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Original Article

GENOTOXIC AND MUTAGENIC STUDIES OF THE ANTIEPILEPTIC DRUG LEVETIRACETAM IN PREGNANT RATS AND THEIR FETUSES

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ABSTRACT

Objective: Levetiracetam (LEV) is an anti-epileptic drug, initially approved as an adjunct therapy in adult patients with partial-onset seizures, and used as monotherapy treatment during pregnancy. However, very few, if none, investigations have been focused on LEV neurotoxicity or hepatotoxicity at the molecular level. This study aimed to evaluate the genotoxic and mutagenic potential of LEV, in liver and brain tissues of treated pregnant rats and their fetuses during pregnancy.

Methods: LEV was administered to pregnant female albino rats at doses 300 and 600 mg/kg b. w, from gestation days 5-18. Comet assay, DNA fragmentation were performed for detection of DNA damage. Single-stranded conformation polymorphism (SSCP) followed by DNA sequencing were accomplished for detecting possible mutagenicity.

Results: Administration of the two tested doses of LEV resulted in a significant increase of DNA damage as detected by alkaline Comet assay, and an appearance of both apoptotic laddered and smeared DNA in the tissues tested. Moreover, a significant incidence of mutations in exon 2 and 3 of Harvey rat sarcoma viral oncogene (*HRAS)* gene, were detected in fetal liver and brain tissues respectively, using single-stranded conformation polymorphism (SSCP) and were confirmed by DNA sequencing.

Conclusion: Maternal and fetal DNA damage induced by LEV was evidenced in our study, even at the commonly used therapeutic dose (300 mg/kg), and thus these side effects should be considered when using LEV for long-term during pregnancy.

Keywords: Mutagenicity, SSCP, *HRAS* gene mutation, Comet assay, Levetiracetam.

INTRODUCTION

Epilepsy is considered as the second common chronic neurological disorder and one of the most important neurological syndromes during pregnancy. It's marked by sudden recurrent episodes of sensory, motor or autonomic disturbance, with or without loss of consciousness or convulsions, associated with brain abnormal electrical activity [1]. Antiepileptic drugs (AEDs) are used to prevent epileptic seizures. Despite the high risk of treating a pregnant woman suffering from epilepsy with AED, due to the more frequent occurrence of complications, it may be dangerous to stop or even change the AED regimen during pregnancy due to the frequency and severity of their underlying epileptic disorder. Thus, prescribing AEDs in pregnancy is a challenge to clinicians.

Levetiracetam (Keppra) is an anti-epileptic drug, initially approved as an adjunct treatment for patients with refractory partial seizures, and is now used as monotherapy in pregnancy [2, 3]. It's mechanism of action implicates blocking N-type calcium channels and indirectly inducing the major inhibitory neurotransmitters GABA and glycine resulting in the blunting of excessive neuronal activity in the brain [4]. As apoptosis is regulated by growth factors and cytokines as well as by neurotransmitters [5], any compound that interferes with these processes may trigger apoptotic death of neurons [6]. Simultaneously, there have been two case reports of hepatic failure secondary to treatment with LEV [7, 8]. Additionally, a reduction in LEV dose by 50% is recommended in patients with severe liver cirrhosis (Child-Pugh Class C), suffering from seizure prophylaxis, due to the drop in the total clearance of the drug by 57% [9]. However, to our knowledge, there is no information concerning the neurotoxicity or hepatotoxicity of LEV at the molecular level.

The majority of antiepileptic drugs are trans-placental xenobiotic [10]. Moreover, The developing fetuses of pregnant women with epilepsy, who are treated with AEDs, might be associated with increased risk of major congenital malformations (MCM), post-natal developmental anomalies, developmental delay, fetal death and adverse pregnancy outcomes [11]. This has drawn the attention to the possible genotoxicity or/and mutagenicity of LEV in treated mothers and their fetuses.

Therefore, the present study was undertaken to determine the potential genotoxic and mutagenic effects of LEV in liver and brain cells of treated pregnant rats and their fetuses during pregnancy. Alkaline Comet and DNA fragmentation assays were conducted to evaluate Levetiracetam DNA damage. The other two test systems specified mutations in exons 2 and 3 of *HRAS* gene, as an example of an oncogene using PCR-SSCP, followed by sequencing of the mutant and normal PCR products. Oncogenes are the mutant forms of proto-oncogenes that regulate the cell proliferation such as (RAS, RET, KIT, MET). Mutations of hot-spots codons of exon 2 or/and exon 3 in HRAS genes may implicate in the development of benign and malignant tumors [12] or Costello syndrome disease in children [13]. Thus, mutagenicity studies that measure these types of mutations can shed some light on the risk assessment of LEV under sub-chronic administration.

