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Genotoxicity assessment of antiepileptic drugs (AEDs) in human embryonic stem cells

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ABSTRACT

Several antiepileptic drugs (AEDs) are administrated during pregnancy according to recent therapeutic protocols. Ten percent of pregnant women with epilepsy give birth to offspring with malformations and teratogenic defects. Since the mechanism of action of AEDs is not yet completely understood, therefore, it could be hypothesized that they may cause cyto- or genotoxicity in embryonic fetus cells.

To investigate this hypothesis, the genotoxicity and cell survival of AEDs treated human embryonic stem cells (hESCs) were investigated by single-cell gel electrophoresis (Comet assay) and MTS assay, respectively. HESCs (Royan H6 cell line) were treated *in-vitro* with high therapeutic doses of Carbamazepine, Gabapentin, Lamotrigine, Levetiracetam or Topiramate as monotherapy or combination therapy of each drug with Folic acid.

After hESCs pluripotency confirmation, the effect of AEDs on cellular DNA damage of hESCs was investigated. levetiracetam and topiramate were found to damage the DNA significantly compared to untreated cells. The amount of DNA damage produced by carbamazepine and lamotrigine was similar while for gabapentin, the amount of DNA migration was very low and produced less DNA damage than the others. A considerable reduction in DNA damages occurred in genotoxicity in the presence of Folic acid in comparison to AEDs monotherapies.

According to our results, all mentioned AEDs caused DNA damage, while Levetiracetam and topiramate caused more extensive DNA damages than the others. Noticeably, the addition of Folic acid to the treated cells decreased the DNA damages considerably.

1. Introduction

Epilepsy is a prevalent and various set of chronic neurological disorders, characterized by recurrent unprovoked seizures (Kamyar and Varner, 2013). Epileptic seizures are usually classified into partial seizures and generalized seizures. Loss of consciousness occurs in generalized seizures. Tonic-clonic is a form of the most generalized seizures (Daroff et al., 2015). Women with epilepsy in reproductive age constitute one-third of people with epilepsy who receive antiepileptic drugs (AEDs) in antenatal clinics. Administration of pharmaceutical compounds for tonic-clonic seizures that are received during pregnancy by epilepsy mothers can cause serious harm and teratogenic effects on the fetus (Tomson et al., 2013). AEDs such as carbamazepine, gabapentin, lamotrigine, levetiracetam, and topiramate are usually prescribed during pregnancy to avoid generalized tonic-clonic seizures (Koda-Kimble, 2012; Wyllie, 2010).

In comparison with the general population, two to three-fold increase in major malformations have been found in the children who were exposed to AEDs in-utero (Adab et al., 2004). Major malformations are structural defects formed during organogenesis that can lead to dysfunction or death. The most commonly observed malformations include neural tube defects, congenital heart disease, orofacial clefts, intestinal atresia, and urogenital defects (Kaneko et al., 1999). Hence the adverse effects of AEDs prescription during the pregnancy makes it

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essential to examine the mechanism of AEDs cyto- or genotoxicity in embryonic cells.

On the other hand, folate is a coenzyme necessary for the accurate function of the central nervous system (Wills, 1931). In 1972, Speidel and Meadow described that in patients who were taking AEDs, the serum folic acid level decreased, leading to malformation risk increase (Speidel and Meadow, 1972). Likewise, folate deficiency was identified in 1990 as a cause of neural tube defects such as spina bifida, bifurcated brain, and anencephaly. Therefore, folic acid supplementation which can reduce the risk of neural tube defects is essential for all women of childbearing age (Bruno and Harden, 2002). Different studies have explored clinical effects of carbamazepine, gabapentin, lamotrigine, levetiracetam and topiramate on the fetus however a lack of evidence on their mechanism in causing malformations and teratogenic effects during pregnancy on embryonic cells of the fetus exists.

To propose a basic hypothesis in terms of the safety of AEDs administration in pregnancy, either genotoxicity or cytotoxicity, we looked at the toxicity effects of the mentioned AEDs and the role of folic acid in the decrease of potential toxicity on embryonic stem cells.

