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EVALUATION OF DNA DAMAGE USING COMET ASSAY IN CHILDREN ON LONG-TERM PHENOBARBITAL MONOTHERAPY FOR THE PREVENTION OF RECURRENT FEBRILE SEIZURES

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ABSTRACT: The majority of children who have febrile seizures will require no treatment other than parental reassurance. But, in some situations, the parents' anxiety level remains very high even after reassurance, and long-term management of febrile seizures may focus on decreasing parental anxiety. In these severe cases, phenobarbital is the most widely prescribed anticonvulsant drug. Its long-term use may have adverse effects on the various organ systems. Among them, the toxic effect on DNA presents one of the most important medical problems.
The aim of the study was to determine the potential mutagenic effects of long term monotherapy with phenobarbital by means of comet assay in the peripheral blood lymphocytes of children with simple febrile seizures. Twenty-seven children with simple febrile seizures between the ages 29-54 months receiving long-term phenobarbital monotherapy for 7 to 27 months were studied. Control group consisted of 20 healthy sister or brothers of the patients who didn't use any long-term drug. No statistical difference between patient and control groups on comet scores were found ($p>0,05$). Some physician may believe that prevention of future febrile seizure is indicated because of the high degree of parental anxiety. In such a situations, we suggest that it is not necessary to consider the potential mutagenic effects of long-term phenobarbital monotherapy.
[Key words : Phenobarbital, febrile seizures, DNA damage, comet assay]

INTRODUCTION

A simple febrile seizure is defined as a brief (<15 minutes) generalized seizure that occurs only once during a 24-hour period in a febrile child who does not have an intracranial infection or severe metabolic disturbance. After the first febrile seizure, approximately 33 % of children will experience one or more recurrences, and about 9 % of children who have febrile seizures will have three or more. The younger the child when the first febrile seizure occurs, the greater the likelihood of recurrence. Most recurrence (75 %) happen within 1 year. An increased risk of recurrence to be associated with a shorter duration of fever before the initial febrile seizure and a lower temperature has been shown. Family history of febrile seizures is another reported risk factor for recurrence (1, 2). Febrile seizure is the most common seizures in children (3). It occurs in approximately 3 to 5 % of children between the ages of six months and five years who are previously healthy and have no defined cause for their seizure except fever (4).

In spite of the high frequency of febrile convulsions in childhood, there has been no consensus on the need for long-term antiepileptic therapy. Moreover, there is no unanimity of opinion about therapeutic interventions (5, 6). No study has demonstrated that treatment for simple febrile seizures can

prevent the later development of epilepsy. Furthermore, there is no evidence that simple febrile seizures cause structural damage and no evidence that children with simple febrile seizures are at risk for cognitive decline (1, 7). On the other hand, recurrent episodes of febrile seizures can cause anxiety in some parents and their children. In situations in which parental anxiety associated febrile seizures is severe, Long-term antiepileptic drug use may be preferred by some physicians.

Phenobarbital is effective in preventing the recurrence of simple febrile seizures. It has been shown that daily therapy with phenobarbital reduced the rate of subsequent febrile seizures from 25 per 100 subjects per year to 5 per 100 subjects per year (8-10). It is the most widely used antiepileptic drug at febrile convulsions. Potential risks of this treatment should be weighed against benefits.

In the present study, we tested the potential mutagenic effects of long term monotherapy with phenobarbital by means of comet assay in the peripheral blood lymphocytes of children with simple febrile seizures.

MATERIALS AND METHODS

Twenty-seven children with simple febrile seizures between the ages 29-54 months receiving long-term phenobarbital monotherapy for 7 to 27 months were studied. Phenobarbital therapy was started after the third febrile convulsion had been occurred in 19 patients but in eight patients, treatments with phenobarbital were given after second febrile convulsion due to the high degree of parental anxiety. Upper respiratory infection was identified at 35 febrile convulsion attacks as the commonest febrile convulsion triggering factor and the other factors were acute otitis media at 21 cases, urinary tract infection at 9 cases, amebiasis at 3 cases but no etiological agent were identified at 5 cases. Control group consisted of 20 healthy sister or brothers of the patients who didn't use any long-term drug. To our knowledge, neither the children on the prophylaxis nor the control subjects were exposed to other mutagenic agents (e.g. , radiation , chemicals, lifestyle, smoking, drugs , or viruses) during the at least one year before the study , and none of them presented chronic or neoplastic diseases. An appropriate institutional review board approved the project and informed consent was obtained from both parents or legal guardian after the nature of the procedures had been explained fully .

