

Effect of levetiracetam on DNA oxidation and glutathione content in a temporal lobe epilepsy model

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Levetiracetam (LEV) is a drug commonly used as an anticonvulsant. However, recent evidence points to a possible role as an antioxidant. We previously demonstrated the antioxidant properties of LEV by significantly increasing catalase and superoxide dismutase activities and decreasing the hydrogen peroxide (H₂O₂) levels in the hippocampus of rats with temporal lobe epilepsy (TLE) showing scavenging properties against the hydroxyl radical. The aim of the present work was to evaluate, the effect of LEV on DNA oxidation, by determining 8-hydroxy-2-deoxyguanosine (8-OHdG) levels, and glutathione content, through reduced (GSH) and oxidized (GSSG) glutathione levels, in the hippocampus of rats with TLE. Male Wistar rats were assigned to the control (CTRL), CTRL+LEV, epileptic (EPI) and EPI+LEV groups. TLE was induced using the lithium-pilocarpine model. Thirteen weeks after TLE induction, LEV was administered for one week through osmotic pumps implanted subcutaneously. The determination of 8-OHdG, GSH and GSSG levels were measured using spectrophotometric methods. We showed that LEV alone significantly increased 8-OHdG and GSSG levels in the hippocampus of control rats compared to those in epileptic condition. No significant differences in GSH levels were observed. LEV could induce changes in the hippocampus increasing DNA oxidation and GSSG levels under nonepileptic condition but not protecting against the mitochondrial dysfunction observed in TLE probably by mechanisms related to changes in chromatin structure, neuroinflammation and alterations in redox components.

Key words: levetiracetam, temporal lobe epilepsy, DNA oxidation, glutathione content

INTRODUCTION

Levetiracetam (LEV), (S-enantiomer pyrrolidine derivative of α -ethyl-2-oxo-1-pyrrolidine acetamide, (S)-2-(2-oxopyrrolidin-1-yl) butanamide) is a common

antiepileptic drug (AED) that is mainly employed to treat partial, myoclonic, tonic-clonic seizures and refractory epilepsy (Farooq et al., 2009). However, LEV has been shown to have other therapeutic effects such as anti-inflammatory, in the treatment of motor or cognitive disease, anticancer and anti-ictogenic ef-

fects (Contreras-García et al., 2022). In the past, several AEDs, such as valproic acid (VPA), LEV, lamotrigine, phenytoin, phenobarbital, carbamazepine, and oxcarbazepine, have been shown to have antioxidant properties and to modulate antioxidant and oxidant systems (Martinc et al., 2010; Işık et al., 2015; Keskin et al., 2016; Beltrán-Sarmiento et al., 2018; Onishi et al., 2023). Our work group recently showed that LEV has antioxidant effects by increasing the superoxide dismutase (SOD) and catalase (CAT) activities and decreasing the hydrogen peroxide (H_2O_2) levels in the hippocampus of rats with temporal lobe epilepsy (TLE). Moreover, we observed that LEV is an *in vitro* scavenger of hydroxyl radical ($HO\cdot$) (Ignacio-Mejía et al., 2023). Therefore, the objective of the present work was to determine, for the first time, the effect of LEV on DNA oxidation, through the determination of 8-hydroxy-2-deoxyguanosine (8-OHdG) levels, and on glutathione content, through the determination of reduced (GSH) and oxidized (GSSG) glutathione levels, in the hippocampus of rats with TLE.

METHODS

Animals

Male Wistar rats weighing 250–300 g (Cinvestav, Mexico) were used. The animals were kept under controlled conditions of temperature ($20\pm 2^\circ C$) and a controlled light/dark cycle (6:00 am / 6:00 pm, light cycle) with food and water *ad libitum*. All procedures were performed in accordance with the National Institutes of Health guide for the care and use of experimental animals and the Official Mexican Standard of the Secretariat of Agriculture (SAGARPA NOM-062-Z00-1999). The protocols were approved by the Institutional Committee of Care and Use of Laboratory Animals of the National Institute of Pediatrics (INP 2022/048). The rats were randomly assigned to the CTRL, CTRL+LEV, EPI and EPI+LEV groups.

