

RESEARCH ARTICLE

# Reproductive toxicity after levetiracetam administration in male rats: Evidence for role of hormonal status and oxidative stress

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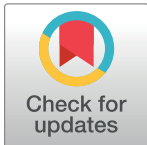
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## Abstract

Levetiracetam (LEV) is an antiepileptic drug commonly used in the treatment of epilepsy because of its excellent safety profile in all age groups. It is remarkable that there are no studies evaluating the toxic effects of this drug on the male reproductive system, as it is commonly used in male patients of reproductive age. From this point of view, our aim was to evaluate the possible toxic effects of LEV on the male reproductive system. Therefore, LEV was administered to male rats orally at 50, 150, and 300 mg/kg for 70 consecutive days. At the end of this period, alterations to body and organ weights were calculated, and sperm concentration, motility, and morphology were investigated by a computer-assisted sperm analysis system. Sperm DNA damage was determined by comet assay and histopathological examination of the testes was carried out. Serum testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) levels were measured by ELISAs to determine the effects of hormonal status, while glutathione, superoxide dismutase, catalase, and malondialdehyde levels in the testes were measured by colorimetric assay kits to determine the role of oxidative status in potential toxicity. According to the results, sperm quality was decreased by LEV treatment in a dose-dependent manner. LEV induced significant DNA damage in the 150 and 300 mg/kg LEV-administered groups. Histopathology of the testes showed that LEV resulted in testicular injury in the 300 mg/kg LEV-administered group. Serum testosterone, FSH, and LH levels were significantly decreased in the 300 mg/kg LEV-administered group. Glutathione, superoxide dismutase, and catalase levels were significantly decreased in all experimental groups while malondialdehyde levels were significantly increased in 150 and 300 mg/kg LEV-administered groups. According to these results, it was determined that LEV administration decreased sperm quality and it was alleged that hormonal alteration and oxidative stress are potential contributors to reproductive toxicity.

## OPEN ACCESS

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## Introduction

Reproductive toxicity has increased in recent years because of enhanced exposure to chemicals like environmental pollutants, industrial products, and pharmaceuticals [1]. Infertility is becoming the focus of interest among all reproductive toxic effects [2]. Results of epidemiological studies have suggested that approximately 50% of infertility cases are due to male-related factors. Pharmaceuticals are considered among the factors that may lead to infertility in males by decreasing the quality of semen [2, 3].

Reproductive toxicity occurs in epilepsy patients owing to the drugs used in treatment, as well as the disease itself [4, 5]. Reproductive dysfunction is observed in 22–67% of males with epilepsy. It is suggested that seizures and antiepileptic drugs change hormone levels and cause reproductive dysfunction by affecting the hypothalamic-pituitary-gonadal axis [6, 7]. Epilepsy affects approximately 50 million people worldwide [8]. It is therefore very important to determine the toxic effects of antiepileptic drugs that are frequently used in patients of reproductive age, during reproductive development and maturation processes [9].

Levetiracetam (LEV) is an antiepileptic drug that is frequently used in the treatment of epilepsy during childhood, adolescence, young adulthood, and adulthood periods [10, 11]. Although LEV is the first-line drug in the treatment of different types of epilepsy, there is insufficient data on the effects of this new-generation antiepileptic [4]. We aimed to assess the possible toxic effects of LEV on the male reproductive system in this study. Accordingly, sperm concentration, motility, morphology, and sperm DNA damage were determined and testis tissue was analyzed in a histopathological manner. In addition to this, serum testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) levels were determined to evaluate the effect of hormonal status on possible toxicity. Glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels in the testis tissue were determined to evaluate the role of oxidative stress.

## Materials and methods

### Materials

LEV (Keppra<sup>®</sup>, UCB Pharma) tablets were used for experiments. FSH, LH, and testosterone levels in serum were determined by ELISA kits from Cusabio Biotech Co. Ltd., Hubei, P. R. C. GSH levels were measured with Cayman<sup>®</sup> Glutathione Assay Kit, MI, USA. Also, MDA, CAT levels and SOD activity were determined by BioVision<sup>®</sup> Lipid Peroxidation (MDA) Colorimetric/Fluorometric Assay Kit, CA, USA; BioVision<sup>®</sup> Catalase Activity Colorimetric/Fluorometric Assay Kit, CA, USA; and BioVision<sup>®</sup> Superoxide Dismutase (SOD) Activity Assay Kit, CA, USA; respectively.

### Animal model and experimental design

Male Wistar rats (12 weeks old, weighing 180–200 g) obtained from the Anadolu University Research Center for Animal Experiments were used for this study. Also this study was approved by the Local Ethical Committee on Animal Experimentation of Anadolu University, Eskisehir, Turkey (File Registration No: 2015–15). The rats were housed in a room at a controlled temperature (24°C) under a 12-h light/12-h dark cycle (lights on at 08:00 h) with free access to standard rat food and water. Animals were acclimatized to the laboratory environment for at least 48 h before the experimental session.

The rats were randomly divided into four groups, each group containing 10 rats. Administration duration of LEV was chosen according to the completion time of spermatogenesis and was in accordance with the guideline OECD 416: Guideline For Testing of Chemicals: Two-

Generation Reproduction Toxicity Study [12], and doses of LEV were determined according to previous studies [13–15]. Also, the therapeutic doses of LEV were between 500 mg/day and 3000 mg/day [11] and the doses we have chosen were in accordance with the guidelines extrapolating human doses to animal doses [16]. LEV doses were administered at a volume of 1 mL/100 g by dissolving in distilled water. The rats were assigned randomly into the following treatment groups: *Control group (C)*, animals receiving distilled water by oral gavage for 70 days; *50 mg/kg LEV-administered group (LEV-50)*, animals receiving a 50 mg/kg dose of LEV by oral gavage for 70 days; *150 mg/kg LEV-administered group (LEV-150)*, animals receiving a 150 mg/kg dose of LEV by oral gavage for 70 days; *300 mg/kg LEV-administered group (LEV-300)*, animals receiving a 300 mg/kg dose of LEV by oral gavage for 70 days.

