	2	3	2.571	0.010
		4	2.646	0.008
Blood TBARS	1	3	2.009	0.045
	2	3	3.021	0.003
		4	2.670	0.008
Tissue glutathione peroxidase	1	3	2.598	0.009
		4	2.382	0.017
	2	3	2.941	0.003
		4	2.662	0.008
Tissue glutathione reductase	1	3	2.502	0.012
		4	2.276	0.023
	2	3	2.310	0.021
		4	1.967	0.049
Tissue glutathione	1	3	2.598	0.009
		4	2.699	0.007
	2	3	2.941	0.003
		4	3.125	0.002
Tissue catalase	1	3	2.598	0.009
	2	3	2.521	0.012
		4	2.646	0.008
Tissue TBARS	1	3	2.791	0.005
		4	2.699	0.007
	2	3	2.836	0.003
		4	2.662	0.008

Group 1: Hyperhomocysteinemia, Group 2: Hyperhomocysteinemia + Folic acid, Group 3: Sham, Group 4: Folic acid. Z: Mann-Whitney U test value.

Table 3. Comparison of relaxation responses between the groups

Acetylcholine dose (M)	Group	Mean	SD	H	p
10^{-9}	1	73.88	9.96	0.716	0.550
	2	76.15	9.28		
	3	76.61	8.83		
	4	69.26	15.68		
$3.16x10^{-9}$	1	85.78	7.95	0.361	0.781
	2	90.32	10.80		
	3	86.16	5.94		
	4	87.43	13.19		
10^{-8}	1	83.36	9.11	0.036	0.990
	2	83.84	9.88		
	3	83.75	6.74		
	4	84.89	12.87		
$3.16x10^{-8}$	1	82.92	9.28	0.021	0.995
	2	82.86	10.06		
	3	83.13	7.00		
	4	84.01	13.81		
10^{-7}	1	82.44	10.46	0.022	0.995
	2	81.89	10.19		
	3	82.55	7.64		
	4	83.28	14.16		
$3.16x10^{-7}$	1	80.99	10.51	0.012	0.998
	2	80.68	10.48		
	3	80.84	8.08		
	4	81.67	14.44		
10^{-6}	1	79.30	9.88	0.020	0.995
	2	79.48	10.82		
	3	80.13	8.46		
	4	80.47	14.06		
$3.16x10^{-6}$	1	77.87	9.95	0.009	0.998
	2	78.30	10.73		
	3	78.68	8.66		
	4	78.54	13.90		
10^{-5}	1	76.78	9.82	0.046	0.986
	2	77.59	9.78		
	3	75.59	10.09		
	4	76.92	13.82		
$3.16x10^{-5}$	1	74.95	9.85	0.022	0.995
	2	75.58	9.17		
	3	74.27	10.82		
	4	75.37	13.35		
10-4	1	74.04	9.79	0.028	0.993
	2	74.10	9.97		
	3	72.68	10.87		
	4	73.54	13.89		

SD: Standard deviation. H: Kruskal-Wallis test value

DISCUSSION

Key Results

This study demonstrated statistically significant differences in the mean blood glutathione reductase, blood glutathione peroxidase, blood catalase, blood TBARS, tissue glutathione peroxidase, tissue glutathione reductase, tissue glutathione, tissue catalase, and tissue TBARS levels between the groups. However, there was no significant difference in the organ bath in terms of relaxation responses between the groups.

Limitations

This was a well-conducted experimental study. However, there is a gap between study conduction and writing, which is due to the personal problems of the primary author. Also, the fact that the degree of endothelial damage was not measured can be considered as a limitation.

Interpretation

Hyperhomocysteinemia can cause several harmful effects in the body, some of which include acting as free radicals and causing endothelial damage. As a result, it causes coagulation-enhancing effects, such as platelet activation, coagulation factors, thrombus formation, oxidation in biological membranes, and induces atherosclerosis-enhancing effects by low-density lipoprotein (LDL) oxidation (22). Furthermore, hyperhomocysteinemia (HHcy) has been reported to increase aortic aneurysm and calcification in aortic valves (23,24).

