Research Article

Selim Gökdemir*, Zeynep Gizem Todurga Seven, Andleeb Shahzadi, Neşet Neşetoğlu, Durişehvar Ünal, Gökhan Akkan and Sibel Özyazgan

Comparative vascular effects of levetiracetam and valproate with hyperhomocysteinemia in rat models

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Abstract

Objectives: Hyperhomocysteinemia (HHcy) a significant risk factor for vascular disease, often emerges in epilepsy with the use of antiepileptic drugs. In this relationship, our study investigates the combined effects of HHcy and antiepileptics on vascular function using a rat model.

Methods: Fourty two rats were included and divided into six groups as, 1-Control, 2-L-Met, 3-LEV injected, 4-LEV-injected + L-Met, 5-VAL-injected, 6-VAL injected + L-Met. L-Methionine (L-Met) was added to drinking water of rats for 1 month to develop HHcy. Simultaneously, intraperitoneal (ip) injections of sodium valproate (VAL) and levetiracetam (LEV) were administered. Effects were comparatively investigated, and noradrenaline (NA), followed by acetylcholine (ACh) and glyceryl trinitrate (GTN) were applied in organ bath system. Agonist doses were expressed as ten base logarithm (M) through 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} mol/L in dose-response graph.

Results: NA contractions between LEV and LEV + L-Met groups showed statistical significance (LEV E_{max}=288.50 ± 46.54, LEV + L-Met E_{max}=480.40 ± 78.83) ($p<0.05$) however, no significance was observed among the other groups. ACh relaxations between Control-L-Met (Control Inh_{max}=12.65 ± 2.09, L-Met Inh_{max}=50.05 ± 7.43) ($p<0.05$), and Control-Val + L-Met (Control E_{max}=328.20 ± 52.83, VAL + L-Met E_{max}=452.60 ± 71.53) ($p<0.01$), groups showed statistical significance. Between other groups, no significance was observed. In GTN relaxations, no statistical significance was observed.

Conclusions: This study highlights the adverse impact of HHcy on aortic relaxation. Further impairment was observed with VAL compared to other treatment and control groups. These findings underscore the importance of considering vascular side effects when selecting antiepileptic drugs. Ultimately, our study contributes valuable insights that may aid the choice of appropriate treatment strategies to mitigate potential vascular complications of HHcy.

Keywords: hyperhomocysteinemia; anticonvulsants; organ bath; vascular damage; epilepsy treatment

***Corresponding author: Selim Gökdemir**, MD, Department of Medical Pharmacology, Cerrahpaşa Medical Faculty, İstanbul University-Cerrahpaşa, Istanbul, Türkiye; and Pharmacology Department, İstanbul Dr. Lütfi Kırdar Şehir Hastanesi, Istanbul, Türkiye,
E-mail: selimgokdemir@yahoo.com. https://orcid.org/0000-0003-4551-3013

Zeynep Gizem Todurga Seven, Andleeb Shahzadi and Sibel Özyazgan, Department of Medical Pharmacology, Cerrahpaşa Medical Faculty, İstanbul University-Cerrahpaşa, Istanbul, Türkiye. https://orcid.org/0000-0002-2529-0118 (Z.G. Todurga Seven). https://orcid.org/0000-0002-2774-2325 (A. Shahzadi). https://orcid.org/0000-0002-2511-3541 (S. Özyazgan)

Neşet Neşetoğlu and Durişehvar Ünal, Department of Basic Pharmaceutical Sciences and Analytical Chemistry, Faculty of Pharmacy, Istanbul University, Istanbul, Türkiye. https://orcid.org/0000-0002-2996-8440 (N. Neşetoğlu). https://orcid.org/0000-0003-0754-1240 (D. Ünal)

Gökhan Akkan, Department of Medical Pharmacology, Cerrahpaşa Medical Faculty, İstanbul University-Cerrahpaşa, Istanbul, Türkiye; and Department of Medical Pharmacology, Medical Faculty, Bezmialem Vakif University Hospital, Istanbul, Türkiye. https://orcid.org/0000-0003-3210-2709

Introduction

Hyperhomocysteinemia (HHcy) is a known risk factor for cardiovascular disease (CVD). It has been reported to promote dyslipidemia [1], oxidative stress [2], vascular smooth muscle cell (VSMC) proliferation [3], and impairment of endothelial regeneration and function [4], all of which are linked to CVD. In epilepsy patients, HHcy has been linked to an increased risk of occlusive vascular disease, brain shrinkage, and seizure aggravation [5–8].