Animal model of temporal lobe epilepsy

The methods used to induce of status epilepticus (SE) and post SE care have been previously published (Pichardo-Macías et al., 2018; Contreras-García et al., 2018). Briefly, the EPI and EPI+LEV groups were treated with lithium chloride (127 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO, USA) 19 h prior to pilocarpine administration. The next day, the animals were treated with methyl-scopolamine bromide (1 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO, USA), 30 min before treatment

with pilocarpine; later, SE was induced with pilocarpine hydrochloride (30 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO, USA). SE was defined as continuous convulsive behavior for more than 5 min. After 90 min, SE was interrupted with diazepam (5 mg/kg, i.m.; PISA, Mexico City, Mexico), and immediately, the animals were placed on ice for one hour. Eight hours after the first application of diazepam, the animals received a second dose (5 mg/kg, i.m.), were hydrated with 5 mL of saline solution (SS; 0.9% s.c.) and were kept at $17^\circ C$ overnight. The following day, the rats were returned to controlled conditions and were fed for 3 days with a nutritional feed supplement (Gliem et al., 2002). The CTRL and CTRL+LEV groups received only SS.

Monitoring of spontaneous behavioral seizures

Twelve weeks after SE induction, behavioral video monitoring of the animals was performed to ensure that all the rats had spontaneous recurrent seizures (SRS) and, therefore, were considered epileptic. For this purpose, the rats were continuously monitored on video (24 h / 7 days), using a 2-camera system (Stereon Model CCTV-970, Mexico) and the fast forward speed (8X) of the video recorder. Subsequently, well-trained researchers, blinded to the experimental groups, reviewed the videos using the H.264. The PlayBack for Windows program (v.1.0.1.15, Infinova, China) was used (Contreras-García et al., 2018; Pichardo-Macías et al., 2018).

Levetiracetam treatment

Thirteen weeks post-SE induction, for subchronic LEV treatment (300 mg/kg/day), ALZET® osmotic mini-pumps were implanted subcutaneously in the CTRL+LEV and EPI+LEV groups for one week. Briefly, LEV was extracted from one tablet of 1000 mg (Pharmalife Laboratories, Atlanta, GA, USA) and dissolved in 3 mL of SS. Then, the solution was sonicated, centrifuged and filtered before use. The osmotic pumps were filled with 2 mL of LEV and incubated for 4 h at $37^\circ C$ in SS. Next, the rats were anesthetized with isoflurane (Sofloran®Vet, PISA, Mexico City, Mexico), and the mini-pumps were implanted subcutaneously. Finally, an acute dose of LEV (200 mg/kg i.p.; Keppra, UCB Laboratories, Brussels, Belgium) was administered to the rats (Gliem et al., 2002; Curia et al., 2008; Contreras-García et al., 2021).

The LEV blood concentration was measured 7 days after the osmotic pump was implanted; for this purpose, 50 μL of blood was collected from the caudal vein and

placed on a Guthrie card (Whatman®903, Maidstone, UK). LEV was extracted from the Guthrie card and analyzed via high-performance liquid chromatography according to a method published previously (Oláh et al., 2012). LEV levels in the CTRL+LEV (26.17±2.07 mg/mL) and EPI+LEV (27.82±3.53 µg/mL) groups were not significantly different. No significant concentrations of LEV were detected in the CTRL or EPI groups.

Tissue sample collection and processing

The brains were removed and sectioned to obtain the hippocampus, and the tissues were frozen and stored at -80°C until use. Both hippocampi were homogenized in 50 mM phosphate buffer with 0.05% Triton X-100 (Sigma, St. Louis, MO, USA) at pH=7.0 at a 1:5 ratio and then were centrifuged at 15,000 × g for 30 min at 4°C. The supernatants were separated and stored in tubes to determine 8-OHdG, GSH and GSSG levels. On the day of sacrifice, none of the animals presented SRS.

