

Protective effect of melatonin against zonisamide-induced reproductive disorders in male rats

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Abstract

Introduction: Zonisamide (ZNS) is a modern antiepileptic drug (AED) that is distinguished from other AEDs by its unique structure and broad mechanistic profile. The pineal hormone melatonin is involved in the regulation of reproductive function, including the timing of the luteinizing hormone (LH) surge. The aim of the present work was to study the protective effect of melatonin against the potential suppression impact of ZNS on reproductive activity.

Material and methods: Ninety adult albino male rats were allocated to several groups treated with melatonin (10 mg/kg BW), ZNS (10, 20 and 50 mg/kg BW) and 10 mg/kg of melatonin plus ZNS (10, 20 or 50 mg/kg BW, respectively). Reproductive hormones (testosterone, LH and follicle-stimulating hormone (FSH)) levels were measured in animal serum. Sperm abnormalities and DNA fragmentation in testis tissues as well as expression alteration of several reproductive-related genes were analyzed.

Results: The results revealed that ZNS decreased the levels of serum free testosterone, LH, and FSH and expression of their encoding genes in male rats. In addition, ZNS treatment increased the sperm abnormalities and DNA fragmentation and inducible nitric oxide synthase (iNOS) in testis tissues as well as GABA level in liver tissues. However, melatonin supplementation inhibited the negative symptoms of ZNS in which it increased the levels of reproductive hormones and expression of their encoding genes in the ZNS-treated rats. Moreover, melatonin decreased the sperm abnormalities, DNA fragmentation, iNOS activity and GABA level in ZNS-treated rats.

Conclusions: The data obtained in this study suggest that melatonin administration confers protection against toxicity inflicted by ZNS, and support the contention that melatonin protection is achieved by its ability as a scavenger for free radicals generated by ZNS.

Key words: zonisamide, melatonin, testosterone, luteinizing hormone, follicle-stimulating hormone, gene expression, DNA damage.

Introduction

Zonisamide (ZNS) (Zonegran[®]; Eisai Inc, Teaneck, NJ, USA) is a newer, broad-spectrum antiepileptic drug (AED) that has been shown to be effective in the treatment of refractory partial seizures [1, 2]. Chemically classified as a sulfonamide, ZNS is characterized by a unique structural, mechanistic, and pharmacokinetic profile that is distinct from that of other AEDs. Zonisamide is a benzisoxazole derivative, originally synthesized in Japan in 1974 during exploratory research on psychiatric drugs, where it

was subsequently identified as having anticonvulsant activity during screening [3]. Preclinical animal studies conducted in Japan revealed the anti-seizure effects of zonisamide on maximal electroshock-induced seizures in rats, mice, rabbits, and dogs.

It is also effective for the management of migraine [4], neuropathic pain [5], essential tremor [6], anxiety [7], and other conditions. Recently, ZNS has been approved as a new adjunctive therapy for motor complications of Parkinson's disease (PD) in Japan [8]. More recently, ZNS was reported to be effective for the management of impulse control behavior in PD [9], suggesting potential effects on non-motor PD symptoms.

Melatonin, N-acetyl-5-methoxytryptamine, is a hormone secreted mainly by the pineal gland. Retinal light exposure reduces the amount of serotonin metabolized to melatonin via neural pathways connecting the retina to the pineal gland. Thus, pineal production of melatonin increases at night, and the amount of melatonin secreted into the plasma is related to the length of exposure to darkness [10]. Melatonin is involved in the modulation of a variety of endocrine, neural, and immune functions [10, 11]. Gonadotropin-releasing hormone (GnRH) neurons represent the final output neurons in the central control of the reproductive system. Gamma-amino butyric acid (GABA), one of the major regulators of GnRH neurons, depolarizes GnRH neurons isolated from adult rats via GABA (A) receptors. The presence of GABA (A) receptors in GnRH neurons has also been demonstrated morphologically [12]. The pineal hormone melatonin is involved in the regulation of reproductive function, including the timing of the luteinizing hormone surge. The suprachiasmatic nucleus and the GABAergic system in the medial pre-optic area are considered as possible sites of the action of melatonin [12].

The effect of melatonin on the hypothalamus pituitary axis and therefore the reproductive system have initiated studies aimed at the use of melatonin as an oral contraceptive [13]. When using high doses of melatonin (75 and 300 mg daily) in combination with norethisterone, no peak in luteinizing hormone (LH) secretion was observed during the menstrual cycle. Furthermore, plasma follicle-stimulating hormone (FSH) levels remained constant. These circumstances prevented ovulation and the increase in progesterone during the luteal phase. The suggested mechanism of action involved antigonadotropic effects on the hypothalamus, such as alteration in the hypothalamic pulsatile GnRH secretion and/or effects on the pituitary release of LH, or even a direct effect on the ovary. Due to melatonin's side effects (especially sleep induction, etc.) this possibility now is viewed rather skeptically [14].

