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Assessment of atherosclerosis risk due to the homocysteine–asymmetric dimethylarginine–nitric oxide cascade in children taking antiepileptic drugs

Hamdi Cihan Emeksiz [a,*], Ayse Serdaroglu [b], Gürsel Biberoglu [c], Ozlem Gulbahar [d], Ebru Arhan [b], Ali Cansu [e], Mustafa Arga [a], Alev Hasanoglu [c]

[a] Department of Pediatrics, Gazi University, TR-06450 Ankara, Turkey
[b] Department of Pediatric Neurology, Gazi University, TR-06450 Ankara, Turkey
[c] Department of Pediatric Nutrition and Metabolism, Gazi University, TR-06450 Ankara, Turkey
[d] Department of Biochemistry, Gazi University, TR-06450 Ankara, Turkey
[e] Department of Pediatric Neurology, Karadeniz Technical University, Trabzon, Turkey

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ABSTRACT

Purpose: The aim of this study was to assess the atherogenicity risk of antiepileptics in children by investigating the cascade, "hyperhomocysteinemia (HHcy) → asymmetric dimethylarginine (ADMA) increase → nitric oxide (NO) decrease", which is thought to contribute to the developmental process of atherosclerosis.
Methods: The participants included 53 epilepsy patients who received either valproic acid (VPA, $n = 26$) or oxcarbazepine (OXC, $n = 27$). Twenty-four healthy sex- and age-matched children served as controls. Fasting plasma total homocysteine (tHcy), ADMA and NO levels were measured.
Results: The differences in Hcy, ADMA, NO, vitamin B_{12} and folate levels between VPA, OXC and control groups were all insignificant ($p > 0.05$ for all). In the patient group (VPA and OXC groups), 22.6% of the children (12/53) had tHcy levels above the normal cutoff (13.1 μmol/l) for children and 17% of the children (9/53) had tHcy levels of greater than 15 μmol/l which is accepted as the critical value for an increased atherosclerosis risk ($p < 0.05$ for both). The difference in rate of HHcy between VPA and OXC groups was statistically insignificant ($p > 0.05$, for both cut off levels of HHCy). There was a positive correlation of tHcy levels and antiepileptic drug treatment duration in the patient group ($r = +0.276$, $p < 0.05$).
Conclusion: HHcy may develop in patients using OXC. Contrary to some previous publications, our data do not suggest that OXC is safer than VPA in terms of HHcy risk. Further prospective, large scale and longer term studies investigating all suggested pathways responsible for development of atherosclerosis due to HHcy should be conducted to define the exact mechanism responsible for AEDs related atherosclerosis.

1. Introduction

During the last few years, it has been shown that risk factors for atherosclerosis appear during childhood and adolescence and are already at that time associated with atherosclerotic changes in vessel walls.[1] As is well known, endothelial dysfunction is considered a precursor phenomenon. The concern is that acceleration of this atherosclerotic process due to a variety of unfavourable factors could lead to the emergence of cardiovascular diseases earlier than would otherwise be expected. An increased risk of fatal cardiovascular disease has been reported in patients with epilepsy[2]. However, any relationship to antiepileptic drugs (AEDs) remains unclear. Over the past two decades, high tHcy levels, an independent risk factor for atherosclerosis, ADMA, lipoprotein(a), and impaired lipid profiles, have been documented in several studies with children on AEDs.[3–9]

The mechanisms underlying the cardiovascular pathophysiology of HHcy are not yet fully understood. Numerous studies in animals and humans have demonstrated that HHcy leads to endothelial dysfunction due to decreased bioavailability of NO. Nitric oxide is a powerful vasodilator and anti-atherogenic agent that is produced in the endothelium from the amino acid L-arginine via the action of endothelial nitric oxide synthase (eNOS). One of the likely mechanisms of the decreased NO bioavailability in

* Corresponding author at: Gazi University, Faculty of Medicine, Department of Pediatric Endocrinology, Besevler, TR-06450 Ankara, Turkey.
Tel.: +90 532 724 81 24; fax: +90 312 213 36 43.
E-mail address: hcemeksiz@gmail.com (H.C. Emeksiz).

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HHcy is the increased concentration of ADMA, an endogenous inhibitor of eNOS, which, in turn, is followed by reduced synthesis of NO.[10]

Asymmetric dimethylarginine is also considered to be a potential risk factor for the development of atherosclerosis.[11] It has been suggested that elevated levels of ADMA are associated with the severity of cardiovascular events.[12,13] Asymmetric dimethylarginine is produced from methylated proteins derived from homocysteine (Hcy) metabolism. It competes with L-arginine and causes a reduction in NO formation in the vascular wall. Plasma tHcy has been shown to increase endothelial cell generation of ADMA by inhibiting the activity of the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which is responsible for ADMA metabolism.[14,15]

Valproic acid (VPA) and oxcarbazepine (OXC) are widely used AEDs in childhood epilepsy. In this study, we assessed whether children on these common antiepileptics had an increased risk of atherosclerotic diseases by investigating NO levels for the first time, in addition to tHcy and ADMA levels, and assessing possible associations among them.