Homocysteine (Hcy) is an amino acid with a sulphur molecule that does not enter the content of proteins. It is normally not found within the diet. It occurs in the process of methionine metabolism as an intermediate product and

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70–80 % of it is bound to plasma albumin. The bound and free portions reflect the total Hcy amount in the blood. The free part is unstable and rapidly turns into Hcy and disulphide [9].

Vitamin deficiencies are recognized as the foremost etiological factors contributing to the excessive formation of HHcy. Vitamins B6, B12 and folic acid are cofactors in Hcy metabolism and their deficiencies in the diet have been reported to cause an increase in Hcy plasma levels [10]. Extensive evidence from both *in vivo* human and animal studies has demonstrated that HHcy leads to dysfunction within the vascular endothelium. This dysfunction manifests as impairments in nitric oxide (NO)-induced vasodilation and inhibition of the endothelial-derived antithrombotic effects. As a consequence of these effects, there's an observed increase in cell proliferation within the vascular smooth muscle, further highlighting the detrimental impact of HHcy on vascular health [11–13].

The incidence of epilepsy, which affects many people worldwide, is reported to be 1–4% and although epilepsy surgery and other alternative treatment methods are developing further, medical treatment is still the main method of treatment today [14]. New generation drugs are added to the medical treatment of epilepsy alongside traditional drugs and currently, many studies continue on the efficacy and safety of these drugs. Furthermore, HHcy has been studied in epilepsy, and it is known that high Hcy levels and some antiepileptic drugs together facilitate epileptic seizures [15]. Long-term use of older antiepileptic drugs can also cause HHcy by lowering vitamin B6, B12 and folate levels in the blood [8, 16]. It has also been shown that HHcy is effective over the oxidative system in epilepsy [17], and this may occur as adverse effect with chronic use of antiepileptics such as sodium valproate (VAL), topiramate, and oxcarbazepine [7]. Similarly, studies conducted in this context showed that levetiracetam (LEV) does not change the level of Hcy and has an anti-inflammatory effect through IL 1-beta inhibition [18–21]. In contrary, literature showed that valproate tends to increase the blood Hcy level [6], and this situation potentiates epileptic seizures, opposite to the antiepileptic effect of the drug [15].

Our aim was to highlight the detrimental effects of HHcy on vascular endothelial damage and to investigate how antiepileptic drug therapy (VAL and LEV) might modify these consequences.

Materials and methods

Experimental animals

Forty-two male Wistar albino rats weighing 350–450 g were included in the study. Rats were obtained from İstanbul Bezmialem Vakıf University Experimental Animals Laboratory and placed in groups of 3 or 4 in cages that were cleaned regularly. Rats were housed at a constant temperature of 21–23 °C, in a ventilated environment, on a free-feed and water-drinking regimen (*ad libitum*) with a 12-h light/dark cycle. The experimental protocols used in the presented study were approved by the Bezmialem Vakıf University Experimental Animals Ethics Committee meeting (2019/221). The sacrification of animals were carried out in Bezmialem Vakıf University Experimental Animals Laboratory, however, the experiments were conducted in Istanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Department of Medical Pharmacology Laboratories. In our study, a total of 42 Wistar albino rats were divided into 6 groups, and over the course of a 1-month period, these rats underwent a series of distinct procedures. These groups were 1-Control, 2-L-Methionine (L-Met) added to drinking water, 3-LEV injected, 4-LEV injected + L-Met, 5-VAL injected, 6-VAL injected + L-Met (Table 1).

Hyperhomocysteinemia animal model

The HHcy animal model was established according to the previously described method in literature [22]. The amount of drinking water was calculated and corresponding amount of L-Met (1 g/kg) (Sigma Aldridge, L-Methionine, Reagent Grade, M9625-100G) was added to the drinking water in order to create HHcy (Target blood Hcy>20 µmol/L). Daily intraperitoneal (ip) injections of LEV (50 mg/kg) [23], VAL (100 mg/kg) [24] and SF were carried out for 1 month period in corresponding groups.

