Genotoxicity Evaluation in Female Patients On Valproic Acid Monotherapy Using Alkaline Single Cell Gel Electrophoresis (Comet Assay)

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Valproic acid is a commonly used antiepileptic drug for chronic therapy and may have adverse effects on gastrointestinal, hematologic and nervous system. Potential mutagenic effects of sodium valproate have been reported, but this effect of valproic acid has still not been clarified yet. Comet assay was performed in peripheral lymphocytes of 24 epileptic adolescent and adult female treated with valproic acid monotherapy for at least one year and in a control group including 16 adolescent and adult healthy, drug free females. The frequencies of comet scores in the valproic acid treated epileptic patients were significantly higher than that of the control group ($p<0.05$) indicating a detectable chromosome damaging effect of valproic acid monotherapy on human lymphocytes. No significant correlation was noted amongst age, drug dose, blood valproic acid levels, duration of valproic acid therapy and the comet scores. Although genotoxic effects of valproic acid was shown by means of sister chromatid exchange and chromosomal aberration assays in previous studies, we report here the first demonstration of genotoxic potential of valproic acid with comet assay. This data supports the conclusion that valproic acid is genotoxic.

Keywords: *Valproic acid, epilepsy, alkaline comet assay, genotoxicity*

The anticonvulsant drug, valproic acid (VPA) is used in a wide spectrum of seizure disorders, for example, it is the most effective agent available for treatment of myoclonic seizures. However it may have many side effects including hepatotoxicity, nausea, vomiting, sedation, ataxia, tremor, thrombocytopenia, impaired platelet function, and occasionally severe bleeding[1,2]. An association between anticonvulsant drug treatment during pregnancy and congenital anomalies was first shown in 1968 by Meadow [3]. The teratogenic effect of valproic acid in certain animals resulting in congenital neural tube defects was reported in 1982 (4). Similar teratogenetic effects have been reported in infants exposed to valproic acid in utero (5-7). Diliberti and colleagues also described typical facial features, including epicanthal folds which continued inferiorly and laterally to form a crease or groove just under the orbit, flat nasal bridge, small upturned nose, long upper lip with a relatively shallow philtrum, a thin upper vermillion border, and downturned angles of the mouth, which were termed as the fetal valproate syndrome and associated with intrauterin valproic acid (VPA) exposure (8). Other congenital features including lung hypoplasia, ompholocele, diastasis recti, duodenal atresia, reduction deformity of limb, postaxial polydactily, arachnodactily, congenital heart disease, and hemangioma associated intrauterin valproate exposure have been described (9-12).

Potential mutagenic effects of sodium valproate have also been reported. A significant increase in chromosomal aberration rates in adults treated with antiepileptic drugs has been reported previously (13-15) although no increase was observed in epileptic children (16, 17). Several workers have studied the genotoxic effects of valproic acid using sister chromatid exchange (SCE) analysis but the results are contradictory. Taneja et al. suspected that the increase in SCE frequency detected in lymphocytes of epileptic children treated with valproic acid was not related to the drug, but instead was associated with the disease state (18). In contrast, Hu et al. reported an increase in SCE frequency in epileptic children that was independent of epilepsy (19). However, the mutagenic effect of sodium valproate has not yet been clarified.

The comet assay (single-cell gel electrophoresis) has been recognized as a sensitive indicator of DNA strand breaks at the level of single cells and is widely used not only as an useful in vitro test to assess the genotoxicity of environmental substances but also as a valuable method for monitoring human population exposed to genotoxic

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agents (20, 21, 22, 23). The comet assay, a rapid, simple, visual and sensitive method suitable for detecting DNA damage of human lymphocytes, has not yet been used in epileptic patients undergoing valproic therapy.

The purpose of this study is to determine the potential genotoxic effects of long term monotherapy with valproic acid using comet assay in the peripheral blood lymphocytes of epileptic patients.

Material and Methods

Subjects

Twenty-four epileptic female patient between the ages of 14 to 40 years (mean 22.50 years) receiving long-term valproic acid monotherapy for 1-7 years (mean 3.25 years) were studied. All patients were non-smoker and had normal menstruel cycles. The valproic acid levels of the patients were within the normal therapeutic levels of 50-100 mg/ml. Control group consisted of 16 healthy, non-smoker female patients, who had normal menstrual cycles and without long-term drug usage. The blood samples were taken from the control and patient groups within 20^{th} and 27^{th} days following the beginning of menstrual bleeding. Both the patient and control groups were informed of the study, written permissions were obtained and reviewed by the ethical committee of the institution.

Genetic assays

The alkaline comet assay

Preparation of peripheral blood lymphocytes

Five ml of blood was carefully layered over 8 ml Lymphocyte Seperation 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 cells/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 frozen at -80°C.

The aplication of alkaline comet assay

All chemicals were purchased from the Sigma Chemical Company unless otherwise stated. Lymphocyte Separation Medium was from ICN Flow and TC-199 from Gibco. Superfrost 1.0 - 1.2 mm thick microscope slides from Merck were used. Normal and low melting point agarose were obtained from Gibco. Dulbecco's phosphate-buffered salts (PBS), without Mg and Ca, was from ICN Flow.