2. Patients and methods

The study group consisted of 53 patients with epilepsy (26 girls, 27 boys) treated in the Department of Paediatric Neurology at Gazi University Faculty of Medicine (Table 1). All patients had received antiepileptic monotherapy (VPA, $n = 26$; OXC, $n = 27$) for at least 1 year. The presence of epilepsy due to cerebrovascular disease as well as mass lesions, history of cardiac or peripheral vascular disease, and renal, hepatic, or thyroid disease were exclusion criteria. Subjects using any other medication or vitamins were also excluded. All patients were judged to be in good health and free of a history of bleeding or thrombosis. A total of 24 healthy children served as the control group. This study was approved by the local ethics committee. Informed consent was obtained from parents.

Fasting venous blood samples were obtained from patients and controls to measure tHcy, NO, and ADMA levels. Serum and plasma were separated as soon as possible by centrifugation (5 min, $3000 \times g$). The samples were stored at $-80\,^{\circ}C$ until assayed. Plasma tHcy concentrations and ADMA levels were measured by high-performance liquid chromatography, and serum nitric oxide levels by an incubation assay method. Plasma tHcy levels $\geq$13.1 μmol/l were deemed to indicate hyperhomocysteinemia.[16]

Table 1
Characteristics of VPA, OXC, and control groups.

	Control group ($n = 24$)	VPA group ($n = 26$)	OXC group ($n = 27$)
Gender M/F (n)	13/11	16/10	11/16
Mean age (±SD, years)	12.1 ± 3.3	10.9 ± 3.1	10.8 ± 3.2
Mean duration of treatment (±SD, years)		3.4 ± 1.7	2.7 ± 1.4
Mean dose (±SD, mg/kg/d)		22.7 ± 7.0	22.5 ± 6.7

2.1. Statistical analyses

All statistical calculations were performed using the SPSS 11.5 software. Descriptive statistics were computed as means ± standard deviations and median (25–75th percentiles). Differences between groups were assessed using one-way analysis of variance, Pearson's chi-squared test, the Mann–Whitney U-test, and the Kruskal–Wallis test. The linear relationships between variables were evaluated using Spearman's rho coefficient. Statistical significance was set at $p < 0.05$.

3. Results

In total, 53 idiopathic epilepsy patients and 24 controls participated. Gender and mean age distributions did not differ significantly between the groups ($p > 0.05$ for both; Table 1). None of the differences in tHcy, ADMA, NO, vitamin B_{12}, and folate levels among the VPA, OXC, and control groups was significant (all $p > 0.05$; Table 2). No significant relationship was found among tHcy, NO, and ADMA levels ($p > 0.05$ for all). In the patient group, 22.6% (12/53) of the children had tHcy levels above the normal cutoff level (13.1 μmol/l), and 17% (9/53) of the children had tHcy levels greater than 15 μmol/l, the critical value for increased atherosclerosis risk. In contrast, no subject in the control group had tHcy levels above these values (Table 3). The difference in HHcy between the VPA and OXC groups was not statistically significant ($p > 0.05$ for both cutoff levels of HHcy). In the patient group, a statistically significant correlation was detected between the duration of treatment and tHcy level ($r = +0.276$, $p < 0.05$), whereas no such correlation was detected for the NO and ADMA levels ($p > 0.05$ for both). No statistically significant difference was found in ADMA and NO levels between patients with tHcy above

Table 2
Comparison of tHcy, ADMA, and NO levels.

	Control group[a] ($n = 24$)	VPA group[a] ($n = 26$)	OXC group[a] ($n = 27$)	p
tHcy (μmol/l)	9.2 (6.6–11.3)	9.2 (6.5–12.8)	9.3 (8.2–13.5)	>0.05[b]
ADMA (μmol/l)	2.1 (2.0–2.3)	2.1 (2.0–2.3)	2.2 (1.8–2.3)	>0.05[b]
NO (μmol/l)	1.8 (1.4–2.3)	1.8 (1.3–2.7)	2.3 (1.4–3.1)	>0.05[b]
Folic acid (ng/ml)	8.87 (7.27–10.66)	7.78 (6.08–9.40)	8.43 (6.45–10.67)	>0.05[b]
Vitamin B_{12} (pg/ml)	499.1 (385.6–678.3)	439.8 (319.7–544.3)	489.9 (378.5–640.9)	>0.05[b]

[a] Values are expressed as median and median (25–75th percentiles).
[b] p-Value, compared with control, VPA, and OXC groups.

Table 3
Comparison of the patients and control group for HHcy according to cutoff values.