Isolation of thoracic aorta and blood sample collection

The experiments were conducted upon the completion of the 1-month period used to establish HHcy animal model. The dosage of anesthetic agents required was determined based on the weight of the rats. Ketamine HCl 40 mg/kg (Ketalar® 50 mg/mL vial, Pfizer Co., Istanbul, Turkey) + Xylazine Hydrochloride 5 mg/kg (Rompun® 23.32 mg/mL, 50 mL vial, Bayer Co., Istanbul, Turkey) was administered ip to provide appropriate dose of anesthesia. The sacrification process was started 5 min after the injection. The rat was fixed and abdomen was opened to get the thoracic aorta. Blood samples were collected from supraclavicular notch, centrifuged at 3,000 g for 20 min. Plasma was separated and stored at −80 °C until assayed. The plasma Hcy levels were measured by fluorescence immunoassay (Abbott IMx analyzer; Abbott Laboratories, Berkshire, UK).

Aortic ring preparation and vascular reactivity measurement

The following procedure was conducted in accordance to previous studies with minor modification [25]. Thoracic aortas were excised out of peripheral fat and connective tissue to be cut into approximately 3 mm rings then placed into Krebs–Henseleit solution (KHS, nM: NaCl 119, KCL 4.70, MgSO4.7$H_2$0 1.20, KH2PO4 1.20, CaCl2 2.50, NaHCO3 25, Glucose 11.1). Rings were mounted between two stainless steel hooks. With a resting tension of 2 g in an isolated organ bath (Ugo-basile, 4050, Italy) containing 25 mL KHS, aerated (95 % O_2 and 5 % CO_2) at 37 °C. Using an integrated force-displacement transducer (BOPAC MP36, USA) and tissue bath system, responses of separate organs were recorded isometrically (Commat, Ankara, Turkey). Concentration-response correlations of the aorta were obtained with dosages of noradrenaline (NA) after an hour of incubation. Up until a maximum response was reached,

NA (10^{-9}–10^{-4} mol/L) was applied cumulatively prior to the next dose, a plateau response was attained following the addition of each dose. Each strip was pre-contracted submaximally (EC90 10^{-5}) by the injection of NA, and a cumulative relaxation curve to acetylcholine (ACh; 10^{-9}–10^{-4} mol/L) was obtained. For each strip that had been pre-contracted submaximally (about EC90 10^{-5} mol/L) by the injection of NA, a cumulative relaxation curve to glyceryl trinitrate (GTN; 10^{-9}–10^{-4} mol/L) was obtained. The tissue was blotted dry, measured, and weighed at the end of each experiment.

Plasma homocysteine level measurement method

A new method for homocysteine analysis in biological fluids was developed and validated according to the EMEA (European Medicines Agency) Bioanalytical Method Validation Guidelines [26]. LC-MS/MS analysis was carried out using Agilent Technologies 6420 Triple Quad LC/MS system comprising Infinity 1260 series binary pump. Agilent Electrospray Ionization (ESI) was used as ion source. Isocratic separation was carried out using a Poroshell EC-C18, 2.7 µm, 50 mm × 3.0 mm analytical column thermosttated at 40 °C. The mobile phase consisted of Merck Millipore LC-MS Grade Methanol and Milli-Q ultra-pure water containing with % 0.2 formic acid (80:20 v/v). The flow rate was set at 0.6 mL/min and injection volume was 3 µL. Tolterodine (TOL) was used as internal standard. Each injected sample contained a known amount of the internal standard at a concentration of 0.1 µg/mL. Mass spectrometric detection was performed with electrospray ionization in positive ion mode using multiple reaction monitoring (MRM). The detection settings for Hcy and TOL are presented in Table 2. Run time was set as 2 min.

Statistics

The contraction response was calculated as mg tension/mg vessel wet weight. Relaxation responses were calculated by taking the percentage of submaximal NA contraction. EC50 graphs were drawn from the contraction and relaxation dose-response curves to find pD2 and maximum contraction and relaxation response values. The data are expressed as the mean ± standard error and were analyzed by Graphpad Prism® (Version 5.0; Graphpad Software, Inc., La Jolla, CA, USA) software. Two-way ANOVA analysis was used for statistics, followed by Tukey's multiple comparison test for isolated organ bath experiments. pD2 (M), E_{max} and Inh_{max} data were calculated by non-linear regression analysis. Data are expressed as mean ± SEM. The significance level was taken as $p<0.05$.

