ORIGINAL ARTICLE

Homocysteine Metabolism and Hematological Parameters in Early Stage of Phenytoin Treated Epileptic Children

Tarun K. Sharma [1], S. K. Vardey [2], S. Sitaraman [3]

[1] Department of Biochemistry, Gajra Raja Medical College, Gwalior, Madhya Pradesh, India
[2] Department of Biochemistry, S.M.S. Medical College, Jaipur, Rajasthan, India
[3] Department of Pediatrics, Sir Padampat Mother & Child Health Institute, S.M.S. Medical College, Jaipur, Rajasthan, India

SUMMARY

Background: Long-term antiepileptic drug (AED) therapy has been associated with metabolic consequences that lead to an increase in risk of atherosclerosis in patients with epilepsy. Earlier published studies showed conflicting results about the levels of hematological parameters, serum homocysteine, folate, and vitamin B_{12}, in epileptics treated with phenytoin monotherapy. Therefore, we evaluated homocysteine metabolism and hematological parameters in early stage of phenytoin treated epileptic children.
Methods: A total of 64 newly diagnosed epileptic children with mean age 10.09 ± 2.56 years were enrolled at the start of study. However, after 3 months follow up, the final total sample size was only 50 epileptic children. Fourteen children dropped out of study due to poor follow up. Serum homocysteine levels were measured by enzyme immunoassay method. Serum folate and vitamin B_{12} levels were estimated by Competitive Chemiluminescent Enzyme Immunoassay method. Hematological parameters were analysed by an automated hematology analyzer (Cell counter), Sysmex XT-1800i, using commercially available reagents.
Results: In our study the anthropometric and hematological parameters did not show any significant difference after phenytoin monotherapy as compared to before therapy in epileptic children. The serum homocysteine level in epileptic children was found to be significantly increased after phenytoin (PHT) monotherapy as compared to before therapy. Moreover, a highly significant decrease was observed in the serum folate and vitamin B_{12} levels after phenytoin monotherapy as compared to before therapy in epileptic children.
Conclusions: Phenytoin monotherapy may cause a significant increase in the levels of serum homocysteine and a significant decrease in the serum folate and vitamin B_{12} levels in children with epilepsy, and the significant changes in above mentioned parameters occur early in the course of treatment. This could be responsible for a higher prevalence of cardiovascular incidents in epileptic children taking phenytoin monotherapy. Therefore, it may be useful to do early screening and treatment of increased serum homocysteine levels in epileptic children under phenytoin monotherapy to prevent atherosclerosis and its complications. Hematological parameters should also be strictly monitored regularly in individuals administered with PHT monotherapy. If there are persistent alterations, the administration of the drugs should be discontinued.
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Correspondence:
Dr. Tarun Kumar Sharma
Assistant Professor
Department of Biochemistry
Gajra Raja Medical College
474009 - Gwalior
(Madhya Pradesh), India
Mobile: +91 7597604023
         +91 9407523057
Email: tarunggc@gmail.com
         sharma_bio82@yahoo.co.in

INTRODUCTION

The relationship between hyperhomocysteinemia and vaso-occlusive diseases has been known for a long time. Antiepileptic drugs (AEDs) lead to hyperhomocysteinemia by affecting the levels of folate and vitamin B_{12} which have a role in the metabolism of homocysteine. Hyperhomocysteinemia in turn causes vascular endothelial dysfunction and results in atherosclerosis [1-4]. Hyperhomocysteinemia has been associated with an increased risk for occlusive vascular disease [5]. Long-term treatment with phenytoin (PHT) may lead to hyperhomocysteinemia by affecting the blood concentrations of folate, which have a role in the metabolism of homocysteine [6,7]. Sener U et al. revealed that patients receiving phenytoin had significantly increased serum homocysteine and significantly decreased folate levels while the effects on the concentrations of vitamin B_{12} remain nonsignificant as compared to healthy controls [8]. Linnebank M et al. reported that patients treated with phenytoin were associated with lower mean serum folate levels or with a higher frequency of folate levels below the reference range in comparison with the entire group of patients, untreated patients, or controls, and this is a risk factor for hyperhomocysteinemia. Oral substitution is effective to restore vitamin and homocysteine levels [9].

