Investigation of DNA Damage in Epileptic Women Treated with Phenobarbital

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✔ Frequency of seizure during pregnancy is increased about one-third of the women with epilepsy. Therefore, seizure is one of the most difficult problems awaiting solution for epileptic female patients during their pregnancy. The main toxic effect of the antiepileptic drugs (AED) to be considered in women is the teratogenicity. Phenobarbital is one of the widely used AED. Its potential toxic effects on DNA have been tested by various methods, but the results were conflicting. In the present study, comet assay, sensitive and reliable test, was used for the first time to detect any teratogenic effects of phenobarbital in thirty epileptic women. 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 30 healthy, nonsmoker female patients, who had normal menstrual cycles and did not use any long-term drugs. The damaged (limited and extensively migrated) cells in patients' group were higher than that of the control group, but this difference was not statistically significant (p>0,05). Our results support that long-term phenobarbital monotherapy in adults does not appear to have any mutagenic effect. This conclusion, however, can not be extended to the developing fetus exposed to the effects of phenobarbital in utero.

Key words: *Phenobarbital, epilepsy, DNA damage, teratogenicity, comet assay*

✔ **Fenobarbital ile Tedavi Edilen Epilepsili Kadınlarda DNA Hasarının Araştırılması**

Gebelik süresince nöbet sıklığı epilepsili kadınların yaklaşık üçte birinde artar. Bu nedenle, epileptik nöbet epilepsili kadınların hamilelik dönemlerinde çözülmesi gereken en zor problemlerden biridir. Antiepileptik ilaçların kadınlarda göz önünde bulundurulması gereken en önemli toksik etkisi teratojenitedir. Fenobarbital en çok kullanılan antiepileptik ilaçlardan biridir. DNA üzerindeki olası toksik etkisi çeşitli yöntemlerle test edilmiş, fakat çelişkili sonuçlara ulaşılmıştır. Bu çalışmada, otuz epileptik kadında fenobarbitalin teratojenik etkisini tespit etmek üzere duyarlı ve güvenilir bir metot olan komet testi ilk kez kullanıldı. Çalışmaya düzenli adet gören, sigara içmeyen ve epilepsiden başka bir sağlık sorunu almayan kadınlar kabul edildi. Kontrol gurubu benzer özellikleri taşıyan ve uzun süreli ilaç kullanmayan 30 sağlıklı kadından oluşturuldu. Hasarlı (sınırlı miktarda ve yoğun olarak yer değiştirmiş) hücreler epileptik grupta kontrole nazaran daha fazla olmakla birlikte fark istatistiksel olarak anlamlı değildi (p>0,05). Sonuçlarımız uzun süreli fenobarbital kullanımının mutajenik etkisi olmadığı yönündeki

1.0-1.2 mm thick microscope slides were purchased from Merck. Normal and low melting point agarose were obtained from Gibco. Dulbecco's phosphate-buffered salts (PBS), without Mg^{2+} and Ca^{2+}, was from ICN Flow. The other chemicals used in the present study were purchased from the Sigma.

Peripheral blood lymphocyte preparation

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 1000xg. Lymphocytes were resuspended at approximately 10^7/mL in TC-199 medium supplemented 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 application of the alkaline comet assay (single cell gel electrophoresis)

The comet assay, as described by Singh et al[10], was used with some modifications[11]. The comet assay protocol was carried out under dim light to prevent any additional DNA damage. Darkin fully frosted microscope slides were covered each with 100 µL of 0.5% normal melting point agarose in Ca^{2+} and Mg^{2+}-free PBS at 45°C. They were immediately covered with a large no. 1 cover slip and then kept at 4°C until the agarose had solidified. Seventy-five µL of 0.5% low melting point agarose (LMA) at 37°C was added to the lymphocytes (1500-10000 cells) suspended in 10 µl of PBS. After gently removing the cover slip, the cell suspension was rapidly pipetted on to the first agarose layer, spreaded using a cover slip, and allowed to solidify at 4°C. A final layer of 75 µL of 0.5% LMA was applied in the same way. The slides were immersed in freshly prepared, cold lysing solution (2.5 M $NaCl_2$, 100 mM Na_2 EDTA, 10 mM Tris, pH 10, 1% sodium sarcosinate 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 (300 mM NaOH and 1 mM Na_2EDTA) 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 then drained, placed on a tray and flooded slowly with 3 changes of neutralization 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 µL of 20 µg/ml ethidium bromide and a cover slip was placed on top. Slides were stored in a closed container at 4°C and analyzed within 24 h, gel dehydration over longer storage times led to detoriation in slide quality.