Results

Blood homocysteine levels

LC-MS/MS analysis of plasma samples showed significant increase in Hcy levels in the L-Met groups when compared with control and treatment groups. (Figure 1; $p<0.001$).

In this study contraction of thoracic aorta with NA were assessed within the organ bath system. During our experiments, NA was first applied to all animal groups from 10^{-9} to 10^{-4} mol/L doses. E_{max} and pD2 values are summarized in Table 3.

Regarding the contraction studies, a statistically significant difference was found between the group injected with LEV only and the group with L-Met and injected with LEV ($p<0.05$). Contraction was recorded to be significantly higher than the isolated LEV group. Apart from this, no statistically significant difference was observed between the groups ($p>0.05$) (Figure 2).

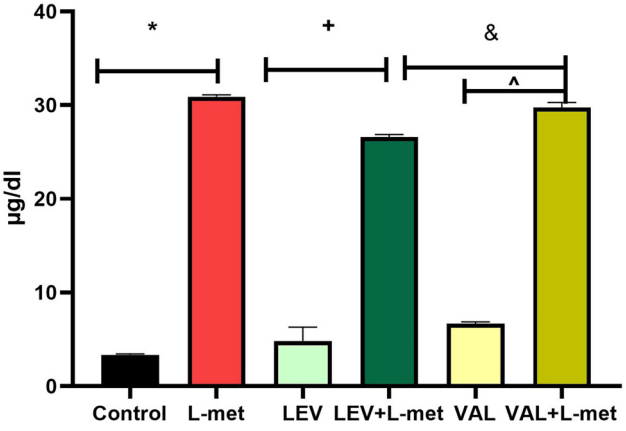

Figure 1: Plasma levels of homocysteine in control, L-Met and treatment groups. *,+,&,^p<0.001.

Table 1: Procedures applied to the groups.

Group	Serum physiologic (SF) injection	LEV injection (50 mg/kg)	VAL injection (100 mg/kg)	L-Met in drinking water (1 mg/kg)	Number of rats
1. Control	+				7
2. L-Met	+			+	7
3. LEV		+			7
4. VAL			+		7
5. LEV + L-Met		+		+	7
6. VAL + L-Met			+	+	7
Total number of rats					42

Table 2: Parameters for detection settings.

Name	IS	Precursor ion	Product ion	Dwell time	Fragmentor voltage (FV)	Collision energy (CE)	Cell accelerator voltage	Polarity
Homo-cysteine	No	136	90,1	150	51	6	7	Positive
Tolterodi-ne	Yes	326.1	147	150	80	24	7	Positive

IS, international standard.

Table 3: pD2 (log M) and E_{max} values (mg/mg ww) for NA-induced contraction of aortic rings from groups.

Group	NA	
	E_{max}, mg/mg	pD_2 (log M)
Control	328.20 ± 52.83	7.57 ± 2.34
L-Met	395.80 ± 65.55	7.07 ± 1.62
LEV	288.50 ± 46.54[a]	7.30 ± 1.06
VAL	467.70 ± 68.07	7.88 ± 3.98
LEV + L-Met	480.40 ± 78.83[a]	7.04 ± 1.14
VAL + L-Met	452.60 ± 71.53	7.03 ± 6.48

[a]p<0.05; LEV vs. LEV + L-Met.

Acetylcholine relaxation response

ACh was applied to all animal groups from 10^{-9} to 10^{-4} mol/L doses. E_{max} and pD2 values are summarized in Table 4.

Following the statistical analysis of vascular relaxation responses between the control group and the L-Met group, a significant difference was observed at the significance level of *p<0.05 at 10^{-5} mol/L dose, **p<0.01 at 10^{-4} mol/L dose (Figure 3).

The statistical comparison of the vascular relaxation responses of the control group and VAL + L-Met group, at 10^{-8}, 10^{-7} and 10^{-5} mol/L ACh doses, significance was determined at +p<0.05 level and ++p<0.01 level at 10^{-4} mol/L ACh dose (Figure 4).