After LEV administration over 70 days, rats were anesthetized with urethane (1.5 g/kg, i.p.) [17]. Under general anesthesia, blood samples were collected via withdrawal of large amounts of blood from the right ventricle of the heart and rats were euthanized by cardiac puncture exsanguination.

Blood samples from the animals were kept at 2–8°C overnight, centrifuged at 1000 × g for 15 min, and the supernatant was collected. The serum samples were separated into 200 µL aliquots and stored at -20°C until levels of FSH, LH, and testosterone were measured.

Following the collection of blood samples, the testis and epididymis tissues were removed. The left testis and epididymis were washed with phosphate-buffered saline (PBS) and weighed. The left testis was then divided into equal parts and stored at -20°C until levels of oxidative stress parameters like MDA, GSH, SOD, and CAT were measured. The right testis was fixed for histopathological examination after washing with PBS. The cauda of the right epididymis was used to determine sperm parameters.

## Collection and evaluation of sperm samples

For sperm sampling, the rat cauda epididymis was immediately washed in PBS after removal, and placed in DMEM/Ham's F-12 at 37°C. The cauda epididymis was cut into small pieces in a Petri dish containing the same medium to remove blood vessels and fat tissue. Half a centimeter of the cauda epididymis was transferred to another Petri dish containing 1 mL medium, and the sperm was allowed to swim up for 1 min.

Five microliters of the sperm suspension was loaded into a Leja slide (Leja Products BV, Nieuw Vennep, Netherlands). A 4× negative phase contrast objective combined with a phase contrast condenser was used to determine sperm motility and concentration via the Motility/Concentration module of the Sperm Class Analyzer<sup>®</sup> version 5.4.0.1 software (Microptic S. L., Barcelona, Spain) at 25 frames/second. For motility analysis, 200 motile spermatozoa were analyzed as recommended [18, 19].

## Assessment of sperm morphology

Five microliters of the sperm suspension was placed on a slide and smeared with another slide. The sperm smears (three samples/animal) were totally dried at room temperature and stained with Spermblyue<sup>®</sup> (Microptic S. L., Barcelona, Spain) according to the method described by [20]. For morphology analysis, 200 spermatozoa were analyzed with the morphometry module of Sperm-Class Analyzer<sup>®</sup> version 5.4.0.1 software (Microptic S. L., Barcelona, Spain). The machine was equipped with a Nikon Eclipse model 50i (Nikon Corporation, Tokyo, Japan) microscope with a 60× bright-field objective and a video camera (Basler, A78075gc, Germany). The abnormalities of the spermatozoa were evaluated according to the criteria from previous studies [21–25].

## Detection of sperm DNA damage by comet assay

For the comet assay, frosted slides were coated with 1% normal melting point agarose in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS. Ten microliters fresh sperm sample (containing  $1 \times 10^5$  sperm/mL) was suspended in 75  $\mu\text{L}$  of 1% low melting point agarose. This mixture was placed on a coated slide, covered with a lamella, and allowed to set at  $4^\circ\text{C}$  for 5 min. After solidification of the gel, the slide was immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 10.0 containing 1% triton X-100 added just before use, and 40 mM dithiothreitol) for 24 h at room temperature. After the first lysis step, proteinase K was added to the lysis solution (0.5 mg/mL) and a second lysis step was performed at  $37^\circ\text{C}$  for 24 h. At the end of these two lysis steps, all slides were washed three times with deionized water to remove salt and detergent from the micro gels. Following the washes, slides were placed on the platform of the electrophoresis apparatus and were allowed to equilibrate for 20 min with running buffer (500 mM NaCl, 100 mM Tris-HCl and 1 mM EDTA, pH 9) before electrophoresis (0.60 V/cm, 250 mA) for 30 min. The slides were then stained with SYBR Green I (1:10,000) for 1 h and covered with cover slips. DNA damage in spermatozoa was determined by measuring the tail moment via software BS 200 ProP, BAB Imaging System (Ankara, Turkey). One hundred randomly selected sperms were analyzed per sample [26].

## Histological analysis of testis tissue

Following the removal of the right testis, tissues were immediately sliced into small pieces ( $\sim 2 \text{ mm}^3$ ) and then fixed in paraformaldehyde (4%) in phosphate buffer pH 7.2. After dehydration of tissues in a graded series of alcohols, the samples were treated with a mixture of LR White resin (Electron Microscopy Sciences, PA, USA) and ethanol (2:1) (v:v) for 1 h at room temperature in order to improve infiltration. The samples were then embedded in LR White resin and sectioned at a thickness of 700 nm using a Leica EM UC7 ultramicrotome (Wetzlar, Germany). Semi-thin sections were stained with 1% toluidine blue/borax (pH 8.4) for 2 min and observed under a Leica DM 750 light microscope (Wetzlar, Germany) [27].

## Determination of serum FSH, LH, and testosterone levels

Blood samples from the animals were kept at  $2-8^\circ\text{C}$  overnight, centrifuged for 15 min (at  $4^\circ\text{C}$  and at 1000 x g) and the serum was separated. The hormonal analyses were performed using commercially available kits and in accordance with the manufacturer's instructions.