2. Materials and methods

2.1. Human embryonic stem cell culture

Human Royan H6 (RH6) cells, a human embryonic stem cell (hESC) line (Baharvand et al., 2006), were cultured (passage number 23–33) as colonies in Matrigel (Sigma, Germany) coated culture dishes at 37 °C and 5% CO2 and passaged every 6–7 days by collagenase/dispase (1:2) solution (Sigma, Germany). The human feeder-free culture medium (HFFC) contained Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 20% knockout serum (Gibco, USA), 1% Insulin-Transferrin-Selenium (Gibco, USA), 1% non-essential amino acids (Gibco, USA), and 1% Glutamax (Gibco, USA).

2.2. Immunostaining analysis of the hESCs

The hESCs were fixed using 4% paraformaldehyde (PFA, Sigma, Germany) for 30 min, permeabilized in 0.1% Triton X-100 for 20 min, blocked in 10% secondary antibody host serum and 0.5% BSA for 1 h, and incubated overnight at 4 °C with primary antibodies anti-Nanog (1:200; Cell signaling; USA; 4903) and anti-Oct4 (1:100; Santa Cruz Biotechnology; USA; SC-5279). Afterward, the cells were washed in PBS and incubated for 2 h with secondary antibodies Rabbit IgG-Alexa Fluor® 488 (1:1000; Invitrogen; USA; A-11008) and Goat IgG-Alexa Fluor® 546 (1:1000; Invitrogen; USA; A-11056). Cell nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI, Sigma, Germany) for 1 min. Images were visualized by an Olympus BX51 fluorescence microscope and captured by an Olympus DP72 digital camera.

2.3. AEDs treatment conditions

To start the AEDs treatments, RH6 day 7 colonies were dissociated into single cells by trypsin (Gibco, USA). Final concentrations of 200,000 cells/ml were transferred to Matrigel coated plates. After 48 h (Fig. 1A-C), the following monotheraphy treatments were performed in a total volume of 1 ml of HFFC medium: untreated hESCs as negative control; 50 mM carbamazepine (Rouzdarou Co., Iran); 0.01 mM gabapentin (Arya Pharmaceutical Co., Iran); 0.07 mM lamotrigine (Rouzdarou Co., Iran); 0.2 mM levetiracetam (Iran Darou Pharmaceutical Co., Iran); 0.01 mM topiramate (Arya Pharmaceutical Co., Iran); and 40 μM hydrogen peroxide (Carlo Erba, France) as positive control. For preparation of each drug, the effective therapeutic serum concentration was considered. For combined drug therapy tests, in addition to each drug 0.002 mM folic acid (Iran Darou Pharmaceutical Co., Iran) was added as well. Afterwards, the plates were incubated at 37 °C, in 5% CO2 for 5 h. A 96 h-treatment was performed with gabapentin (0.01 mM) and lamotrigine (0.07 mM) random selected compounds to determine the maximum period time of the study (Fig. 1E).

2.4. Comet assay

Following harvesting the treated cells, 100 μl (50,000 cells) of each cell sample was suspended in 200 μl of 1.5% low melting point agarose in 1X tris-borate EDTA buffer immediately. Subsequently, 50 μl of the suspension was spread on a slide and left to harden at 4 °C for 20 min. Afterward, the slides were immersed in pre-chilled lysis buffer (2.5 M sodium chloride, 10 mM EDTA, 10 mM Tris base, 1% Triton X-100) for 30 min at 4 °C and immediately drip-dried. The slides were then placed in 100 ml of 1X tris-borate EDTA buffer for 30 min at room temperature in the dark. The slides were electrophoresed in 1X tris-borate EDTA buffer at 75 V for 20 min to separate DNA segments. To fix DNA on the slides, the slides were rinsed by 70% methanol for 5 min and allowed to dry. Each slide was stained with 30 μl ethidium bromide dye, diluted in 1X tris-borate EDTA before use, and inspected visually under a fluorescent microscope (OLYMPUS IX71) (Deutsch et al., 2001). The analysis of the tail moment was performed using OpenComet software (Cometbio, 2017). For this purpose, around 50 cells per drug sample was examined and all specimens were evaluated with negative and positive controls.

2.5. Cell viability assay

In brief, 15,000 cells were seeded per well in a 96-well plate with HFFC media. Fifty mM carbamazepine, 0.01 mM gabapentin, 0.06 mM lamotrigine, 0.2 mM levetiracetam and 0.01 mM topiramate were added separately to the mentioned wells. A negative control containing the corresponding solvent was also included for each drug. After the foregoing treatment for 24 h, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)2-(4-sulfophenyl)-2H-tetrazolium (MTS) was added to the wells. Followed by incubation at 37 °C for 3 h, the absorbance was measured at 490 nm wavelength using Multiskan spectrum (Thermo scientific, USA) (Yuan et al., 2013).

