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ATP mitigates high-dose lamotrigine-induced oxidative ovarian injury in rats more effectively than melatonin: insights from biochemical parameters, histopathology, and 8-OHdG, dityrosine, and COX-1/COX-2 double immunofluorescence

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Abstract

Background Lamotrigine has been shown to alter intracellular adenosine triphosphate (ATP) levels depending on the administered dose, thereby promoting reactive oxygen species (ROS) generation and oxidative tissue injury. However, its ovarian effects and the protective role of exogenous ATP remain unexplored. This study investigated the ovarian effects of low- and high-dose lamotrigine and compared exogenous ATP and melatonin, a well-established antioxidant, in lamotrigine-induced oxidative ovarian injury.

Methods Forty-two female Wistar albino rats were allocated into seven groups ($n=6$): healthy control, low-dose lamotrigine (10 mg/kg, oral), high-dose lamotrigine (30 mg/kg, oral), and combination groups receiving ATP (5 mg/kg, intraperitoneal) or melatonin (10 mg/kg, oral) with either lamotrigine dose. Both agents were administered one hour prior to lamotrigine daily for 30 days. Ovarian oxidant and antioxidant biomarkers were quantified. Serum anti-Mullerian hormone (AMH) was measured. Histopathological injury and double-immunofluorescence immunopositivity for 8-hydroxy-2′-deoxyguanosine [8-OHdG], dityrosine, cyclooxygenase-1 [COX-1], and cyclooxygenase-2 [COX-2] were graded semiquantitatively.

Results Low-dose lamotrigine produced no detectable oxidative, histopathological, or immunofluorescence alterations. High-dose lamotrigine significantly elevated oxidant markers and reduced antioxidant levels and AMH, accompanied by severe follicular degeneration and pronounced 8-OHdG, dityrosine, and COX-2 immunopositivity with concurrent COX-1 suppression. ATP pre-administration more effectively attenuated these alterations than melatonin.

Conclusion ATP mitigates high-dose lamotrigine-induced oxidative ovarian injury more effectively than melatonin, likely by restoring cellular energy homeostasis and modulating inflammatory signaling. These findings support further translational investigation of ATP-mediated ovarian protection during chronic high-dose lamotrigine exposure and highlight cellular bioenergetic restoration as a potential adjunctive therapeutic strategy.

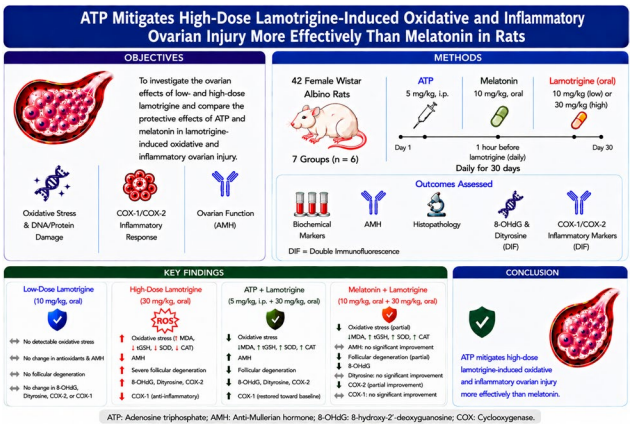
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Graphical Abstract



Keywords Adenosine triphosphate · Fluorescent antibody technique · Lamotrigine · Melatonin · Ovarian diseases/chemically induced · Oxidative stress · Rats

Introduction

Lamotrigine is an antiepileptic drug widely used in the maintenance treatment of bipolar I disorder, as well as in partial and primary generalized tonic-clonic seizures. However, it also has off-label uses, including acute bipolar depression, fibromyalgia, schizophrenia, and unipolar depression [1]. Lamotrigine, a triazine derivative, has been shown to selectively inhibit voltage-gated sodium channels, stabilize presynaptic neuronal membranes, and suppress presynaptic glutamate and aspartate release. In addition, previous studies have shown that it may also interact with voltage-gated calcium channels [1, 2]. Although rare, potentially life-threatening idiosyncratic dermatologic reactions have been reported during lamotrigine use [3]. Moreover, case reports in the literature have described acute porphyric attack, multiple organ failure, and disseminated intravascular coagulation associated with lamotrigine therapy [4, 5]. In animal experiments, lamotrigine has also been shown to induce oxidative liver injury [6].

Previous studies suggest that the use of antiepileptic drugs may increase the risk of infertility [7]. In an experimental study conducted in this context, maternal exposure to lamotrigine was reported to significantly reduce the number of oocytes in female embryos [8]. In addition, various abnormalities related to the reproductive endocrine system have been reported in patients using lamotrigine and other antiepileptic drugs [9]. It has been reported that maternal exposure to lamotrigine at a dose of 20 mg/kg during gestation may exert deleterious effects on the fetal gonads [8]. Furthermore, reproductive endocrine abnormalities have been documented in patients receiving antiepileptic agents, including lamotrigine [9]. Anti-Mullerian hormone (AMH), a key reproductive endocrine hormone, is widely recognized as a reliable marker of ovarian reserve. It exerts regulatory influence over successive stages of folliculogenesis and is further characterized by neuroendocrine actions [10]. It has been emphasized that antiepileptic drugs may lead to hormonal and reproductive disorders in the reproductive endocrine system through their effects on the hypothalamic–pituitary–ovarian axis. Changes in hormone metabolism and effects on hormone-related signaling pathways have also been noted to play a role in this process [9]. More recently, animal studies have shown that lamotrigine treatment leads to marked structural abnormalities in ovarian and uterine tissues and that these changes are associated with increased oxidative stress [11]. In another study involving biochemical and histological examinations of ovarian tissue, an increase in the number of atretic and cystic follicles was detected in groups receiving high-dose lamotrigine [12].

Despite these findings, the mechanisms underlying lamotrigine toxicity have not yet been fully elucidated. Studies show that lamotrigine might affect cellular adenosine triphosphate (ATP) levels. For instance, in lymphocytes from children treated with lamotrigine, increased ATP production was observed at low doses, whereas decreased ATP production was reported at high doses [13]. A reduction in intracellular ATP production increases the generation of reactive oxygen species (ROS), thereby leading to the development of oxidative stress [14]. Elevated ROS levels interact with deoxyribonucleic acid (DNA) and cause oxidative damage. One of the important biomarkers of this damage is 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is considered a product of oxidative DNA damage [15].

In parallel with the reduction in cellular ATP levels, intracellular Ca^{2+} concentration has been reported to increase [16]. This increase in intracellular Ca^{2+} activates phospholipase A2, thereby enhancing the release of arachidonic acid from membrane phospholipids; this process contributes to the activation of the cyclooxygenase-2 (COX-2) pathway [17]. Taken together, these findings suggest that high-dose lamotrigine might cause oxidative damage in various tissues, particularly in the ovaries. Therefore, ATP supplementation might potentially be used to manage dose-dependent lamotrigine toxicity.

As is well known, ATP is a nucleoside triphosphate composed of a nitrogenous base, a ribose sugar, and three phosphate groups [18]. It serves as a fundamental energy source for the synthesis of antioxidants [19, 20]. In addition, ATP exhibits anti-inflammatory effects [21]. Taken together, these findings suggest that high-dose lamotrigine exposure may lead not only to disruption of the oxidant/antioxidant balance in ovarian tissue, but also to an increase in oxidative DNA and protein damage as well as in the inflammatory response, thereby adversely affecting ovarian function. In particular, it may be proposed that alterations in ATP levels, one of the fundamental determinants of cellular energy metabolism, may play a critical role in the interaction between oxidative stress and inflammation. However, no experimental study has investigated the possible protective effect of ATP against lamotrigine-induced ovarian toxicity or its relationship with AMH levels, a key indicator of ovarian reserve, as well as oxidative stress markers, oxidative DNA/protein damage, and the COX-1/COX-2-mediated inflammatory response. In this context, the aim of this study was to investigate the effects of low- and high-dose lamotrigine on rat ovarian tissue, and to evaluate the effect of ATP on potential lamotrigine-induced ovarian damage in comparison with melatonin, an agent whose antioxidant activity is well established.

Materials and methods

Animals and housing

To ensure homogeneity among the experimental groups and to minimize possible confounding effects arising from age- and body weight–related physiological variability, 42 female Wistar albino rats, confirmed at baseline assessment to be 9–10 weeks old and weighing 255–270 g, were included in the study. All animals used in the study were obtained from the Experimental Animal Application and Research Center of Erzincan Binali Yıldırım University (Erzincan, Turkey). The rats were randomly assigned to seven experimental groups, each consisting of six individuals, with care taken to maintain a balanced distribution of baseline body weight across the groups in order to minimize the impact of potential confounding variables. Before the experimental procedures were initiated, the rats were allowed a one-week acclimatization period. During this period, the animals were housed in standard laboratory polycarbonate cages with stainless steel wire lids (Cat. No.: 129 H Eurostandard Type III H; Tecniplast S.p.A., Buguggiate, VA, Italy) measuring 425 mm × 260 mm × 185 mm (base area: 800 cm^2), with six animals per cage.

Throughout the study, environmental parameters were carefully maintained. The light/dark cycle was set at 12 h, the ambient temperature was maintained at 22 °C ± 2 °C, and the relative humidity was kept between 30% and 70%. During the experimental period, all animals were provided ad libitum access to standard laboratory chow (Bayramoglu Feed and Flour Industry Inc., Erzurum, Turkey) and drinking water. Before the start of the

experimental procedures, each animal was confirmed to be clinically healthy, and no findings suggestive of systemic or local pathology were observed.

All experimental procedures were performed in accredited laboratories within the Experimental Animal Application and Research Center of Erzincan Binali Yıldırım University. The applied protocols were designed and conducted in full compliance with the European Parliament Directive 2010/63/EU [22] on the use of animals for scientific purposes (Approval No. 2016-24-199) and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines [23]. During the study, efforts were made to minimize pain and stress in the animals in accordance with the principle of refinement. In addition, the principle of using the minimum number of animals necessary to meet scientific validity criteria was carefully observed.