MATERIALS AND METHODS

Chemicals

Levetiracetam (LEV) was purchased from UCB Pharmaceutical Sector (Chemin du Foriest, Belgium). All other chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

Female albino rat (*Rattus Norvegicus*) were obtained from the animal house of Theodor-Bilharz Research Institute, (Giza, Egypt). All procedures involving rats followed the specifications recommended in The Guide for the Care and Use of Laboratory Animals [14]. Animals were housed (5/cage), the temperature in the experimental animal room was about 24 °C and relative humidity about 60%. The light cycle was 12 h light/12 h dark. Animals were

allowed free access to tap water and certified laboratory rodent chow. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Science, Cairo University, Egypt (CUFS/S/phy/14/14).

Experimental design

Fifteen Females of 11-13 w old (275±24 g body weight (b. w)) were selected for the present study, and vaginal smears were prepared every morning and examined under the light microscope according to the method of Snell [15] for 5 d to select the rat in the proestrus.

Each two females with regular estrus cycle were selected in the proestrus stage and caged together with one male overnight under controlled environmental conditions of temperature, humidity and light. The first day of gestation was determined by the presence of sperms in the vaginal smear [16]. The pregnant rats were then randomly allocated into three groups of five each. LEV was administrated intragastrically daily, from the 5th day to the 18thday of gestation at 9:00 a. m (±30 min) to treatment groups; Group A received 300 mg/kg (equivalent low therapeutic dose) while Group B received 600 mg/kg (equivalent greater therapeutic dose) of LEV. Group C (the control group) received distilled water by intragastric route. The present study was done in healthy (non-epileptic) pregnant rats. The rats were weighed daily, and the doses of LEV were adjusted according to their bodyweight. On the 18th day of gestation, all pregnant rats of groups (A, B, and C) were euthanized under chloroform vapor and sacrificed by decapitation after being fasted overnight. Fetuses recovered through caesarian section were removed from their membranes and were separated from their placentae. The brain was extracted as follows; the scalped skull was opened carefully by fine scissors, starting laterally from the outer ear foramen and moving forwards. By using forceps, the roof of the skull bones was carefully removed in pieces. The brain was finally separated from the skull base after cutting all the cranial nerves. Parts of the liver and brain tissues of pregnant rats and fetuses of different groups were stored at-20 ˚C for further investigations.

Alkaline comet assay

DNA damage baseline level was estimated in Pregnant rats, and their fetuses in both brain and liver tissues of rats injected with two different doses of LEV using alkaline (pH>13) comet assay according to Tice *et al.* [17]. Preparation of slides, lysis, and electrophoresis, were conducted under red light in order to prevent additional DNA damage. Cells from 10 µl aliquot of homogenized brain or liver tissue in cold mincing solution (Hanks balanced Salt Solution (HBSS) Ca++and Mg++free with 20 mM EDTA, 10% Dimethyl sulphoxide (DMSO) was mixed with 75 µl of 0.5% low melting point agarose (Sigma) and was added to a fully frosted microscope slide pre-coated with 1% of normal melting agarose. After solidification, slides were placed in a cold fresh lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, to which 1% Triton X-100 and 10% DMSO were freshly added) for 24 h at 4 °C in the dark. Subsequently, the slides were incubated in freshly-made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH>13) for 20 min. Electrophoresis was performed for 20 min at 300 mA and 25 V (0.90 V/cm), and then the alkali was neutralized with 0.4 m Tris (pH7.5), fixed in 100% cold ethanol and air dried. Finally, the DNA was stained with ethidium bromide (2 mg/ml). Using a fluorescence microscopy equipped with an integrated digital camera, images of 50 randomly selected cells were analyzed for: The Tail Moment (product of the proportion of the tail's intensity and the displacement of the tail's center of mass relative to the center of the head), tail Length (the extent of DNA damage away from the nucleus and expressed in µm, and % DNA in the tail were evaluated using Comet Assay IV software (Perceptive Instruments, Suffolk, UK) at 200x magnification.