Melatonin exhibits some protective functions for cells against noxious agents, acts as a powerful antioxidant and scavenger of free radicals, and

counteracts the generation of free radicals by inhibiting the activity of inducible nitric oxide synthase (iNOS) [15]. Melatonin shows a similar activity in purification of peroxide radicals (ROO^-), a consequence of lipid peroxidation, thus being a more powerful antioxidant than vitamin E [16, 17]. Therefore, we hypothesize that melatonin could be highly specific against lipid peroxidation for several reasons: it is highly lipophilic, it is usually found in high concentrations in cell membranes and, like vitamin C and E, it reduces the oxidation of lipids due to its purifying activity towards the OH^- radical [18]. Although the protective mechanism of melatonin has not been completely clarified, in most cases it is of great interest to investigate whether melatonin could prevent the potential cytotoxic effects of ZNS in male rats. Therefore, several reproductive parameters including alterations in serum levels of free testosterone, LH, and FSH and their encoding gene expression, sperm abnormalities and DNA fragmentation in male rats were examined.

Material and methods

Experimental animals

Ninety adult albino male rats (100–120 g), purchased from the Animal House Colony, Giza, Egypt, were maintained on a standard laboratory diet (protein 16.04%, fat 3.63%, fiber 4.1%, and metabolic energy 0.012 MJ) and water *ad libitum* at the Animal House Laboratory, National Research Centre, Dokki, Giza, Egypt. After an acclimation period of 1 week, animals were divided into groups (10 rats/group) and housed individually in filter-top polycarbonate cages, housed in a temperature-controlled ($23 \pm 1^\circ\text{C}$) and artificially illuminated (12 h dark/light cycle) room free from any source of chemical contamination. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Centre, Egypt.

Experimental design

The male rats were randomly allocated to 9 groups ($n = 10$ per group) and treated intragastrically for 4 week as follows: group 1, control group: animals were treated with solvent vehicle control (NaCl); group 2, animals were treated with melatonin (10 mg/kg BW, [19]); groups 3–5, animals were treated with 10, 20 and 50 mg/kg BW of zonisamide (ZNS, [20]), respectively; groups 6–8, animals were treated with melatonin (10 mg/kg BW) plus ZNS (10, 20 and 50 mg/kg BW, respectively); group 9, animals were injected intraperitoneally (*i.p.*) with cyclophosphamide (CP) alone (25 mg/kg BW, as a single injection) 24 h prior to their sacrifice.

All animals were sacrificed by cervical dislocation after 24 h of CP injection. Half of the animals per

group ($n = 5$) were used for hormone analysis, DNA fragmentation and gene expression analyses. The other half of the animals were kept for 35 days after the last dose of treatment for sperm morphology.

Biochemical analyses

Quantitative estimation of free testosterone was carried out in the sample of rat's serum using enzyme-linked immunosorbent assay (ELISA) according to McCann and Kirkish [21]. Quantitative estimation of FSH was carried out in the sample of rat's serum using ELISA according to Knobil [22]. Quantitative estimation of LH in the sample of rat's serum was carried out using ELISA according to Wakabayashi [23]. The kits used for determination of free testosterone, FSH and LH in rat serum were obtained from Gloryscience Co., Ltd.

γ -Aminobutyric acid measurements

For determination of the content of GABA liver tissues were used to carry out the analysis. Liver tissues (100 mg) were homogenized on ice with ice-cold phosphate buffer saline. Afterwards, liver cells were washed twice with developmental buffer (DB: 5 mM Na_2HPO_4 , 5 mM KH_2PO_4 , 0.2 mM CaCl_2 , 2 mM MgSO_4 , pH 6.5), and then suspended in 100 μl of DB. The suspension was shaken gently at 120 rpm and 22°C. After that, the suspension was centrifuged at 1,500 g for 1 min. A volume of 40 μl of supernatant was analyzed for GABA via high-performance liquid chromatography (HPLC). Each measurement was performed at least in triplicate.

Determination of inducible nitric oxide synthase activity

Inducible nitric oxide synthase (iNOS) activity was determined by an NOS assay kit. The NOS assay kit is to use the method of catalyzing L-arginine to measure NOS activity in testis tissues. Hundred milligrams of testis tissue samples from all the groups of rats were placed in a centrifuge tube, adding 10 volumes of ice-cold phosphate buffer saline (PBS, pH 7.4), and immediately homogenized for 1–2 min on ice; the homogenate was then centrifuged at 3000 r/min for 15 min at 4°C. The supernatant was collected and aliquoted. One of the aliquots was used for detection and the rest were stored at -80°C for future study. Then, the protein concentration was determined by bicinchoninic acid (BCA) Protein Assay Kit. The supernatant was incubated with 0.6 ml of reaction buffer combined with the addition of inhibitor 1 mmol/l EGTA (ethylene glycol tetraacetic acid, it is iNOS Ca^{2+} independent and can complex Ca^{2+} in samples). The reaction was terminated after 15 min at 37°C. Photometric measurement of the absorbance at 530 nm was applied to determine total iNOS activity.