## Determination of GSH and MDA levels in testis tissue

The left testis was divided into equal parts and stored at  $-20^\circ\text{C}$  after freezing in liquid nitrogen. The GSH, CAT, SOD, and MDA levels in the testis were determined using commercially available kits and in accordance with the instructions of the manufacturer.

## Statistical analysis

All data are expressed as mean  $\pm$  standard deviation. Statistical analyses of the groups were performed on the SigmaPlot v.10 package (Systat Software, CA, USA). All values were verified to be normally distributed. In the sperm comet assay, a Dunnett T3 test was performed as a post hoc test. In the other experiments, one-way ANOVA following a Tukey test as a post hoc test was performed.  $P < 0.05$  was considered statistically significant.

**Table 1. Effect of LEV administration on weight gain percentiles and relative organ weights.**

Experimental Groups	Weight Gain %	Relative Testis Weights (g/100 g Body Weight)	Relative Epididymis Weights (g/100 g Body Weight)
C	71.35 ± 23.44	0.55 ± 0.10	0.26 ± 0.04
LEV-50	48.24 ± 16.15 <sup>(a)</sup>	0.52 ± 0.06	0.27 ± 0.04
LEV-150	47.18 ± 16.09 <sup>(a)</sup>	0.52 ± 0.09	0.27 ± 0.05
LEV-300	34.63 ± 12.41 <sup>(aaa)</sup>	0.51 ± 0.07	0.26 ± 0.03

C: Control group; LEV-50: 50 mg/kg levetiracetam administered group; LEV-150: 150 mg/kg levetiracetam administered group; LEV-300: 300 mg/kg levetiracetam administered group; Results are Mean ± SD, n = 10.

<sup>(a)</sup> Different from control group ( $P < 0.05$ ),

<sup>(aaa)</sup> Different from control group ( $P < 0.001$ ).

<https://doi.org/10.1371/journal.pone.0175990.t001>

## Results

### Effect of LEV administration on weight gains and relative testis/epididymis weights

Effects of LEV administration on weight were recorded as weight gain percentiles, and testis/epididymis weight changes were shown as relative testis/epididymis weights.

Relative testis/epididymis weights were expressed as g/ 100 g body weight. No differences were seen when comparing testis/epididymis weights in LEV-administered groups to the control group (Table 1).

Weight gain percentiles in LEV-administered groups showed a significant decrease when compared with the control group. The most dramatic changes were observed in the LEV-300 group in comparison with the control group (Table 1).

### Effect of LEV administration on sperm parameters

**Sperm concentration.** As shown in Table 2, sperm concentration significantly decreased in the LEV-administered groups in comparison with the control group. While sperm concentration in the control group was  $2.68 \pm 0.88 \times 10^6$ /mL, sperm concentrations in the LEV-50, LEV-150, and LEV-300 groups were  $0.83 \pm 0.50 \times 10^6$ /mL,  $0.82 \pm 0.38 \times 10^6$ /mL, and  $0.81 \pm 0.37 \times 10^6$ /mL, respectively.

**Sperm motility.** The percentage of motile sperm in the control group was  $84.54 \pm 9.08\%$ , while it was determined as  $78.83 \pm 8.16\%$ ,  $80.49 \pm 3.89\%$ , and  $69.78 \pm 13.02\%$  in the LEV-50, LEV-150, and LEV-300 groups, respectively. According to these results, a significant decrease was observed in the LEV-300 group compared with the control group. No significant differences were observed among LEV-administered groups in terms of sperm motility (Table 2).

**Table 2. Effect of LEV administration on sperm concentration and motility.**

Experimental Groups	Sperm Concentrations ( $10^6$ /mL)	Sperm Motility (%)
C	2.68 ± 0.88	84.54 ± 9.08
LEV-50	0.84 ± 0.22 <sup>(a)</sup>	78.83 ± 8.16
LEV-150	0.82 ± 0.38 <sup>(a)</sup>	80.49 ± 3.89
LEV-300	0.81 ± 0.37 <sup>(a)</sup>	69.78 ± 13.02 <sup>(a)</sup>

C: Control group; LEV-50: 50 mg/kg levetiracetam administered group; LEV-150: 150 mg/kg levetiracetam administered group; LEV-300: 300 mg/kg levetiracetam administered group; Results are Mean ± SD, n = 10.

<sup>(a)</sup> Different from control group ( $P < 0.05$ ).

<https://doi.org/10.1371/journal.pone.0175990.t002>

**Table 3. Effect of LEV administration on sperm morphology.**

Experimental Groups	Abnormal Sperm (%)	Head Abnormality (%)	Tail Abnormality (%)	Multiple Abnormality (%)
C	24.85 ± 4.88	8.75 ± 3.22	12.4 ± 3.74	3.7 ± 1.58
LEV-50	38.7 ± 2.98	13.55 ± 3.24	18.3 ± 2.09	6.5 ± 2.04
LEV-150	46.3 ± 7.02 <sup>(a)</sup>	13.3 ± 3.14	25.85 ± 3.34 <sup>(a)</sup>	7.15 ± 2.75 <sup>(a)</sup>
LEV-300	59.25 ± 7.21 <sup>(aaa,b)</sup>	15.05 ± 3.59 <sup>(a)</sup>	35.5 ± 4.47 <sup>(aaa,b)</sup>	8.7 ± 3.72 <sup>(a)</sup>

C: Control group; LEV-50: 50 mg/kg levetiracetam administered group; LEV-150: 150 mg/kg levetiracetam administered group; LEV-300: 300 mg/kg levetiracetam administered group; Results are Mean ± SD, n = 10.