2.6. Statistical analysis

Statistical analysis of the data was performed using Sigma plot software. All samples were normalized to the positive control group and data was presented as a percentage. Multiple comparisons among data were statistically evaluated by a one-way ANOVA test. P-value < 0.05 was considered statistically significant. Three independent repeats were placed for each test and all results were presented as mean ± SEM.

3. Results

3.1. hESCs characterization

To evaluate the initial efficiency of hESCs pluripotency, protein expression of pluripotency markers, Nanog and Oct4, were analyzed 48 h after single cell seeding. Immunofluorescent staining verified that the cells are completely pluripotent (Fig. 1D).

3.2. Genotoxicity effects of AEDs on hESCs

To address the effect of AEDs on cellular DNA damage of hESCs, the pharmaceutical compounds were exposed to 48 h cultured-hESCs and their effects were analyzed using Comet assay. A 96 h-treatment was performed with gabapentin and lamotrigine to determine the maximum period time of the study (Fig. 1E). After this time of the treatment, most of the cells were died.

In this study, the number of double-strand breaks in DNA was measured by assessment of the formed tail moments (Fig. 2A-F). When the test was performed on cultured hESCs, levetiracetam and

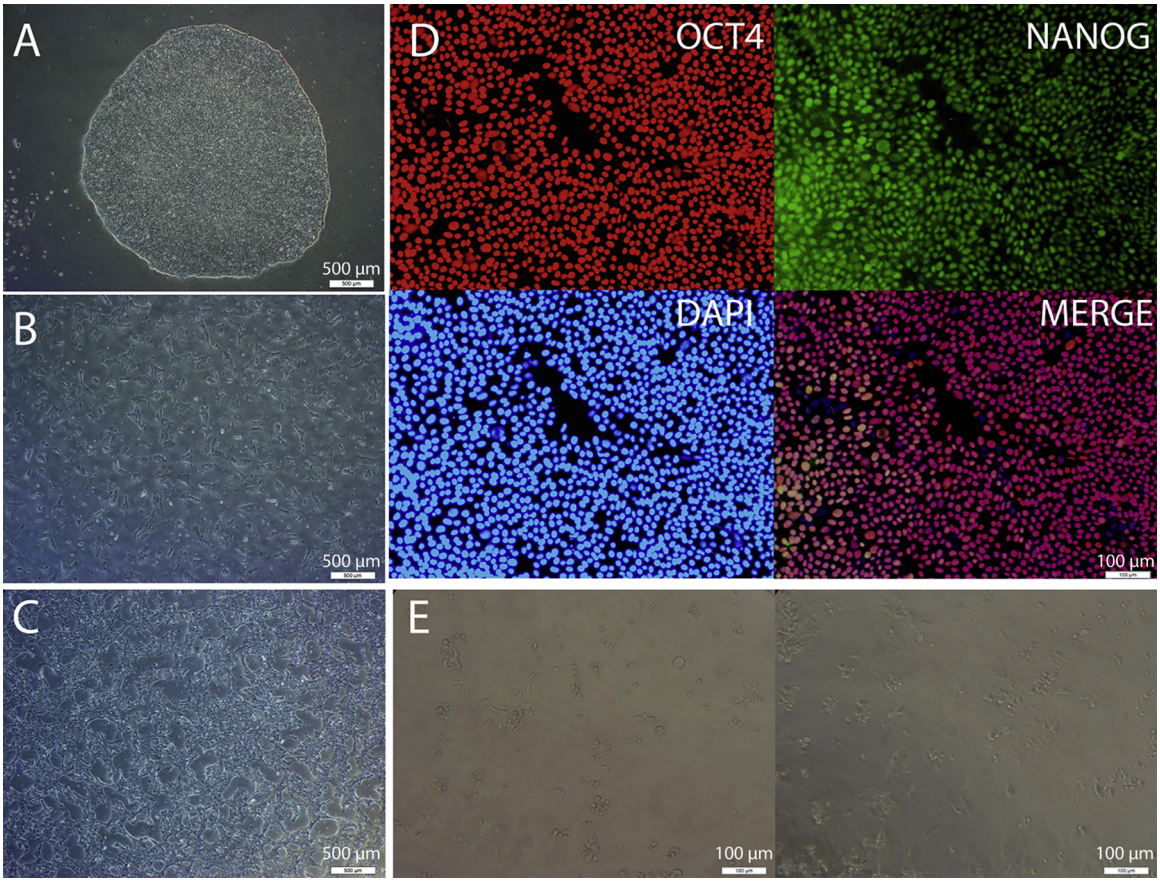

Fig. 1. Characterization of human embryonic stem cells (Royan H6) in HFFC media. A) Morphology of a day 7 RH6 colony on Matrigel with defined border and small and compact cells. Scale bar: 500 µm. B) Morphology of RH6 cells 24 h after dissociation with the concentration of 200,000 cells/ml, cultured on Matrigel. Scale bar: 500 µm. C) Morphology of RH6 cells 48 h after passage, with higher than 90% confluence, ready to start the different pharmaceutical compound treatments. Scale bar: 500 µm. D) Immuno-cytofluorescence staining for pluripotency