Sperm abnormalities

Thirty-five days after the last dose (duration of spermatogenesis) [24], half of the animals of each treated group (5 animals per group) were sacrificed by neck vertebra luxation. The epididymides and testes from each rat were removed and weighed. Sperm were collected as quickly as possible when each rat was dissected. To release sperm, the cauda epididymides were cut in a pre-warmed Petri dish containing 1 ml of saline solution at 37°C. After mincing with scalpels, the suspension was stirred and dropped on a grease-free clean slide to determine the motility of sperm using a microscope. Spermatozoa were counted using a hemocytometer and a drop of a homogenate smeared on a cleaned slide allowed to air dry and stained with approximately 0.05% aqueous eosin Y. The slides were coded and used for the examination of sperm head and tail abnormalities. For each animal 500 sperm were examined for morphological abnormalities according to the criteria of Jeong *et al.* [25].

DNA fragmentation analysis

Apoptotic DNA fragmentation was qualitatively analyzed by detecting the laddering pattern of nuclear DNA as described according to Lu *et al.* [26]. Briefly, liver tissues were homogenized, washed in PBS, and lysed in 0.5 ml of DNA extraction buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% Triton, and 100 $\mu\text{g}/\text{ml}$ proteinase K, pH 8.0) overnight at 37°C. The lysate was then incubated with 100 $\mu\text{g}/\text{ml}$ DNase-free RNase for 2 h at 37°C, followed by three extractions of an equal volume of phenol/chloroform (1 : 1 v/v) and a subsequent re-extraction with chloroform by centrifuging at 15,000 rpm for 5 min at 4°C. The extracted DNA was precipitated in two volumes of ice-cold 100% ethanol with 1/10 volume of 3 M sodium acetate, pH 5.2 at -20°C for 1 h, followed by centrifuging at 15,000 rpm for 15 min at 4°C. After washing with 70% ethanol, the DNA pellet was air-dried and dissolved in 10 mM Tris-HCl/1 mM EDTA, pH 8.0. The DNA was then electrophoresed on 1.5% agarose gel and stained with ethidium bromide in Tris/acetate/EDTA (TAE) buffer (pH 8.5, 2 mM EDTA, and 40 mM Tris-acetate). A 100-bp DNA ladder (Invitrogen, USA) was included as a molecular size marker and DNA fragments were visualized and photographed by exposing the gels to ultraviolet transillumination.

Expression of aromatase, luteinizing hormone and follicle-stimulating hormone genes

Isolation of total RNA

Total RNA was isolated from brain and testis tissues of male rats by the standard TRIzol[®] Reagent extraction method (cat#15596-026, Invitrogen, Ger-

many). Briefly, tissue samples were homogenized in 1 ml of TRIzol[®] Reagent per 50 mg of the tissue. Afterwards, the homogenized sample was incubated for 15 min at room temperature. A volume of 0.2 ml of chloroform per 1 ml of TRIzol[®] Reagent was added. Then the samples were vortexed vigorously for 15 s and incubated at room temperature for 3 min. The samples were centrifuged at no more than 12,000x g for 15 min at 4°C. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase. Therefore, the upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. A volume of 0.5 ml of isopropyl alcohol was added per 1 ml of TRIzol[®] Reagent used for the initial homogenization. Afterwards, the samples were incubated at 15°C to 30°C for 10 min and centrifuged at not more than 12,000x g for 10 min at 4°C. The RNA was precipitated, which was often invisible before centrifugation, forming a gel-like pellet on the side and bottom of the tube. The supernatant was removed completely. The RNA pellet was washed once with 1 ml of 75% ethanol. The samples were mixed by vortexing and centrifuged at no more than 7,500x g for 5 min at 4°C. The supernatant was removed and the RNA pellet was air-dried for 10 min. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing the solution a few times through a pipette tip.

Total RNA was treated with 1 U of RQ1 RNase-free DNase (Invitrogen, Germany) to digest DNA residues, and re-suspended in DEPC-treated water. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT).

Reverse transcription reaction

The complete poly(A)⁺ RNA isolated from male rat tissues was reverse transcribed into cDNA in a total volume of 20 µl using RevertAid[™] First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5 µg) was used with a reaction mixture, termed the master mix (MM). The MM consisted of 50 mM MgCl₂, 5x RT buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM of each dNTP), 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M-MuLV reverse transcriptase. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and the

reaction was stopped by heating for 5 min at 99°C. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative real time-polymerase chain reaction (sqRT-PCR).