<sup>(a)</sup> Different from control group ( $P < 0.05$ ),

<sup>(aaa)</sup> Different from control group ( $P < 0.001$ ),

<sup>(b)</sup> Different from LEV-50 ( $P < 0.05$ ).

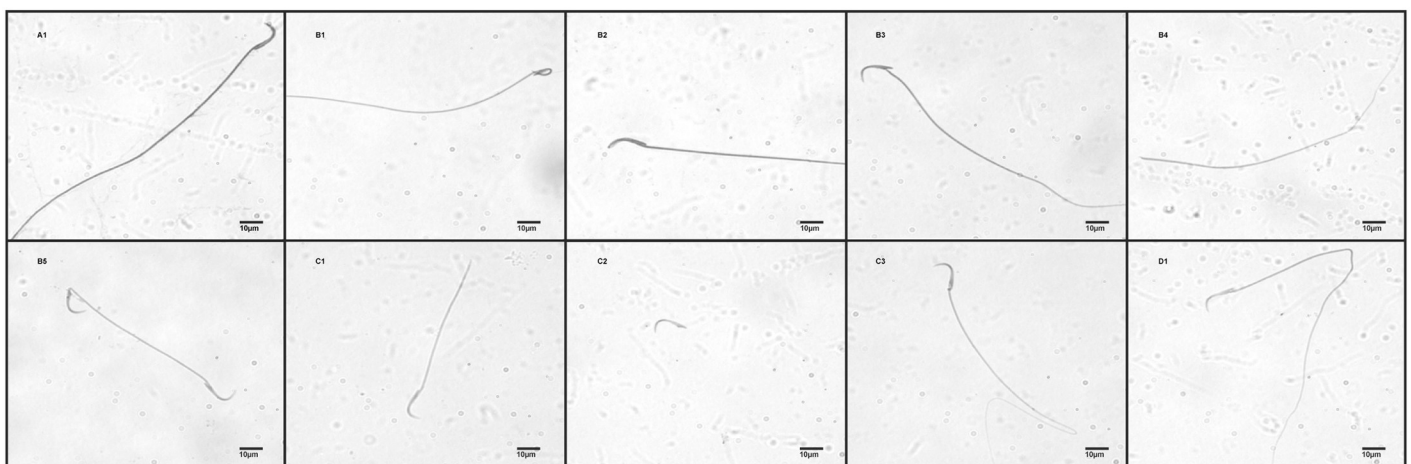
<https://doi.org/10.1371/journal.pone.0175990.t003>

**Sperm morphology.** The percentage of abnormal sperm in the LEV-150 and LEV-300 groups significantly increased when compared to the control group. Among LEV-administered groups, the LEV-300 group showed a significantly higher abnormal sperm percentage when compared to the LEV-50 group (Table 3).

The LEV-300 group showed a significant increase in head abnormalities when compared with the control group. According to percentage of tail abnormalities, significant increases were observed in the LEV-150 and LEV-300 groups when compared with the control group. Among LEV-administered groups, the LEV-300 group showed a significantly higher percentage of tail abnormalities when compared to the LEV-50 group. Percentage of multiple abnormalities in the LEV-150 and LEV-300 groups were significantly higher than the control group (Fig 1, Table 3).

### Sperm DNA damage

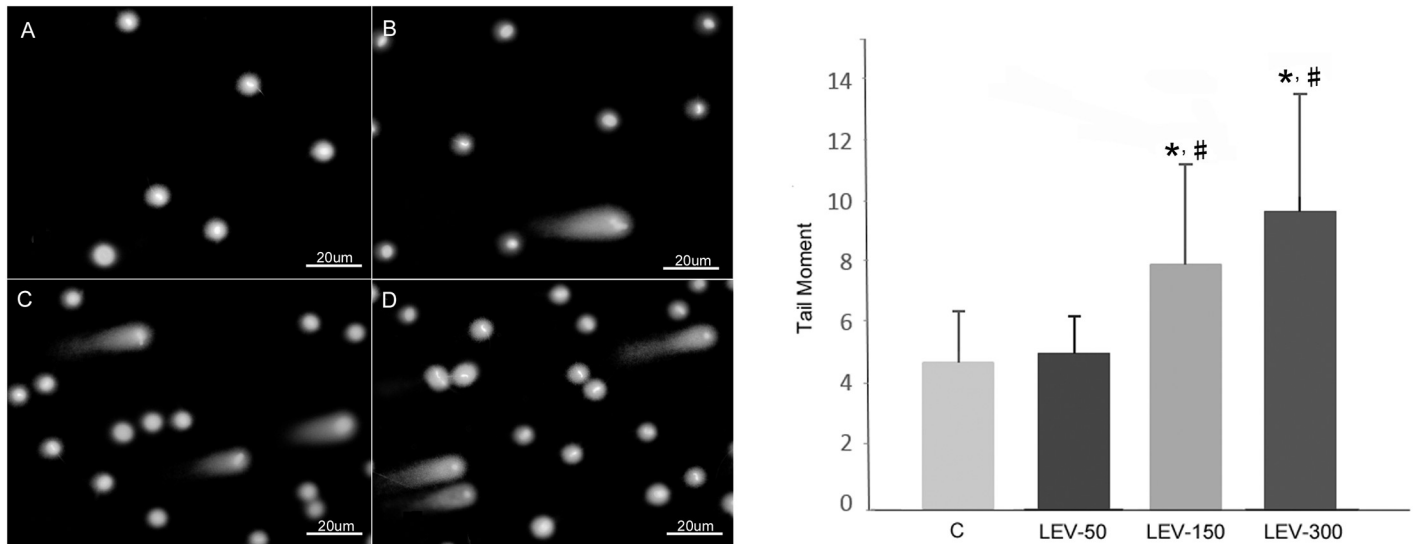
The results expressed as tail moment (extent tail moment: tail length x tail%DNA / 100) for sperm exposed to different doses of LEV and for control group are shown in Fig 2. Exposure to 150 and 300 mg/kg of LEV increased the tail moment by about 67.35% and 104.75% (about