All subjects were healthy at the time of sampling. Five ml of blood was carefully layered over 8 ml Lymphocyte Separation Medium and centrifuged at 2000 x g for 15 min. After the plasma layer was removed and saved, the buffy coat was carefully removed and the cells were washed with TC-199 medium and then collected by 10 min centrifugation at 1000 x g. Lymphocytes were resuspended at approximately 10^7 / ml in TC-199 medium with 20% v/v plasma and 10% v/v plasma and v/v DMSO. Lymphocytes were transferred to microfuge tubes and stored at -20°C. The comet assay was performed as described previously (11). Comets from as broken ends of the negatively charged DNA molecule becomes free to migrate in the electric field towards the anode. The assay provides direct determination of the extend of DNA damage in individual cells and the extend of DNA damage can be assessed from the length of DNA migration which is derived by subtracting the diameter of the nucleus from the total length of the image. The degree of damage was determined by grading the cells as; normal (undamaged - no migration), limited migration (at low damage levels, stretching of attached strands of DNA, rather than migration of individual pieces is likely to occur), and extensive migration (with increasing numbers of breaks, DNA pieces migrate freely into the tail forming comet images). A minimum of 100 cells were analyzed for each subject. Slides were scored blindly by two independent investigators. Statistical comparisons between the grade of DNA damages in control/patient groups were analyzed by using Student t test which assumes Gaussian populations with equal SD's and two sided P values were used.

RESULTS

The data including ages, duration of treatment and comet scores of the 27 children with simple febrile convulsion and 20 controls are shown in table 1 and 2 respectively. The mean ages of patients were 43,703±7,086 (±SD,n=27) months and the mean ages of controls were 39,450±7,646 (±SD,n=20) months and there is no statistical difference between ages of the patient and control group (p>0,05). Sex distributions of two groups were also similar (p>0,05). The mean duration of treatment was 17,259±5,741 (±SD,n=27) months. No patients had new febrile convulsion attacks after starting phenobarbital prophylaxis. No statistical difference between patient and control groups on comet scores were found (p>0,05).

Table 1: Individual data (age, duration of treatment, grade of DNA damage by comet assay) from children treated with phenobarbital.

Subject Number	Sex	Age (months)	Duration of treatment (months)	Grade of damage in 100 cells Undamaged (no migration)	Limited migration	Extensive migration
1	F	34	7	92	7	1
2	F	42	19	89	9	2
3	M	54	22	90	6	4
4	F	38	18	97	3	0
5	M	47	23	99	1	0
6	M	50	17	94	6	0
7	M	29	9	92	7	1
8	F	39	11	98	1	1
9	M	45	9	96	4	0
10	M	42	14	88	6	6
11	F	51	25	96	3	1
12	F	50	20	93	4	3
13	M	53	27	90	7	3
14	M	44	25	98	1	1
15	M	47	26	98	2	0
16	M	30	10	95	4	1
17	M	49	23	94	5	1
18	M	45	12	90	8	2
19	F	34	16	91	7	2
20	M	46	22	92	6	2
21	F	36	19	95	5	0
22	F	43	13	97	1	2
23	M	40	15	96	2	2
24	M	39	11	91	4	5
25	F	54	19	97	3	0
26	M	49	16	96	2	2
27	M	50	18	94	3	3
Mean		43,703	17,259	94	4,333	1,666
SD		7,086	5,741	3,150	2,353	1,568

Table 2: Individual data (age, grade of DNA damage by comet scores) of control group.

Subject number	Sex	Age (months)	Grade of damage in 100 cells		
			Undamaged (no migration)	Limited migration	Extensive migration
1	M	34	92	6	2
2	F	31	94	4	2
3	F	37	98	2	0
4	M	39	89	7	4
5	M	41	93	6	1
6	M	36	90	8	2
7	F	33	98	1	1
8	M	47	95	3	2
9	F	40	92	6	2
10	F	32	94	5	1
11	F	44	93	5	2
12	M	30	97	3	0
13	M	37	96	3	1
14	F	46	98	1	1
15	F	27	100	0	0
16	M	59	98	1	1
17	M	48	95	4	1
18	F	46	99	1	0
19	F	38	91	5	4
20	M	44	97	3	0
Mean		39,450	94,950	3,700	1,350
SD		7,646	3,170	2,273	1,182

Table 3: Statistical comparisons between the grade of DNA damages in controls/children on prophylaxis.