	Control group ($n = 24$)	Patient group ($n = 53$)		p
		VPA group ($n = 26$)	OXC group ($n = 27$)	
tHcy > 13.1 (μmol/l), n (%)	–	6 (23.1)	6 (22.2)	0.014[a]
tHcy > 15.0 (μmol/l), n (%)	–	5 (19.2)	4 (14.8)	0.049[a]

[a] p-Value, compared between control and patient groups.

13.1 μmol/l or 15.1 μmol/l and patients with tHcy levels below these cutoff values ($p > 0.05$, for both).

4. Discussion

To our knowledge, this is the first reported study to evaluate atherosclerosis risk in children receiving AEDs associated with the HHcy → ADMA → NO cascade. Hyperhomocysteinemia was determined in approximately one-quarter of the patients taking AEDs, and the level of HHcy was the same in the VPA and OXC groups. Additionally, there was a positive correlation between tHcy levels and antiepileptic drug treatment duration in the patient group. There was no significant difference in tHcy, serum ADMA, and NO levels among the VPA, OXC, and control groups.

Hyperhomocysteinemia is an independent risk factor for cardiovascular and cerebrovascular diseases and thrombosis.[18] Many reported studies have proposed that AEDs may result in HHcy, but its frequency varies over a wide range in different population-based studies. In paediatric studies, HHcy has been observed in as many as 40% of the children receiving AEDs.[18,19] It is difficult to build a consensus for a cutoff level for HHcy due to variations in ethnicity, food consumption, and life styles among populations.[20] There are also limited data on tHcy levels in healthy Turkish children. To our knowledge, the report by Dinleyici et al.[16] is the only one that provides a paediatric cutoff (13.1 μmol/l) in Turkish children for HHcy. In our study, 12 of 53 patients (22.6%) had HHcy (>13.1 μmol/l), and nine of 53 patients (17%) had tHcy levels greater than 15 μmol/l, accepted as the critical value for increased atherosclerosis risk.[17] Although it is known to induce HHcy, VPA is still widely prescribed in neurology clinics. The mechanism by which VPA induces HHcy is not fully understood.[19,20] Moreover, data about the probable effects of new-generation AEDs, such as OXC, lamotrigine (LTG), levatiracetam (LEV), and topiramate (TPM), on Hcy metabolism are still limited.[21,22] These drugs may be safer than old-generation AEDs in children with epilepsy who are at increased risk for early atherosclerosis development, such as those with familial hyperlipidaemia or genetic disorders of Hcy metabolism. However, as yet, there are too few studies in the literature comparing new- and old-generation AEDs on this issue.[19] Oxcarbazepine possesses chemical features similar to those of its parent compound carbamazepine (CBZ). Compared with CBZ, OXC has a reduced capacity for inducing hepatic cytochrome P450 enzymes and thus of interacting with other drugs.[23] As a result, OXC has replaced CBZ for certain indications.[21] One purpose of the present study was to evaluate whether OXC affected tHcy levels and to compare OXC with VPA in terms of HHcy. In our study, HHcy was detected in six patients (22.2%), which was not different from HHcy in the VPA group. Kurul et al.[22] reported that no child using OXC developed HHcy in their study. However, a major limitation of their study was the small sample size. A study of adults conducted by Belcastro et al.[21] did reveal that new-generation AEDs such as OXC and TPM may induce HHcy. They also proposed that LTG and LEV, also classified as new-generation AEDs, had no effect on Hcy metabolism. Considering these results, HHcy may develop due to OXC treatment. Thus, clinicians should be careful about prescribing OXC in patients with additional risks for atherosclerosis and instead, safer new-generation AEDs, such as LTG and LEV, which are believed to have no deleterious effect on Hcy metabolism, should be used.

Deficiencies in vitamins acting as cofactors and genetic polymorphisms in genes encoding enzymes involved in Hcy metabolic pathways may result in HHcy.[19] Two important cofactors, vitamin B_{12} and folate, are tightly connected with Hcy metabolism.[19,20,24] Reduced plasma levels of these cofactors have been reported in children under VPA or CBZ treatment.[25,26] However, the exact mechanism(s) by which different AEDs exert their effects on vitamin metabolism and thus result in HHcy are incompletely understood.[19] The proposed mechanisms responsible for vitamin depletion in patients under AED treatment can be summarised as interference with vitamin absorption from the intestinal mucosa, increased requirements of vitamins due to dysfunction in Hcy metabolism, and the induction of liver enzymes responsible for increased vitamin metabolism.[20,27] There are also contrary reports in the literature revealing unchanged or increased levels of these vitamins in patients taking AEDs.[28–31] In our study, no vitamin B_{12} or folate deficiency was detected in patients receiving AEDs. Moreover, there was also no significant difference in vitamin B_{12} and folate levels between patients with HHcy and without HHcy. Some studies conducted in patients receiving AEDs found increased Hcy levels in patients carrying methylenetetrahydrofolate reductase (MTHFR) and cystathionine beta-synthase (CBS) gene polymorphisms.[19,20] Additionally, an experimental study conducted on pregnant Wistar rats demonstrated that VPA caused HHcy by directly impairing methionine synthase (MS) activity.[32] A major limitation of our study was that we did not perform a genetic work-up of our patients for the MTFR and CBS genes, which are known to play a role in Hcy metabolism. Further studies concerning the probable mechanisms by which OXC causes HHcy and/or examining whether OXC is related to genetic polymorphisms involved in Hcy pathways may help in better understanding the actions of OXC in Hcy metabolism.