Qualitative DNA fragmentation assay

DNA fragmentation was determined according to the standard protocol described by Sam brook [18]. Liver and brain Cells were suspended in 100 µl lysis buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 10 mM EDTA and 1% SDS, incubated with 50 µg/ml proteinase K at 56 °C overnight, and then centrifuged at 10,000 rpm for 30 min. Soluble DNA in the resulting supernatant was precipitated with ethanol at-20 °C, The DNA pellet was then rinsed with 70% ethanol and dissolved in sterile ddH_2O. Fragmented DNA was detected by running 5 µg of genomic DNA on a 1.5% ethidium bromide treated agarose gel. The gel run at 80 Volt (Power Supply Biorad, Model 200/2.0), and visualized under UV transilluminator (Stratagene, USA)to visualize intranucleosomal DNA fragmentation (laddering), characteristic of apoptosis.

Single strand conformation polymorphism (SSCP) analysis

Isolation of genomic DNA

Genomic DNA was prepared from rat liver and brain tissues using salting-out procedure performed as previously described [19].

Polymerase chain reaction (PCR)

Primers for *HRAS* gene exons (2, 3) were designed to include codon 12 (normal (N)=GGA), 13(N=GGC) in exon 2 and codon 61(N=CAA) in exon 3 as follow: exon 2 forward: 5'CAGGAGCT CCTGGATTGG, reverse: 5'AAAATGGTTCTGG ATCAG and exon 3 forward: 5'GACTCCTACCGGAAA CAG, reverse: 5'GTGCGCATGTACTGGTC CCC3'. These hot spot codons are the most frequent position for a point mutation leading to an amino acid substitution in the encoded protein, and thus continuous transduction of inappropriate growth signal [20]. The size of the amplified region is 119 and 110 of exon 2 and exon 3 respectively. 100 ng of extracted DNA, 10 pmol of both forward and reverse primers and 2 x master mixes were mixed. Samples were initially denatured at 94°c for 2 min followed by 35 cycles of 1-minute denaturation at 94°c, 2 min annealing at 58°c and 2 min elongation at 72 °c ended with a final extension step at 72°c for 7 min, and then cooling to 4°c. Cycling was carried out using Thermal Cycler (PTC-100™ thermal cycler, Watertown, MA, USA). The final products of PCR were separated and visualized by electrophoresis, through 2% ethidium bromide treated agarose gel using UV trans-illuminator (Stratagene, USA).

SSCP analysis

A mixture of 5 µl aliquot of the amplified PCR products, 5 µl of denaturing loading dye (95% formamide, 0.1% bromophenol blue, 0.1% Xylene cyanol FF and 0.5 µl 15% Ficoll) and 5 µl of TE buffer was heat denatured at 95 °C for 5 min and then chilled on ice for 10 min. The denatured PCR products was electrophoresed on a 20% polyacrylamide gel electrophoresis (acrylamide/bis-acrylamide = 49:1, v/v) at 90 volt for 2 h. The gel was stained by shaking for 10 min in 100 ml of 1× TBE with 10 µl ethidium bromide (10 mg/ml) to visualize the DNA bands. The stained gels were placed on a UV transilluminator (Stratagene, USA) and photographed using Medidoc gel documentation system (Herolab, Wiesloch, Germany).

Direct sequencing

Samples that showed different band pattern compared with their corresponding normal control, using SSCP analysis, were considered to harbor mutations. Their PCR products were electrophoresed on 2% agarose gel, and purified using QIAquick® Gel Extraction Kit (QIAGEN, USA), according to the manufacturer's recommendations. The samples were sequenced by the dideoxy chain termination method, using the original set of primers. DNA sequencing was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA), using ABI PRISM®BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA). Forward and reverse Sequences were compared against the corresponding rat gene sequence in NCBI database (Genbank accession number NM_001130441.1), for *HRAS* exon 2 (119 base pair (bp)) and exon 3(110bp), (132-249 bp, 278-387 bp on the corresponding mRNA respectively).

Statistical analysis

The effect of different parameters on the tail moment was tested using Univariate Analysis of Variance. Duncan's test was used to evaluate the significant difference of tail moment between all groups. Regression analysis was also done to investigate the correlation between different LEV concentrations and tail moment. Chi-square (χ^2) test was applied to clarify the effect of different doses of LEVon the studied gene mutations. In order to test the

significant differences of mutations between the experimental groups, Mann-Whitney test was applied. Statistics were carried out using the Statistical Package for the Social Sciences (SPSS) version 20 package software.