The comet assay was used with minor modifications as previously described (24). The comet assay protocol was carried out under dim light to prevent any additional DNA damage. Darkin fully frosted microscope slides were each covered with 100 ml of 0.5% normal melting point agarose in Ca^{+2} and Mg^{+2} - free PBS at 45°C. The agarose layer was immediately covered with a large no. 1 cover slip and then kept at 4°C until the agarose had solidified. Seventy-five ml of 0.5% low melting point agarose (LMA) at 37°C was added to the lymphocytes (1500-100000 cells) suspended in 10 ml of PBS. After gently removing the cover slip, the cell suspension was rapidly pipetted on to the first agarose layer, spread using a cover slip, and allowed to solidify at 4°C. A final layer of 75 mL of 0.5% LMA was applied in the same way. The slides were immersed in freshly prepared, cold lysing solution (2.5 M NaCl2, 100mM Na2 EDTA, 10 mM Tris, pH 10, 1% sodium sancosinate with 1% Triton X-100 and 10% DMSO added just before use) for 1 hr at 4°C. Slides were removed from the lysing solution, drained and placed in a horizontal gel electrophoresis tank side by side with the agarose end facing the anode. The tank was filled with fresh electrophoresis buffer (300mM NaOH and 1 mM Na2 EDTA) at 12-15°C to a level approximately 0.25 cm above the slides. The slides were left in the alkaline buffer (pH 13) for 20 min to allow unwinding of the DNA to occur before electrophoresis. Electrophoresis was conducted for 20 min at 25 V adjusted to 300 mA by raising or lowering the buffer level in the tank. Slides were than drained, placed on a tray and flooded slowly with 3 changes of neutralisation buffer (0.4 M Tris, pH 7.5) for 5 min each, to remove alkali and detergents. The slides were again drained before being stained with 50 ml of 20 mg/ml ethidium bromide and a cover slip was placed on top. Slides were stored in a closed container at 4°C and analysed within 24 h, gel dehydration over longer storage times led to deterioration in slide quality.

Comet capture and analysis

Analysis was performed immediately after staining, using a 200 X objective with a Zeiss Optiphot® microscope equipped with an excitation filter of 515-560 nm from a 100-W mercury lamp and a barrier filter of 590 nm. Comets form 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 extent of DNA damage in individual cells and the extent of DNA damage can be assessed from the length of DNA migration which is derived by substracting the diameter of the nucleus from the total length of the image. Several software systems are now available commercially, and can be configured to estimate total DNA content and comet image length. The tail length can also be measured from the trailing edge of the nucleus to the leading edge of tail, using a calibrated scale in the ocular of the microscope. We determined the degree of damage 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)

Table 1: Individual data (age, duration of treatment, drug dose, drug blood level, grade of DNA damage by comet assay

Subject Number	Age (years)	Duration of treatment (years)	Dose mg/kg/day	Blood Level µg/ml	Grade of damage in 100 cells		
					Undamaged (no migration)	Limited migration	Extensive migration
1	16	1	30	55	82	10	8
2	22	3	30	66	88	6	6
3	19	4	50	69	91	4	5
4	21	5	35	61	90	4	6
5	14	3	35	83	90	6	5
6	22	1	30	50	90	5	5
7	26	4	45	60	88	5	7
8	14	7	50	77	87	6	7
9	35	5,5	35	60	90	6	4
10	40	3	30	65	87	6	7
11	20	6,5	50	78	83	9	8
12	16	2	25	60	91	4	5
13	21	2,5	40	52	82	7	11
14	22	3	60	100	88	6	6
15	24	3,5	25	66	90	5	5
16	15	5	30	74	87	5	8
17	29	2,5	35	82	87	6	7
18	17	4	35	70	90	6	4
19	33	3	30	69	82	8	10
20	32	1	30	88	80	9	11
21	26	1,5	30	50	79	9	12
22	20	3	45	97	81	9	10
23	21	3	25	57	82	8	10
24	15	1	20	64	86	5	9

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

Subject number	Age (years)	Grade of damage in 100 cells		
		Undamaged (no migration)	Limited migration	Extensive migration
1	17	91	5	4
2	20	91	6	3
3	35	96	4	-
4	28	97	2	1
5	29	93	5	2
6	34	96	3	1
7	27	98	2	-
8	24	92	7	1
9	36	95	4	1
10	24	92	7	1
11	21	93	3	4
12	27	94	5	1
13	22	97	3	-
14	15	100	-	-
15	20	97	2	1
16	18	98	2	-

A minimum of 100 cells were analysed for each sample population. Slides were scored blind by two independent investigators and cells were graded as normal (undamaged), limited migration and extensive migration.

Statistics

Statistical comparisons between the grade of DNA damages in control/patient groups were analysed by using student t-test. The effects of age, durations of treatments, drug doses and blood levels of the drug in the patient group were investigated by correlation analysis. Statistical results were summarised in table 3 and 4.