Results of glyceryl trinitrate relaxation responses

Initially, GTN was administered to all animal groups from 10^{-9} to 10^{-5} mol/L doses. E_{max} and pD2 values are summarized in Table 5.

Discussion

In the present study, we utilized a HHcy induced rat model. We evaluated the adverse effects of HHcy on vascular endothelial damage and investigated how these effects could be influenced by the administration of antiepileptic drugs (VAL and LEV).

We demonstrated in our study that HHcy leads to notable vascular damage. The VAL + L-Met group exhibited signs of impaired vascular relaxation responses, likely attributed to its hyperhomocysteinemic effect. In contrast, the LEV groups did not display significant deterioration. Our findings suggest that LEV might be a more favorable option compared to VAL for epilepsy patients with HHcy.

We selected VAL and LEV due to their specific effects on vascular health and frequent usage in the treatment of epilepsy. In addition, a growing body of data suggests an

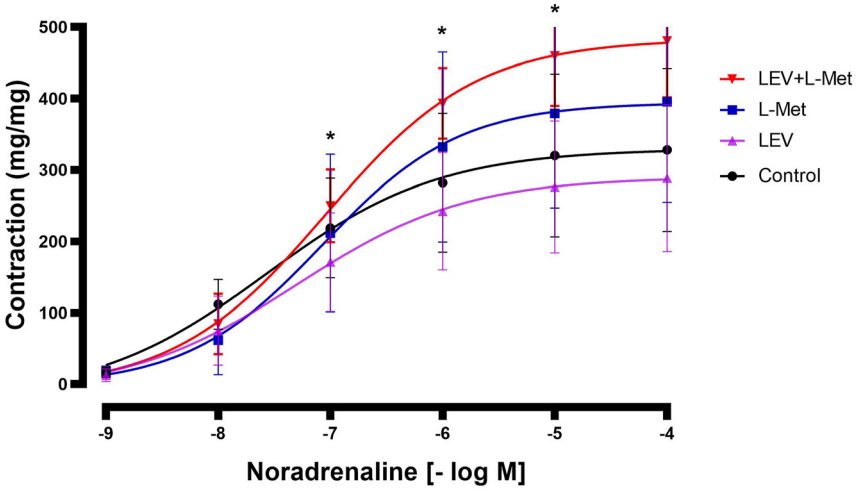

Figure 2: Noradrenaline contraction response. Noradrenaline contraction/-log M (control; R2=0.765, L-Met; R2=0.750, LEV; R2=0.7622, LEV + L-Met; R2=0.944 groups) *p≤0.05; LEV vs. LEV + L-Met.

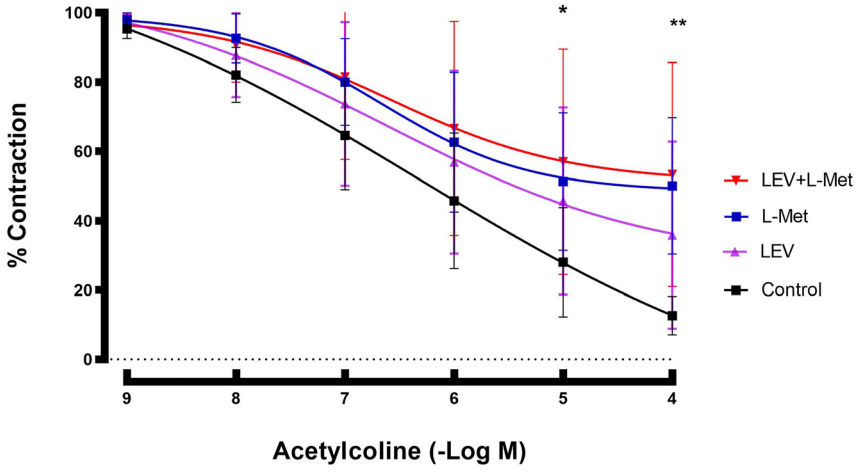

Figure 3: Acetylcholine relaxation response. Acetylcholine relaxation/-log M graph for control, LEV, L-Met and LEV + L-Met groups (control; R2=0.857, LEV; R2=0.540, L-Met; R2=0.642, LEV + L-Met; R2=0.336) *p<0.05; **p<0.01.