Semi-quantitative real time-polymerase chain reaction

Polymerase chain reaction reactions were set up in 25 µl reaction mixtures containing 12.5 µl of 1x SYBR[®] Premix Ex Taq[™] (TaKaRa, Biotech. Co. Ltd., Germany), 0.5 µl of 0.2 µM sense primers, 0.5 µl of 0.2 µM antisense primer, 6.5 µl of distilled water, and 5 µl of cDNA template. The reaction program was allocated to 3 steps. The first step was at 95.0°C for 3 min. The second step consisted of 40 cycles in which each cycle was divided into 3 steps: (a) at 95.0°C for 15 s; (b) at 55.0°C for 30 s; and (c) at 72.0°C for 30 s. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 s up to 95.0°C. At the end of each sqRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers. Each experiment included a distilled water control.

The semi-quantitative values of RT-PCR (sqRT-PCR) of aromatase (Cyp19-F: 5'-ATA CCA GGT CCT GGC TAC TG-3', Cyp19-R: 5'-TTG TTG TTA AAT ATG ATG CC-3' [27]), LH (LH -F: 5'-TCT CAC CACCAC CGT CTG TA-3', LH-R: 5'-TGC AGT CGT TGT AGT CCA TC-3' [27]), and FSH (FSH-F: 5'-GGG CCA GGA ACT GTG AAA TA-3', FSH-R: 5'-TCT CAG AAC TGC CGA GGT TT-3' [27]) genes were normalized on the bases of β-actin (β-actin-F: 5'-TTG CCG ACA GGA TGC AGA A-3', β-actin-R: 5'-GCC GAT CCA CAC GGA GTA CT-3' [27]) expression.

Calculation of gene expression

First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formulae [28]: $Ef = 10^{-1/\text{slope}}$, efficiency (%) = $(Ef - 1) \times 100$. The relative quantification of the target to the reference was determined by using the ΔCT method if E for the target (Cyp19, LH and FSH) and the reference primers (β-actin) are the same [28]:

$$\text{ratio}_{(\text{reference}/\text{target gene})} = Ef^{CT(\text{reference}) - CT(\text{target})}$$

Statistical analysis

All data were analyzed using the General Linear Models (GLM) procedure of Statistical Analysis System [29] followed by the Scheffé test to assess significant differences between groups. The values are expressed as mean ± SEM. All statements of significance were based on probability of $p < 0.05$.

Table I. Concentrations of testosterone, LH, and FSH in melatonin- and/or ZNS-treated rats

Groups	Free testosterone [ng/l]	LH [mIU/ml]	FSH [mIU/ml]
Control	5.82 ±0.32 ^a	1.92 ±0.10 ^a	1.43 ±0.07 ^a
Melatonin	7.48 ±0.24 ^a	2.34 ±0.11 ^a	1.72 ±0.03 ^a
ZNS10	5.24 ±0.34 ^{ab}	1.74 ±0.10 ^a	1.13 ±0.11 ^a
ZNS20	3.46 ±0.22 ^b	1.23 ±0.07 ^{ab}	0.78 ±0.06 ^b
ZNS50	2.82 ±0.14 ^b	0.74 ±0.10 ^b	0.57 ±0.10 ^{bc}
Melatonin + ZNS10	5.95 ±0.52 ^a	1.89 ±0.12 ^a	1.21 ±0.07 ^a
Melatonin + ZNS20	5.12 ±0.13 ^{ab}	1.69 ±0.10 ^a	1.10 ±0.04 ^a
Melatonin + ZNS50	4.96 ±0.16 ^b	1.41 ±0.07 ^{ab}	0.94 ±0.06 ^b
CP	1.46 ±0.21 ^c	0.51 ±0.06 ^b	0.41 ±0.03 ^c

^{a, b, c}Mean values within columns with unlike superscript letters are significantly different ($p < 0.05$, Scheffé-Test). ^{a, b, ba, bc}Mean values within columns with similar superscript letters are not significantly different ($p > 0.05$).

Results

Hormonal assessment

The results depicted in Table I show the effect of ZNS exposure and/or melatonin on serum levels of free testosterone, LH, and FSH of adult male rats. The results indicated that there were significant decreases in serum free testosterone, LH, and FSH in male rats treated with medium and high doses of ZNS compared with the control group (Table I). However, the levels of free testosterone, LH, and FSH in rats treated with a low dose of ZNS were relatively similar to the control group. In contrast, treatment of male rats with melatonin increased

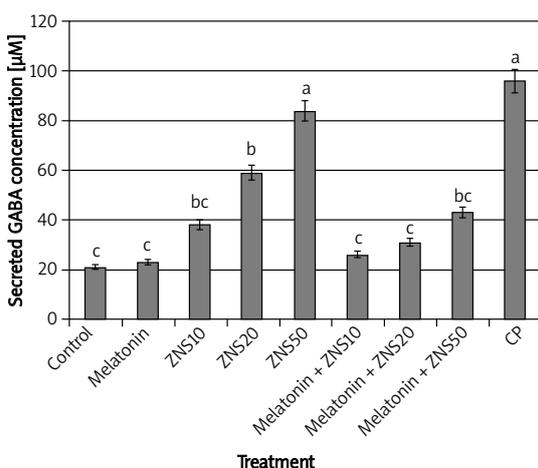


Figure 1. Comparison of GABA secretion in liver tissues of male rats after exposure to ZNS and/or melatonin. Mean values in the same column with different superscript letters differ significantly ($p < 0.05$) ZNS – Zonisamide, iNOS – inducible nitric oxide synthase, CP – cyclophosphamide.