8-OHdG levels determination

8-OHdG levels were determined using a DNA Damage ELISA Kit according to the manufacturer's instructions (Enzo Life Sciences®, Butler Pike Plymouth Meeting, PA, USA). In brief, 50 µL of hippocampal supernatant was diluted 1:8, a blank or 8-OHdG standard was used, and 50 µL of anti-8-OHdG was added to each well, the plate was incubated and washed, and 100 µL of HRP-conjugated anti-mouse IgG was added to each well. The plate was incubated and washed and then 100 µL of tetramethylbenzidine (TMB) substrate was added to each well. After of the plate was incubated in the dark, 100 µL of stop solution was added to each well. The absorbance was immediately measured at 450 nm. 8-OHdG levels were expressed in ng/mg of protein.

GSH and GSSG levels determination

GSH and GSSG levels were determined using a glutathione colorimetric detection kit according to the manufacturer's instructions (Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA). In brief, 50 µL of previously prepared hippocampal supernatants were diluted 1:8, and blank or previously prepared oxidized GSH standards, 25 µL of colorimetric detection reagent and 25 µL of reaction mixture were added to each well and incubated at room temperature for 20 min. The absorbance was immediately measured at 405 nm and GSH and GSSG levels were expressed in µM.

Statistical analysis

All the data are presented as the mean±standard deviation (SD) for the animals in each group (n=6). The Shapiro-Wilk normality test was performed based on the null hypothesis that the distribution was normal. To determine differences between the groups, the data were analyzed by one-way ANOVA and Tukey's *post hoc* test. The determinations were performed in triplicate. A $p < 0.05$ was considered to indicate a significant difference. All the data were analyzed using Sigma Plot v. 9.5.1 (GraphPad Software Inc, San Diego, CA, USA).

RESULTS

Effect of LEV on DNA oxidation in the hippocampus of rats with TLE

Regarding DNA oxidation (8-OHdG levels), the levels in the CTRL, CTRL+LEV, EPI, and EPI+LEV groups were 4.917±0.64, 5.502±0.64, 4.396±0.35, and 4.710±0.85 ng/mg of protein, respectively. 8-OHdG levels were significantly greater (1.25-fold) in the CTRL+LEV group than in the EPI group (Fig. 1, $F_{3,18}=3.356$, $P=0.0419$, a Tukey *post*

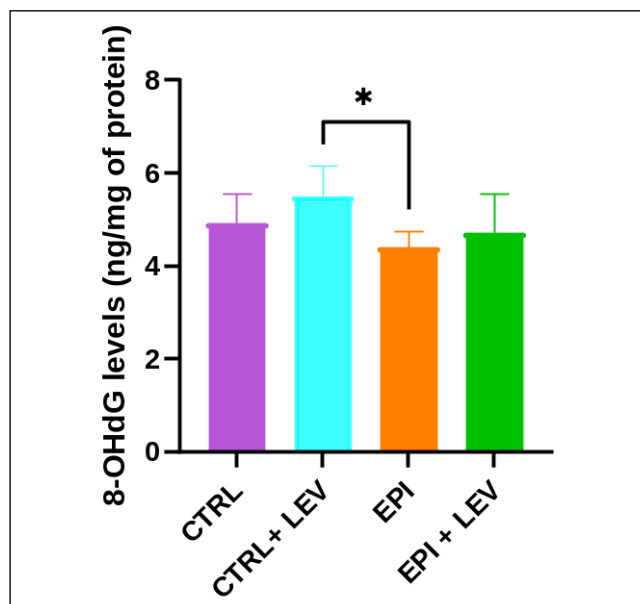


Fig. 1. 8-OHdG (ng/mg of protein) levels determined in the hippocampus of the rats. For all the measurements, each quantification was performed in triplicate, using data from six rats in each group; the values are represented as the mean ± SD. Differences were analyzed using one-way ANOVA and Tukey's *post hoc* test. The brackets indicate the groups that are significantly different. We observed significant differences in 8-OHdG levels between the CTRL+LEV and the EPI groups. The brackets indicate the groups that are significantly different. $P=0.0272$ represented a significant difference. * P value in the graph for Tukey's *post hoc* test.