**Fig 1. Sperm morphology observed under 60 x magnification following LEV administration.** A1: Normal; B: Head abnormalities; B1: Amorphous; B2: Banana; B3: Bent neck; B4: Headless; B5: Two headed; C: Tail abnormalities; C1: Broken tail; C2: Detached; C3: Bent tail; D: Multiple abnormalities; D1: Banana + Bent tail.

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**Fig 2. Effect of LEV on sperm DNA damage.** (A) Sperm comet assay photo of control group, (B) Sperm comet assay photo of LEV-50, (C) Sperm comet assay photo of LEV-150, (D) Sperm comet assay photo of LEV-300; Tail moment graph: C: Control group; LEV-50: 50 mg/kg LEV administered group, LEV-150: 150 mg/kg LEV administered group, LEV-300: 300 mg/kg LEV administered group. \* Significant differences when compared with control group ( $P < 0.05$ ); # Significant differences when compared with LEV-50 group ( $P < 0.05$ ).

<https://doi.org/10.1371/journal.pone.0175990.g002>

two-fold), respectively, when compared to the control. Tail moment also increased in these groups by 58.20% and 93.55%, respectively, when compared to the LEV-50 group.

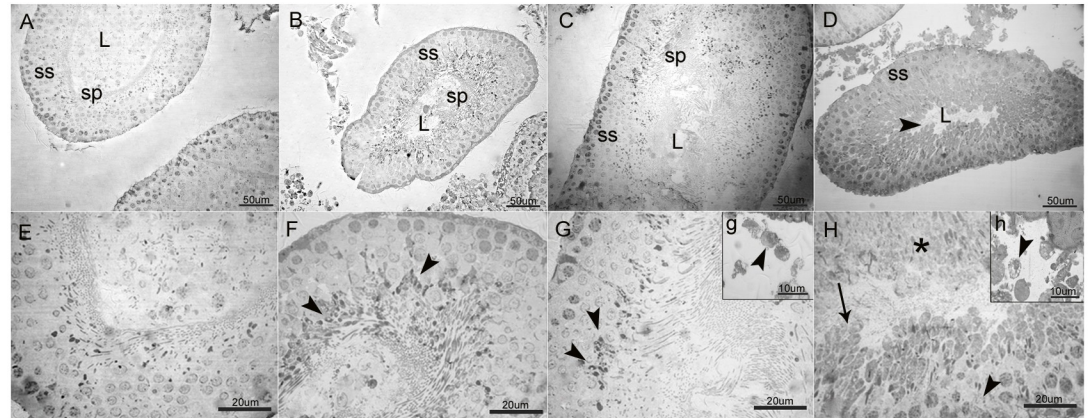
### Histological analysis of testis tissue

Control group animals showed normal architecture of the seminiferous tubules with regularly arranged rows and complete set of germinal epithelium (Fig 3A and 3E). Tubular degeneration was not observed in the LEV-50 group; however, there was a slight vacuolation in late stage spermatids (Fig 3B and 3F). In the LEV-150 group, late stage spermatids and mature spermatozoa were relatively fewer and showed increased vacuolation as was also observed in Leydig cells (Fig 3C, 3G and 3g). LEV (300 mg/kg) administration dramatically increased the lesions which resulted in tubular degeneration and loss of cellular architecture in spermatogenic series. Late stage spermatids and many early spermatids were swollen and vacuolated, and necrosis was observed in some parts of the seminiferous tubules. Very few mature spermatids were observed in the tubular lumen (Fig 3D, 3H and 3h). In accordance with these findings, Johnsen's scoring of the 50 mg/kg LEV-administered group was similar to that of the control ( $9.49 \pm 0.22$  and  $9.68 \pm 0.16$  respectively). However, 150 and 300 mg/kg LEV administration resulted in a significant decrease in these scores, with  $8.93 \pm 0.34$  in LEV-150 group and  $7.51 \pm 0.31$  in LEV-300 group, indicating the increase in the impairment of spermatogenesis at higher doses (Table 4).

### Effect of LEV administration on serum hormone levels

When serum FSH levels were compared between groups, LEV-300 group showed a significant decrease in comparison with the control group. Additionally, FSH levels of the LEV-300 group were significantly reduced compared to the LEV-50 and LEV-150 groups (Table 5).

According to the serum levels of LH in our study, serum LH level of the LEV-300 group decreased significantly compared to the control group. A significant decrease was also observed in the LEV-300 group compared to the LEV-50 and LEV-150 groups (Table 5).