The tendency toward lower folate and higher total homocysteine (tHcy) concentrations may put children on AEDs at special risk for atherosclerosis [9]. Compared to adults, children taking valproic acid (VPA) suffer from folate depletion and hyperhomocysteinemia, which is an atherosclerotic risk factor [9,10]. The factors of early childhood origin of adult cardiovascular risks are now known and demand early intervention. In Asian Indian children who are genetically more exposed to CVD (cardiovascular disease) risks, AED therapy is an additional risk for future development of CVDs. Elevated tHcy levels due to AED use can alter the circulatory markers of vascular risk, such as common carotid artery intima thickness which is positively correlated with the duration of AED therapy [11]. Studies reported that in those on AEDs, the standardized mortality ratio (SMR) due to cerebrovascular disease attributed to atherosclerosis was high [12]. Children born to women taking AEDs during the first trimester of pregnancy have been shown to have higher chances of cardiovascular malformations and neural tube defects. The impacts of folic acid deficiency are higher during childhood when there is increased cell division [13]. The children on AED have to take it for many years, and tHcy elevation itself has an epileptogenic potential and can cause the risk of resistance to treatment leading to development of refractory epilepsy [14]. The association of folate depletion and hyperhomocysteinemia with reduced cognitive performance in children was reported [13]. Antiepileptic drugs are hematotoxic i.e., there is a decrease in hemoglobin concentration, red blood cell (RBC) and white blood cell (WBC) counts after long term anti-

epileptic therapy [15]. In contrast to these findings some studies mention that AEDs do not have any effect on the hematological parameters of epileptic patients [16]. However, there are limited studies about the early effect of phenytoin monotherapy on the hematological parameters, fasting serum homocysteine, folate, and vitamin B_{12} levels. However, only few studies are available on the effect of phenytoin monotherapy on serum homocysteine, folate, and vitamin B_{12} levels in newly diagnosed epileptic children, as most of the studies were between healthy controls and epileptic children after phenytoin monotherapy. Moreover, to the best of our knowledge, no such study, which observed the early effects of phenytoin monotherapy on the levels of hematological parameters, serum homocysteine, folate, and vitamin B_{12}, before and after phenytoin monotherapy, has been reported until now. Therefore, the present study is designed to evaluate the effect of a 12-week period of phenytoin monotherapy on the hematological parameters, serum homocysteine, folate, and vitamin B_{12} levels in epileptic children after initiation of phenytoin monotherapy.

MATERIALS AND METHODS

Study design

The present observational study was conducted in the Department of Biochemistry, in association with the Department of Pediatrics, Sir Padampat Mother & Child Health Institute, S.M.S. Medical College and attached Hospitals, Jaipur, India.

A total of 64 newly diagnosed epileptic children in the age group of 2 - 15 years were enrolled at the start of study. However, after 3 months follow up, the final total sample size was only 50 epileptic children (26 males, 24 females, mean age: 10.09 ± 2.56 years). Fourteen children dropped out due to poor follow up. Phenytoin monotherapy doses were given according to standard dose schedule [17]. The diagnosis of epilepsy was made by a qualified neurologist and treatment was initiated by him using standard protocol.

Epileptic children receiving antiepileptic drugs other than phenytoin (PHT) monotherapy, or already taking any antiepileptic drug (AED) and/or other drugs for chronic problems known to affect homocysteine levels, or vitamin supplements were excluded. Epileptic children with development delay, any metabolic problems, chronic systemic disease (liver or kidney disease) like hepatitis/nephrotic syndrome, HIV infection etc. and severe anemia were excluded from the study. Children with seizures due to meningitis or head injury were excluded from the study. A detailed history and examination was carried out.

The protocol was approved by the institutional CTSC (Clinical Trial and Screening Committee) and Ethics committee. An informed and written consent was obtained from all subjects' guardians after explaining the objectives of the study.