Chemicals and reagents

All chemical agents and reagents employed throughout this study were of analytical grade and procured from certified commercial sources. Thiopental sodium (Pental Sodium®, 0.5 g powder for solution for injection; Cat. No.: 8699508270385) was provided by Menarini Health and Pharmaceutical Industry Trade Inc. (Istanbul, Turkey). Lamotrigine (Lamictal DC®, 25 mg dispersible chewable tablet; Cat. No.: 8699522072538) was obtained from GlaxoSmithKline (GSK) Pharmaceuticals Inc. (Istanbul, Turkey). ATP (ATP®, 10 mg/mL injectable solution; Cat. No.: 4820117741513) was purchased from Zdorovye Narodu Pharmaceutical LLC (Kharkiv, Ukraine). Melatonin (Melatonina LEK-AM®, 3 mg tablet; Cat. No.: 5909990884926) was supplied by Lek-AM Pharmaceutical Company Ltd. (Zakroczym, Warsaw, Poland).

Experimental design and randomization

Sample size determination was conducted in strict adherence to the principles of the 4R framework—Reduction, Refinement, Replacement, and Responsibility—which aims to minimize the number of animals used while ensuring scientific validity and reproducibility [24]. Exclusion criteria were predefined before the initiation of experimental procedures and were addressed in two separate phases: pre-experimental and during/post-experimental.

Within the pre-experimental exclusion criteria, animals exhibiting abnormal body posture, reduced spontaneous motor activity, or signs of injury resulting from aggressive interactions with cage mates were identified. Any animal meeting any of these criteria was marked for exclusion before randomization and the administration of experimental treatments. The exclusion criteria applicable during and after the experiment were as follows: unexpected death before reaching the predefined experimental endpoints; adverse events related to anesthesia or drug administration; technical failures, such as unsuccessful oral gavage or extravasation at the injection site; noncompliance with the established treatment protocol or incomplete administration of the study compounds; loss exceeding 15% of baseline body weight; clinical findings indicative of dehydration or systemic disease; and uncontrollable pain or severe distress manifested by persistent vocalization or self-injurious behavior. In addition, tissue integrity defects arising during sample collection or processing that could compromise the reliability and accuracy of biochemical, histopathological, or double immunofluorescence analyses were also considered valid grounds for exclusion.

Monitoring of all predefined exclusion criteria was maintained continuously and consistently throughout the intervention period and the subsequent data evaluation process. No animal met any of these criteria at any stage of the experimental procedure; therefore, no animals were excluded from the final analyses, and all individuals were included in the statistical evaluations. No a priori power analysis was performed in this study, and the sample size was determined based on previous experimental studies [11]. Randomization was performed by an independent researcher who was not involved in outcome assessment, using a computer-generated randomization sequence. To minimize potential confounding variables and sources of systematic bias, each cage and each animal was assigned a unique numerical identification code, which was maintained

consistently throughout the experiment. All biochemical, histopathological, and double immunofluorescence evaluations were performed by researchers blinded to group allocation.

Experimental groups

Seven distinct experimental groups were established: a healthy control group (HG), which received no treatment; two lamotrigine monotherapy groups that received oral lamotrigine at doses of 10 mg/kg (LTG-10) and 30 mg/kg (LTG-30); two ATP–lamotrigine combination groups that received intraperitoneal ATP (5 mg/kg) combined with oral lamotrigine at either 10 mg/kg (ATLT-10) or 30 mg/kg (ATLT-30); and two melatonin–lamotrigine combination groups that received oral melatonin (10 mg/kg) combined with oral lamotrigine at either 10 mg/kg (MLLT-10) or 30 mg/kg (MLLT-30).

The ATP dose (5 mg/kg, intraperitoneal, once daily) and the melatonin dose (10 mg/kg, oral, once daily) were each selected based on prior studies using identical dose and administration schedules that demonstrated efficacy in suppressing oxidative stress markers and providing organ-protective, antioxidant effects in drug-induced toxicity models [25, 26]. The 10 mg/kg lamotrigine dose corresponds to the dose range used in humans and was previously shown to produce no significant change in oxidative stress biomarkers or in overall follicle and corpus luteum counts in rat ovarian tissue, despite a modest increase in atretic follicles [12]. The 30 mg/kg lamotrigine dose was selected as a supratherapeutic exposure, consistent with dose-dependent pharmacological effects reported in rats at this dose [27]. This dose exceeds the highest dose evaluated in long-term oral dosing studies of lamotrigine in rats (10–15 mg/kg/day) [28]. Consistent with this, applying Paget's interspecies scaling equation (animal equivalent dose = human dose × Km ratio, Km = 6.2), as used by Rezk et al. [11], the 30 mg/kg dose corresponds to a human equivalent of approximately 4.8 mg/kg (≈ 339 mg/day for a 70-kg adult), near the upper end of the approved human dosing range of up to 500 mg/day [28], reinforcing its classification as a supratherapeutic exposure. Using the same Km ratio and a representative human dose of 250 mg, the mean of the 25–500 mg range reported across lamotrigine's approved indications, Rezk et al. [11] had earlier derived an animal-equivalent dose of 22.14 mg/kg, rounded to 20 mg/kg, which over a 90-day administration period was confirmed to induce marked ovarian and uterine toxicity, manifesting as oxidative stress, hormonal disruption, and histopathological damage. Given the shorter 30-day exposure period in the present study, the dose was raised to 30 mg/kg to reliably ensure toxicity induction within this timeframe.

Experimental procedure

Animals assigned to the ATLT-10 ($n = 6$) and ATLT-30 ($n = 6$) groups received intraperitoneal injections of ATP at a dose of 5 mg/kg, while melatonin (10 mg/kg) was administered orally via gastric gavage to animals in the MLLT-10 ($n = 6$) and MLLT-30 ($n = 6$) groups. Animals in the HG ($n = 6$), LTG-10 ($n = 6$), and LTG-30 ($n = 6$) groups received an equivalent volume of distilled water as a vehicle control. Equivalent volumes were administered across all groups to minimize route-related variability. One hour following the administration of ATP, melatonin, or vehicle, lamotrigine was delivered orally by gastric gavage at a dose of 10 mg/kg to the ATLT-10, MLLT-10, and LTG-10 groups, and at 30 mg/kg to the ATLT-30, MLLT-30, and LTG-30 groups. All compounds were administered once daily for a period of 30 consecutive days. Lamotrigine and melatonin tablets were finely powdered using a sterile mortar and pestle, and the required amounts corresponding to the intended mg/kg doses were calculated based on the active pharmaceutical ingredient content. The powders were suspended in distilled water and vortexed immediately prior to administration to ensure homogeneity. All doses were adjusted individually according to body weight and administered via oral gavage. ATP was used as a commercially available injectable solution without further modification.

At the conclusion of the treatment period, blood samples were collected from the tail vein under brief manual restraint, without chemical anesthesia, given the minimally invasive nature of this procedure, at a

consistent time point across all groups and analyzed for serum anti-Mullerian hormone (AMH) levels. For the terminal procedure, deep surgical anesthesia was first induced in all animals via a single intraperitoneal injection of thiopental sodium (50 mg/kg); anesthetic depth was confirmed by the loss of the corneal [29] and pedal withdrawal [30] reflexes before any further manipulation. Once areflexia was confirmed, animals were humanely euthanized by cervical dislocation, performed while animals remained under deep anesthesia, and death was confirmed by the cessation of respiration and heartbeat, after which ovarian tissues were surgically excised. This protocol was selected in accordance with the AVMA Guidelines for the Euthanasia of Animals (2020) [31] and Annex IV of European Parliament Directive 2010/63/EU [22], ensuring a rapid and painless death. The excised tissues were subsequently subjected to biochemical analysis for the quantification of malondialdehyde (MDA) and total glutathione (tGSH) levels, as well as superoxide dismutase (SOD) and catalase (CAT) activities. Ovarian tissues were additionally processed for histopathological and double immunofluorescence (8-OHdG, dityrosine, COX-2 and cyclooxygenase-1 [COX-1]) examinations.

Biochemical analyses

Preparation of ovarian tissue samples for MDA, tGSH, SOD, CAT, and protein analyses

After euthanasia, ovarian tissues from each animal were surgically dissected, and approximately 100 mg of tissue from each sample was weighed on a precision balance. The tissue samples were washed with ice-cold 0.9% sodium chloride solution to remove residual blood and possible contaminants from their surfaces. The tissues were then cut into small pieces, flash frozen in liquid nitrogen, and finely pulverized using a prechilled mortar and pestle.

The resulting tissue powder was homogenized by suspending it in phosphate-buffered saline (PBS, pH 7.4) at a ratio of 1:10 (w/v). After vortexing the homogenates for 10 s, they were centrifuged at 10,000 × g for 20 min at 4 °C using a refrigerated centrifuge (Allegra X-30R; Beckman Coulter Inc., Brea, CA, USA) [32]. Following centrifugation, the supernatant was carefully separated and stored at − 80 °C until biochemical analyses were performed. MDA and tGSH levels were expressed as nmol/mg protein, whereas SOD and CAT enzyme activities were expressed as U/mg protein.

Determination of MDA, tGSH, SOD, CAT, and protein levels

MDA and tGSH levels, as well as SOD and CAT enzyme activities, in ovarian tissue were measured using rat-specific commercial assay kits (MDA: Cat. No. YLA0029RA, Lot No. YLA83HUF; tGSH: Cat. No. YLA0121RA, Lot No. YL321478; SOD: Cat. No. YLA0115RA, Lot No. YLNBI278; CAT: Cat. No. YLA0123RA, Lot No. YL65523; Shanghai YL Biotech Co., Ltd., Shanghai, China) according to the manufacturer's protocol [32].

Total protein concentration was determined using the Bradford method [33]. In this method, Coomassie Brilliant Blue G-250 dye (Sigma-Aldrich Chemie GmbH, Taufkirchen, Bavaria, Germany; Catalog No. 115444) bound to proteins, resulting in a characteristic shift in the absorbance spectrum, and measurements were performed at a wavelength of 595 nm using a spectrophotometer (DU 730 UV/Vis; Beckman Coulter Inc., Brea, CA, USA).

Determination of serum AMH levels

Serum AMH concentrations were quantitatively determined using a commercial assay kit based on an immunochromatographic sandwich principle (Unicell-AMH; Yhlo Biotech Co., Ltd., Shenzhen, China) [34]. The method is based on the principle that the intensity of the generated fluorescent signal is directly proportional to the AMH level in the sample. According to the manufacturer's data, the analytical sensitivity of the kit was 0.1 ng/mL, and the measurement range was reported to be 0.1–25.0 ng/mL.