Results and discussion

The demographic data including age, smoking habit, duration of treatment, drug dose and blood level of the drug, cytogenetic evaluation, and the comet scores of the

Tablo 3:Statistical comparisons between the grade of DNA damages in control/patient groups

Group		N	Mean	Std. Deviation	Std.Error Mean	t	p(sig)
Age	Patient	24	22,5000	7,0249	1,4346	-1,053	0,299
	Control	16	24,8125	6,4521	1,6130		
No Migration	Patient	24	86,2917	3,8615	,7882	-7,764	,000
	Control	16	95,0000	2,7809	,6952		
Limited Migration	Patient	24	6,4167	1,7917	,3657	4,114	,000
	Control	16	3,7500	1,9833	,4958		
Extensive migration	Patient	24	7,3333	2,3713	,4840	7,227	,000
	Control	16	1,2500	1,3416	,3354		

Table 4: Statistical comparisons between patient age, valproic acid dose, duration of treatment, valproic acid blood level and comet

	Drug Dose		Age		Duration of Treatment		Drug Blood Level	
	Pearson Correlation	p (Sig.) 2-tailed	Pearson Correlation	p (Sig.) 2-tailed	Pearson Correlation	p (Sig.) 2-tailed	Pearson Correlation	p (Sig.) 2-tailed
No Migration	,065	,763	,056	,729	,295	,161	-,460	,831
Limited Migration	,027	,902	,037	,823	-,211	,322	,141	,510
Extensive Migration	-,127	,555	-,119	,466	-,324	,122	-,013	,951

24 patients and 16 controls are shown in table 1 and 2. The mean ages of subjects were 22.50 years in patients and 24.81 years in controls. The statistical comparison of the ages of the patients and the controls showed no significant difference ($p>0.05$). Cytogenetic evaluation of the patients and the controls were performed and all subjects were 46, XX. Table 1 and 2 show the number of undamaged and damaged cells. The statistical comparison of the epileptic patients and controls demonsrated a significant difference in number of damaged cells. Damaged (limited and extensive migrated) cells in epileptic patients receiving valproic acid were higher than those of controls ($p<0.05$). Undamaged cells in epileptic patients were lower than those of controls ($p<0.05$). No significant correlation was noted between drug doses, blood valproic acid levels and the comet scores. No relationship was observed between the frequency of the patients comet scores and duration of valproic acid therapy. The age of the patients and controls also had no significant effect on the comet scores.

Data about cytogenetic analysis of peripheral lymphocytes from epileptic patients receiving valproic acid are conflicting and the mutagenic effect of this drug has not been established clearly. Hu et al. evaluated the potential mutagenic effects of valproic acid using the SCE and chromosome aberration assay systems and reported a significantlincrease in SCE rates and a slight but not significant increase in chromosome aberration compared to the control groups (19). Curatolo et al. studied the mutagenic effects of long term monotherapy of antiepileptic drugs including valproic acid in epileptic children in comparison to a control group of untreated epileptic children and found a significant increase in chromosomal aberration rates in the epileptic children undergoing long-term antiepileptic drug (25). In contrast, Schumann et al. reported no significant increase of sister chromatid exchanges in peripheral lymphocyte cultures of adult patients with epilepsy treated chronically with valproic acid, and lack of mutagenic potential of valproic acid within the dose range (26). Taneja et al. also found no significant difference in SCE frequency between valproic acid treated and untreated patients, and they suggested the disease itself may be associated with an increased frequency of SCEs (18).

The single-cell gel electrophoresis assay (comet assay) has been used in both in vitro and in vivo studies to assess DNA damage and repair induced by various agents in a variety of mammalian cells. The alkaline single-cell gel electrophoresis 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 (20).

Several studies have been published about genotoxic effects of valproic acid but none of them is based on the comet assay analysis. In the present study, in vivo effects of valproic acid were investigated by the comet assay in female patients. In the present study, the comet scores between the patients receiving valproic acid and the control group was significantly different ($p<0.05$). We found a

significant increase in cell damage in comet assay of peripheral lymphocytes of the epileptic patients undergoing long-term valproic acid monotherapy. Previous studies showed that aging increased the SCEs and comet scores in human population. Although the epileptic patients were younger than controls in our study, comet scores in epileptic patients were higher than controls and age did not effect comet scores in the patient group. There was no effect of dose and blood level of valproic acid and treatment duration on the comet scores. These observations suggest that valproic acid have genotoxic effect in epileptic females receiving valproic acid monotherapy so valproic acid increases mutagenesis and carcinogenesis risk in these individuals. Dickinson et al. previously showed the ability of valproic acid to cross the placenta (27). Thus, genotoxic effects of valproic acid may occur in offspring of epileptic women receiving valproic acid.

Although, the genotoxic effects of valproic acid have been demonstrated using some SCE and chromosomal aberration assays in previous studies, we showed for the first time this effect of valproic acid by means of the comet assay. We suggest that valproic acid has genotoxic effects that can occur at any blood level of valproic acid in therapeutic ranges, and that the damage can be detected within the first year of valproic acid treatment.

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