HHcy can be defined as having total homocysteine (Hcy) above 15 μmol/L (25). There can be many causes of hyperhomocysteinemia. Some of these can be listed as folic acid, cyanocobalamin, pyridoxal phosphate deficiency, or various enzyme abnormalities (26,27).

There is a complicated relationship between plasma homocysteine, glutathione peroxidase (an antioxidant enzyme, containing selenocysteine), and endothelial dysfunction. It has been claimed that glutathione peroxidase may play a role in the harmful effects of homocysteine. It has even been suggested that homocysteine partially decreases GPx-1 expression and, therefore, may increase endothelial dysfunction. There is even evidence that increasing the level of GPx can reduce endothelial dysfunction (28). In our study, glutathione peroxidase levels were observed lower in homocysteine groups, in line with previous publications, which is an encouraging finding of the experiment.

Detoxification of hydrogen peroxide (H2O2), which passes from mitochondria to cytosol, is carried out by the enzyme catalase, synthesized by peroxisomes (29). It has been noted that increased hydrogen peroxide levels may have contributed to the emergence of the pathological effects of homocysteine because it inhibits catalase (30). In our study, the fact that the level of catalase in the homocysteine groups was lower than the other groups supports this view.

Biomembranes and intracellular organelles are susceptible to the attack of oxidants due to the unsaturated fatty acids in membrane phospholipids. Malondialdehyde (MDA), which is one of the vital products of lipid peroxidation, affects ion exchange through cell membranes, leads to cross-linking of the compounds in the membrane, and causes unwanted results, such as change of ion permeability and enzyme activity. MDA can react with the nitrogenous bases of DNA and is, therefore, mutagenic, genotoxic, and carcinogenic for cell cultures (31). On the other hand, in pharmacological doses, folic acid has been reported to lower MDA levels and increase antioxidant capacity (32).

Determination of MDA, one of the lipid peroxidation products, is based on the principle that thiobarbituric acid reacts with MDA to give rise to a colored compound that can be measured at a wavelength of 532 nm. In this method, substances that react with thiobarbituric acid are analyzed and included in the literature as TBARS (33). In our experiment, the lower TBARS levels in groups without hyperhomocysteinemia were evaluated as in harmony with the previous research.

Hyperhomocysteinemia causes vascular dysfunction mainly due to oxidative stress, reduced vasodilators such as nitric oxide, damaging the vascular matrix, supporting smooth muscle proliferation, and reducing vascular constriction (34). The fact that no significant difference was detected between the groups in both blood and tissue nitric oxide levels in our study suggested that the adverse effects of homocysteine may not have been fully revealed in our experiment.

In an animal study with pulmonary arteries, it was reported that hyperhomocysteinemia significantly affected both the contraction and relaxation responses of the vessels compared to the control group (26). It has been stated that high doses of folic acid improve endothelial function and vascular reactivity after four weeks of supplementation, which may reduce the risk of cardiovascular disease (35). It has been claimed that folic acid can improve endothelial function even without lowering homocysteine levels (36). Although interventions for reducing the increased homocysteine concentration to desired levels vary depending on the underlying causes, folic acid has been reported to be very useful (37).

Reviewing the studies on this issue has created an expectation that giving folic acid in rats with hyperhomocysteinemia should increase vascular reactivity. In our study, a moderate hyperhomocysteinemia model was created in rats. However, no significant improvement in vascular reactivity was observed in rats given folic acid compared to the control group. Perhaps the duration of the experiment was not sufficient for the reactive effects to occur in the vessels. On the other hand, the folic acid dose might not have been high enough, and the supposed damage caused by homocysteine could not have been adequately established.

CONCLUSION

In the moderate hyperhomocysteinemia model, five-week folic acid administration increases antioxidant levels and decreases levels of damaging agents such as TBARS. However, there was no significant decrease in vasodilating nitric oxide levels. Likewise, folic acid therapy failed to produce a substantial improvement in vascular reactivity. This result may be due to insufficient vascular damage or inadequate folic acid doses. To elucidate this speculation, further studies are needed with higher doses of folic acid, ensuring that sufficient damage is generated in the vascular endothelium.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study protocol was approved by Adnan Menderes University Animal Experiments Local Ethics Committee (IRB number: 2011-001, Date: 09.02.2011).

Informed Consent: All patients signed the free and informed consent form.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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