Histopathological evaluation

All ovarian tissue samples were fixed in 10% neutral buffered formalin to preserve the morphological integrity required for light microscopic examination. After fixation, the tissue samples were placed in cassettes and washed under running tap water for 24 h to remove residual fixative. The tissues were then dehydrated through a graded ethanol series (70%, 90%, and 100%), cleared with xylene, and embedded in paraffin blocks. Serial sections of 4–5 µm thickness were obtained from the paraffin blocks using a rotary microtome (Leica RM2255; Leica Biosystems Nussloch GmbH, Nussloch, Germany), mounted on glass slides, and stained with hematoxylin and eosin (H&E) for routine histopathological evaluation [32].

Ovarian tissues from six different animals from each experimental group ($n=6$) were examined, and one paraffin section obtained from each animal was used for morphological analysis. Microscopic evaluation was performed on a single representative field randomly selected from each section and examined under 40× objective magnification. Representative fields were selected randomly to minimize sampling bias. With this approach, a total of six photomicrographs were obtained per group, with one image from each animal. Imaging was performed using an Axiolab 5 microscope, an Axiocam 305 color camera, and a Colibri 3 LED illumination system (all components: Carl Zeiss Microscopy GmbH, Jena, Germany); image acquisition and processing were conducted using the ZEISS ZEN 3.1 (Blue Edition) software (Carl Zeiss Microscopy GmbH, Jena, Germany).

Histopathological damage was evaluated primarily based on the presence of follicular degeneration. The degree of tissue injury was scored using a four-point semiquantitative scoring system presented in Table 1 (0: none, 1: mild, 2: moderate, 3: severe) [32]. To minimize observer bias during the evaluation process, all sections were analyzed by a single experienced pathologist blinded to group allocation.

Double immunofluorescence method

Five-micrometer-thick tissue sections mounted on poly-L-lysine-coated slides were deparaffinized and rehydrated through xylene and a graded alcohol series, and then washed with PBS. To unmask antigenic epitopes in the tissues, antigen retrieval was performed in citrate buffer (pH 6.0) in a microwave oven (Bosch BEL554MS0; BSH Hausgeräte GmbH, Munich, Germany) at 800 W for 2 × 5 min. After being washed twice with PBS for 10 min each time, the sections were incubated for 10 min in a solution containing PBS/gelatin (0.25%)/Triton X-100 (0.25%) to permeabilize the tissue and reduce nonspecific binding.

Table 1 Semiquantitative histopathological evaluation of ovarian follicular degeneration in experimental groups

Group	Follicular Degeneration Score
HG	0 (0–0)
LTG-10	0 (0–1)
LTG-30	3 (2–3) [a, b]
ATLT-10	0 (0–1)
ATLT-30	1 (1–2) [a, c]
MLLT-10	0 (0–1)
MLLT-30	2 (1–2) [a, c, d]

Histopathological data are expressed as median (min–max). Follicular degeneration was graded on a four-point scale: 0 = absent, 1 = mild, 2 = moderate, 3 = severe. [a] indicates $p<0.05$, vs. HG; [b] indicates $p<0.05$, vs. LTG-10; [c] indicates $p<0.05$, vs. LTG-30; [d] indicates $p<0.05$, vs. ATLT-30. Pairwise comparisons were performed using the Mann–Whitney U test (asymptotic significance, 2-tailed). HG was compared with each of the remaining six groups; LTG-10 was compared with LTG-30; additionally, groups receiving 10 mg/kg lamotrigine (LTG-10, ATLT-10, MLLT-10) were compared among themselves, and groups receiving 30 mg/kg lamotrigine (LTG-30, ATLT-30, MLLT-30) were compared among themselves. Each group consisted of six animals ($n=6$)

Abbreviations: *HG*, healthy control group; LTG-10, lamotrigine (10 mg/kg)-only group, *LTG-30* lamotrigine (30 mg/kg)-only group, *ATLT-10* ATP + lamotrigine (10 mg/kg) group, *ATLT-30* ATP + lamotrigine (30 mg/kg) group, *MLLT-10* melatonin + lamotrigine (10 mg/kg) group, *MLLT-30* melatonin + lamotrigine (30 mg/kg) group, *ATP* adenosine triphosphate

The sections were then blocked with 5% bovine serum albumin (BSA) for 1 h to prevent nonspecific protein binding. After blocking, the sections were sequentially incubated on the same slide with two different pairs of primary antibodies, with intermediate washing twice with PBS for 10 min each time and intermediate blocking with 5% BSA for 1 h at each step to prevent cross-reactivity. The first pair consisted of monoclonal anti-3,3-dityrosine (Jaica, Nikken SEIL Co., Ltd., Shizuoka, Japan; Cat. No. MDT-020P) and polyclonal 8-OHdG antibodies (Bioss Antibodies Inc., Woburn, MA, USA; Cat. No. bs-1278R). The second pair consisted of monoclonal COX-1 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; Cat. No. sc-19998) and polyclonal COX-2 antibodies (BT-Lab, Shanghai Korain Biotech Co., Ltd., Shanghai, China; Cat. No. BT-AP08027). All primary antibodies were prepared at a 1:200 dilution, and the sections were incubated overnight at 4 °C. After each incubation step, the sections were washed twice with PBS for 10 min each time. Sections not subjected to primary antibody treatment were also included in the protocol as negative controls.

Following primary antibody incubation, the sections were incubated for 45 min with a secondary antibody cocktail prepared in 1% BSA and diluted 1:100. The cocktail consisted of equal proportions of Goat Anti-Mouse fluorescein isothiocyanate (FITC; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; Cat. No. 115-095-003) and Anti-Rabbit Alexa Fluor 594 (Cell Signaling Technology, Inc., Danvers, MA, USA; Cat. No. 8889 S). The sections were then washed twice with PBS for 10 min each time and treated with 10 mM $CuSO_4$/50 mM NH_4Cl solution for 10 min to reduce autofluorescence.

The sections were washed with distilled water, treated with DAPI (4′,6-diamidino-2-phenylindole) for nuclear staining, and examined under 40× magnification using an Axiolab 5 fluorescence microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), an Axiocam 305 color camera system (Carl Zeiss Microscopy GmbH, Jena, Germany), and a Colibri 3 light source (Carl Zeiss Microscopy GmbH, Jena, Germany) [32].

During the evaluation process, 8-OHdG and COX-2 immunopositivities were analyzed as red fluorescence, whereas dityrosine and COX-1 immunopositivities were analyzed as green fluorescence. Images were scored using a four-point semiquantitative scoring system as follows: absent (0), mild (1), moderate (2), and severe (3), using the ZEISS ZEN 3.1 (Blue Edition) software (Carl Zeiss Microscopy GmbH, Jena, Germany). Scoring was performed by a single experienced observer blinded to group allocation.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows (Version 27.0, 2020; IBM Corp., Armonk, NY, USA). Biochemical data were presented as mean ± standard error of the mean, and graphs were generated using GraphPad Prism (Version 8.0.1, 2018; GraphPad Software, Inc., San Diego, CA, USA). Before analysis, the normality assumption for the biochemical data was tested using the Shapiro–Wilk test (Supplementary Table 1), and the homogeneity of variances assumption was tested using Levene's test (Supplementary Table 2). When both assumptions were satisfied, intergroup differences were evaluated using one-way analysis of variance, and multiple comparisons were performed using Tukey's Honestly Significant Difference test (Supplementary Table 3). When the distribution was normal, but the homogeneity of variances assumption was not met, Welch's analysis of variance was applied, followed by post hoc comparisons using the Games–Howell test (Supplementary Table 3).

The use of parametric statistical methods was considered inappropriate for histopathological damage scores and double immunofluorescence signal intensity ratings because these data are ordinal variables. For these variables, pairwise comparisons between groups were performed using the two-tailed Mann–Whitney U test. Histopathological and double immunofluorescence findings were reported as median (minimum–maximum). For all statistical tests conducted in this study, the level of statistical significance was set at $p < 0.05$.

Results

Biochemical findings

Evaluation of ovarian tissue MDA levels

High-dose lamotrigine significantly increased ovarian MDA levels, and this increase was attenuated by both ATP and melatonin co-treatment, though to differing degrees. Ovarian MDA levels were comparatively evaluated across the experimental groups (Fig. 1). Compared with the healthy control group (HG, 4.53 ± 0.09), MDA levels were significantly increased in the high-dose lamotrigine group (LTG-30, 7.14 ± 0.13; $p < 0.001$), whereas no significant change was observed in the low-dose lamotrigine group (LTG-10, 4.44 ± 0.06; $p = 0.952$). A statistically significant difference was also observed between the LTG-10 and LTG-30 groups ($p < 0.001$).

In the ATP-combined lamotrigine groups, MDA levels in the ATLT-10 group (4.37 ± 0.15) did not differ significantly from those in the HG ($p = 0.950$) or LTG-10 groups ($p = 0.999$). In the ATLT-30 group (4.79 ± 0.08), MDA levels were significantly lower than those in the LTG-30 group ($p < 0.001$), while no significant difference was observed compared with the HG ($p = 0.392$).