Comet capture and analysis

Analysis was performed immediately after staining, using a 200 X objective with a Zeiss optiphot equipped with an excitation filter of 515-560 nm from a 100-W mercury lamp and a barrier filter of 590 nm. Comets from as the negatively charged, damaged 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 substracting the diameter of the nucleus

Table I. Clinical Data and Comet Scores Obtained From The Epileptic Women Treated with Phenobarbital.

Subject number	Age (years)	Duration of treatment (years)	Grade of damage in 100 cells		
			Undamaged (no migration)	Limited migration	Extensive migration
1	24	1	98	2	0
2	21	2	94	3	3
3	31	4	95	3	2
4	30	7	89	8	3
5	33	4	90	7	3
6	24	2	95	4	1
7	27	3	96	3	1
8	28	2	95	3	2
9	34	2	94	4	2
10	33	1	97	2	1
11	31	3	93	5	2
12	20	4	91	5	4
13	25	3	93	4	3
14	21	2	91	6	3
15	23	3	96	4	0
16	24	5	90	5	5
17	26	2	96	3	1
18	34	2	95	2	3
19	28	3	96	2	2
20	32	1	97	1	2
21	29	3	95	4	1
22	29	3	94	6	0
23	25	3	96	3	1
24	21	3	95	3	2
25	20	4	96	2	2
26	22	1	99	1	0
27	27	3	98	1	1
28	30	2	93	4	3
29	33	2	92	5	3
30	21	3	99	1	0
Mean	26.87	2.73	94.60	3.53	1.87
SD	4.58	1.29	2.63	1.80	1.28
SEM	.84	.24	.48	.33	.23

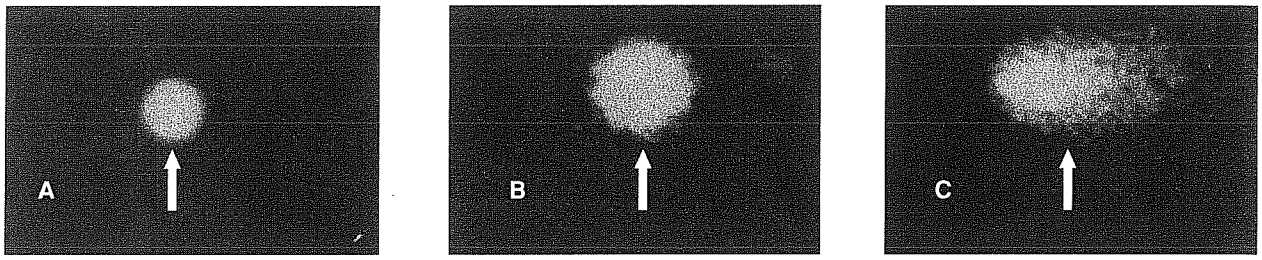

Figure. Comet assay for the detection of DNA damage produced by the various effects.
A: normal cell (undamaged-no migration), B: Limited migrated cell (at low damage levels, stretching of attached strands of DNA, rather than migration of individual pieces is likely to occur), and C: Extensively migrated cell (with increasing numbers of breaks where DNA pieces migrate freely into the tail forming comet images).

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[8,10,27].

Several studies evaluated the effects of phenobarbital 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 phenobarbital on DNA in the peripheral lymphocytes of female epileptic patients treated with phenobarbital. The factors that may have influence on the comet scores (age, sex, race, nutrition, environ etc.) were similar in both groups. Another factor that affects the frequency of SCE is the physiological status of the donor; it has been demonstrated that increased frequencies of SCE and chromosome aberrations during "ovulatory" and "estrogenic" stages as compared with those of "progestogenic" stage of the menstrual cycle in women, and it has been suggested that female subjects are more influenced by biologic rhythms because of specific hormonal cycles and are sensitive to genetic damage during ovulatory/estrogenic stages of the menstrual cycle[28]. Considering this fact, we made sure that all the subjects (patients and the control group) were at the same phase of their menstrual cycles (within 20^{th} and 27^{th} days following the beginning of menstrual bleeding) at the time of sampling.

The number of damaged (limited and extensively migrated) cells in epileptic patients receiving phenobarbital were higher than that of the control group. But, statistical analyses did not reveal any significant differences between the comet scores of the patients and the healthy women, indicating the lack of phenobarbital toxicity on DNA as expressed by the migrations of the cells in the comet assay. Our results support that long-term phenobarbital monotherapy in adults does not appear to have any mutagenic effect. This conclusion, however, can not be extended to the developing fetus exposed to the effects of phenobarbital in utero.

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