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ANALYSIS OF DNA DAMAGE USING THE COMET ASSAY IN FEMALE PATIENTS TREATED WITH PHENYTOIN FOR EPILEPSY

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ABSTRACT: Women with epilepsy have been encouraged to consider marriage and child bearing in recent years. Antiepileptic drugs are mostly used such as phenytoin, but its effect on fetus health and its long term effects on DNA have not been enough clear yet. The decision to continue or initiate pharmacotherapy for epilepsy during pregnancy becomes complicated. Therefore it was aimed to determine the potential toxic effects of long term phenytoin monotherapy on the peripheral blood lymphocytes of female patients with epilepsy using the comet assay. Twenty-three female patients on a long-term treatment of phenytoin monotherapy for 2-6 years were studied. The epileptic female patients who had normal menstrual cycles, and who were in, otherwise, good health were accepted. They were also nonsmokers. Control group consisted of 23 healthy, nonsmoker female patients, who had normal menstrual cycles and did not use any long-term drugs. The blood samples were taken from the control and patient groups within 20^{th} and 27^{th} days following the beginning of their menstrual bleeding. As a result, the statistical comparison of the comet scores of two groups demonstrated that there is a significant difference in number of damaged cells. Damaged (limited and extensive migrated) cells in the epileptic women who were taking phenytoin were higher than the control group ($p<0,0001$).
[Key words : Phenytoin, epilepsy , pregnancy, DNA damage, comet assay,]

INTRODUCTION

In recent years, women with epilepsy have been encouraged to consider marriage and childbearing. With this new societal tolerance have come new problems; as more women with epilepsy become pregnant and bearing children, it has become clear that they are at greater risk of adverse pregnancy outcomes, including complications that affect the fetus. These risks are well documented and fall into four general areas: death, malformations, dysmorphism, and developmental delay.

Congenital malformations have received the most attention; infants of mothers with epilepsy are at greater risk of developing congenital malformations than are those in the general population (1, 2, 3). The types of malformations observed are fairly diverse (4). The specific variables associated with these increased risks are not clear. Three of the known variables are important: maternal seizures during pregnancy, maternal epilepsy itself, and antiepileptic drugs.

The decision to continue or initiate pharmacotherapy for epilepsy during pregnancy is complicated by the need to balance maternal well-being with fetal safety. Although the first trimester of pregnancy, in particular week 2 to 8 after conception, is the most critical period for drug-induced malformations, the brain and some organs develops throughout pregnancy and some defects may occur after the first trimester (5). Although phenytoin is one of the most frequently prescribed antiepileptic drugs, its

toxicity on DNA has not been enough clear yet.

The purpose of this study was to determine the potential toxic effects of long term phenytoin monotherapy in the peripheral blood lymphocytes of female patients with epilepsy using the comet assay.

MATERIALS AND METHODS

Twenty-three female patients on a long-term treatment of phenytoin monotherapy for 2-6 years (mean=3,547±1.171 years; ±SD,n=23) were studied. The epileptic female patients who had normal menstrual cycles, and who were in, otherwise, good health were accepted. They were also nonsmokers. Control group consisted of 23 healthy, nonsmoker female patients, who had normal menstrual cycles and did not use any long-term drugs. Both, the patient group and control group were informed about the study. The informed consent of the patients and the necessary permissions from the ethical committee were obtained. The blood samples were taken from the control and patient groups within 20^{th} and 27^{th} days following the beginning of their menstrual bleeding. To our knowledge, neither the patients receiving phenytoin nor the control group were exposed to any other mutagenic agents (e.g., radiation, chemicals, lifestyle, smoking, drugs, or viruses) during the at least one year before the study. All subjects were healthy at the time of sampling. Five ml of blood was carefully layered over eight ml Lymphocyte Separation Medium and centrifuged at 2000 x g for 15 min. After the plasma layer was removed and saved, the buff coat was carefully removed and the cells were washed with TC-199 medium. Then they were collected by a 10 min centrifugation at 1000 x g. Lymphocytes were re-suspended 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 they were stored at -20°C. The comet assay was performed as described previously (6). Comets from as broken ends of the negatively charged DNA molecule become free to migrate in the electric field towards the anode. The assay provides direct determination of the extent of DNA. Damage in individual cells and the extent of DNA damage can be assessed from the length of DNA migration. And, this 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). Minimum of 100 cells were analyzed for each study subject. Slides were scored blindly by the independent investigator. 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 ages of the patient group ranged from 24 to 39 years (30,652±4,829; ±SD,n=23). The ages of the controls ranged from 23 to 39 years (29,826±5,882; ±SD,n=23). The statistical comparison of the ages in two groups showed no significant difference (p>0,05). The comet scores and clinical data of the patient and control groups are summarized in Table-1. The statistical comparison of the comet scores of two groups demonstrated that there is a significant difference in number of damaged cells. Damaged (limited and extensive migrated) cells in the epileptic women who were taking phenytoin were higher than those of the controls (p<0,0001).