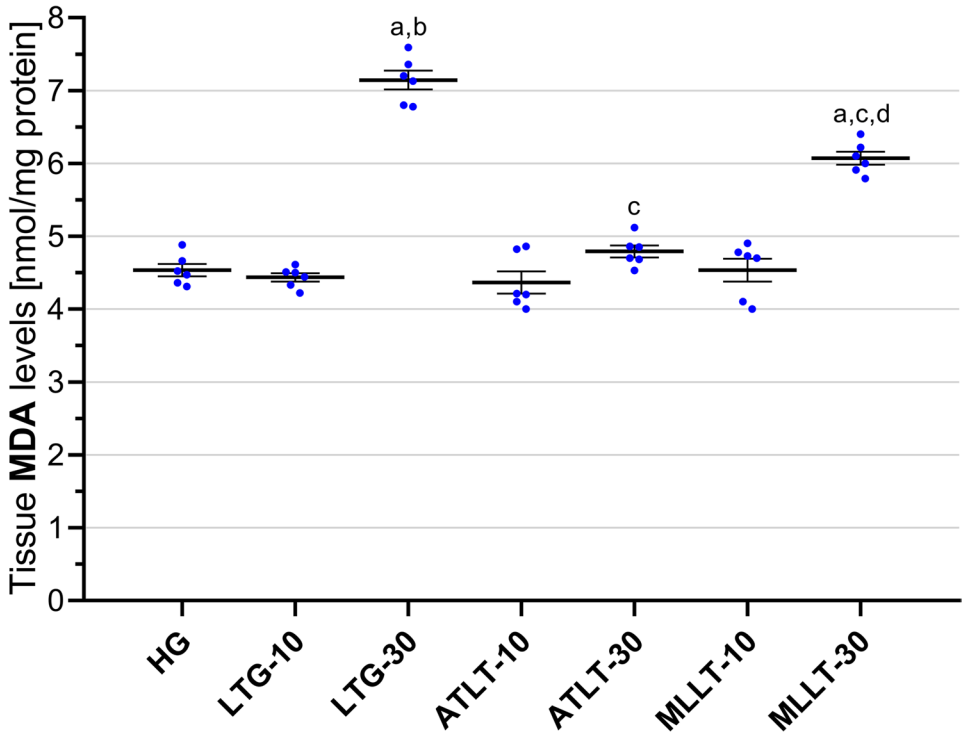


Fig. 1 Modulatory effects of ATP, melatonin, and lamotrigine on MDA levels in rat ovarian tissue. Footnote: Values are presented as mean ± standard error of the mean (SEM). Intergroup statistical comparisons were performed using Welch's analysis of variance (ANOVA) in conjunction with the Games–Howell post hoc test. HG was compared with each of the six treatment groups; LTG-10 was compared with LTG-30; groups administered 10 mg/kg lamotrigine (LTG-10, ATLT-10, MLLT-10) and groups administered 30 mg/kg lamotrigine (LTG-30, ATLT-30, MLLT-30) were each compared among themselves. Statistical significance is indicated as follows: a, $p < 0.001$ vs. HG; b, $p < 0.001$ vs. LTG-10; c, $p < 0.05$ vs. LTG-30; d, $p < 0.001$ vs. ATLT-30. Each experimental group consisted of six animals ($n = 6$). Abbreviations: HG, healthy control group; LTG-10, lamotrigine (10 mg/kg)-only group; LTG-30, lamotrigine (30 mg/kg)-only group; ATLT-10, ATP + lamotrigine (10 mg/kg) group; ATLT-30, ATP + lamotrigine (30 mg/kg) group; MLLT-10, melatonin + lamotrigine (10 mg/kg) group; MLLT-30, melatonin + lamotrigine (30 mg/kg) group; ATP, adenosine triphosphate; MDA, malondialdehyde

In the melatonin-combined lamotrigine groups, MDA levels in the MLLT-10 group (4.54 ± 0.16) did not differ significantly from those in the HG ($p > 0.999$) or LTG-10 groups ($p = 0.994$). MDA levels in the MLLT-30 group (6.07 ± 0.09) were significantly lower than those in the LTG-30 group ($p = 0.001$), yet remained significantly higher than those in the HG ($p < 0.001$). Furthermore, MDA levels in the MLLT-30 group were significantly higher than those in the ATLT-30 group ($p < 0.001$).

Evaluation of ovarian tissue tGSH levels

High-dose lamotrigine significantly reduced ovarian tGSH levels, and ATP preserved this antioxidant more effectively than melatonin. Ovarian tGSH levels were comparatively assessed across experimental groups (Fig. 2). Relative to the HG (5.73 ± 0.09), tGSH levels were significantly reduced in the high-dose lamotrigine group (LTG-30, 2.39 ± 0.08; $p < 0.001$), whereas no significant change was observed in the low-dose lamotrigine group (LTG-10, 5.69 ± 0.08; $p > 0.999$). The difference between the LTG-10 and LTG-30 groups was likewise statistically significant ($p < 0.001$).

In groups co-administered ATP and lamotrigine, tGSH levels in the ATLT-10 group (5.74 ± 0.05) did not differ significantly from either the HG ($p > 0.999$) or the LTG-10 group ($p > 0.999$). In the ATLT-30 group (5.33 ± 0.12), tGSH levels were significantly higher than those in the LTG-30 group ($p < 0.001$), while the difference relative to the HG did not reach statistical significance ($p = 0.053$).

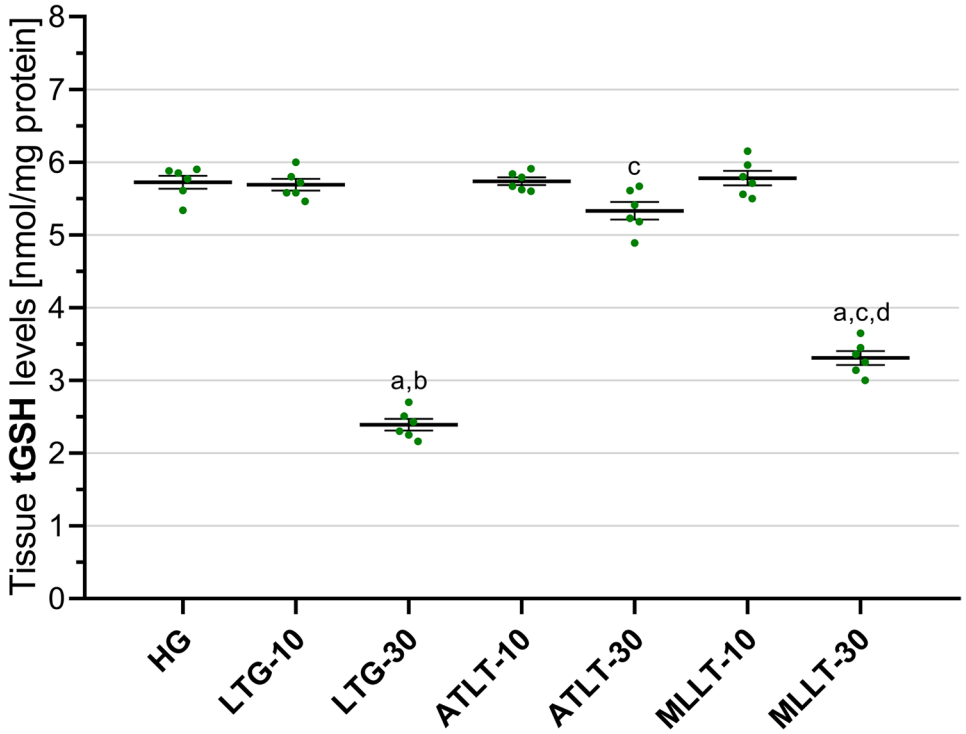


Fig. 2 Modulatory effects of ATP, melatonin, and lamotrigine on tGSH levels in rat ovarian tissue. Footnote: Values are presented as mean ± standard error of the mean (SEM). Intergroup statistical comparisons were performed using one-way analysis of variance (ANOVA) in conjunction with Tukey's honestly significant difference (HSD) post hoc test. HG was compared with each of the six treatment groups; LTG-10 was compared with LTG-30; groups administered 10 mg/kg lamotrigine (LTG-10, ATLT-10, MLLT-10) and groups administered 30 mg/kg lamotrigine (LTG-30, ATLT-30, MLLT-30) were each compared among themselves. Statistical significance is indicated as follows: a, $p < 0.001$ vs. HG; b, $p < 0.001$ vs. LTG-10; c, $p < 0.001$ vs. LTG-30; d, $p < 0.001$ vs. ATLT-30. Each experimental group consisted of six animals ($n = 6$).Abbreviations: HG, healthy control group; LTG-10, lamotrigine (10 mg/kg)-only group; LTG-30, lamotrigine (30 mg/kg)-only group; ATLT-10, ATP + lamotrigine (10 mg/kg) group; ATLT-30, ATP + lamotrigine (30 mg/kg) group; MLLT-10, melatonin + lamotrigine (10 mg/kg) group; MLLT-30, melatonin + lamotrigine (30 mg/kg) group; ATP, adenosine triphosphate; tGSH, total glutathione

In groups co-administered melatonin and lamotrigine, tGSH levels in the MLLT-10 group (5.78±0.10) were comparable to both the HG ($p=0.999$) and the LTG-10 group ($p=0.991$). In the MLLT-30 group (3.31±0.09), tGSH levels were significantly higher than those in the LTG-30 group ($p<0.001$); however, they remained significantly below HG levels ($p<0.001$). Moreover, tGSH levels in the MLLT-30 group were significantly lower than those in the ATLT-30 group ($p<0.001$).

Evaluation of ovarian tissue SOD activity

High-dose lamotrigine suppressed SOD activity, an effect that was substantially reversed by ATP co-treatment and partially attenuated by melatonin. Ovarian tissue SOD activity was measured and comparatively evaluated across experimental groups (Fig. 3). SOD activity was significantly lower in the high-dose lamotrigine group (LTG-30, 4.36±0.08) than in the healthy control group (HG, 7.49±0.09; $p<0.001$), whereas no significant difference was observed between the low-dose lamotrigine group (LTG-10, 7.53±0.08) and the HG ($p>0.999$). A statistically significant difference in SOD activity was also identified between the LTG-10 and LTG-30 groups ($p<0.001$).