hoc test showed a significant difference at $P=0.0272$). No significant changes were observed in the CTRL or CTRL+LEV groups. However, 8-OHdG levels in the CTRL+LEV group were observed that to be 1.1- and 1.16-fold greater than those in the CTRL and EPI+LEV groups, respectively.

Effect of LEV on the glutathione content in the hippocampus of rats with TLE

Concerning GSSG and GSH levels in the CTRL, CTRL+LEV, EPI, and EPI+LEV groups, there were

30.31±5.24, 38.75±6.69, 26.67±6.55, and 31.67±3.42 μM values for GSSG levels, respectively and 171.1±29.8, 180.3±33.65, 164.4±52.5, and 155±24.53 μM values for GSH levels, respectively. GSSG levels were significantly greater (1.45-fold) in the CTRL+LEV group than in the EPI group (Fig. 2A; $F_{3,16}=3.872$, $P=0.0295$, a *post hoc* Tukey test showed significant difference at $P=0.0179$). No significant changes were observed for the CTRL or CTRL+LEV groups. Like those in DNA oxidation, the GSSG levels in the CTRL+LEV group were 1.27- and 1.22-fold greater than those in the CTRL and EPI+LEV groups, respectively. No significant differences in GSH levels were detected among all groups (Fig. 2B;