Group	N	Mean	Std. Deviation	P values
Age	27	43,703	7,086	
Patient	20	39,450	7,646	0,0553
Control				
No Migration	27	94,000	3,150	
Patient	20	94,950	3,170	0,4794
Control				
Limited Migration	27	4,333	2,353	
Patient	20	3,700	2,273	0,4452
Control				
Extensive migration	27	1,666	1,568	
Patient	20	1,360	1,182	0,1038
Control				

DISCUSSION

The most widely used antiepileptic drug at febrile convulsions is phenobarbital (5-ethyl-5-phenylbarbituric acid, PB). Phenobarbital has been used as a hypnotic, a sedative and a major anticonvulsant drug for years. Its long-term use may have adverse effects on the various organ systems; it has been found that the mean IQ was 8,4 points low in the phenobarbital group than in the placebo group at the end of two-year study (12). Phenobarbital is associated with impairment of short-term memory and concentration and worsening of behavior (13, 14).

Potential mutagenic effects of Phenobarbital also have been tested by various methods in adults and children, but the results of these studies are controversial; Phenobarbital was reported to induce chromosome damage in the germ cells of male mice (15), micronuclei in the bone marrow cells (16), and dominant lethal mutations in the germ cells (17). In an other investigation, a significant increase in chromosomal aberration rates has been found in the epileptic children undergoing long-term antiepileptic drug therapy including phenobarbital compared to those observed in control group of epileptic children without treatment (18). In some studies also, it has been reported that phenobarbital has toxic effects on DNA (19-21).

On the other hand, no elevation of chromosome aberrations was observed in Chinese hamster ovary cells with and without metabolic activation (22). Phenobarbital monotherapy does not to be associated with any observable increase in carcinogenesis (23). There was no increase in chromosomal aberrations in an in vivo study of patients treated with phenobarbital (24). In an other study, no mutagenic effect of phenobarbital could be demonstrated as revealed by SCE, but , all the subjects in this study (epileptic and non-epileptic) were cases of cerebral palsies due to perinatal asphyxia (25). Some studies also reported that phenobarbital has no toxic effect on DNA (26-28).

The single cell gel electrophoresis (SCGE) assay that is also known as comet assay is a rapid, simple, visual and sensitive technique for measurement and analyzing DNA strand breakage at the level of single cells in monitoring human cells exposed to mutagenic agents (29-31). The single-cell gel electrophoresis assay (comet assay) was conceived in 1984 (32) and subsequently modified by Singh and colleagues in 1988 (33). The alkaline single-cell gel electrophoresis assay has been used in both in vitro and in vivo studies to assess damage and repair at DNA exposed to various agents in a variety of mammalian cells. Comet assay is now widely used for measuring DNA damage in somatic cells and has been successfully applied to monitor for DNA damage in lymphocyte samples from human populations. The single-cell gel electrophoresis assay is a potentially sensitive system to assess induced genotoxic damage in vivo and in vitro (30,33,34). There is no study; until now, evaluated the toxic effects on DNA of long-term monotherapy with phenobarbital in children using the comet assay.

In the present study, we used the comet assay which is more sensitive to investigate the mutagenic effects of long-term phenobarbital treatment. We could not detect any toxic effects on DNA of this treatment. In our study, the control group consisted of sisters or brothers of the children on the prophylaxis. All the subjects in both groups were of the same environ and socioeconomic status. We believe that long-term monotherapy with phenobarbital has no mutagenic effects.

Because treatment with anticonvulsants will not prevent the development of epilepsy, the goal of the treatment is to prevent recurrent febrile seizures. There are some families for whom febrile seizures are extremely frightening events, and educational and emotional support could not alleviate some of these concerns. Some physician may believe that prevention of future febrile seizure is indicated because of the high degree of parental anxiety. In such a situations, we suggest that it is not necessary to

consider the potential mutagenic effects of long-term phenobarbital monotherapy.

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