**Fig 3. General view of the seminiferous tubules (A-D) and high magnification of germinal epithelium (E-H) in LEV administered groups and control.** (A-D; L: lumen; ss: spermatogenic series; sp: spermatozoa, scale bar: 50  $\mu$ m). A: Normal architecture of the seminiferous tubule associated with complete spermatogenic series (ss) in control. B & C: Relatively preserved structure of seminiferous tubules and considerable number of spermatozoa in the tubular lumen in 50 mg/kg and 150 mg/kg LEV administered groups, respectively. D: Tubular degeneration with disorganisation of spermatogenic series and markedly decreased number of sperms (▶) in the tubular lumen in 300 mg/kg LEV administered group. (E-H; scale bar: 20  $\mu$ m). E: Intact layers of spermatogenic series in control. F: Slight vacuolation at late stage spermatids (▶) in 50 mg/kg LEV administered group. G: Increased vacuolation and slight degeneration at late stage spermatids (▶) in 150 mg/kg LEV administered group. H: Loss of cellular architecture in spermatogenic series (▶), swelling, vacuolation (→) and necrosis (\*) of the spermatids in 300 mg/kg LEV administered group. g & h: High magnification of Leydig cells (Scale bar: 10  $\mu$ m). g: Vacuolation in Leydig cells (▶) in 150 mg/kg LEV administered group. h: Increased vacuolation and cellular degeneration in Leydig cells (▶) in 300 mg/kg LEV-administered group.

<https://doi.org/10.1371/journal.pone.0175990.g003>

**Table 4. Johnsen’s scores and semi-quantitative comparison of pathology at the cellular level.**

Experimental Groups	Johnsen’s score	Spermatogonial swelling	Cytoplasmic vacuolation	Deformation of cellular architecture
C	9.677 $\pm$ 0.160	-	-	-
LEV-50	9.485 $\pm$ 0.215	-	+	-
LEV-150	8.934 $\pm$ 0.343 <sup>(a)</sup>	-	++	+
LEV-300	7.514 $\pm$ 0.309 <sup>(a)</sup>	++	+++	+++

C: Control group; LEV-50: 50 mg/kg levetiracetam administered group; LEV-150: 150 mg/kg levetiracetam administered group; LEV-300: 300 mg/kg levetiracetam administered group; Results are Mean  $\pm$  SD, n = 160.

<sup>(a)</sup> Different from control group ( $P < 0.05$ ).

<https://doi.org/10.1371/journal.pone.0175990.t004>

**Table 5. Effect of LEV administration on reproductive hormones FSH, LH, and testosterone.**

Experimental Groups	FSH (IU/L)	LH (mg/dL)	Testosterone (IU/L)
C	12.63 $\pm$ 2.73	18.16 $\pm$ 3.73	4.87 $\pm$ 2.6
LEV-50	11.26 $\pm$ 1.76	16.14 $\pm$ 2.91	3.05 $\pm$ 1.85
LEV-150	10.44 $\pm$ 3.55	15.70 $\pm$ 3.05	3.44 $\pm$ 1.39
LEV-300	6.34 $\pm$ 1.77 <sup>(a,b,c)</sup>	11.13 $\pm$ 2.90 <sup>(a,b,c)</sup>	2.48 $\pm$ 1.26 <sup>(a)</sup>

C: Control group; LEV-50: 50 mg/kg levetiracetam administered group; LEV-150: 150 mg/kg levetiracetam administered group; LEV-300: 300 mg/kg levetiracetam administered group; Results are Mean  $\pm$  SD, n = 10.

<sup>(a)</sup> Different from control group ( $P < 0.05$ ),

<sup>(b)</sup> Different from LEV-50 ( $P < 0.05$ ),

<sup>(c)</sup> Different from LEV-150 ( $P < 0.05$ ).

<https://doi.org/10.1371/journal.pone.0175990.t005>



**Table 6. Effect of LEV administration on oxidative stress parameters SOD activity, CAT, GSH, and MDA levels in testis tissue.**

Experimental Groups	SOD Activity (%)	CAT (mU/mL)	GSH (µm)	MDA (Nmol/g Tissue)
C	97.94 ± 1.13	60.47 ± 12.97	217.48 ± 15.95	0.56 ± 0.16
LEV-50	95.5 ± 2.15 <sup>(a)</sup>	37.65 ± 12.13 <sup>(a)</sup>	120.99 ± 7.92 <sup>(a)</sup>	0.82 ± 0.43
LEV-150	92.16 ± 4.17 <sup>(a)</sup>	35.94 ± 7.23 <sup>(a)</sup>	101.07 ± 11.62 <sup>(a)</sup>	0.94 ± 0.49 <sup>(a)</sup>
LEV-300	86.82 ± 6.67 <sup>(a,b,c)</sup>	33.56 ± 4.91 <sup>(a)</sup>	92.64 ± 10.09 <sup>(aaa,b)</sup>	1.09 ± 0.48 <sup>(a)</sup>

C: Control group; LEV-50: 50 mg/kg levetiracetam administered group; LEV-150: 150 mg/kg levetiracetam administered group; LEV-300: 300 mg/kg levetiracetam administered group; Results are Mean ± SD, n = 10.

<sup>(a)</sup> Different from control group ( $P < 0.05$ ),

<sup>(aaa)</sup> Different from control group ( $P < 0.001$ ),

<sup>(b)</sup> Different from LEV-50 ( $P < 0.05$ ),

<sup>(c)</sup> Different from LEV-150 ( $P < 0.05$ ).

<https://doi.org/10.1371/journal.pone.0175990.t006>

When serum testosterone levels were compared across the different groups, testosterone levels in the LEV-300 group were lower than the control group. The results were not significantly different among LEV-administered groups regarding serum testosterone level (Table 5).

### Effect of LEV administration on oxidative stress parameters in testis tissue

SOD activity in the LEV-150 and LEV-300 groups was significantly decreased compared to the control group. The LEV-300 group showed significant differences compared with the LEV-50 and LEV-150 groups (Table 6).