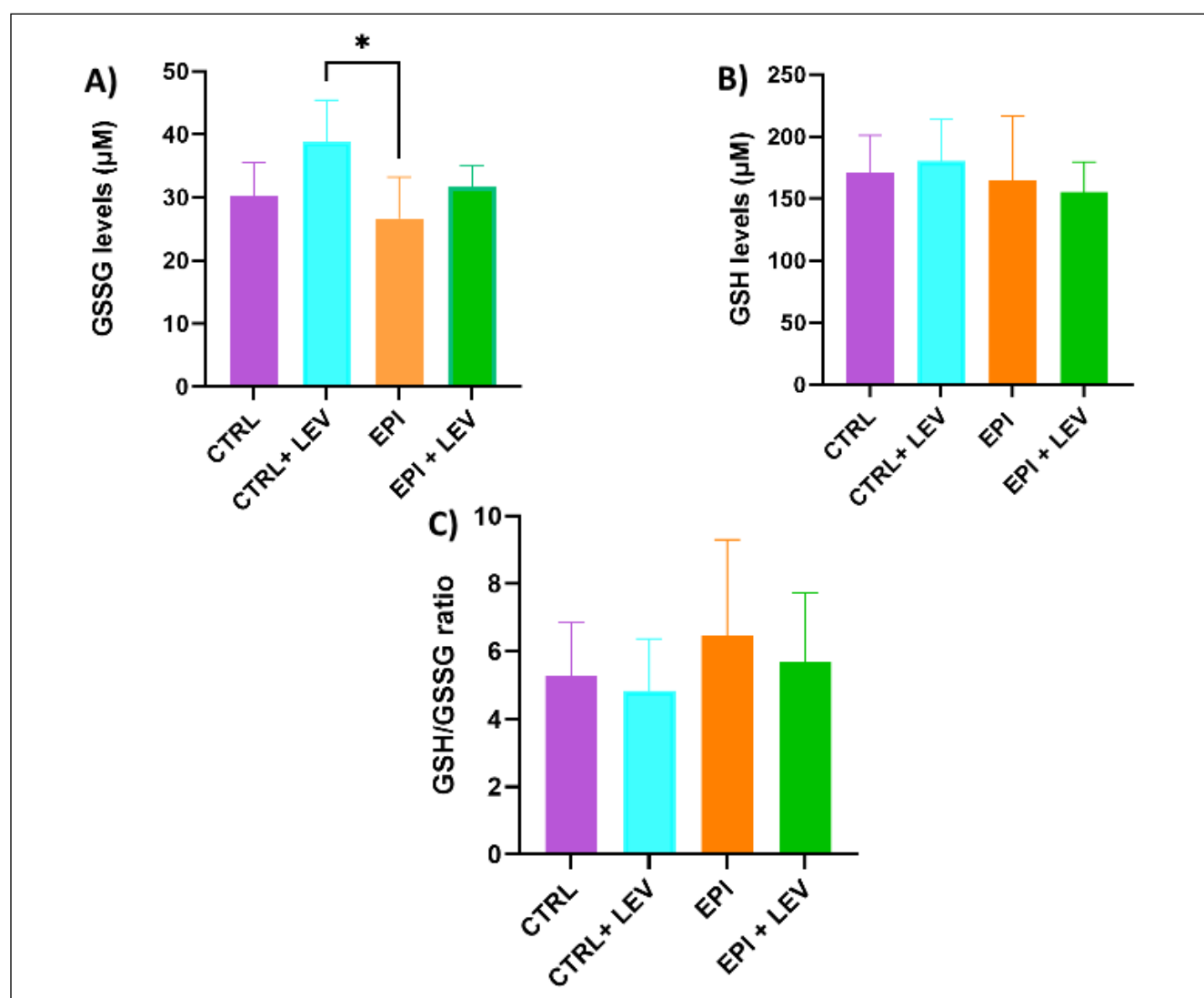


Fig. 2. GSH and GSSG levels (μM) determined in the hippocampus of the rats. (A) GSSG levels; (B) GSH levels and (C) GSH/GSSG ratio. For all the measurements, each quantification was performed in triplicate, using data from six rats in each group; the values are presented as the mean \pm SD. Differences were analyzed using one-way ANOVA and Tukey's *post hoc* test. We observed significant differences in GSSG levels between the CTRL+LEV and the EPI groups. The brackets indicate the groups which that significantly different. * $P=0.0179$ represents a significant difference. * P value in the graph for Tukey's *post hoc* test.

$F_{3,16}=0.3871$, $P=0.7638$); moreover, in the CTRL+LEV group, the GSH levels were slightly greater 1.05-, 1.1- and 1.16-fold than those in the CTRL, EPI and EPI+LEV groups, respectively.

In addition, the GSH/GSSG ratios in the CTRL, CTRL+LEV, EPI, and EPI+LEV groups were 5.28 ± 1.59 , 4.84 ± 1.53 , 6.47 ± 2.83 , and 5.68 ± 2.06 , respectively. No significant differences were observed in any of the groups (Fig. 2C; $F_{3,16}=0.5092$, $P=0.6815$). However, in the EPI group, the GSH/GSSG ratio was 1.22-, 1.33- and 1.13-fold greater than that in the CTRL, CTRL+LEV and EPI+LEV groups, respectively. Similarly, in the EPI+LEV group, the ratios were 1.07 and 1.17-fold greater than those in the CTRL and CTRL+LEV groups, respectively.