RESULTS

Clinical signs of toxicity

No mortality was observed in any of the treated groups. All female rats dosed with the control substances or dosed with LEV at concentrations of 300 or 600 mg/kg b.w, appeared normal throughout the experiment. Furthermore, LEV did not cause miscarriage in female rats. In addition, intragastric LEV exposure at the tested doses did not affect the gestation duration**.**

Comet assay

The statistical significant increase ($p<0.001$) in DNA migration from the nucleus (fig. 1), as assessed by tail moment (TM), indicated that the administration of LEV at 300 and 600 mg/kg b. w has induced DNA damage when compared with negative control group in the maternal and fetal liver and brain tissues. Simultaneously, hepatic cells showed a significant increase in DNA damage when compared with neural cells in both pregnant rats and their fetuses ($p<0.001$). Overall results showed a significant increase ($p<0.001$) in TM in pregnant rats compared to their fetuses in the tested tissues at both doses (table 1).

Regression analysis indicated a strong positive correlation between tail moment and different LEV concentrations in all maternal and fetal tissues tested (fig. 2).

The effect of different factors (doses, organs, cases) on TM, using Univariate ANOVA test, have caused a significant effect on the TM at $p<0.001$; except (cases, organs) and (doses, organs, cases) which showed an insignificant effect at $p>0.05$ (table 2).

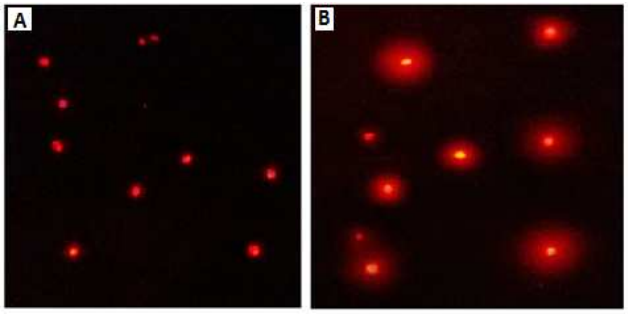

Fig. 1: Representative photo for DNA damage inductions by LEV doses (B) compared with undamaged DNA (A), (200 x)

Table 1: The effect of Levetiracetam (LEV) different concentrations on tail moment (TM) in the hepatic and neural cells of treated pregnant rats and their fetuses after daily injection from gestation days 5-18

Doses of LEV	Fetuses			Pregnant rats			(%)◄	(%)►
	Liver	Brain	(%)·	Liver	Brain	(%)☼		
0 mg/kg	0.0765±0.006^{A}	0.0807±0.005^{A}	-5.5%	0.1600±0.016^{B}	0.0774±0.002^{A}	+51.6%	(+109.2%)	(-4.1%)
300 mg/kg	0.3018±0.022^{C}	0.1056±0.006^{AB}	+65.0%	0.4593±0.017^{EF}	0.3264±0.029^{D}	+28.9%	(+52.2%)	(+209.1%)
% of change⊕	(+294.5%)	(+30.9%)		(+187.1%)	(+321.7%)			
600 mg/kg	0.5216±0.037^{EF}	0.2475±0.003^{C}	+52.6%	0.7300±0.048^{G}	0.4423±0.043^{DE}	+39.4%	(+40.0%)	(+78.7%)
% of change⊕	(+581.8%)	(+206.7%)		(+356.3%)	(+471.5%)			

Data are represented as a mean of five rats±standard error of the mean (SEM), Mean values marked with the same letter are similar (insignificant, $P>0.05$), ⊕: Percentage of changes in comparison with the corresponding control, ·, ☼: Percentage of changes of the liver TM in comparison with the corresponding TM in neural cells of the fetuses and pregnant rats respectively, ◄, ►: Percentage of changes of the mother hepatic and brain TM in comparison with those of the corresponding fetuses, respectively.

Table 2: The effect of different doses of LEV (0,300,600 mg/kg b. w), organs (liver, brain), cases (fetuses, pregnant rats) and their interactions on the tail moment in the treated albino rats using Multivariate ANOVA test

Source	Type III Sum of squares	Df	Mean square	F	Sig.
Corrected Model	1.463[a]	11	.133	71.944	p<0.001
Intercept	3.114	1	3.114	1684.468	p<0.001
Doses	.897	2	.449	242.764	p<0.001
Organs	.235	1	.235	127.013	p<0.001
Case	.186	1	.186	100.421	p<0.001
Doses * Organs	.088	2	.044	23.723	p<0.001
Doses * case	.048	2	.024	13.110	p<0.001
Organs * case	.000	1	.000	.186	p>0.05
Doses * Organs * case	.008	2	.004	2.284	p>0.05
Error	.044	24	.002		
Total	4.621	36			
Corrected Total	1.507	35			

$P>0.05$: insignificant effect; $P<0.001$: significant effect at $\alpha= 0.01$.