Some reports have noted a progressive increase in tHcy levels as the use of AEDs was prolonged and/or involved multiple-AED treatment.[18,25,29] Ono et al.[27] reported an increased risk of HHcy in patients receiving multiple and prolonged (>7 years) AED medication. Similarly, Vilaseca et al.[29] reported an increase in tHcy level in parallel with the duration of AED use in children with seizure disorder. We also detected a weak but significant correlation between tHcy level and duration of AED use. All patients in our study received AED monotherapy. Children with epilepsy may require long-term, even life-long AED treatment. Because HHcy itself can cause an epileptic seizure, patients with epilepsy may require multiple AEDs to prevent seizures.[19] Prospective studies may be designed to determine the time required until the cutoff value for HHcy is reached by certain AEDs that are already known to induce HHcy. Thus, long-term morbidity due to these drugs may be preventable by switching the drugs before reaching the critical cutoff value for HHcy.

Hyperhomocysteinemia gives rise to enhanced ADMA production. A higher plasma level of ADMA exerts its atherogenic effect by lowering NO levels. This is one of the major suggested mechanisms by which ADMA and Hcy may lead to atherosclerosis.[33,34] To our knowledge, only a few reported studies have investigated ADMA as a marker of endothelial dysfunction and its relation with Hcy in patients receiving AEDs.[4,5] Oz et al.[4] first reported significantly elevated ADMA levels in adult patients taking VPA. Similar results have also been reported in children. Ozdemir et al.[5] noted increased serum ADMA levels and increased tHcy levels in epileptic children receiving VPA monotherapy. However, in neither study a significant relationship between Hcy and ADMA was reported. Moreover, no significant ADMA elevation accompanying HHcy was detected in patients with vascular disease and under long-term multiple AED treatment.[35,36] Similarly, we found no significant difference in ADMA levels between the study and control groups or between patients with and without HHcy. We also found no significant relationship between Hcy and ADMA levels. These results may be explained in two different ways. It has been proposed that more than one mechanism plays a role in the development of atherosclerosis and vascular disease due to HHcy. The Hcy–ADMA–NO cascade evaluated in our study is one of these. Other mechanisms suspected in atherosclerosis development due to HHcy are summarised as oxidative modifications of low density lipoproteins, direct oxidative damage to endothelial cells, promotion of thrombosis due to loss of endothelial cell anti-thrombotic

function, and platelet activation and induction of the inflammatory process of atherogenesis.[19,37] Another reason for the results noted above may be related to factors affecting ADMA metabolism, as noted in previous reports.[35,36]

NO is the major molecule that contributes to the anti-atherosclerotic effects of the endothelium through its biological properties, such as vasodilatation, inhibition of platelet aggregation, leucocyte adhesion to the vascular endothelium, and protection of plasma LDL against oxidation.[10] Thus, a decrease in NO levels contributes to the destructive process. However, to date, no reported study has investigated NO levels together with Hcy and ADMA levels in children taking AEDs. We found no significant difference in NO levels between control and patient groups. Moreover, no significant correlation with Hcy–ADMA–NO levels was found. In our study, the absence of a significant increase in ADMA levels between groups may be one reason for our not finding a significant change in NO levels. Our study design was cross sectional. Thus, we did not know baseline Hcy, ADMA, and NO values of the patients before the start of AED treatment. By taking these factors into consideration, further studies with a relatively larger sample size may clarify whether the suggested association occurs among Hcy, ADMA, and NO in children taking AEDs.

5. Conclusion

A significant level of hyperhomocysteinemia may develop in patients using OXC, which is a new-generation AED. Thus, contrary to some reports, OXC may not be a safe choice for patients who already possess additional risk factors for the development of HHcy. Although we found no significant differences in Hcy, NO, and ADMA levels between study and control groups, further prospective large-scale and longer term studies investigating all pathways suggested to be responsible for the development of atherosclerosis due to HHcy should be conducted to define the exact mechanism(s) responsible for AEDs related atherosclerosis.

Conflicts of interest: The authors declare that there is no conflict of interest in this paper.

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