the levels of free testosterone, LH, and FSH compared with control rats. Moreover, treatment of male rats with melatonin significantly increased the secretion of free testosterone, LH, and FSH in rats treated with low and medium doses of ZNS compared with the levels in rats treated with ZNS alone (Table I). In addition, the levels of free testosterone, LH, and FSH were increased with melatonin plus high dose of ZNS compared with ZNS alone; however, the levels were not significantly different (Table I). On the other hand, the levels of free testosterone, LH, and FSH were significantly decreased in rats treated with CP compared with the control group (Table I).

Secretion of γ -aminobutyric acid

The secretion of GABA after treatment of male rats with ZNS and/or melatonin was determined in liver tissues (Figure 1). The results revealed that GABA concentration in melatonin treated rats was similar to that in the control group. However, treatment of male rats with medium and high doses of ZNS significantly increased the GABA level in liver tissues compared with the control group (Figure 1). In the same trend, the level of GABA increased in male rats treated with CP compared with the control group. In contrast, treatment of ZNS-treated rats with melatonin significantly decreased the concentration of GABA in all groups of rats treated with ZNS plus melatonin compared with those treated with ZNS alone (Figure 1).

Determination of inducible nitric oxide synthase activity

Compared with the control group, iNOS activity was significantly high in testis tissues of male rats treated with all doses of ZNS or with CP (Figure 2). However, treatment of male rats with melatonin did not increase the iNOS activity compared with the control group. Moreover, treatment of ZNS-treated rats with melatonin significantly decreased the activity of iNOS in all groups of rats treated with ZNS plus melatonin compared with those treated with ZNS alone (Figure 2).

Assessment of sperm abnormalities

The mean values of different types of abnormalities of sperm morphology are shown in Table II. The most frequently observed abnormality was head without hock and coiled tail sperm.

The results revealed that melatonin treatment did not increase the frequencies of the morphologically abnormal sperm compared with the control group (Table II). In addition, there was no difference in the frequencies of the morphologically

abnormal sperm at the low dose treatment of ZNS compared with the control group. However, the frequency of sperm abnormalities increased significantly with the medium and high doses of ZNS compared with the control group (Table II).

On the other hand, treatment of male rats with melatonin decreased the frequency of sperm abnormalities in ZNS-treated rats. However, the frequency of sperm abnormalities increased significantly with CP treatment compared with control and other treatment groups (Table II).

DNA fragmentation

Quantitative DNA fragmentation was determined in rats exposed to ZNS alone or in combination with melatonin. The DNA damage was examined in testis tissues collected from ZNS-treated groups using gel electrophoresis laddering assay (Figure 3). The results of this assay revealed that ZNS treatment increased the DNA damage compared with the control group (Figure 3). DNA damage was observed with medium and high doses of ZNS. However, the low dose of ZNS did not induce damage in the DNA of the testis tissues of male rats.

On the other hand, the DNA remained without damage when the male rats were treated with melatonin. Furthermore, treatment of ZNS-treated rats with melatonin decreased the DNA damage compared with rats treated with ZNS alone. In contrast, the enhancement of the DNA damage with CP treatment was more effective than ZNS treatment (Figure 3).

Expression analysis of CYP19, LH and FSH genes

The effect of melatonin and ZNS on the expression of CYP19 in the testis tissues and LH as well

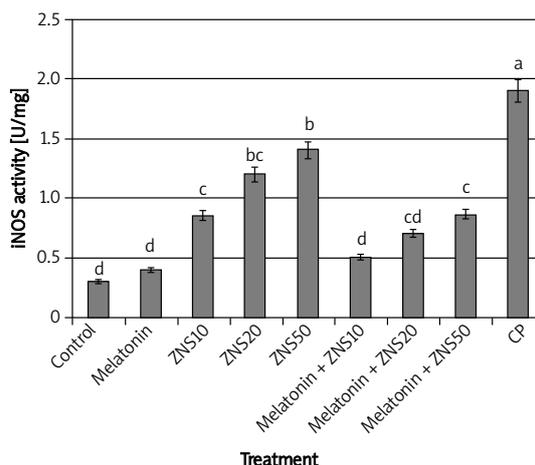


Figure 2. Comparison of iNOS activity in testis tissues of male rats after exposure to ZNS and/or melatonin. Mean values in the same column with different superscript letters differ significantly ($p < 0.05$) ZNS – Zonisamide, iNOS – inducible nitric oxide synthase, CP – cyclophosphamide.

as FSH genes in pituitary tissues of adult male rats is summarized in Figures 4–6.