In groups co-administered ATP and lamotrigine, SOD activity in the ATLT-10 group (7.56±0.09) did not differ significantly from either the HG ($p=0.998$) or the LTG-10 group ($p>0.999$). In the ATLT-30 group (7.32±0.10),

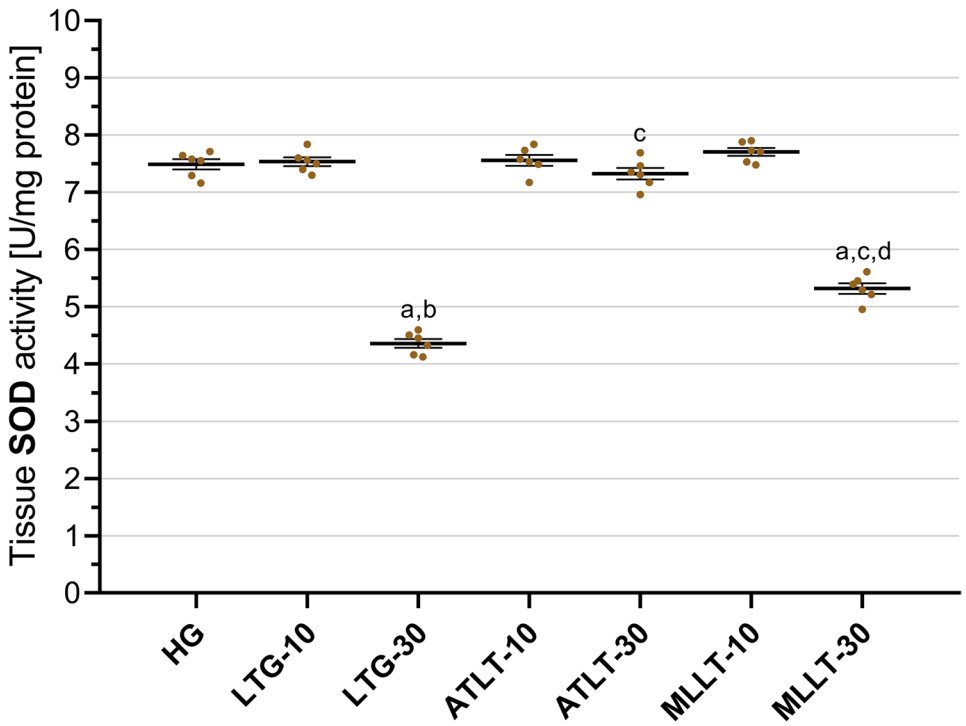


Fig. 3 Modulatory effects of ATP, melatonin, and lamotrigine on SOD activity in rat ovarian tissue. Footnote: Values are presented as mean±standard error of the mean (SEM). Intergroup statistical comparisons were performed using one-way analysis of variance (ANOVA) in conjunction with Tukey's honestly significant difference (HSD) post hoc test. HG was compared with each of the six treatment groups; LTG-10 was compared with LTG-30; groups administered 10 mg/kg lamotrigine (LTG-10, ATLT-10, MLLT-10) and groups administered 30 mg/kg lamotrigine (LTG-30, ATLT-30, MLLT-30) were each compared among themselves. Statistical significance is indicated as follows: a, $p<0.001$ vs. HG; b, $p<0.001$ vs. LTG-10; c, $p<0.001$ vs. LTG-30; d, $p<0.001$ vs. ATLT-30. Each experimental group consisted of six animals ($n=6$). Abbreviations: HG, healthy control group; LTG-10, lamotrigine (10 mg/kg)-only group; LTG-30, lamotrigine (30 mg/kg)-only group; ATLT-10, ATP + lamotrigine (10 mg/kg) group; ATLT-30, ATP + lamotrigine (30 mg/kg) group; MLLT-10, melatonin + lamotrigine (10 mg/kg) group; MLLT-30, melatonin + lamotrigine (30 mg/kg) group; ATP, adenosine triphosphate; SOD, superoxide dismutase

SOD activity was significantly higher than that of the LTG-30 group ($p<0.001$); moreover, no significant difference was detected relative to the HG ($p=0.823$).

In groups co-administered melatonin and lamotrigine, SOD activity in the MLLT-10 group (7.71 ± 0.07) showed no significant difference compared with either the HG ($p=0.573$) or the LTG-10 group ($p=0.795$). In the MLLT-30 group (5.32 ± 0.09), SOD activity was significantly higher than that of the LTG-30 group ($p<0.001$); however, it remained significantly below HG levels ($p<0.001$). Furthermore, SOD activity in the MLLT-30 group was significantly lower than that of the ATLT-30 group ($p<0.001$).

CAT analysis results for the ovarian tissue

High-dose lamotrigine suppressed CAT activity, an effect that was nearly fully reversed by ATP co-treatment and partially attenuated by melatonin. Ovarian tissue CAT activity was comparatively evaluated across experimental groups (Fig. 4). CAT activity was significantly lower in the high-dose lamotrigine group (LTG-30, 4.46 ± 0.11) than in the HG (8.16 ± 0.05; $p<0.001$), whereas CAT activity in the low-dose lamotrigine group (LTG-10, 8.24 ± 0.11) did not differ significantly from that in the HG ($p=0.992$). A statistically significant difference in CAT activity was also observed between the LTG-10 and LTG-30 groups ($p<0.001$).

In groups co-administered ATP and lamotrigine, CAT activity in the ATLT-10 group (8.26 ± 0.16) did not differ significantly from either the HG ($p=0.994$) or the LTG-10 group ($p>0.999$). In the ATLT-30 group (7.98 ± 0.04), CAT activity was significantly higher than that of the LTG-30 group ($p<0.001$), while no significant difference was detected relative to the HG ($p=0.165$).

In groups co-administered melatonin and lamotrigine, CAT activity in the MLLT-10 group (8.05 ± 0.12) did not differ significantly from either the HG ($p=0.981$) or the LTG-10 group ($p=0.920$). In the MLLT-30 group (5.51 ± 0.06), CAT activity was significantly higher than that of the LTG-30 group ($p<0.001$), yet remained significantly below HG levels ($p<0.001$). Furthermore, CAT activity in the MLLT-30 group was significantly lower than that of the ATLT-30 group ($p<0.001$).

Serum AMH analysis results

High-dose lamotrigine significantly reduced serum AMH levels, an effect that was largely attenuated by ATP co-treatment, whereas melatonin failed to produce a significant improvement. Serum AMH levels were quantified and compared across experimental groups (Fig. 5). AMH levels were significantly lower in the high-dose lamotrigine group (LTG-30; 0.143 ± 0.003) compared with the HG (0.209 ± 0.003) ($p<0.001$), whereas no significant difference was observed between HG and the low-dose lamotrigine group (LTG-10, 0.214 ± 0.002) ($p=0.957$). A significant difference was also detected between LTG-10 and LTG-30 ($p<0.001$).

In the groups receiving combined ATP and lamotrigine treatment, AMH levels in ATLT-10 (0.211 ± 0.003) did not differ significantly from either HG ($p>0.999$) or LTG-10 ($p=0.996$). In contrast, AMH levels in ATLT-30 (0.197 ± 0.003) were significantly higher than LTG-30 ($p<0.001$), while the difference versus HG did not reach statistical significance ($p=0.088$).

In the melatonin co-treatment groups, AMH levels in MLLT-10 (0.206 ± 0.003) did not differ significantly from either HG ($p=0.991$) or LTG-10 ($p=0.633$). AMH levels in MLLT-30 (0.148 ± 0.003) were comparable to LTG-30 ($p=0.877$) but remained significantly lower than HG ($p<0.001$). Furthermore, AMH levels in MLLT-30 were significantly lower than those in ATLT-30 ($p<0.001$).

Collectively, these biochemical findings indicate that high-dose lamotrigine disrupted the ovarian oxidant/antioxidant balance and reduced serum AMH levels, and that ATP co-treatment consistently offered protection across all measured parameters, whereas melatonin provided only partial protection against oxidative stress markers and no significant protection against the reduction in AMH.

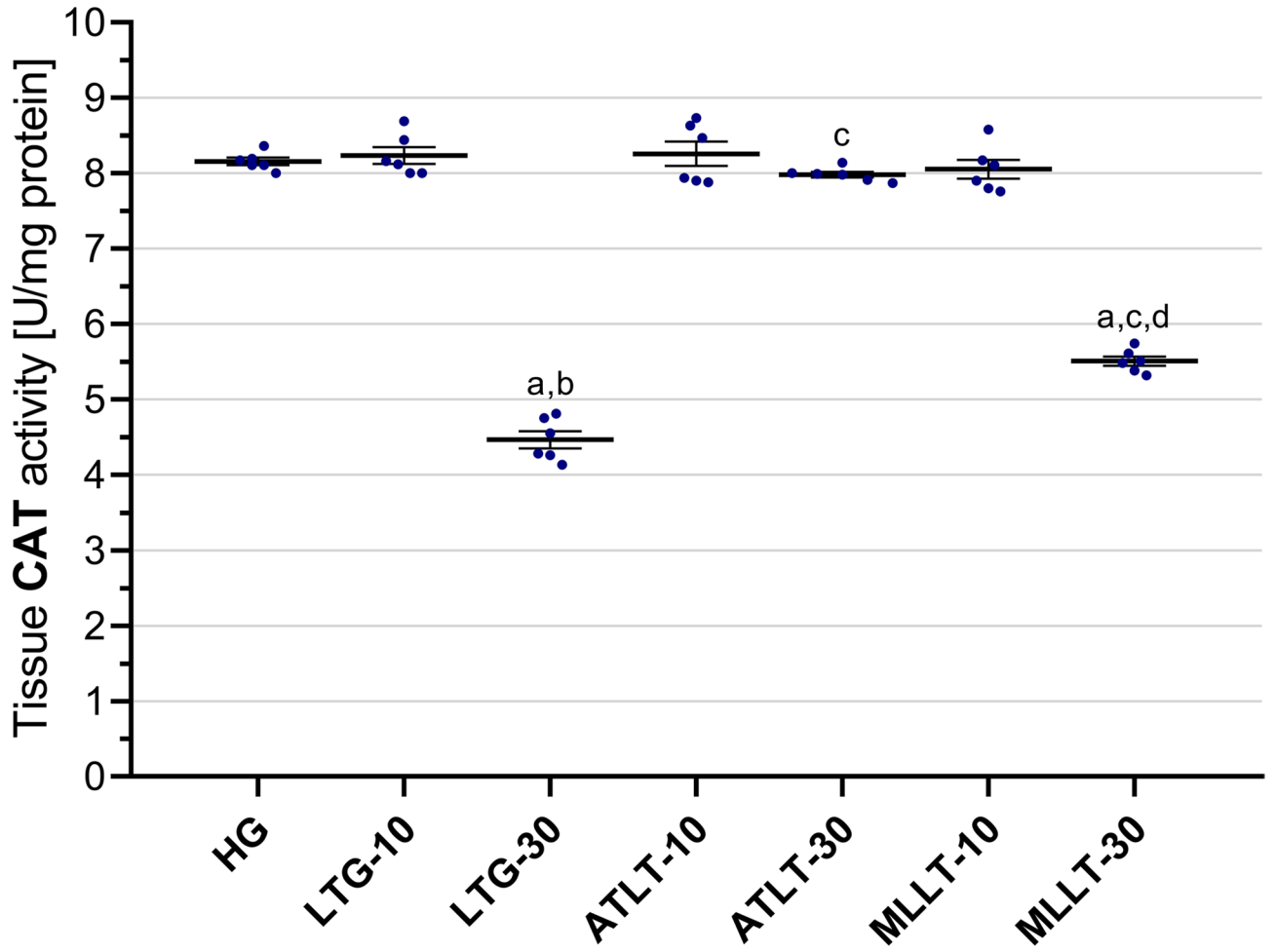


Fig. 4 Modulatory effects of ATP, melatonin, and lamotrigine on CAT activity in rat ovarian tissue. Footnote: Values are presented as mean ± standard error of the mean (SEM). Intergroup statistical comparisons were performed using Welch's analysis of variance (ANOVA) in conjunction with the Games–Howell post hoc test. HG was compared with each of the six treatment groups; LTG-10 was compared with LTG-30; groups administered 10 mg/kg lamotrigine (LTG-10, ATLT-10, MLLT-10) and groups administered 30 mg/kg lamotrigine (LTG-30, ATLT-30, MLLT-30) were each compared among themselves. Statistical significance is indicated as follows: a, $p < 0.001$ vs. HG; b, $p < 0.001$ vs. LTG-10; c, $p < 0.001$ vs. LTG-30; d, $p < 0.001$ vs. ATLT-30. Each experimental group consisted of six animals ($n = 6$). Abbreviations: HG, healthy control group; LTG-10, lamotrigine (10 mg/kg)-only group; LTG-30, lamotrigine (30 mg/kg)-only group; ATLT-10, ATP + lamotrigine (10 mg/kg) group; ATLT-30, ATP + lamotrigine (30 mg/kg) group; MLLT-10, melatonin + lamotrigine (10 mg/kg) group; MLLT-30, melatonin + lamotrigine (30 mg/kg) group; ATP, adenosine triphosphate; CAT, catalase