markers (OCT4 & NANOG) on RH6 single cells before compound treatment. The staining indicates the pluripotency of the cells before treatment. Scale bar: 100 µm. E) RH6 cells morphology after the 96 h-treatment with gabapentin (0.01 mM) and lamotrigine (0.07 mM). Most of the cells were died after this time. Scale bar: 100 µm.

topiramate were found to damage the DNA extensively and significantly (68% and 63% respectively) compared to untreated cells (0.1%); however, it was less than what was found for hydrogen peroxide (positive control; 100%). Levetiracetam and topiramate also caused extensive DNA damages on hESCs in comparison with carbamazepine, gabapentin, and lamotrigine (p-value ≤ 0.001). The amount of DNA damage produced by carbamazepine (34%) and lamotrigine (31%) was similar while for gabapentin, the amount of DNA migration was very low and produced less DNA damage (26%) than the other pharmaceutical compounds (Table 1 & Fig. 3).

3.3. Preventive effect of folic acid on genotoxicity of AEDs

Analyzing the tail moments (Fig. 2G-L) revealed that when AEDs pharmaceutical compounds were combined with folic acid, a considerable reduction in DNA damages occurred in comparison to AEDs monotherapy (Fig. 3). The reduction in genotoxicity in the presence of folic acid was 0.38%, 0.2%, 1.89%, 16.16% and 25.3% for gabapentin, carbamazepine, lamotrigine, levetiracetam, and topiramate pharmaceutical compounds, respectively (Table 1).

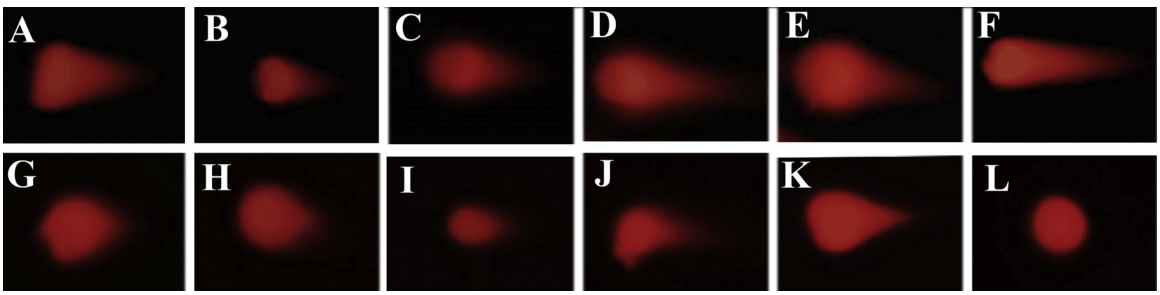

Fig. 2. Detection of DNA damages in RH6 cells by neutral Comet assay. The cells were treated with (A): Carbamazepine, (B): Gabapentin, (C): Lamotrigine, (D): Levetiracetam, (E): Topiramate, (F): Hydrogen peroxide (positive control), (G): Carbamazepine + Folic acid, (H): Gabapentin + Folic acid, (I): Lamotrigine + Folic acid, (J): Levetiracetam + Folic acid, (K): Topiramate + Folic acid, or (L): HFFC media (negative control).