Collection and analysis of blood samples

Overnight fasting blood samples were collected from all study subjects under sterile conditions at time of enrolment and after 3 months of phenytoin monotherapy. Blood was collected in different correspondingly labeled tubes [plain tube and EDTA tube]. The blood sample was immediately allowed to clot for no more than 30 minutes before centrifugation and separation of serum for analysis of serum homocysteine, folate, and vitamin B_{12} levels. Blood samples were immediately processed in order to prevent artificial variations of homocysteine due to the products of *in vitro* erythrocyte metabolism. Since the level of homocysteine is strongly influenced by the pre-analytical conditions, serum homocysteine level was estimated immediately after clotting process of blood sample. The serum was separated from the clotted sample by centrifugation at 3000 rpm and used for analysis. Samples with signs of hemolysis were discarded. Serum aliquots were quickly separated and frozen at -20°C until analysis.

Serum homocysteine levels were measured by enzyme immunoassay method (EIA method) [18] using the kits provided by Axis-Shield Diagnostics Ltd (Dundee DD2 1XA, United Kingdom) with a precision of 10%, as per the instruction provided by the manufacturer. Serum folate (competitive immunoassay) [19] and serum vitamin B_{12} (competitive chemiluminescent enzyme immunoassay) [20] were estimated using an Immulite 2000 analyzer (SIEMENS). Hematological parameters were analysed by an automated hematology analyzer (cell counter), Sysmex XT-1800i, using commercially available reagents. The procedures given in the manuals, accompanying the kits, were strictly followed.

Statistical analysis

The presentation of the results is in the form of mean ± standard deviation. SPSS for windows (version 19, Chicago, IL, USA) was used for the analysis of data collected. The means of epileptic children were compared at beginning and end of PHT monotherapy using the paired-samples *t*-test. Chi square test was applied to check the male and female ratio in all groups. For all statistical assessments, a value of $p < 0.05$ was accepted to be significant.

RESULTS

In our study the anthropometric and hematological parameters did not show any significant difference after phenytoin monotherapy as compared to before therapy in epileptic children (Table 1 and 2). Table 3 and Figure 1, 2, and 3 show the comparison of serum homocysteine, folate, and vitamin B_{12} levels in epileptic children, before and after phenytoin monotherapy. The mean value of serum homocysteine before therapy was 7.30 ± 1.65 µmol/L and after therapy was found to be increased (9.16 ± 2.93 µmol/L), which was highly significant ($p = 0.000$). Moreover, a highly significant decrease was observed in the mean values of serum folate ($p = 0.000$) and vitamin B_{12} ($p = 0.000$) in epileptic children after phenytoin monotherapy (5.96 ± 2.16 ng/mL and 362.10 ± 137.47 pg/mL, respectively) as compared to before therapy (7.66 ± 1.50 ng/mL and 397.82 ± 123.90 pg/mL, respectively).

DISCUSSION

Patients on anti-epileptic drugs are prone to high concentrations of plasma homocysteine and low status of folate and vitamin B_6 [21-23]. The consequences of this metabolic disturbance may be of clinical importance. First, in addition to its adverse effects on atherogenic lipid levels [24], increased homocysteine levels associated with anti-epileptic drugs may be a risk factor for systemic vascular events including stroke. Because the use of the anti-epileptic drug may be extended over years, chronic vascular toxicity may be an important concern. Second, increased homocysteine and low folate status may contribute to the development of anti-epileptic drug related side effects, such as impaired cognitive function and fetal malformations including neural tube defect [25], for which hyperhomocysteinemia (HHcy) is an established independent risk factor [26]. Third and the most speculative, increased homocysteine may also be involved in poor seizure control in epileptic patients, based on the fact that its systemic application is known to cause an animal model of epilepsy [27]. Folate is important for cells and tissues that rapidly divide e.g., bone marrow [28]. Anti-folic acid activity of phenytoin is responsible for bone marrow depression that results in blood dyscrasias such as thrombocytopenia, leucopenia, and aplastic anemia [29]. Bhosale UA et al. observed that platelet count was significantly reduced in epileptic treated with carbamazepine, phenytoin, phenobarbitone, and valproate (66.6%) as monotherapy or combination therapy compared to the newer AED combination therapy (33.3%). This toxicity was prominent in valproate, phenytoin, and phenobarbitone treated epileptics singly or in combination, and at the same time leucopenia was significant in phenytoin and carbamazepine monotherapy treated patients (26.6%). AED monotherapy has overall less toxic effects on the lipid profile, hemoglobin (Hb) %, and RBC count compared to AED combination therapy, although it was found less promising than newer AED combination therapy pertaining to platelet count and WBC count [30].