The expression levels of CYP19, LH and FSH genes were increased with melatonin compared with the control group; however, the increase of the expression levels was significant in CYP19 and FSH genes (Figures 4–6).

On the other hand, treatment of male rats with low and medium doses of ZNS slightly decreased the levels of CYP19, LH and FSH genes compared with the control group. However, the levels of CYP19, LH and FSH genes decreased significantly with high dose of ZNS compared with the control group (Figures 4–6).

Supplementation of ZNS-treated rats with melatonin increased the expression levels of CYP19, LH and FSH genes. The expression levels in ZNS-treated rats exposed to melatonin were relatively simi-

Table II. Mean values of different types of sperm abnormalities induced by melatonin and/or ZNS in male rats

Treatment	Sperm abnormalities*				
	Head without hock	Amorphous head	Banana head	Coiled tail	Total
Control	0.4 ±0.1 ^c	0.0 ±0.0	0.2 ±0.0 ^b	0.2 ±0.1 ^c	0.8 ±0.1 ^d
Melatonin	0.4 ±0.0 ^c	0.0 ±0.0	0.2 ±0.1 ^b	0.3 ±0.1 ^c	0.9 ±0.1 ^d
ZNS10	0.6 ±0.1 ^c	0.3 ±0.0 ^c	0.4 ±0.1 ^b	0.5 ±0.1 ^c	1.8 ±0.2 ^c
ZNS20	0.8 ±0.0 ^b	0.6 ±0.1 ^b	0.5 ±0.1 ^{ab}	0.5 ±0.1 ^{bc}	2.4 ±0.1 ^c
ZNS50	1.2 ±0.2 ^{ab}	0.7 ±0.1 ^b	0.6 ±0.1 ^{ab}	0.7 ±0.1 ^b	3.2 ±0.2 ^b
Melatonin + ZNS10	0.5 ±0.1 ^{bc}	0.2 ±0.0 ^c	0.3 ±0.1 ^b	0.3 ±0.1 ^c	1.3 ±0.2 ^{cd}
Melatonin + ZNS20	0.7 ±0.0 ^b	0.4 ±0.1 ^{bc}	0.3 ±0.1 ^b	0.3 ±0.1 ^c	1.7 ±0.1 ^c
Melatonin + ZNS50	0.8 ±0.2 ^b	0.5 ±0.1 ^{bc}	0.5 ±0.1 ^{ab}	0.4 ±0.1 ^c	2.2 ±0.1 ^c
CP	1.7 ±0.2 ^a	1.2 ±0.1 ^a	0.9 ±0.1 ^a	1.4 ±0.2 ^a	5.2 ±0.3 ^a

*After 35 days of treatment the other 5 animals of each group were used to assess the sperm morphology. a, b, c, d Mean values within columns with unlike superscript letters were significantly different ($p < 0.05$, Scheffé test). b, bc, c, d, dc Mean values within columns with similar superscript letters were not significantly different ($p > 0.05$).

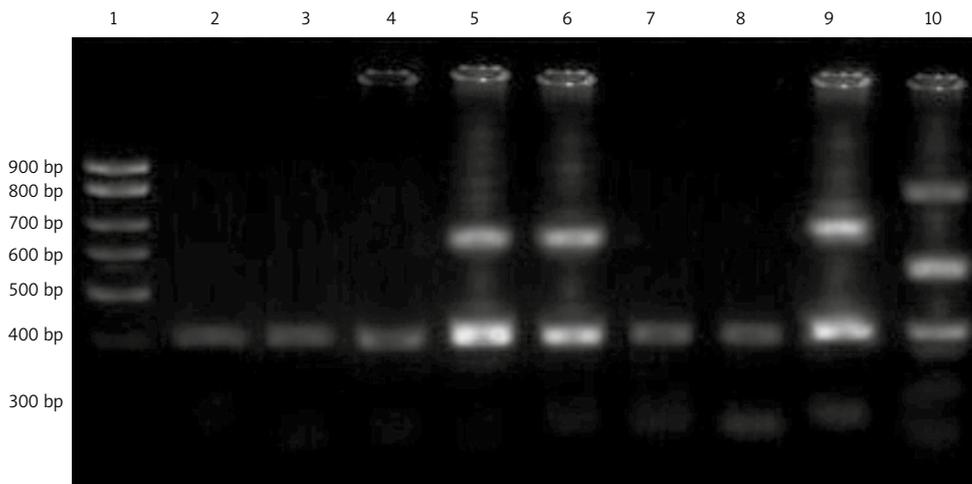


Figure 3. DNA fragmentation detected with agarose gel of DNA extracted from testis tissues of rats exposed to ZNS and/or melatonin analyzed by DNA gel electrophoresis laddering assay. Lane 1 represents DNA ladder. Lane 2 represents control samples. Lane 3 shows DNA fragmentation in testis tissues following melatonin exposure. Lanes 4–6 show DNA fragmentation in testis tissues following 10, 20 and 50 mg/kg of ZNS, respectively. Lanes 7–9 show DNA fragmentation in testis tissues following melatonin plus ZNS as in lanes 4–6. Lane 10 represents CP-treated samples

lar to the control group (Figures 4–6). In contrast, the expression levels of CYP19, LH and FSH genes were decreased significantly with CP treatment (Figures 4–6).