Histopathological findings

Histopathological analysis revealed statistically significant intergroup differences in ovarian morphology (Table 1). Pairwise comparisons were performed between the healthy control group (HG) and the six treatment groups, among groups administered 10 mg/kg lamotrigine, among groups administered 30 mg/kg lamotrigine, and between the LTG-10 and LTG-30 groups.

Histopathological damage was evaluated in terms of granulosa cell disorganization, cellular dissociation accompanied by disruption of follicular architecture and cellular organization, the emergence of degenerative alterations in follicular cells, and loss of normal follicular morphology. High-dose lamotrigine caused severe follicular degeneration, which was significantly attenuated but not fully normalized by ATP co-treatment, and only partially attenuated by melatonin, with ATP providing significantly greater protection than melatonin (Table 1).

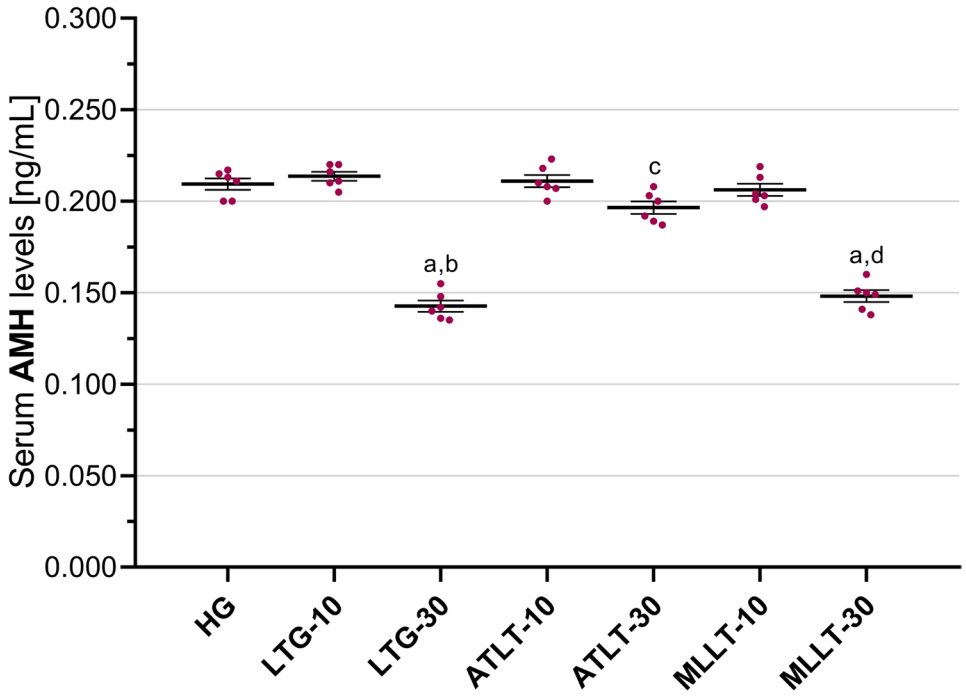


Fig. 5 Modulatory effects of ATP, melatonin, and lamotrigine on AMH levels in rat serum. Footnote: Values are presented as mean ± standard error of the mean (SEM). Intergroup statistical comparisons were performed using one-way analysis of variance (ANOVA) in conjunction with Tukey's honestly significant difference (HSD) post hoc test. HG was compared with each of the six treatment groups; LTG-10 was compared with LTG-30; groups administered 10 mg/kg lamotrigine (LTG-10, ATLT-10, MLLT-10) and groups administered 30 mg/kg lamotrigine (LTG-30, ATLT-30, MLLT-30) were each compared among themselves. Statistical significance is indicated as follows: a, $p < 0.001$ vs. HG; b, $p < 0.001$ vs. LTG-10; c, $p < 0.001$ vs. LTG-30; d, $p < 0.001$ vs. ATLT-30. Each experimental group consisted of six animals ($n = 6$).Abbreviations: HG, healthy control group; LTG-10, lamotrigine (10 mg/kg)-only group; LTG-30, lamotrigine (30 mg/kg)-only group; ATLT-10, ATP + lamotrigine (10 mg/kg) group; ATLT-30, ATP + lamotrigine (30 mg/kg) group; MLLT-10, melatonin + lamotrigine (10 mg/kg) group; MLLT-30, melatonin + lamotrigine (30 mg/kg) group; ATP, adenosine triphosphate; AMH, anti-Mullerian hormone

The HG, LTG-10, ATLT-10, and MLLT-10 groups demonstrated preserved ovarian architecture without histological evidence of secondary follicle degeneration. Degenerative alterations were evident in the secondary follicles of the groups administered 30 mg/kg lamotrigine. Specifically, in the LTG-30 group, granulosa cells exhibited severe disorganization, accompanied by extensive and severe cellular dissociation, resulting in severe disruption of follicular architecture and cellular organization (grade 3). In contrast, the ATLT-30 group exhibited mild disorganization of granulosa cells, with limited/focal cellular dissociation, accompanied by mild disruption of follicular architecture and cellular organization; mild degenerative alterations were also observed in follicular cells (grade 1). The MLLT-30 group, however, exhibited pronounced disorganization of granulosa cells, accompanied by more extensive cellular dissociation, along with moderate disruption of follicular architecture and cellular organization (grade 2). All intergroup differences reached statistical significance ($p < 0.05$) (Fig. 6A–G).

Double immunofluorescence findings

Double immunofluorescence analysis revealed statistically significant differences in the expression of 8-OHdG, dityrosine, COX-2, and COX-1 in ovarian tissues, as determined by semiquantitative scoring (Table 2). Pairwise comparisons were performed between the healthy control group (HG) and the six treatment groups, among groups administered 10 mg/kg lamotrigine, among groups administered 30 mg/kg lamotrigine, and between the LTG-10 and LTG-30 groups.

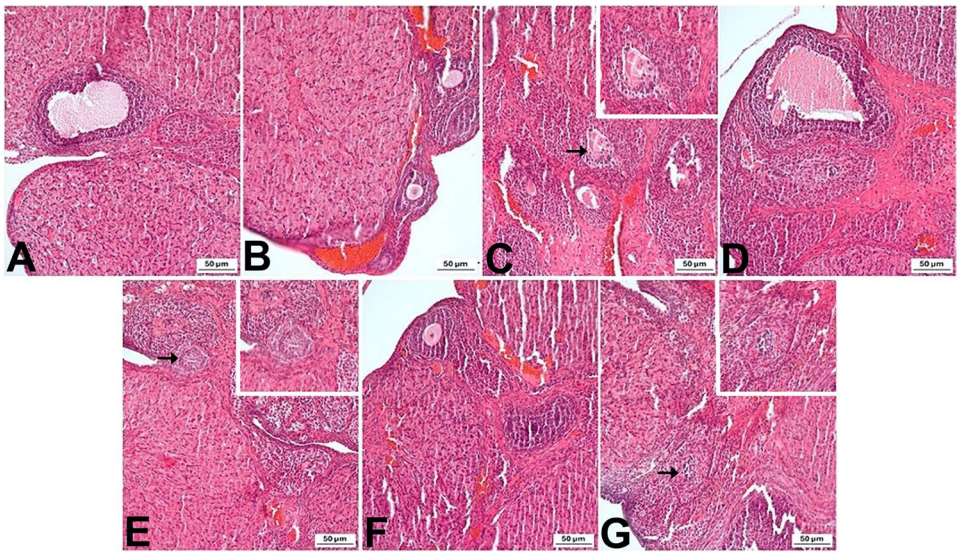


Fig. 6 Representative photomicrographs of ovarian tissue sections illustrating histopathological findings across the experimental groups. (**A**) HG group: normal ovarian histological architecture. (**B**) LTG-10 group: normal follicular morphology with no degenerative changes. (**C**) LTG-30 group: severe degenerative changes (grade 3) in secondary follicles (arrows). (**D**) ATLT-10 group: normal follicular morphology with preserved histological architecture. (**E**) ATLT-30 group: mild degenerative changes (grade 1) in secondary follicles (arrows). (**F**) MLLT-10 group: normal follicular morphology with no observable degenerative alterations. (**G**) MLLT-30 group: moderate degenerative changes (grade 2) in secondary follicles (arrows). H&E. ×40

Table 2 Semiquantitative assessment of double immunofluorescence expression of ovarian injury markers

Group	8-OHdG	Dityrosine	COX-2	COX-1
HG	0 (0–0)	0 (0–0)	0 (0–0)	3 (2–3)
LTG-10	0 (0–1)	0 (0–1)	0 (0–1)	3 (2–3)
LTG-30	3 (3–3) [a, b]	2 (1–2) [a, b]	3 (3–3) [a, b]	0 (0–1) [a, b]
ATLT-10	0 (0–1)	0 (0–1)	0 (0–1)	3 (2–3)
ATLT-30	1 (1–2) [a, c]	1 (1–1) [a, c]	1 (0–1) [a, c]	2 (2–2) [a, c]
MLLT-10	0 (0–1)	0 (0–1)	0 (0–1)	3 (2–3)
MLLT-30	2 (1–2) [a, c, d]	2 (1–2) [a, d]	2 (1–2) [a, c, d]	0 (0–1) [a, d]