Folic acid is itself not biologically active, but its biological importance is due to tetrahydrofolate and other derivatives after its conversion to dihydrofolate in the liver by dihydrofolate reductase. This reductase is said to be blocked by AEDs [31]. Anti-folate activity can also lead to decreased hemoglobin (Hb) % and homocysteine accumulation [32] which further affect lipid metabolism [33]. In earlier studies AEDs have minimal or no effect on hematological parameters [16].

Table 1. Comparison of anthropometric parameters in epileptic children before and after phenytoin (PHT) monotherapy (mean ± SD).

Variable	Epileptic children before PHT (n = 50)	Epileptic children after PHT (n = 50)	p-value
Gender (male/female)	26/24	26/24	
Age (years)	10.09 ± 2.56		
Weight (kg)	27.55 ± 10.08	27.68 ± 10.01	0.086 [†]
BMI (kg/m^2)	15.64 ± 2.41	15.72 ± 2.35	0.088 [†]
Waist Hip Ratio	0.8381 ± 0.03759	0.8382 ± 0.03787	0.816 [†]

[†] - Non significant.

Table 2. Comparison of hematological parameters in epileptic children before and after phenytoin (PHT) monotherapy (mean ± SD).

Variable	Epileptic children before PHT (n = 50)	Epileptic children after PHT (n = 50)	p-value
Hemoglobin (g/dL)	13.32 ± 1.19	13.20 ± 1.15	0.286 [†]
RBC count (mill/cumm)	4.63 ± 0.39	4.56 ± 0.46	0.161 [†]
PCV (%)	40.11 ± 3.45	39.73 ± 3.59	0.186 [†]
MCV (fL)	86.82 ± 4.60	87.49 ± 6.22	0.312 [†]
MCH (pg)	28.80 ± 1.48	29.04 ± 1.80	0.170 [†]
MCHC (g/dL)	33.12 ± 0.99	33.25 ± 1.41	0.603 [†]
RDW-CV (%)	12.41 ± 0.50	12.50 ± 0.61	0.276 [†]
Platelet count (10^3/µL)	254.14 ± 56.07	260.02 ± 51.88	0.598 [†]
TLC (1000/cumm)	8.36 ± 1.69	8.01 ± 1.64	0.187 [†]

[†] - Non significant.
List of abbreviations: RBC count - red blood cell count, PCV - packed cell volume, MCV - mean corpuscular volume, MCH - mean corpuscular hemoglobin, MCHC - mean corpuscular hemoglobin concentration, RDW-CV - red cell distribution width - coefficient of variation, TLC - total leucocyte count.

Table 3. Comparison of serum homocysteine, folate, and vitamin B$_{12}$ in epileptic children before and after phenytoin (PHT) monotherapy (mean ± SD).

Variable	Epileptic children before PHT (n = 50)	Epileptic children after PHT (n = 50)	p-value
Serum homocysteine (µmol/L)	7.30 ± 1.65	9.16 ± 2.93	0.000 [*]
Serum folate (ng/mL)	7.66 ± 1.50	5.96 ± 2.16	0.000 [*]
Serum vitamin B$_{12}$ (pg/mL)	397.82 ± 123.90	362.10 ± 137.47	0.000 [*]

[*] - Significant.