Table 1
DNA damage percentage measured in Comet assays of embryonic stem cells after treatment with Carbamazepine (CBZ), Gabapentin (GAB), Lamotrigine (LAM), Levetiracetam (LEV), Topiramate (TOP) and Folic acid (FA). Untreated embryonic stem cells and hydrogen peroxide were negative control (NC) and positive control (PC), respectively.

Letter used in Fig. 2	Treatment	Adjusted tail moment	DNA damage (%)
L	NC	0.047	0.07
F	PC	60.79	100
A	CAR	20.60	33.89
B	GAB	15.63	25.72
C	LAM	19.00	31.26
D	LEV	41.27	67.89
E	TOP	38.19	62.83
G	CAR + FA	0.20	0.33
H	GAB + FA	0.23	0.38
I	LAM + FA	1.89	3.12
J	LEV + FA	16.16	26.58
K	TOP + FA	25.30	41.63

3.4. Cell cytotoxicity effect of AEDs

To analyze the cytotoxicity effect of AEDs, the hESCs were exposed to AEDs for 24 h before cell viability assessment by MTS assay. Cell viability percentage for carbamazepine, gabapentin, lamotrigine, levetiracetam, and topiramate was 87.25%, 86.62%, 86.48%, 92.21% and 92.36%, respectively. The exposed cells to hydrogen peroxide (positive control) found to have approximately 40% viability. Data has indicated that there was no significant decrease in cell viability of hESCs which were treated by AEDs compared to the negative control (untreated hESCs, Fig. 4).

4. Discussion

Epilepsy must be considered as a critical condition in pregnancy, because when seizures occurs, both mother and fetus are exposed to vital risks (Yerby, 2003). Basically, medical treatment such as carbamazepine, gabapentin, lamotrigine, levetiracetam and topiramate is considered as AEDs to control this condition. (Tomson et al., 2013). It has been shown that many different AEDs increase the risk of fetal malformations particularly neural tube defects (Hernandez-Diaz et al., 2012). According to studies, exposure to carbamazepine during pregnancy causes some malformations including cleft palate, neural tube defects, hypospadias and cardiovascular defects (Czeizel et al., 1992; Jentink et al., 2010; Källén, 1994). The risk of oral clefts is higher among infants who were exposed to topiramate (Hernandez-Diaz et al., 2012). Moreover, gabapentin and lamotrigine showed a lower risk of malformation than conventional AEDs (Mølgaard-Nielsen and Hviid, 2011; Sabers et al., 2004). For levetiracetam, a clinical study found that the risk of major malformation was very low (Hernandez-Diaz et al., 2012). Other findings have shed light on the monotherapy as the first choice for pregnant women with epilepsy since taking multiple AEDs provide a higher risk of bearing babies with malformations. (Oguni and Osawa, 2004).

There are different procedures to evaluate DNA damage extent, among which single cell gel electrophoresis (Comet) assay is mostly selected due to its high sensitivity and accuracy (identifying break among thousands of strands) (Lee and Steinert, 2003). Comet assay is widely used to measure genotoxic damage, especially single and double strand breaks and baseless sites in DNA (Deutsch et al., 2001). In this method, a fluorescent microscope is used to quantify DNA damages that are drawn from nuclei during electrophoresis that form comet-like images (Jackson et al., 2013).

In this study, conventional AEDs were exposed as monotherapy on hESCs and their potential DNA damages, genotoxic effects or cytotoxicity were evaluated by Comet and MTS assays. Besides, the effect of folic acid combination therapy was assessed on the reduction of AEDs genotoxicity.

Carbamazepine is considered as the first-generation of anti-epileptic drugs and is still being used to control seizures caused by epilepsy during pregnancy despite the appearance of newer generation of these drugs (Koda-Kimble, 2012). Conducted clinical studies have shown that using carbamazepine during pregnancy leads to malformations such as cleft palate, neural tube defects, hypospadias and cardiovascular defects (Czeizel et al., 1992; Jentink et al., 2010; Källén, 1994). In this research, it was found that although carbamazepine drug caused Comet forming in comparison with its control group (untreated hESCs) the genotoxic effects were less than the newer generation of anti-epileptic drugs such as levetiracetam and topiramate.