DISCUSSION

Phenytoin has been used extensively due to the low cost, once-daily dosing, efficacy, and IV formulation. It is associated with fetal hydantoin syndrome characterized by a wide

spectrum of malformations including digit and nail hypoplasia, polidactyly, growth retardation, typical facial appearance, rib anomalies, abnormal palmar creases, hirsutism, and low hairlines and anomalies of the heart and central nervous system and rarely ambigious genitalia in infants born to women taking phenytoin during pregnancy (7). Antiepileptic drugs, particularly phenytoin, have been suspected to be toxic on DNA in humans and experimental animals. It forms a highly reactive arenoxide intermediate known to have cytotoxic, teratogenic and carcinogenic properties, during its biotransformation. It is reported that the genotoxic effects of phenytoin mediated by this intermediatwe metabolite (8). In some investigations, It has been reported increased SCE frequencies exposed to higher phenytoin concentrations (9, 10), increased the frequencies of chromosomal aberations (11), frameshift mutations in salmonella typhimurium (12), and SCE induction by in vitro phenytoin treatment (13). In an other study also, it has been found that phenytoin when added to cultures of normal human lymphocytes increased the frequency of SCE, and this increase was dose dependent (14).

On the other hand, it has been reported that phenytoin, phenobarbital, carbamazepine and primidone and their metabolites showed no effect on SCE frequency (15). In an other study also, no mutagenic effect of phenytoin could be demonstrated as revealed by SCE (there was no difference in the frequency of SCE between treated and untreated groups), but , all the subjects in this study (epileptic and non-epileptic) were cases of cerebral palsies due to perinatal asphyxia (16).

The single cell gel electrophoresis (SCGE) assay also known as comet assay is a rapid simple, visual and sensitive technique for measurement and analyzing DNA breakage in mammalian cells. One of the advantages of SCGE assay is that it can be used to measure DNA breaks in virtually any cell type. DNA damage is known as responsible from teratogenity and cancerogenesis (17-22). Several studies evaluated the effects of phenytoin on DNA, but none of them has used the comet assay until now. For the first time, we used the comet assay to investigate the toxic effects of phenytoin on DNA in the peripheral lymphocytes of female epileptic patients treated with only phenytoin. We found a significant difference in the comet scores between the patients and the healthy women. The factors that may have influence on the comet scores (age, sex, race, nutrition, environ etc.) were similar in both groups. Physiological factors that may have effects on DNA are reproductive hormones; evaluation of SCE frequencies during a normal menstrual cycle demonstrated a higher rate of ovulation, and in the luteal phase as compared to the early follicular phase (23). In our study, all the subjects (patients and the control group) were at the same phase of the menstrual cycle (within 20^{th} and 27^{th} days following the beginning of menstrual bleeding) at the time of sampling. Therefore, we think that the difference in the comet scores was induced by the exposure to the phenytoin. These results support the idea that the exposure to phenytoin is associated with DNA damage which may be associated with teratogenity. We suggest that this possible toxic effect of phenytoin should be considered in the treatment of epilepsy, especially in women who may be pregnant.

Table 1. Clinical data and comet scores obtained from the patients and control subjects.

Subject	EPILEPTIC WOMEN TREATED WITH PHENITOIN					CONTROL GROUP			
	Age (years)	Usage periode (years)	Undamaged (no miggration)	Limited migration	Extensive migration	Age (years)	Undamaged (no migration)	Limited migration	Extensive migration
1	27	2.5	90	7	3	35	96	2	2
2	24	2.8	79	12	9	39	95	3	2
3	34	3.5	87	9	4	24	100	-	-
4	31	4	86	8	6	29	95	4	1
5	36	3.7	89	8	3	25	98	2	-
6	37	5.9	79	13	8	27	95	4	1
7	25	6	90	7	3	26	93	6	1
8	28	5	87	9	4	28	99	1	-
9	29	3	85	9	6	29	94	5	1
10	32	2.5	84	8	8	24	97	2	1
11	34	2.7	90	7	3	23	99	1	-
12	35	3.5	91	6	3	38	91	6	3
13	26	3.1	83	11	6	37	92	6	2
14	39	2.5	88	9	3	39	89	8	3
15	27	3.5	85	11	4	29	94	5	1
16	26	3.7	86	8	6	25	100	-	-
17	25	6	85	8	7	24	97	3	-
18	29	4	87	8	5	25	90	7	3
19	34	2	91	7	2	26	93	5	2
20	36	2.8	88	9	3	24	97	2	1
21	38	2.6	85	9	6	39	92	6	2
22	24	3.8	83	11	6	37	96	3	1
23	29	2.5	87	9	4	34	92	6	2
Mean	30,652	3,547	86,304	8,826	4,869	29,826	94,956	3,782	1,260
SD	4,829	1,171	3,308	1,749	1,961	5,882	3,154	2,295	1,009
n	23	23	23	23	23	23	23	23	23

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