DNA fragmentation

DNA obtained from maternal tissues treated with different doses was detected as dense smears at size 500 and 100 bp compared to intact genomic DNA in the negative group (fig.3). However, low level of DNA degradation was detected as less dense smears in the fetal tissues at (100 bp). The degree of DNA fragmentation at dose 600 mg/kg was higher than that at 300 mg/kg dose in both maternal and tested fetal tissues.

SSCP analysis

PCR products of exon 2 and 3 of *HRAS* gene measuring 119bp, 110bp respectively (fig.4), were analyzed by SSCP to identify DNA harboring mutation, all of which were identified either as an extra lower band in exon 2 or as band shifts in exon 3 (fig. 5A and 6A). Liver and brain tissues of five pregnant rats and their thirty fetuses were examined for each dose and exons. In the treated maternal brain tissue, SSCP analysis of exon 3 showed no mutation, while exon 2 revealed two mutations out of five (40%) at dose300 mg/kg b. wand three mutations out of five (60%) at dose 600 mg/kg b.w. No abnormal SSCP pattern was detected in the treated maternal liver tissue at the doses tested for both exons. In fetuses, five mutations out of thirty (16.6%) were observed for each exon, concerning exon 2, mutations observed only in liver tissue at dose 300 mg/kg b. w, while those in exon 3 appeared only in brain tissue at dose 600 mg/kg b.w.

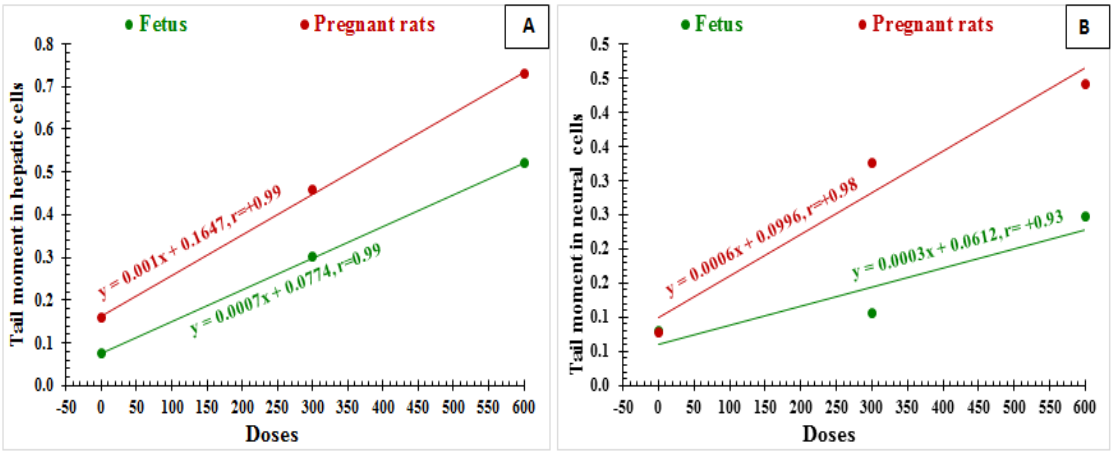

Fig. 2: Regression lines and correlation coefficients for a tail moment affected by daily injection of LEV different concentrations in hepatic (A) and neural cells (B) of treated pregnant rats and their fetuses from 5th-18th days of gestation. Results are expressed as mean

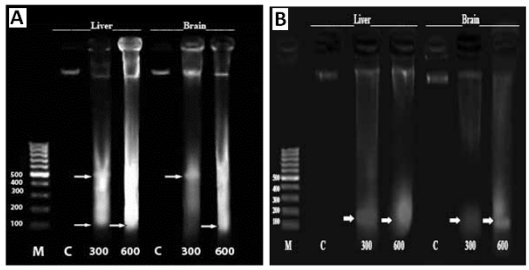

Fig. 3: DNA fragmentation using agarose gel electrophoresis of DNA isolated from liver and brain of pregnant rats (A) and their fetuses (B) rat, Compared with DNA marker (M) and their control (C) at the tested doses (300 or 600 mg/kg b. w) Fragmented DNA was indicated by arrows