DISCUSSION

To our knowledge, this is the first study in which the effects of LEV on glutathione content and DNA oxidation in the hippocampus of rats with TLE were assessed. In the present work, we observed that LEV did not protect against DNA oxidation in the hippocampus of rats with TLE and that LEV alone significantly increased the 8-OHdG levels in comparison with those in epileptic rats. In relation to the GSH or GSSG levels, we observed a significant increase in the GSSG levels in the CTRL+LEV group compared with the EPI group. No significant changes in GSH levels or the GSH/GSSG ratio were observed. Epilepsy is a condition that generates oxidative stress, which contributes to mitochondrial dysfunction causing nuclear or mitochondrial DNA oxidation (Ercegovac et al., 2010; Yang et al., 2020). It has been shown that epilepsy can promote DNA oxidation and decrease the GSH levels in different experimental models and humans (Beltrán-Sarmiento et al., 2018; Abdel-Wahab et al., 2015a; Essawy et al., 2022; Sarecka-Hujar et al., 2022; Wang et al., 2022). A clinical study showed that children with SE, compared with controls, exhibited significantly increased cerebrospinal fluid 8-OHdG levels, which was correlated with brain damage and clinical conditions (Fukuda et al., 2008). These results are consistent with the findings of another clinical study in the brains of patients with TLE in which excessive oxidative stress was shown to be mediated via mitochondrial superoxide ($O_2^{\cdot-}$) and H_2O_2 via dysfunction of mitochondrial complex I in CA neurons and marked ultrastructural abnormalities in mitochondria (Kunz et al., 2000). In relation to the effect of LEV, some studies have evaluated the role of this drug in DNA oxidation and GSH levels, but not in models related to TLE. LEV mitigated doxorubicin-induced DNA ox-

idation in neurons reducing the DNA double-strand breaks and mitigating synaptic and neurite loss. In another study, LEV was shown to reduce the DNA damage caused by amyloid-beta in an Alzheimer's disease mouse model and increase the GSH levels in a rotenone-induced Parkinson's disease rat model (Suberbielle et al., 2013; Erbaş et al., 2016; Manchon et al., 2016). In epileptic models, LEV decreases the 8-OHdG levels and increases the GSH levels in the brains of rats (Al-Shorbagy et al., 2013; Abdel-Wahab et al., 2015b; Mazhar et al., 2017). However, we observed that LEV alone increased 8-OHdG levels in the hippocampus compared with those in TLE patients. Relatedly, therapeutic doses of LEV have been shown to increase 8-OHdG levels in the serum of children with epilepsy (Haznedar et al., 2019). The authors concluded that this evidence explains the adverse effects of LEV via oxidative damage. These results suggest that LEV can cause oxidative damage to DNA through a mechanism that has yet to be determined. However, LEV could have the nuclear DNA as an intracellular site of insult as a result of some stage of its metabolism as was proposed for VPA (Schulpis et al., 2006). On the other hand, LEV modulates histone deacetylase levels (such as VPA) silencing the O6-methylguanine-DNA methyltransferase activating apoptotic pathway in glioblastoma cells (Peruca, 2013; Scicchitano et al., 2018). LEV could cause alterations in the chromatin structure, not necessarily by oxidation process, that could have an effect in the cellular genetic material due to chromatin decondensation inducing then DNA hidroxylation by this way (Baysal et al., 2017; Schulpis et al., 2006; Marchion et al., 2005).

A recent study showed that epileptic patients treated with antiepileptic drugs (AEDs) such as LEV, VPA, or carbamazepine had significantly greater levels of 8-nitroguanine, a DNA nucleobase product of nitrosative stress, than did control groups. The authors concluded that a neuroinflammatory component persists in epilepsy even with AED treatment, as indicated by the increase in the level of 8-NG, a mutagenic lesion that occurs during inflammation, remains elevated (Ersan et al., 2020; Jones, 2012). Consistent with these findings, the TLE rats treated with LEV, particularly those in the EPI+LEV group, still exhibited persistent inflammation and mitochondrial dysfunction. Under epileptic conditions, LEV was unable to reverse or halt existing damage, contrasting with its effect on healthy tissue where it has been shown to induce pro-DNA modifications.