Discussion

The results of the present study revealed that ZNS decreased the levels of serum free testosterone, LH, and FSH in male rats, especially with the medium and high dose. In addition, the frequency of sperm abnormalities increased significantly with the medium and high doses of ZNS compared with the control group. To our knowledge, there are no published data regarding the effect of ZNS on the

fertility hormone and sperm abnormalities in male rats. So, the mechanism of action of ZNS on the fertility symptoms remained not understood. However, the effects of ZNS on neurotransmission and intracellular calcium have been evaluated in several preclinical studies. Results from these studies suggest that ZNS reduces calcium-dependent, potassium-evoked extracellular glutamate release in the hippocampus [30, 31]. Consistently with this, the current results revealed that ZNS increased the extracellular GABA concentration in ZNS-treated rats compared with control rats. Since glutamate is an excitatory neurotransmitter, and excessive release of glutamate may produce seizures, a reduc-

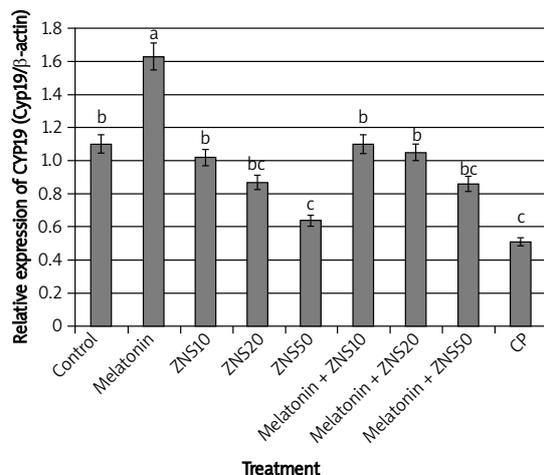


Figure 4. Relative expression of CYP19 gene in testis of male rats after exposure to ZNS and/or melatonin. Mean values in the same column with different superscript letters differ significantly ($p < 0.05$) ZNS – Zonisamide, CP – cyclophosphamide.

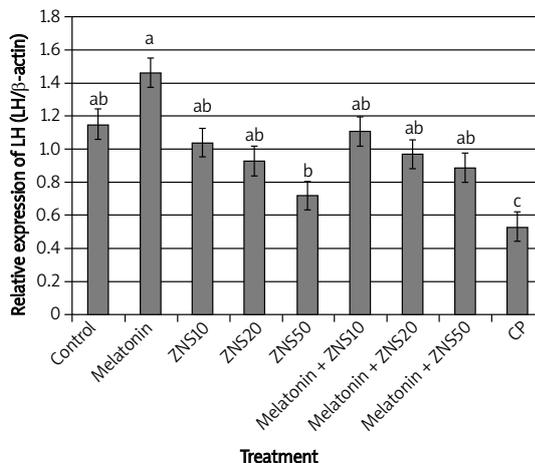


Figure 5. Relative expression of LH gene in brain tissues of male rats after exposure to ZNS and/or melatonin. Mean values in the same column with different superscript letters differ significantly ($p < 0.05$) ZNS – Zonisamide, CP – cyclophosphamide.

tion in glutamate release may reduce seizure activity and may affect epileptogenesis [32]. Therefore, due to the negative effect of ZNS on free testosterone, LH, and FSH levels and sperm viability it seems likely that ZNS has a negative feedback effect on the hypothalamus which affected the secretion of testosterone, LH, and FSH-releasing hormone. In agreement with our suggestion, Ajayi and Akhigbe [33] reported that suppression of free testosterone and FSH is responsible for the decrease in sperm viability, motility, and morphology in male rats.

The results of the present study indicated that ZNS treatment increased the DNA damage compared with the control group. A little information regarding the effect of ZNS on DNA fragmentation was published. In disagreement with our study, Komatsu *et al.* [34] reported that ZNS, in concentrations of 50 and 100 mg/kg body weight, prevented the increase of 8-OHdG levels, as a marker for DNA damage, within the cerebrum 30 min after iron solution injection. However, the difference in the Komatsu *et al.* [34] and our results is that they used only single doses of 50 mg/kg body weight and 100 mg/kg body weight of ZNS but we used a daily intake of ZNS for 4 weeks. So the main cause of ZNS-induced DNA damage in testis tissues of male rats in our results was the high concentrations of ZNS compared with the low concentrations of Komatsu *et al.* [34].