Gabapentin has low teratogenic effects on the hESCs (Hernandez-Diaz et al., 2012). In a study in 2017, it was mentioned that this drug is an acceptable option to treat pregnant women with epilepsy (Veroniki et al., 2017). Our finding in this study has also approved the previous arguments that gabapentin has the least percentage of genotoxicity in comparison with the other AEDs.

Few studies have been performed on teratogenic effects of lamotrigine, a new generation of AEDs, in comparison with the AEDs first-

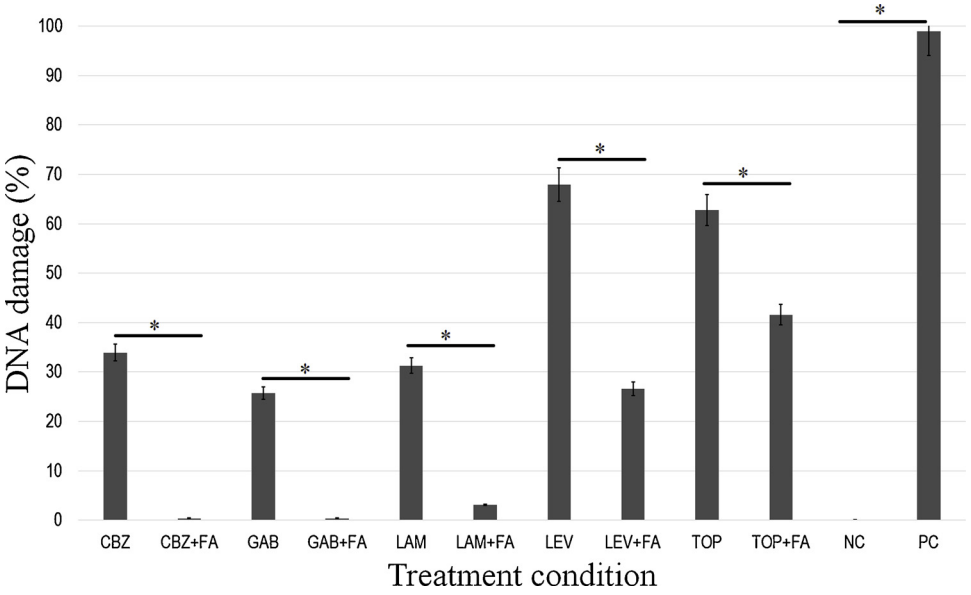

Fig. 3. Genetic damage percentage of hESCs treated with each of the antiepileptic drugs (Carbamazepine, Gabapentin, Lamotrigine, Levetiracetam, and Topiramate) alone or in the presence of folic acid in Comet assay. All drugs, when used with folic acid, were significantly lower in genotoxicity amount (p-value ≤ 0.05) in comparison with their monotherapy treatments (without folic acid). Error bars are indicated as mean ± SEM. N: three independent repeats, Control-: HFFC media, Control+: Hydrogen Peroxide, CBZ: Carbamazepine, GABA: Gabapentin, LEV: Levetiracetam, TPM: Topiramate.

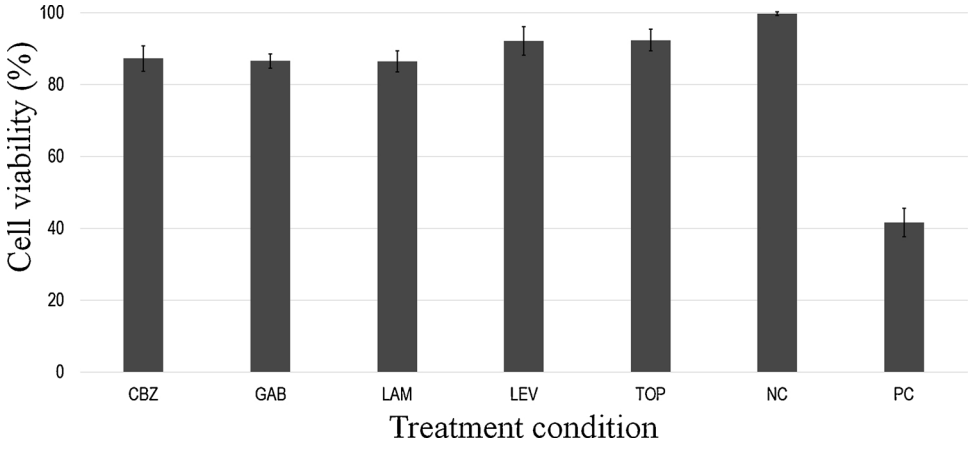

Fig. 4. The cell viability percentage of hESCs treated with Carbamazepine, Gabapentin, Lamotrigine, Levetiracetam, and Topiramate by MTS assay. Error bars are indicated as mean ± SEM. N: three independent repeats, Control-: HFFC media containing drug solvents, Control+: Hydrogen Peroxide, CBZ: Carbamazepine, GABA: Gabapentin, LEV: Levetiracetam, TPM: Topiramate.