In relation to GSH levels, in the past, LEV was shown to increase the protein level of the cystine/glutamate exchange transporter (xCT) protein in the hippocampus of rats, as measured by *in vivo* evalua-

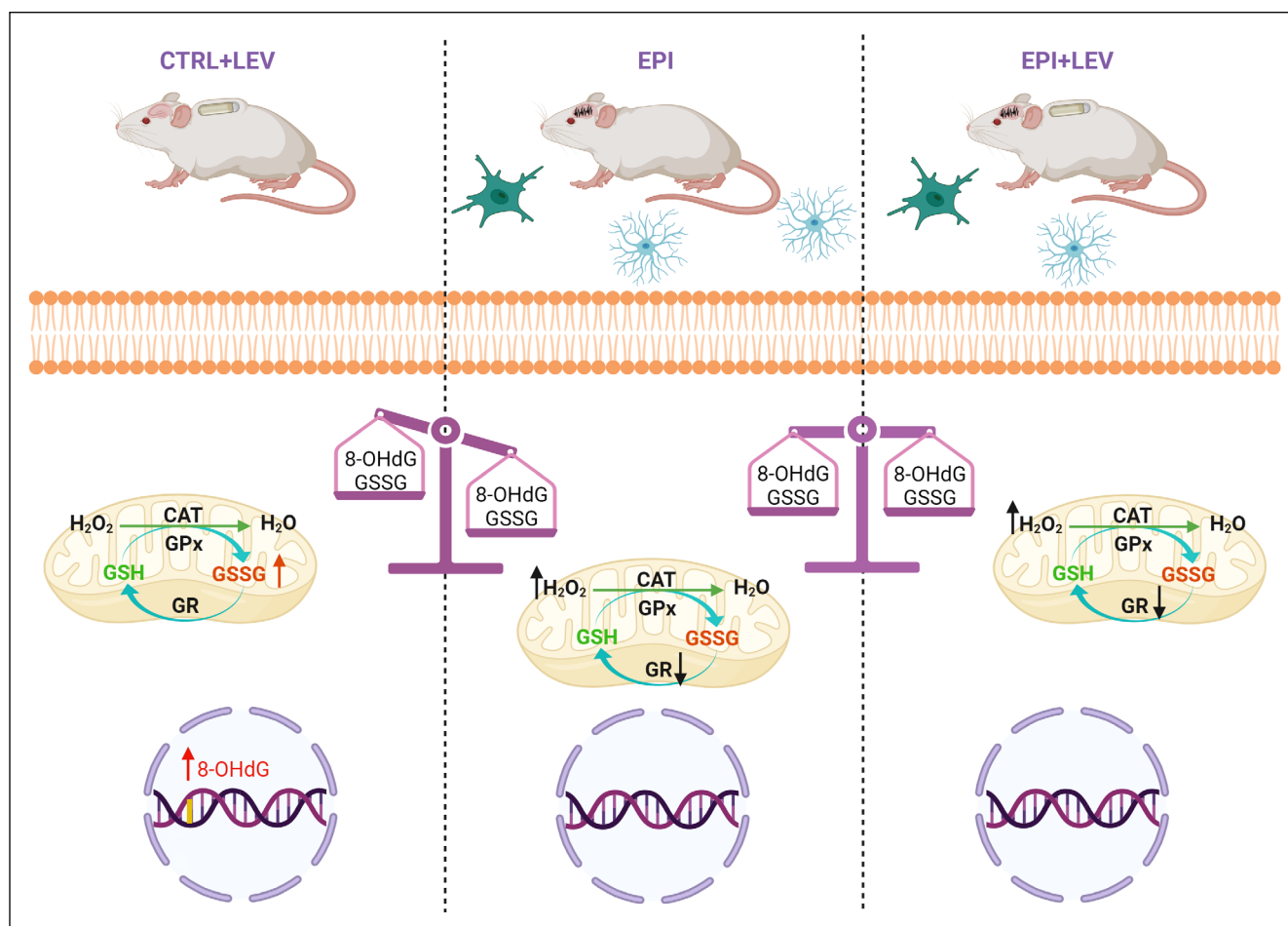


Fig. 3. Effect of LEV on the hippocampus of TLE rats. LEV increases 8-OHdG and GSSG levels.