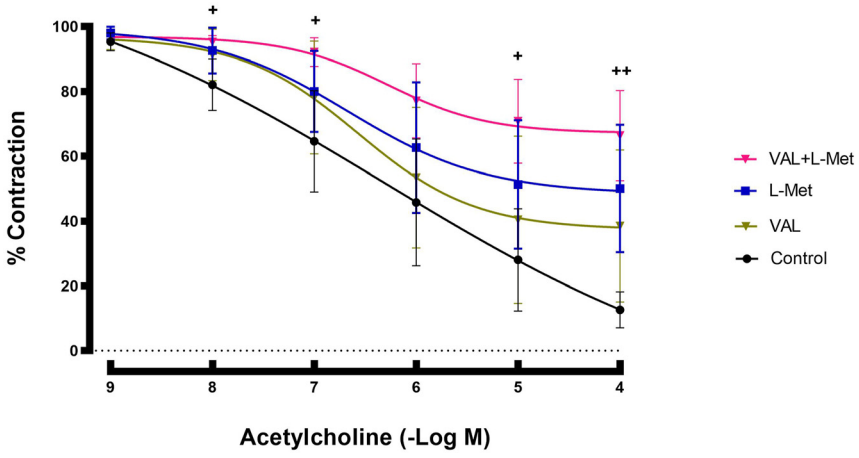

Figure 4: Acetylcholine relaxation response. Acetylcholine relaxation/-log M graph for control, L-Met, VAL, and VAL + L-Met groups (control; R2=0.857, L-Met; R2=0.642, VAL; R2=0.6496, LEV + L-Met; R2=0.663) +p<0.05, ++p<0.01.

Table 4: Inh_{max} and PD2 values for ACh.

	ACh	
	Inh_{max}, %	**pD_2 (log M)**
Control	12.65 ± 2.09	6.30 ± 0.79
L-Met	50.05 ± 7.43[a]	6.68 ± 0.38
LEV	35.86 ± 10.20[a]	6.62 ± 0.95
VAL	38.50 ± 8.87[a]	6.57 ± 0.32
LEV + L-Met	53.32 ± 12.22[a]	6.58 ± 0.85
VAL + L-Met	66.38 ± 5.26[a]	6.27 ± 0.28

[a]p<0.001 when compared with control.

Table 5: Inh_{max} and pD2 values for GTN by groups.

	GTN	
	Inh_{max}, %	**pD_2 (log M)**
Control	6.71 ± 2.49	7.25 ± 0.19
L-Met	6.46 ± 2.90	8.05 ± 0.47
LEV	11.91 ± 2.78	7.38 ± 0.19
VAL	12.76 ± 2.31	7.85 ± 0.88
LEV + L-Met	8.31 ± 1.77	7.52 ± 0.10
VAL + L-Met	33.48 ± 11.74	6.29 ± 1.17

No statistically significant difference was found in the relaxation responses obtained with GTN between all groups (p>0.05).

association between these drugs and HHcy, emphasizing the importance of investigating their combined effects on vascular function [15, 21].

This study compared the impacts on contraction and relaxation responses between LEV, known not to influence blood Hcy levels [21], and VAL which has been linked to elevated Hcy levels [15]. The reason for creating six groups was to illustrate the combined effects of the two drugs and HHcy on the vascular endothelium. Initially, we administered NA to the rat aortas in each group, followed by GTN. These applications aimed to demonstrate the intact contraction and relaxation of the suspended aortic ring within controlled *in vitro* conditions.

Our study showed that HHcy leads to notable vascular damage supporting the existing literature [9, 13, 27, 28]. The

VAL + L-Met group exhibited signs of impaired vascular relaxation responses, likely attributed to its hyperhomocysteinemic effect. In contrast, the LEV groups did not display significant deterioration. According to our research, LEV might be a better choice for epilepsy patients with HHcy than VAL.