The anthropometric and hematological parameters did not show any significant difference after phenytoin monotherapy as compared to before therapy in epileptic children (Table 1, 2). The possible reason may be that the duration of the study was short for the hematological parameters to change; moreover, ethical considera-
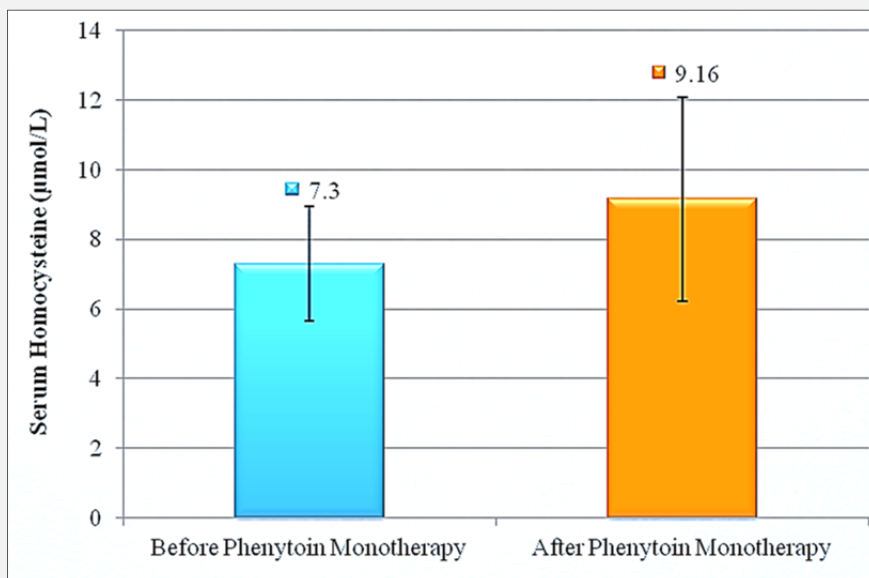
Figure 1. Comparison of serum homocysteine in epileptic children before and after Phenytoin (PHT) monotherapy.
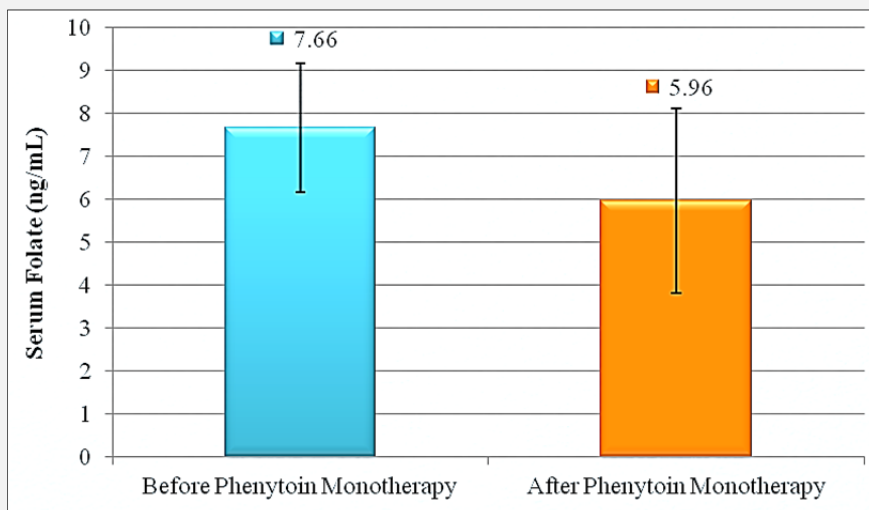

Figure 2. Comparison of serum folate in epileptic children before and after phenytoin (PHT) monotherapy.

tion required the author not to withhold the folic acid and vitamin supplementation.