The current results demonstrated that the levels of CYP19, LH and FSH gene expression decreased significantly with the high dose of ZNS compared with the control group. The inhibitory effect of ZNS on gene expression may be attributed to its negative feedback effect on the hypothalamus and/or its effect on the DNA damage. The integrity of genomic information is critical to the survival and propagation of all cellular organisms. DNA damage that compromises genomic stability can result from environmental stresses and from cellular processes that occur during normal growth. Gasch *et al.* [35] reported that the multiple facets of the DNA damage response include cell-cycle arrest, alterations in gene expression, DNA damage repair, and cell death. Therefore, damage of the DNA influences the expression of many genes in the organism.

The present study indicated that melatonin increased the levels of serum free testosterone, LH, and FSH, as well as expression of their encoding genes in male rats. Furthermore, melatonin improved the negative effect of ZNS on the secretion of free testosterone, LH, and FSH, as well as expression of their encoding genes. In agreement with our findings, Díaz *et al.* [36] found that exogenous melatonin injection stimulated the secretion of LH and FSH pituitary responsiveness to luteinizing

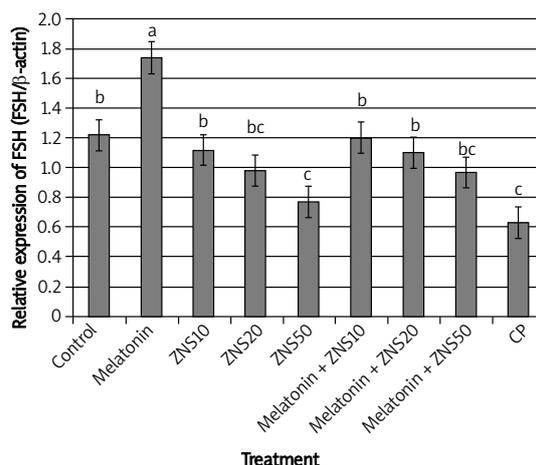


Figure 6. Relative expression of FSH gene in brain tissues of male rats after exposure to ZNS and/or melatonin. Mean values in the same column with different superscript letters differ significantly ($p < 0.05$) ZNS – Zonisamide, CP – cyclophosphamide.

ing hormone-releasing hormone (LHRH). In addition, Cagnacci *et al.* [37] reported a stimulatory effect of melatonin on the gonadotrophin response to physiological or submaximal doses of LHRH during the follicular phase of the menstrual cycle in women. Moreover, according to Li *et al.* [38] melatonin treatment resulted in 11% increase in the expression of the GnRH gene in young male rats. They suggested that the decrease in GnRH gene expression which is likely involved in the decline of reproductive functions in aging can be totally reversed by short-term administration of melatonin. However, understanding how melatonin augments this pituitary response to GnRH remains unknown. The presence of specific melatonin receptors in the pars tuberalis in rodents [39] and humans [40] supports a direct effect of melatonin on pituitary cells. However, the effect of melatonin on the hypothalamic nuclei mentioned above, which directly or indirectly influences the pituitary response to GnRH, cannot be disregarded.

The effect of melatonin on sperm morphology indicated in the present study that melatonin decreased the oxidative impact of ZNS-induced sperm abnormalities. Consistently, Koksai *et al.* [41] found that melatonin is a potent antioxidant agent in preventing testicular post-ischemic reperfusion injury, as shown by the increased Johnsen's score and changed abnormal sperm rate in male rats. In addition, Kurcer *et al.* [42] reported that melatonin treatment significantly decreased the sperm abnormalities when compared with the ischemia/reperfusion injured samples in male rats.

Our current study showed that melatonin decreased the DNA fragmentation in testis tissues induced by ZNS. This correlates with the study of Vargas *et al.* [43], who found that the quality of

sperm DNA, fragmentation, unpacking and DNA stability diminished in male mice exposed to hypoxia and treated with melatonin. The mechanism of melatonin in preventing DNA damage was suggested by Espino *et al.* [44], who posited that the preventive role of melatonin against damage induced by the mutagens possibly takes place via melatonin receptor- and extracellular signal-regulated kinase-mediated pathways. In addition, they found that the damage in the DNA of the sperm was attributed to increased lipid peroxidation in sperm cells. However, pre-treatment with melatonin 30 min before the administration of diazinon was able to completely prevent the increase of lipid peroxidation in epididymal sperm cells from mice treated with diazinon. In the present study, melatonin decreased the sperm abnormalities, which can probably be attributed to decreased lipid peroxidation in the sperm cells. Moreover, the current study found that melatonin decreased the oxidation stress in the form of iNOS in testis tissues of male rats treated with ZNS plus melatonin compared with those treated with ZNS alone.

In conclusion, the administration of melatonin prior to exposure to ZNS prevents the alteration of reproductive parameters including serum free testosterone, LH, and FSH and expression of their encoding genes, sperm morphology and DNA fragmentation in male rats, possibly due to its antioxidant properties. Melatonin could be used to reduce the toxic damage of ZNS and it is recommended to be taken prior to ZNS supplementation against PD.

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Conflict of interest

The authors declare no conflict of interest.

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