generation anti-epileptic drugs such as carbamazepine. A study in 2012 (Hernandez-Diaz et al., 2012), reported few teratogenic capacities of lamotrigine, about 1–2.5 in 1000 people. The results obtained in the present study confirmed the data of this clinical study which shows a low percentage of genotoxicity for lamotrigine.

Topiramate is also a member of the new generation of anti-epileptic drugs that its administration has been increased in the last decade (Mølgaard-Nielsen and Hviid, 2011). Previous studies have shown that topiramate causes hypospadias (Vajda et al., 2013) and oral clefts (Hernandez-Diaz et al., 2012) in newborns and has broad teratogenic effects. The obtained results in this study showed that this drug has significant genotoxic effects in comparison with the other mentioned AEDs, but its genotoxicity is less than levetiracetam.

Levetiracetam is another member of the new generation of AEDs. Two clinical trials for this pharmaceutical compound have been conducted in pregnant women in 2006 and 2011, with no reported abnormalities (Hunt et al., 2006; Mølgaard-Nielsen and Hviid, 2011). Another study in 2012, reported that levetiracetam usage leads to about 2.4% malformation in pregnant women (Hernandez-Diaz et al., 2012). However, in this study, the results showed that levetiracetam and topiramate cause extensive DNA damages on hESCs, compared to carbamazepine, gabapentin, and lamotrigine (p-value ≤ 0.001) (Fig. 3).

Speidel et. al showed that AEDs during pregnancy reduced the serum concentration of folic acid which increased the risk of malformation (Speidel and Meadow, 1972). Other studies have also shown that using 4–5 mg per day of folic acid during pregnancy decreases teratogenic affects up to 50%. This research has shown that when AEDs exposure is combined with folic acid (0.002 mM), DNA damage was clearly reduced and the genotoxicity reduction of AEDs was between 1.5 (topiramate) to 100 (carbamazepine) fold (Table 1). Based on these results, it can be concluded that the addition of folic acid to anti-epileptic therapeutic regime during pregnancy is crucial for the safety of the fetus.

On the other hand, according to our results, the tested doses of these AEDs have not caused a significant decrease in cell viability. Therefore, these compounds may have very little lethal effects on the fetus development process via cell cytotoxicity and their mechanism of adverse effect can be due to the genetic effects they exert.

Placental passage of AEDs due to higher fetus plasma concentrations are associated with increased risk of neonatal complications. This passage has been studied for some AEDs and a high level of transfer was determined (Bank et al., 2017; De Santis et al., 2011). However, pharmacogenetics factors may affect the fetal risks from exposure to AEDs (Atkinson et al., 2007). The *in vitro* results of our study may propose a start point to corelate fetus genotoxicity and maternal plasma concentration of ADEs. Accordingly, more *in vivo* studies should be performed to find the safe dose of these medicines in pregnancy.

5. Conclusions

The results indicate that gabapentin which is used to control tonic-clonic seizures as monotherapy has a lower DNA damage between the studied AEDs in our *in vitro* hESCs-based study; although, its' clinical effects should be investigated to have an *in vivo*-based result. Noticeably, the addition of folic acid to the AEDs treated hESCs decreased the DNA damages significantly. Therefore, it will be of interest if future studies focus on the combinational treatment of these AEDs in the presence of folic acid in both *in vitro* and *in vivo* conditions.

Determination of correlation between therapeutic serum concentrations of AEDs in mother and fetus via the transplacental passage could be considered in clinical pharmacology of AEDs.

It is hence suggested that clinical trials seek optimum epilepsy therapy protocols for pregnancy conditions based on future *in vitro* studies.

Declaration of Competing Interest

The authors have declared they have no conflict of interests.

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