CAT levels were decreased in all LEV-administered groups compared with the control group. There were no significant differences in CAT levels among the LEV-administered groups (Table 6).

According to GSH levels in testis tissue, GSH levels reduced in LEV-administered groups compared to the control group. In addition, GSH levels in the LEV-300 group were significantly lower than the LEV-50 group (Table 6).

MDA levels were increased significantly in all LEV-administered groups compared with the control group. No difference was observed among LEV-administered groups regarding MDA levels (Table 6).

### Discussion

In the present study, we evaluated the reproductive toxicity of LEV in male rats, independently of other risk factors related to infertility, and with repeated pharmacological dosing. The results showed that LEV decreased sperm concentration, motility, normal sperm morphology, increased sperm DNA damage, and induced histopathological changes in testicular tissue in a dose-dependent manner. These toxic effects induced by LEV have been accompanied by induced oxidative stress in testicular tissue and the alteration of serum hormone levels, which play a role in the spermatogenesis process.

Body and organ weights are sensitive biomarkers that are used to determine toxicity related to chemical exposure [28]. Changes in the weight of related tissues reflect the disruptions in the functions of the male reproductive system [29]. In our study, significant differences in relative testis and epididymis weights among groups after administration of LEV were not observed. At this point, it must be emphasized that relative weight differences may escape notice because of factors like edemas occurring in testis and epididymis tissue, or congestion

of efferent channels [30]. Another finding regarding weight changes in our study was the dose-dependent decrease in weight change % after administration of LEV. The effect of antiepileptic drugs on weight loss has been studied for many years [31, 32]. Significant weight loss has been reported after LEV treatment and it is suggested that LEV may cause weight loss at levels that disrupt quality of life in a dose-dependent manner [33].

Sperm concentration, motility, and morphology are biomarkers used to evaluate semen quality and determine reproductive system toxicity [34]. In our study, dose-dependent decreases in sperm concentration in rats have been observed after administration of LEV. In support of this finding, decreasing spermatid counts in tubular lumen were seen in histopathological analyses. Sperm motility was significantly decreased in our high-dose group. Sperm motility is needed for the sperm to reach the cervix and fallopian tubes, and unite with the oocyte. Thus, a decrease in sperm motility is considered an important factor of chemical-induced infertility [29, 35]. Sperm morphology development is another important parameter in determining semen quality, and continues into the late stages of spermatogenesis [34]. Therefore, sperm morphology is a result of the maturation process of sperm in the testes, and is considered as an important biomarker to evaluate testicular functions [29]. Regulatory authorities like EPA, FDA, OECD, WHO, and ICH stress the importance of the abnormalities in the sperm head in the study of male reproductive toxicity [26]. In addition to this, abnormalities in sperm midpiece and tail may also be a factor contributing to infertility [20]. Sperm tail abnormalities, especially twisted tail and bent/spiral tail abnormalities, are considered to be related to infertility [4]. In our study, abnormalities in sperm morphology were detected related to LEV treatment, particularly in the LEV-150 and LEV-300 groups. Head abnormalities that were considered as important biomarkers regarding infertility were observed in the LEV-300 group and tail abnormalities were observed in both LEV-150 and LEV-300 groups. Even though data in regarding LEV's effects on sperm parameters are insufficient, studies have shown that other antiepileptic drugs cause decreases in sperm concentration, motility, and normal sperm morphology [4, 36–38]. Therefore, although LEV is considered safer than other antiepileptic drugs because of its different mechanism of action, its potentially adverse effects on semen quality are similar to other antiepileptic drugs and should not be ignored. In light of these results, it can be suggested that LEV treatment may negatively affect fertility by decreasing semen quality in males.

FSH, LH, and testosterone have roles in the maintenance of male reproductive functions and therefore determining levels of these hormones is important in reproductive toxicity studies [39]. It is known that LH and FSH are secreted under the control of hypothalamic gonadotropin-releasing hormone from the anterior pituitary. LH stimulates the release of testosterone from Leydig cells, and testosterone is necessary for the development of secondary sexual characteristics, spermatogenesis, and the transfer/storage of spermatozoa in the epididymis. FSH regulates the production of spermatozoa in Sertoli cells [40]. The hypothalamic-pituitary-gonadal axis can be affected by many factors. Chemicals including drugs can decrease fertility and even lead to infertility in both males and females by disrupting the normal function of this axis [41]. It has been shown that antiepileptic drugs also influence hypothalamic-pituitary-gonadal axis and cause reproductive dysfunction [6, 36–38, 42–44]. In our study, decreased serum FSH, LH, and testosterone levels were observed in our high-dose group. Studies showed that LH and FSH levels were not altered [44, 45], while testosterone levels were increased [44–46] in male epileptic patients due to LEV treatment. Epilepsy treatment with LEV in prepubertal children did not cause a meaningful change in hormone levels [47]. A decrease in serum LH levels can directly decrease the testosterone production from Leydig cells [40]. Additionally, a decrease in testosterone levels is considered to be related to the severity of spermatogenic cell damage and disruptions in spermatogenesis, and can therefore cause reproductive

dysfunction [40, 41]. Histopathological findings in our study also indicated that vacuolization that was mildly observed in our low-dose group was followed by cellular swelling, spermatid damage, degeneration, and necrosis becoming more pronounced in a dose-dependent manner. Decreased testosterone levels can also be a cause of decrease in semen quality [29]. Therefore, decreased semen quality in our LEV-administered groups could be considered as a consequence of decreased testosterone levels. The basis of the effective mechanism which differentiates LEV from other antiepileptic drugs is that it binds to the synaptic vesicle glycoprotein 2A (SV2A). SV2A is distributed in endocrine tissues as well as in the central nervous system. Previous studies stressed that LEV's endocrine effects can be mediated by this protein [44, 46]. Additionally, it must be emphasized that antiepileptic drugs cause reproductive and sexual disorders by modulating serotonergic transmission [6]. It has been proposed that LEV can lead to sexual dysfunctions by disrupting dopamine/serotonin balance [48]. Increased serotonin levels may disrupt the feedback mechanism of the hypothalamic-pituitary-gonadal axis [49]. Therefore, the possible effects of LEV on the serotonergic pathway may contribute to changes in FSH, LH, and testosterone levels.