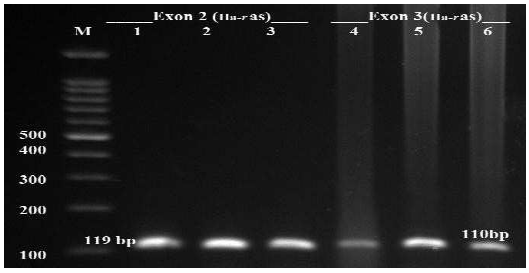

Fig. 4: PCR products of *HRAS* gene exons 2 and 3 electrophoresed on 1.5% agarose, stained with ethidium bromide. M: the ladder marker, lanes (1, 2, 3): PCR products of *HRAS* exon 2 at 119 bp, lanes (10, 11, 12): PCR products of *HRA S*exon 3 at 110 bp

Sequence analysis

PCR products of 8 treated samples that showed abnormal patterns in SSCP than control were randomly selected. Two PCR products of exon 3 from fetal liver tissue treated with 600 mg/kg b. wand six samples of exon 2, (two treated samples of maternal brain tissue for each tested doses and two treated samples of fetal liver tissues at dose 300 mg/kg b. w) were chosen. In addition, two treated samples that showed no difference in SSCP pattern compared to their control were sequenced as well for more conformation and their sequence alignment showed no difference from that of their controls or Genbank data sequence. In exon 2, same point mutations were observed in the six tested samples out of the coding region, at nucleotide (nt) positions 24, 155 (C/G) and 35, 166 (G/T).

Frameshift mutation was found due to insertion font (C) at position 43, 174 (second base of codon 3) (fig. 5C). Synonymous point mutations were detected in exon 3 at nucleotide positions 21, 298 (A/G, Valine to Valine) and 39,316 (G/A, Leucine to Leucine) and 43, 320(T/C, Threonine to Threonine) in the third base of codon 44, in the third base of codon 50, and in the first base of codon 52 respectively (fig. 6C), no mutation has been observed in any hot spot codons of exons 2 or 3.

According to results of Chi-square test, different injected doses did not induce any statistical significant alterations ($p>0.05$) in band pattern in both exons 2 and 3 in the maternal organs tested when compared with the negative controls values. On the other hand, statistical significant alterations ($P<0.05$) in band pattern in exon 2 and exon 3 in the liver and brain fetal tissues respectively, have been observed (table 3).

DISCUSSION

Although, lots of drugs were discovered using animal models, a big portion of patients still remain resistant to the available antiepileptic drugs [21]. The growing importance of LEV in medical practice today, as an AED of low incidence of side effects and proven efficacy, and considering that AEDs in general are transplacental agents [22], beside the absence of enough information about its drug metabolism interaction, have implied the urge to investigate its potential genotoxic effects in maternal and fetal tissues. In the present study, the experimental animals are provided with the therapeutic and the higher doses of the test compound, to reach the plasma concentrations that are in accordance with those expected under clinical conditions.

The results of the comet assay have evidenced that both tested concentrations have induced strand breaks as indicated by the significant elevations in a tail moment in a concentration-dependent manner. The appearance of the smeared pattern of genomic DNA fragments in the range of 20–300 kb, is an early sign of cellular apoptosis. That profits the generation of low molecular weight oligonucleosomal fragments [23], further evidenced DNA damage inductions and fragmentation by the two tested LEV concentrations. The significant increase of tail moment in fetal hepatic and neural cells is an indication of the transplacental genotoxic effects of this drug. Although the exact mechanism for LEV–induced DNA damage in neural cells is not known, it has been postulated as being caused by apoptosis, which is regulated by growth factors and cytokines as well as by neurotransmitters and is accomplished by a number of intracellular proteins [5]. As studies speculate that LEV may involve induction of GABA and glycine-mediated inhibition, and/or synaptic protein binding which alters neurotransmitter release [4], interference with these processes by any compound including LEV may trigger apoptotic death of neurons and cause DNA damage [6].