In the NA contraction studies, a statistically significant difference was found between the group injected with only LEV and the group with L-Met and injected with LEV ($p<0.05$). Contraction was recorded to be significantly higher than the isolated LEV group. However, no statistical significance was observed between the groups. In phase 0 toxicology experiments conducted for FDA approval of LEV, it was reported that LEV had no effect on rat aortic contraction with NA [29]. On the other hand, it has been reported that HHcy damages vascular structures through oxidative stress, hardens the vessel wall and impairs vascular relaxation by reducing NO release with endothelial damage. The vascular tone is regulated by basal NO released from the endothelium [27, 28]. The observed significant differences can be attributed to the heightened vascular tone resulting from diminished basal NO release due to endothelial damage. Consequently, these findings align with a shift towards vessel contraction. However, further detailed studies are required to understand the underlying mechanisms. Apart from this, no further significance was found in the statistical evaluations.

The most important step in this study is relaxation responses with ACh. In all groups receiving L-Met, the expectation is that L-Met causes HHcy in rat metabolism and affects vascular relaxation responses, resulting in a decrease in NO release and a significant impairment of relaxation. Primarily, we compared the vessel relaxation responses of the control group and the group receiving isolated L-Met, a statistically significant difference was found at the level of $p<0.01$. This situation was evaluated in parallel with the detrimental effects of HHcy on the endothelium, which is the basis of the study and is frequently mentioned in the literature [9, 27, 28]. When we compared the control group and the VAL + L-Met group, a statistically significant difference was found at the $p<0.01$ level. No statistical significance was found when the control and LEV + L-Met groups were compared. As a result, relaxation defect was detected in the group in which HHcy was created by administering L-Met however, no statistical significance was found in vascular relaxation responses when the LEV + L-Met group was compared with the control group. This situation made us conclude that LEV [18], whose anti-inflammatory effects were reported, had an effect on reducing the detrimental effects of HHcy on the vessel.

When relaxation responses were evaluated with GTN, no statistical significance was found between the groups in terms of these responses. Since, the relaxation responses obtained with nitrates are independent of the endothelium; these results were thought to be in the expected direction.

This study evaluated whether the antiepileptics used in rats caused vascular effects. From this point of view, no statistical significance was found between control group and isolated VAL group. Although there are many publications in the literature about VAL increasing blood Hcy levels [15, 30], both the dose and the duration are considered insufficient to produce such a long-term effect in our study. While a statistical significance was detected for the comparison between control and VAL + L-Met group, the absence of any significance for the comparison between control and LEV + L-Met group indicates that VAL changes vascular responses significantly more than LEV in case of HHcy. In a research done by Attilakos and colleagues [21], they found that administration of LEV alone to treat children with epilepsy for up to a year does not have detrimental effects on Hcy metabolism, unlike older epilepsy drugs. This study suggested that LEV could be a safer choice in preventing complications associated with long-term use of antiepileptic drugs. Our study also has revealed important data on avoiding VAL in patients with HHcy in terms of protecting vascular health and not to increase the vascular effects caused by HHcy.

In medical treatment of epilepsy, it is important for epileptologists to choose the right drugs in the right combinations as well as to be well aware of some vascular side effects that may exacerbate with the chosen medical treatment. In this context, we have designed our study to delve nto the underlying mechanisms of HHcy in epilepsy, and to shed light on the possible exacerbation of vascular side effects linked to the specific drugs chosen for epilepsy treatment. Through these efforts, we aim to enhance our comprehension of the intricate interplay between epilepsy, HHcy, and the selection of medications, ultimately facilitating more effective and informed clinical management. Through the selection of drugs in the treatment of epilepsy, the patient's blood Hcy levels should be evaluated within the blood tests. This evaluation is of great importance in terms of the side effects of the treatment and to avoid some antiepileptic drugs such as VAL, which increase blood Hcy levels, in patients already with HHcy. In cases where the use of drugs that increase the blood Hcy level such as VAL is inevitable because of resistant epilepsy, administering VAL together with the supplements of vitamins B6, B12 and folic acid will ensure that the vascular changes that may occur are kept at a minimal level.

Research ethics: The experimental protocols used in the presented study were approved by the Bezmialem Vakıf University Experimental Animals Ethics Committee meeting (2019/221).
Informed consent: Not applicable.
Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: Authors state no competing interests.
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Data Availability: The raw data can be obtained on request from the corresponding author.

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