It has been suggested that oxidative stress is an important factor in the decrease of semen quality, and male infertility [50, 51]. Oxidative stress causes a decrease in intracellular ATP levels, release of apoptogenic factors (pro-caspase cytochrome C, apoptosis-inducing factors) into the cytosol as a result of disruption of the mitochondrial membrane, enzyme dysfunction, disruption of protein phosphorylation, increase in membrane permeability, and formation of spermicidal products, and therefore decreases semen quality [50]. The particularly high polyunsaturated fatty acids content of sperm membrane makes it sensitive to oxidative stress [34, 51]. Additionally, insufficiency of the antioxidant defense mechanism in sperm contributes to this vulnerability [52]. In our study, reduced SOD, CAT, and GSH levels and increased MDA levels were detected after administration of LEV indicating that this drug resulted in the induction of oxidative stress in testicular tissue. Studies with opposing results have shown that administration of LEV had antioxidant effects in pathologies where oxidative stress is induced [53–57]. However, in another study, an increase in oxidative stress in the brain tissue of rats after administration of LEV was detected, similarly to that in our study [58]. In support of LEV-induced oxidative stress a previous study has shown decreases in antioxidant parameters and increases in oxidant parameters due to LEV treatment in epileptic patients [59]. Hence, the detected decrease in semen quality in the LEV-administered groups in our study can be consequent to the oxidative stress induced by LEV in testicular tissue.

Sperm DNA integrity is a sign of the reproductive capability of the sperm [60]. Therefore, the DNA's structural integrity needs to be investigated in order to evaluate sperm function and its structural changes [34]. The neutral comet assay is easier, more cost-effective, and more frequently used than other methods in determining DNA damage in human sperm, as it is considered as a more sensitive method in detecting double-strand breaks [34, 61]. The most commonly used parameters in the quantitation of DNA damage by comet method are tail length, DNA percentage in tail, and tail moment. It is argued that tail length can be used to detect the initial damage caused when exposed to a genotoxic agent, while tail moment and DNA percentage in tail can be used to detect the severity of DNA damage [62, 63]. However, many studies concluded that tail moment is a better measure to assess DNA damage [64–67]. According to the tail moment data from our study, an increase in sperm DNA damage in the LEV-administered groups was detected by neutral comet assay. Another study which used comet assay showed that administration of LEV in female rats during their 5<sup>th</sup>-18<sup>th</sup> days of pregnancy resulted in an increase of DNA damage in brain and liver tissues of both female rats and their fetuses [68]. It is argued that ROS may cause sperm DNA damage and lead to the development of infertility in males [34]. Insufficiency of sperm DNA repair mechanisms

increases the sensitivity of sperm DNA to oxidative stress even further [52]. In support of these findings, the most important evidence that suggests oxidative DNA damage in sperm is related to male infertility, is high levels of 8-hydroxydeoxyguanosine, which is a product of oxidative DNA observed in infertile males [34]. It is also known that LEV causes histone modifications in DNA by inhibiting the histone deacetylase enzyme, and this results in chromatin decondensation [69, 70]. Thus, LEV-induced DNA damage observed in our study may be the result of oxidative stress, and DNA histone modification by LEV may be a factor promoting this situation. Furthermore, sperm head morphology is considered to be an indirect sign of chemically induced mutagenic effects on sperm DNA. In a previous study, a positive correlation between sperm head abnormalities and sperm DNA damage was detected, and it was argued that defects in sperm head morphology resulted from damages in the genetic material of the sperm [26]. In our study, sperm head abnormalities increased by administration of LEV can be considered as a sign of LEV-induced DNA damage.

According to the results of our study, LEV, which is a first-line drug in the treatment of epilepsy, adversely affected sperm parameters by reducing sperm concentration and motility, increasing abnormal sperm morphology and sperm DNA damage, and caused damage in the testicular structure of male rats. It is suggested that the mechanism of LEV toxicity in the male reproductive system arises from the promotion of oxidative stress and alterations in hormonal status. However, there is still a need for additional human data to investigate the potential risk of LEV on reproductive function in male patients. Further studies may also be conducted to investigate the effects of LEV on fertilization. It is also emphasized that investigating reproductive toxicity and fertility levels in patients using LEV through clinical studies is necessary. On the other hand, we suggest that sperm DNA damage and oxidative status in semen can be determined in addition to traditional sperm parameters.

## Author Contributions

**Conceptualization:** OA.

**Data curation:** OA SI.

**Formal analysis:** OA VK.

**Investigation:** MB SI GK VK SU OA.

**Methodology:** MB SI GK VK OA.

**Project administration:** MB SI OA.

**Supervision:** OA.

**Validation:** MB GK VK OA SI.

**Visualization:** MB VK GK OA SI.

**Writing – original draft:** MB SI OA.

**Writing – review & editing:** MB SI GK VK SU OA.

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