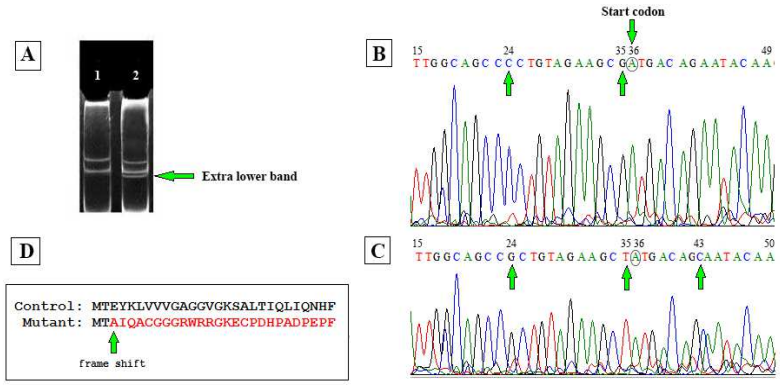

Fig. 5: (A): Representative 10% polyacrylamide gel showing a PCR-SSCP pattern for *HRAS* gene exon 2 (119bp) with the extra lower band (lane 2) relative to control (lane 1). (B): Sequence graph of control of region flanking (15-49bp) showing the normal nucleotide (nt) using a forward primer. (C): Sequence graph of region flanking mutations (15-50bp) showing substitution of nt C/G and G/T and at positions 24, 155 and 35,166 respectively, and insertion of nt C at position 43, 174 using forward primer.(D): Amino acid translation and alignment between mutant coding sequence (starting from 36, 167bp till the end of exon 2) and the control showing frame shift starts at codon three indicated by arrow

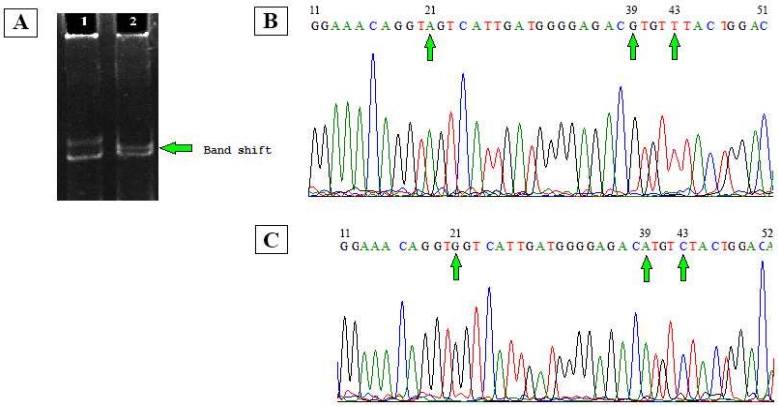

Fig. 6: (A) Representative 10% polyacrylamide gel showing a PCR-SSCP pattern for *HRAS* gene exon 3 (110bp) with band shift (lane 2) relative to control (lane 1). (B): Sequence graph of control of region flanking (11-51) showing the normal nucleotide (nt) using a forward primer. (C): Sequence graph of region flanking mutation (11-52) showing substitution of nt A/G, G/A and T/C at positions (21,298); (39,316) and (43, 320) respectively using forward primer

Table 3: Chi-square to clarify the effect of the different doses of LEV on the *HRAS* gene alterations observed in exon 2 and exon 3 in the liver and brain of treated pregnant rats and their fetuses after daily injection from 5th-18th days of gestation

	Fetal Liver		Fetal Brain		Maternal Liver		Maternal brain	
	EX 2	EX 3	EX 2	EX 3	EX 2	EX 3	EX 2	EX 3
Chi-Square	10.471	0.000	0.000	10.471	0.000	0.000	3.920	0.000
Df	2	2	2	2	2	2	2	2
P-value	0.005	1.000	1.000	0.005	1.000	1.000	0.141	1.000

P>0.05: insignificant effect; P<0.05: significant effect at α= 0.05.

The present results are concurrent with many researchers [24-26], that studied the neurotoxic effects of AEDs in infant rodents. They found that most AEDs cause apoptotic neurodegeneration in the developing rat brain, at doses and plasma concentrations relevant for anticonvulsant treatment [24-26]. Kim *et al.*[27] study on postnatal 8 d-old rats, in contrary to our findings, demonstrated that LEV alone did not cause cell death in the rat brain when given in therapeutic doses, and did not act together with other drugs as well.

The present study has shown no significant increase in the tail moment between treated fetuses at a dose (300 mg/kg) and the reference group in brain tissues (% of change=+30.9%). This is may be contributed to MDRPs (multidrug resistance proteins) that transport AEDs toward the blood compartment, confined to the apical membrane of brain capillary endothelial cells, thereby retained them within the brain, and limiting their penetration [28].

Nevertheless, this was not the case in maternal tissues, as they revealed a significant increase in the tail moment at such does with the referent group (p<0.05) (% of change=+321.7%). Consequently, it was concluded that distribution and bioaccumulation of AEDs, are different in fetus and mother, which revealed the different susceptibility between them.