The mean value of serum homocysteine before therapy was 7.30 ± 1.65 µmol/L and after phenytoin monothera-py was found to be increased (9.16 ± 2.93 µmol/L) in epileptic children, which was highly significant (p = 0.000) [Table 3, Figure 1]. Sener U et al. reported a sig-nificant increase in the levels of serum homocysteine
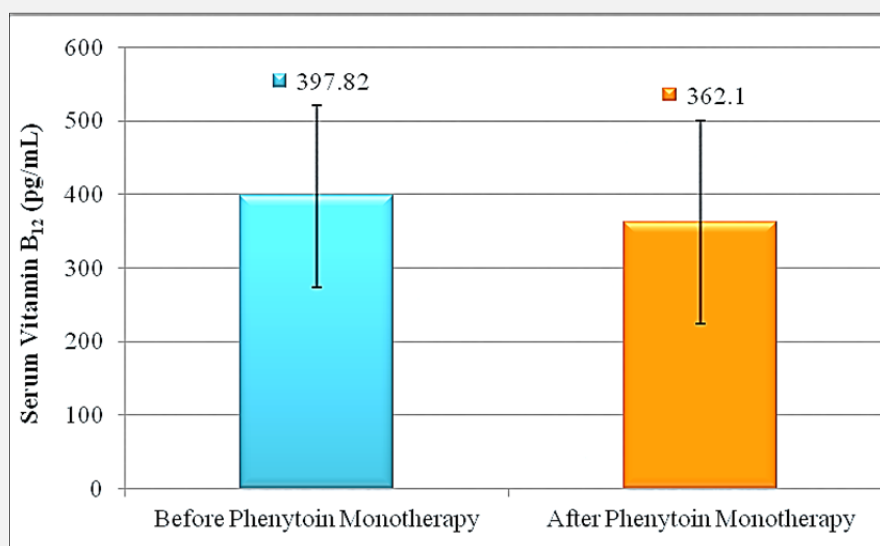
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Figure 3. Comparison of serum vitamin B12 in epileptic children before and after phenytoin (PHT) monotherapy.

in phenytoin treated patients as compared to untreated patients. No difference was observed between mean homocysteine concentrations measured in the newly-diagnosed untreated patients and healthy controls [8]. Hyperhomocysteinemia is an independent risk factor for cardiovascular disease and thrombosis. A 5 µmol/L higher total homocysteine (tHcy) level is associated with a 27% higher risk for venous thrombosis in prospective studies [34], and children with stroke have a higher prevalence of the methylenetetrahydrofolate reductase (MTHFR) 677TT genotype [35]. Endothelial dysfunction, as present in hyperhomocysteinemia, is a precursor of atherosclerosis [36]. It is not completely understood how homocysteine impairs endothelial function, but the induction of oxidative stress and the suppression of nitric oxide synthesis may be due to an increase in asymmetrical dimethylarginine concentrations [37] and seems to play a crucial role. Independent of its homocysteine-lowering capacity, folate may improve endothelial function: the folate metabolite 5-methyltetrahydrofolate appears to enhance endothelial nitric oxide synthesis in healthy adults [38], and flow mediated vasodilatation improved in children with type I diabetes after oral supplementation with folate [39]. Therefore, the tendency toward lower folate and higher tHcy concentrations may put children taking antiepileptic drugs at special risk for atherosclerosis. Other important reasons to monitor tHcy and folate in epileptic patients are the epileptogenic potential of tHcy [40] and the association of folate depletion and hyperhomocysteinemia with reduced cognitive performance [41].

Table 3 and Figure 2 show the comparison of serum fo-late in epileptic children, before and after phenytoin monotherapy. The highly significant decrease was observed in the mean value of serum folate (p = 0.000) in epileptic children after phenytoin monotherapy (5.96 ± 2.16 ng/mL) as compared to before therapy (7.66 ± 1.50 ng/mL). Sener U et al. reported significantly lower serum folate levels in phenytoin treated patients as compared to the healthy controls. There was no difference in serum folate levels among newly-diagnosed untreated patients and healthy controls. A significant negative correlation was found between the levels of homocysteine and folic acid (r = -0.332, p < 0.05) in patients using phenytoin and in entire study population [8]. Linnebank M et al. reported that phenytoin monotherapy was associated with lower mean folate serum levels compared with untreated patients and healthy controls, and patients treated with phenytoin monotherapy had serum folate levels below the reference range more often than untreated patients and controls. Correlation analysis of AED dose with folate levels did not reveal significant results for patients with monotherapy [9].