Data are expressed as median (min–max). Double immunofluorescence signal intensity and immunopositivity were evaluated semiquantitatively using a four-point scale (0 = absent, 1 = mild, 2 = moderate, 3 = severe). Pairwise comparisons were performed using the Mann–Whitney U test (asymptotic, two-tailed). [a] $p < 0.05$ vs. HG; [b] $p < 0.05$ vs. LTG-10; [c] $p < 0.05$ vs. LTG-30; [d] $p < 0.05$ vs. ATLT-30. HG was compared with each of the six treatment groups; LTG-10 was compared with LTG-30; groups administered 10 mg/kg lamotrigine (LTG-10, ATLT-10, MLLT-10) and groups administered 30 mg/kg lamotrigine (LTG-30, ATLT-30, MLLT-30) were each compared among themselves. Each group comprised six animals ($n = 6$)

Abbreviations: *HG* healthy control group, *LTG-10* lamotrigine (10 mg/kg)-only group, *LTG-30* lamotrigine (30 mg/kg)-only group, *ATLT-10* ATP + lamotrigine (10 mg/kg) group, *ATLT-30* ATP + lamotrigine (30 mg/kg) group, *MLLT-10* melatonin + lamotrigine (10 mg/kg) group, *MLLT-30* melatonin + lamotrigine (30 mg/kg) group, *ATP* adenosine triphosphate, *8-OHdG* 8-hydroxy-2'-deoxyguanosine, *COX-2* cyclooxygenase-2, *COX-1* cyclooxygenase-1

High-dose lamotrigine significantly increased 8-OHdG, dityrosine, and COX-2 immunopositivity while suppressing COX-1 immunopositivity. ATP co-treatment significantly reduced the lamotrigine-induced increases in 8-OHdG, dityrosine, and COX-2 immunopositivity and significantly increased COX-1 immunopositivity toward baseline, whereas melatonin significantly reduced only the 8-OHdG and COX-2 increases, providing no significant protection against the dityrosine increase or the COX-1 suppression (Table 2). No detectable immunopositivity for 8-OHdG and dityrosine was observed in the HG, LTG-10, ATLT-10, and MLLT-10 groups (Fig. 7), whereas positive immunofluorescence signals were detected in the groups receiving 30 mg/kg lamotrigine. Specifically, 8-OHdG expression was classified as severe (grade 3) in the LTG-30 group, mild (grade 1) in the

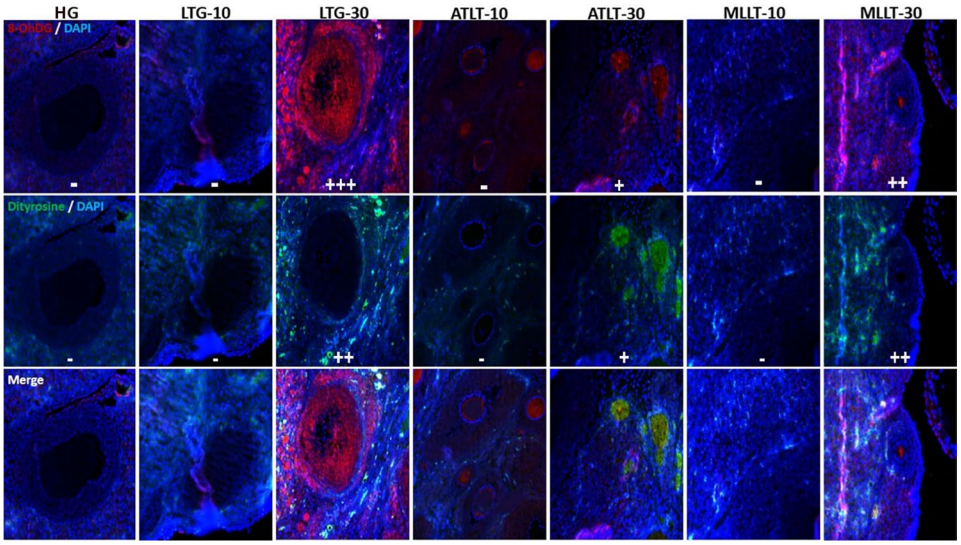


Fig. 7 Representative immunofluorescence photomicrographs showing 8-OHdG (8-hydroxy-2′-deoxyguanosine, a marker of oxidative DNA damage) and dityrosine (a marker of protein oxidation) immunoreactivity in ovarian tissue sections across the HG, LTG-10, LTG-30, ATLT-10, ATLT-30, MLLT-10, and MLLT-30 experimental groups. Immunoreactive staining intensity was assessed by semiquantitative scoring and classified into four grades: absent (−), mild (+), moderate (++), and severe (+++). 8-OHdG immunoreactivity was detected using Alexa Fluor 594, dityrosine immunoreactivity was visualized using fluorescein isothiocyanate (FITC), and nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole). ×40

ATP-treated group (ATLT-30), and moderate (grade 2) in the melatonin-treated group (MLLT-30). Dityrosine immunopositivity was moderate (grade 2) in both the LTG-30 and MLLT-30 groups and mild (grade 1) in the ATP-treated group (ATLT-30) (Fig. 7).

No detectable immunopositivity for COX-2 was observed in the HG, LTG-10, ATLT-10, and MLLT-10 groups, whereas positive signals were identified in the groups receiving 30 mg/kg lamotrigine. COX-2 expression was classified as severe (grade 3) in the LTG-30 group, mild (grade 1) in the ATP-treated group (ATLT-30), and moderate (grade 2) in the melatonin-treated group (MLLT-30) (Fig. 8).

COX-1 immunopositivity was severe (grade 3) in the HG, LTG-10, ATLT-10, and MLLT-10 groups, moderate (grade 2) in the ATP-treated group (ATLT-30), and undetectable in the LTG-30 and MLLT-30 groups (Fig. 8).

Discussion

In this study, the protective effects of ATP against ovarian damage in rats administered low- or high-dose lamotrigine were investigated and compared with those of melatonin. To our knowledge, this is the first experimental study to evaluate the protective potential of ATP against lamotrigine-induced ovarian toxicity using melatonin as a reference antioxidant. Our study further integrated biochemical assessment, serum AMH measurement as an indicator of ovarian reserve, histopathological evaluation, and double immunofluorescence analysis of two complementary marker pairs, 8-OHdG and dityrosine for DNA and protein oxidation, and COX-1 and COX-2 for the inflammatory response. Our biochemical findings showed that low-dose lamotrigine did not affect oxidant and antioxidant markers in ovarian tissue; however, high-dose lamotrigine shifted the oxidant/antioxidant balance in favor of oxidants. Previous studies have shown that lamotrigine treatment is associated with oxidative stress–related structural abnormalities in ovarian and uterine tissues [11]. In the present study, the absence of damage in ovarian tissue following low-dose lamotrigine and the adverse effects of high-dose lamotrigine on follicles were consistent with the literature [12].

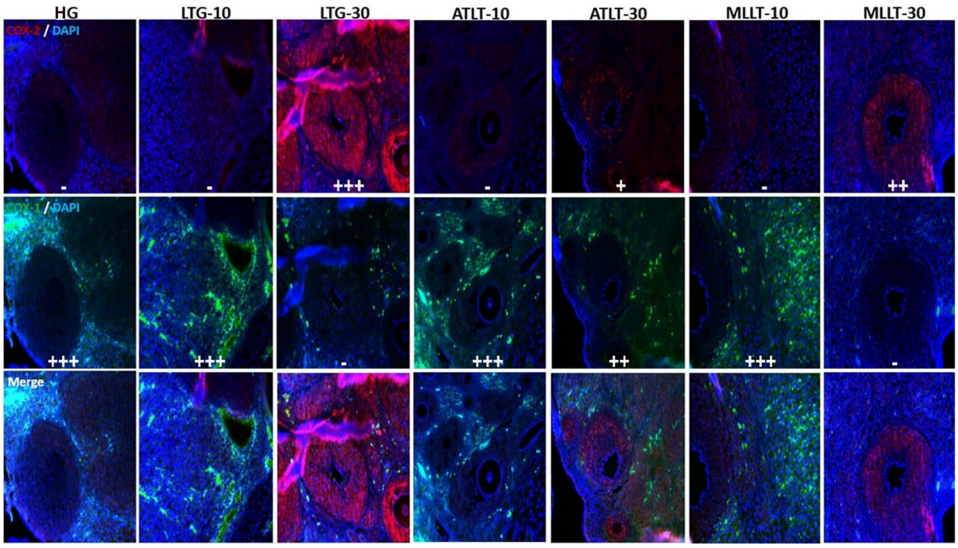


Fig. 8 Representative immunofluorescence photomicrographs showing COX-2 (cyclooxygenase-2) and COX-1 (cyclooxygenase-1) immunolabeling patterns observed in ovarian tissue sections across the HG, LTG-10, LTG-30, ATLT-10, ATLT-30, MLLT-10, and MLLT-30 experimental groups. The degree of immunoreactive staining was assessed through semiquantitative grading, with expression levels classified as absent (−), mild (+), moderate (++), or severe (+++). Alexa Fluor 594 was employed for the visualization of COX-2 immunoreactivity, fluorescein isothiocyanate (FITC) for COX-1 immunolabeling, and DAPI (4′,6-diamidino-2-phenylindole) was applied as a nuclear counterstain. ×40

MDA, tGSH, SOD, and CAT levels were measured to evaluate oxidative stress in ovarian tissue. MDA is the primary and most extensively studied toxic product of polyunsaturated fatty acid peroxidation (LPO). Therefore, MDA is widely used as an indicator of oxidative status [35]. Glutathione (GSH) participates in ROS scavenging and antioxidant defense; disruption of the ROS/GSH balance might lead to oxidative damage to biomacromolecules and cell death [36]. SOD and CAT are key antioxidant enzymes that play sequential and complementary roles in ROS detoxification. SOD catalyzes the dismutation of the superoxide anion to oxygen and hydrogen peroxide, whereas the resulting hydrogen peroxide is detoxified by CAT through its breakdown into water and oxygen [37]. These findings obtained from ovarian tissue were consistent with reports indicating that decreased enzymatic and nonenzymatic antioxidant levels suggest an insufficient antioxidant defense system and might lead to tissue damage [37, 38]. Notably, the concurrent rise in MDA and decline in tGSH, SOD, and CAT indicate that high-dose lamotrigine induced oxidative stress in ovarian tissue.