Concerning hepatocytes injury, several studies confirming that LEV use was unavoidably linked to hepatic failure [29, 7, 8]. Based on the biopsy, Tan *et al.*, [7] reported that LEV had caused hepatocyte death without producing any structural changes to the liver. In addition, Huda and Muna [30]showed that the hepatocellular lesions, congestion of central veins, focal necrosis, dilatation and congestion of portal veins, were noticed in the liver sections of rats, treated with a high dose of LEV (70 mg/kg body weight). All previously mentioned studies support the present findings of hepatocyte injury,

assessed by a significant increase of DNA damage, in maternal and fetal hepatic cells. That could be rationalized by either decreased free radical neutralization and/or an increased production of free radicals, which may explain DNA damage in the liver. This hypothesis was further reinforced by Skopp *et al.* [9] and Ozden's [31] reports. The oxidative metabolism and the formation of reactive intermediates or metabolites could be another elucidation, which has been proposed as possible mechanisms of the teratogenicity for several clinically important AEDs, including CBZ and PHT [32]. Reactive metabolites from AED can, in some cases, cause direct cytotoxicity and liver cell necrosis [33]. Cytotoxic effect of LEV was distinguished using acid phosphatase assay (AP), only at higher concentrations. AP is a functional marker of the lysosomal compartment, and the lysosomal enzymes are known to recruit cell death [34]. All of which can cause DNA damage in liver tissues. However, the mechanism of LEV-induced liver stays puzzling.

Genetic variations, either resulting from spontaneous mutations or as a cause of xenobiotic exposure, may disturb the normal patterning and lead to a wide variety of skeletal alterations [35]. Limited studies on LEV, have reported that it's genotoxic and teratogenic effect risk are low in case it is used in pregnancy [36, 37]. On the other hand, fetal skeletal anomalies are seen in several studies at high doses of LEV [38, 39]. Moreover, exposure of rat pups to LEV doses ≥350 mg/kg/day, throughout their development, had increased the incidence of minor skeletal abnormalities and growth retardation, whereas doses of 1,800 mg/kg/day also led to increased embryonic mortality [36]. These reports reflect the possible mutagenic potential of LEV.

As no current publications regarding LEV mutagenicity exists, this study was carried out to reveal the potential mutagenic effect of this drug. Different SSCP patterns and mutations in exon 2 and 3 of *HRAS* gene were shown. According to chi-square test, maternal brain tissues exhibit insignificant incidence of mutation in exon 2, at both tested doses compared to negative control. This is may be attributed to the short time of administration. On the other hand, the significant mutation induction ($P<0.05$) that were detected in fetal liver and brain tissues, indicated higher susceptibility of fetuses. Mutations in exon 3 detected in brain tissues of fetuses at dose 600 mg/kg could be explained by the water solubility of LEV, and the crossing of the blood-brain barrier [40]. Taking into consideration that 66% of the administered LEV dose is excreted unchanged in the urine, with an additional 24% of the administered dose excreted as the corresponding inactive acid metabolite 2-pyrrolidinone-*N*-butyric acid (PBA) in human [41]. Besides the two high-PBA dosages (600 and 1,200 mg/kg/day), specifically increased the incidence of hypoplastic phalanges as reported by Nina *et al.*[40]. Thus, the mutagenic impact of LEV may be attributed either to its metabolite PBA effects, although at present no distributed data exists with respect to its reproductive safety, or to the induction of oxidative stress as reported by Ozden [31]. Redundant free radicals have been shown to interact with biomolecules including proteins, enzymes, membrane lipids and DNA which could be oxidized, destructured and ultimately dysfunctional [42]. Reactive oxygen species (ROS) can oxidize double bonds on fatty acid tails of membrane phospholipids, peroxidized fatty acids can trigger reactions that generate other free radicals, leading to more cell membrane and DNA damage, including DNA strand breaks, cross-linking, and adducts of the bases or sugars, single base and sugar phosphate damage [43]. Previously mentioned studies confirmed results of the present study and rationalized the different patterns of SSCP and types of mutations in *HRAS* gene exons 2, 3.

The reason behind exhibiting 5% mutant infants after exposure to LEV while the remaining 95% were unaffected are not clearly understood. However, there is an increasing evidence that the placenta has the ability to protect the fetus from drugs and xenobiotics in the maternal circulation [44]. Therefore, there may be a relationship between the protective function of the placenta, and the susceptibility to birth imperfections in some fetuses.

Our findings demonstrate that LEV-induced geno toxicity and mutagenicity in developing rat embryos at the therapeutic dose, and caused a significant increase in the tail moment, and DNA fragmentation at dose exceeded the therapeutic one. That may provide unsafely use for the pregnant, epileptic population after chronic administration. Future studies should address the underlying molecular mechanisms and implications of the observed prenatal effects of Levetiracetam.

CONFLICTS OF INTERESTS

There is no potential conflict of interest or competing interest.

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