tion *via* brain microdialysis (Ueda et al., 2009). LEV activated the upregulation of the xCT, which was mediated by astrocytes, and increased GSH levels in the striatum of mice with Parkinson's disease, which was probably induced by nuclear factor erythroid 2-related factor 2 (Nrf-2) (Miyazaki et al., 2011; 2016). Our results showed that GSH levels did not significantly change, although, a significant increase in GSSG levels was observed in the CTRL+LEV group compared with the EPI group. Moreover, we also observed that the GSH/GSSG ratio was not significantly greater in the EPI group than in the other study groups. LEV administration in the CTRL+LEV group could cause an increase in GSSG levels by acting on the mitochondria in the hippocampus of rats compared with than in the EPI group, where LEV also increased this biomarker in the EPI+LEV group. This process is based on the ability of pilocarpine to induce mitochondrial DNA oxidation in the rat hippocampus and possibly to oxidize GSH, increasing its concentration from

7 mM in the cytoplasm to 10–12 mM (Fernández-Checa et al., 1997; Shin et al., 2009; Lin et al., 2010). This was confirmed by the increase in the GSH/GSSG ratio in the EPI group compared to that in the CTRL+LEV group, in which this ratio was noticeably lower. Moreover, in a recent study, we observed that glutathione reductase activity (GR) was significantly lower in the CTRL+LEV and the EPI+LEV groups than in the EPI group (Ignacio-Mejía et al., 2023). GR is the enzyme responsible for supplying GSH, thereby determining the optimal redox control conditions within a cell. GR function is also critical for maintaining GSH levels within organelles that lack synthesis machinery, such as mitochondria and the endoplasmic reticulum (Couto et al., 2016). The observed decrease in this enzyme might also explain the significant increase in GSSG levels in the CTRL+LEV group. GSH serves as a biological redox switch, regulating mitochondrial potential; changes in this antioxidant could lead to subsequent alterations in the hippocampus (Huynh et

al., 2021). Moreover, considering that seizures induce an alteration in antioxidant defenses and a significant increase in H_2O_2 levels in TLE (Ignacio-Mejía et al., 2023), LEV likely does not positively modulate or increase GR activity in the dysfunctional hippocampus of TLE rats, as indicated by the nonsignificant changes in GSH and GSSG levels, and the GSH/GSSG ratio, in the EPI+LEV group (Fig. 3).

Based on our observations, LEV induces mitochondrial DNA alterations without increasing GR activity in the hippocampus of nonepileptic rats; thus, LEV does not protect against mitochondrial dysfunction or change GSH levels in TLE. In a SE model, LEV not only decreased GSH levels in various hippocampal regions but also reduced the activities of α -ketoglutarate dehydrogenase, aconitase, citrate synthase, and complex I after 5 h of SE, indicating that LEV alone does not protect against SE-induced mitochondrial dysfunction (Gibbs et al., 2006; 2007).

Finally, our study has limitations. A randomized controlled trial study is needed to assess the effect of LEV on oxidative activity in patients with epilepsy. Moreover, additional studies are needed to further characterize the pro- and antioxidative effects of this AED.

CONCLUSION

In conclusion, LEV alone caused significant increases in 8-OHdG levels and in GSSG levels in the hippocampus of rats compared with those in TLE conditions. Moreover, LEV did not induce significant changes in GSH levels. LEV may induce changes in the hippocampus, increasing DNA oxidation through chromatin structure alterations and elevating GSSG levels due to GR activity depletion under nonepileptic condition. Moreover, LEV does not significantly modify the redox parameters when is administered under epileptic condition, probably by a persistent neuroinflammation and a decrease in GR activity not protecting against mitochondrial dysfunction present in TLE.

ACKNOWLEDGEMENTS

This research was funded by E022 Program of the Instituto Nacional de Pediatría (protocol 2022/048). I.J.C.G., C.B., I.I.M., L.A.P.M., J.G.M.-T., S.G.-M. and N.C.-R. are members of the National Research System (SNI) from CONAHCyT in Mexico. We are grateful to María Sara Navarrete-Hernández for providing technical assistance.

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