Table 3 and figure 3 show the serum vitamin B_{12} (p = 0.000) levels were significantly lower in epileptic children, after phenytoin monotherapy (362.10 ± 137.47 pg/mL) as compared to before therapy (397.82 ± 123.90 pg/mL). Sener U et al. had reported non-significant changes in the levels of serum vitamin B_{12} between newly diagnosed untreated patients and phenytoin treated patients or newly-diagnosed untreated patients and healthy controls [8]. Linnebank M et al. reported that the frequency of vitamin B_{12} levels below the refer-

ence range in the entire group of patients treated with phenytoin did not significantly differ from untreated patients or controls [9].

We have also previously shown that epileptic children treated with carbamazepine monotherapy may exhibit early and significant reduction in cofactors, such as serum folate and vitamin B_{12}, and a significant increase in serum homocysteine level in these children [42]. Therefore, the present study suggests that children taking AEDs that induce cytochrome P450 isozymes (phenytoin) are associated with a significant elevation in homocysteine concentration, as well as a significant reduction in serum folate and vitamin B_{12} concentrations. The proposed mechanisms for the folate and vitamin B_{12} depletion and increase in homocysteine levels with AEDs (phenytoin monotherapy) in the present study may be (i) interference with the intestinal absorption of folate by increasing the pH, (ii) interference with folate transport into tissues, and (iii) hepatic microsomal induction and increased folate catabolism [43,44]. Other potential mechanisms through which this may occur include: the hepatic induction of these cytochrome P450 isozymes impairing intestinal absorption of folate through a competitive interaction between folate coenzymes and drugs, the accelerated degradation or depletion of folate, the increase or dysfunction of homocysteine metabolism in the liver, the acceleration of vitamin metabolism, and the interference in the metabolism of folate coenzymes [40,45]. Little is known about how phenytoin exerts its effects on homocysteine metabolism. It has been suggested that phenytoin, as an enzyme inducer, can directly modulate the activity of different liver enzymes. Liver enzyme induction may cause depletion of the cofactor involved, folate, pyridoxal 5'-phosphate, and vitamin B_{12}, leading to the alterations observed in homocysteine status [46].

The difference in the study results might be because of the different genetic make-up of our population. Individuals with C677T variant in the MTHFR (methylenetetrahydrofolate reductase) gene have higher levels of homocysteine as compared to normal MTHFR gene variants [47]. In our study, the genetic variation was not determined. Homocysteine levels are known to be different in different populations. Our results demonstrated that children treated with PHT monotherapy may exhibit early and significant reduction in cofactors, such as serum folate and vitamin B_{12}. Thus, the early occurrence of increased serum homocysteine level in these patients can be secondary to reduced cofactor levels, although more studies are needed to clarify this issue.

Limitations and future outlook

A limitation of the present study is that the epileptic children were followed for only 3 months, not until the end of therapy. The changes in the values of all the parameters studied in the majority of epileptic children were within the reference range of parameters for the age though the difference was significant only for serum homocysteine, folate, and vitamin B_{12} not for hemato-

logical parameters. Therefore, long term studies are required to evaluate the maximum effect of phenytoin monotherapy on the levels of hematological parameters, serum homocysteine, folate, and vitamin B_{12}.

This study has shown changes in the values of hematological parameters, serum homocysteine, folate, and vitamin B_{12} in a short period of 12 weeks but long duration may show how the effects persist and the mechanism by which it is brought about.

Further studies are required to consider the effects of different dosage schedules, polytherapy, and vitamin supplementation on the parameters studied here.

CONCLUSION

In this study, the epileptic children taking phenytoin monotherapy undergo some considerable changes especially at the level of an atherogenic component (serum homocysteine). We can put forward the hypothesis that increased atherosclerotic risk in epileptic children taking phenytoin monotherapy may be related to altered levels of the serum homocysteine, folate, and vitamin B_{12}.

Thus, it would be better to remember the importance of serum homocysteine, folate, and vitamin B_{12} assay in epileptic children taking phenytoin monotherapy to prevent further atherosclerosis and cardiovascular incidents. Hematological parameters should also be strictly monitored regularly in individuals administered with PHT monotherapy. If there are persistent alterations, the administration of the drugs should be discontinued.

Declaration of Interest:
The authors declare that they have no conflict of interests.

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