In the literature, ATP has been reported to protect ovarian tissue against oxidative damage by suppressing lipid peroxidation and strengthening the antioxidant defense system [39]. Previous studies have shown that lamotrigine increases ATP production at therapeutic (low) doses but decreases ATP levels at high doses [13]. These findings suggest that high-dose lamotrigine toxicity may be associated with cellular ATP depletion. A comparable mitochondrial mechanism, involving membrane potential disruption and reduced ATP production, has been proposed to promote oxidative stress in other xenobiotics [40], raising the possibility that this pathway also contributes to lamotrigine-induced ovarian toxicity. In the present study, ATP was found to antagonize the increase in oxidants and the decrease in antioxidants associated with high-dose lamotrigine. The marked reduction in MDA levels in the groups receiving ATP together with lamotrigine supports the view that ATP suppresses oxidative stress, and this finding was consistent with evidence showing that ATP depletion leads to increased mitochondrial ROS production [41]. A decrease in intracellular ATP and an increase in mitochondrial ROS levels indicate impaired cellular metabolism. Therefore, cell survival depends largely on the continuous provision of sufficient ATP [42]. It should be noted that intracellular ATP concentrations were not directly quantified in this study; therefore, the proposed ATP-centered mechanism remains inferential and is based on indirect biochemical, endocrine, histopathological, and double immunofluorescence evidence.

The same set of oxidant and antioxidant parameters was next evaluated in the melatonin-treated groups. Melatonin significantly reduced MDA levels upregulated by high-dose lamotrigine. This effect of melatonin may be related to its ROS-scavenging and chain-breaking antioxidant properties [43]. Moreover, the significant suppression of MDA levels by melatonin was consistent with its ability to support ATP synthesis [44]. However, MDA levels in the melatonin group were higher than those in the ATP group. In addition, ATP was shown to suppress the reduction in tGSH, SOD, and CAT levels induced by high-dose lamotrigine in ovarian tissue more effectively than melatonin. As already stated, ATP is a fundamental energy source for the synthesis of antioxidants [19, 20]. This finding may indicate that the decrease in intracellular ATP levels induced by high-dose lamotrigine could not be adequately compensated by melatonin treatment.

To evaluate functional impairment associated with genital organ pathology, AMH levels were measured. Serum AMH level is used as an indicator of ovarian dysfunction in conditions such as primary ovarian insufficiency, polycystic ovary syndrome (PCOS), and granulosa cell tumors [45]. Decreased serum AMH levels have been reported in various reproductive organ pathologies, including gonadal dysgenesis, ovarian injury, and primary ovarian insufficiency [46]. In the high-dose lamotrigine group treated with ATP, the AMH level was higher than in the melatonin group. In addition, melatonin was not able to significantly prevent the lamotrigine-induced decrease in AMH levels. ATP's superior preservation of AMH levels extends its protective effect from biochemical and histopathological outcomes to endocrine function, an aspect not previously examined in the context of lamotrigine-induced ovarian toxicity. These findings were consistent with reports showing that intracellular ATP depletion leads to mitochondrial dysfunction [16] and that mitochondrial DNA (mtDNA) reduction secondary to mitochondrial dysfunction adversely affects AMH levels [47], suggesting an indirect correlation between intracellular ATP depletion and AMH levels.

In the present study, the results of biochemical experiments were also consistent with histopathological, 8-OHdG, and dityrosine double immunofluorescence findings. The severity of the morphological abnormalities observed in ovarian tissue showed a positive correlation with the severity of 8-OHdG immunopositivity. As is well known, 8-OHdG is a biomarker widely used in the assessment of oxidative stress, reflecting oxidative DNA damage caused by ROS [48]. In the literature, 8-OHdG is accepted as an oxidized form of mutated DNA [49]. ATP significantly prevented the increase in 8-OHdG immunopositivity induced by high-dose lamotrigine in ovarian tissue compared with melatonin. Our experimental results were consistent with findings reporting that intracellular ATP depletion increases ROS production, leading to DNA damage and the formation of modified bases such as 8-OHdG [16]. The 8-OHdG immunopositivity observed in ovarian tissue was further supported by the corresponding dityrosine immunopositivity detected by double immunofluorescence. Dityrosine, one of the indicators of protein oxidation, is formed through radical-mediated cross-linking of tyrosine residues [50] and is increased particularly in peroxynitrite- [51] and myeloperoxidase [50]-mediated oxidative processes. In particular, increased dityrosine immunoreactivity has been associated with oxidative protein damage [52] and functional impairment [53]. Taken together, these findings suggest that high-dose lamotrigine causes extensive oxidative damage at both DNA and protein levels. Oxidative DNA damage such as that observed in the present study has been reported in other reproductive toxicity models to activate apoptotic signaling cascades, including ATM/p53-mediated pathways and caspase activation [54], with concurrent autophagic vacuole formation also documented alongside these apoptotic features in granulosa cells exposed to oxidative-stress-inducing agents [55]. Although the present study did not directly assess autophagic or apoptotic signaling molecules, these reports raise the possibility that similar degradative pathways may contribute to the follicular degeneration observed here under high-dose lamotrigine exposure.

The immunofluorescence findings obtained in this study showed that high-dose lamotrigine administration caused an imbalance in COX isoforms in ovarian tissue. In particular, the marked increase in COX-2 expression and the concomitant pronounced decrease in COX-1 expression in the high-dose lamotrigine group indicate the development of inflammatory damage. These findings were consistent with previous data indicating that COX-1 is constitutively expressed and plays a role in tissue homeostasis, whereas COX-2 is an inducible enzyme that becomes activated during the inflammatory process [56, 57]. It is known that increased ROS levels enhance

proinflammatory COX-2 expression [56, 58]. Because COX-1 is a key enzyme constitutively expressed in nearly all tissues and contributes to the maintenance of tissue homeostasis, severe oxidative damage and cell loss associated with high-dose lamotrigine might suppress COX-1 expression [56, 57].

In the ATP-treated groups, this balance between COX isoforms was observed to be largely preserved. In the ATP-treated group, the markedly low COX-2 expression and the COX-1 expression remaining close to normal levels suggest that ATP may not only reduce oxidative stress but may also modulate the inflammatory response [21]. Previous studies have reported that decreased intracellular ATP levels, together with pro-carcinogenic stimuli, exacerbate endoplasmic reticulum stress and consequently elicit a pronounced proinflammatory response, reflected by COX-2 induction [59]. Overall, our data were consistent with evidence showing that ATP depletion leads to an increase in COX-2 expression. Although a reduction in COX-2 expression was also observed in the melatonin-treated groups, this effect was notably less pronounced than that elicited by ATP. This finding indicates that, despite melatonin being a potent free radical scavenger [60], unlike ATP, it may not directly support cellular energy metabolism. Notably, melatonin significantly attenuated the immunopositivity of only two of these four oxidative and inflammatory markers, namely 8-OHdG and COX-2, while leaving the increase in dityrosine immunopositivity and the suppression of COX-1 immunopositivity unaffected, whereas ATP significantly attenuated the increases in 8-OHdG, dityrosine, and COX-2 immunopositivity and increased COX-1 immunopositivity toward baseline. This marker-specific pattern, made visible only through the paired double immunofluorescence design used here, indicates that ATP's protective action is mechanistically broader than melatonin's, rather than simply more potent along the same pathway. Collectively, these biochemical, endocrine, histopathological, and double immunofluorescence findings support ATP as a broadly protective agent against high-dose lamotrigine-induced ovarian injury, exceeding the protection afforded by melatonin across multiple independent outcome domains.

Conclusion

Our findings demonstrate that 30-day administration of low-dose lamotrigine did not induce significant oxidative stress or structural injury in ovarian tissue, whereas high-dose lamotrigine administered over the same period produced marked oxidative-inflammatory ovarian injury in rats. This injury was characterized by disruption of redox homeostasis, depletion of antioxidant defense systems, reduction in serum AMH levels, severe follicular degeneration, and an enhanced inflammatory response accompanied by oxidative DNA and protein damage.

ATP provided substantially greater protection than melatonin across this comprehensive panel of biochemical, endocrine, histopathological, and double immunofluorescence outcome measures. ATP suppressed the increase in oxidant levels, preserved endogenous antioxidant defense systems and AMH levels, and attenuated follicular injury. In addition, it reduced oxidative DNA and protein damage and mitigated the inflammatory response. The concordant changes observed across these independent outcome measures support a consistent protective effect of ATP against high-dose lamotrigine-induced ovarian injury.

In conclusion, this study identifies ATP as a promising experimental approach for attenuating high-dose lamotrigine-associated ovarian damage. The findings further support the need for mechanistic investigations into the role of cellular bioenergetic pathways in the protective effects of ATP. Future studies should more comprehensively examine mitochondrial bioenergetic mechanisms, reproductive outcomes, longer-term exposure models, and the translational relevance of ATP-mediated ovarian protection in women receiving lamotrigine therapy.

Limitations and future perspectives

This study has several major limitations. First, the findings are based on a Wistar albino rat model and cannot be directly extrapolated to human physiology, so their clinical validity requires confirmation via human studies. Second, intracellular ATP levels and mitochondrial bioenergetic parameters were not directly measured, so the ATP-centered mechanistic conclusions rely on indirect evidence. Third, molecular markers of autophagy and apoptosis (e.g., LC3, Beclin-1, caspase-3, ATM/p53) were not evaluated in this study; direct assessment of these markers in future work could better document the cellular pathways underlying the observed follicular degeneration. Fourth, only a 30-day subacute exposure period was evaluated, leaving the effects of chronic lamotrigine use on ovarian tissue unexamined. Finally, functional reproductive outcomes such as oocyte quality, follicular development, estrous cyclicity, fertility, pregnancy, and live birth rates were not assessed, limiting the translational relevance of the protective effects observed. Addressing these limitations in future studies will strengthen the clinical relevance of our findings.

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Data availability All data supporting the findings of this study are available within the paper and its Supplementary Information.

Declarations

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