Melatonin Pretreatment Protects Against Status epilepticus, Glutamate Transport, and Oxidative Stress Induced by Kainic Acid in Zebrafish

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Abstract

Status epilepticus (SE) develops from abnormal electrical discharges, resulting in neuronal damage. Current treatments include antiepileptic drugs. However, the most common drugs used to treat seizures may sometimes be ineffective and have many side effects. Melatonin is an endogenous physiological hormone that is considered an alternative treatment for neurological disorders because of its free radical scavenging property. Thus, this study aimed to determine the effects of melatonin pretreatment on SE by inducing glutamatergic hyperstimulation in zebrafish. Seizures were induced in zebrafish using kainic acid (KA), a glutamate analog, and the seizure intensity was recorded for 60 min. Melatonin treatment for 7 days showed a decrease in seizure intensity (28%), latency to reach score 5 (14 min), and duration of SE (29%). In addition, melatonin treatment attenuated glutamate transporter levels, which significantly decreased in the zebrafish brain after 12 h of KA-induced seizures. Melatonin treatment reduced the increase in oxidative stress by reactive oxygen species formation through thiobarbituric acid reactive substances and 2',7'-dichlorofluorescin, induced by KA-seizure. An imbalance of antioxidant enzyme activities such as superoxide dismutase and catalase was influenced by melatonin and KA-induced seizures. Our study indicates that melatonin promotes a neuroprotective response against the epileptic profile in zebrafish. These effects could be related to the modulation of glutamatergic neurotransmission, recovery of glutamate uptake, and oxidative stress parameters in the zebrafish brain.

Keywords  Epilepsy · Melatonin · Oxidative stress · Glutamate · Zebrafish

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| DCF          | 2',7'-Dihydrodichlorofluorescein diacetate |
| CAT          | Catalase |
| CNS          | Central nervous system |
| GPx          | Glutathione peroxidase () |
| GR           | Glutathione reductase |
| SOD          | Superoxide dismutase |
| TBA-RS       | Thiobarbituric acid-reactive species |

Highlights

• Pre-treatment with melatonin reduces seizure intensity and Status epilepticus time in zebrafish.
• Pre-treatment with melatonin increases latency time for seizures, induced by kainic acid.
• TBA-RS and DCFH levels are attenuated in zebrafish pre-treated with melatonin.
• Glutamate uptake is reduced in control KA fish, reaching basal levels when pretreated with melatonin.

Introduction

Epilepsy is the second most common neurological disorder with the highest incidence worldwide, characterized by recurrent seizures and abnormal EEG recordings in the brain [1, 2]. Epileptogenesis is the underlying process in which a normal brain becomes epileptic due to numerous factors such as trauma, genetics, infections, and metabolic abnormalities [3].

The glutamatergic system plays an important role in the initiation and propagation of Status epilepticus (SE) [4]. Seizures emerge from abnormal hyperexcitability and hypersynchronization of neurons, resulting in unprovoked excessive electrical discharges causing glutamatergic excitotoxicity, oxidative stress, and neuronal apoptosis [5].
Hyperexcitability leads to excessive glutamate release in the synaptic cleft, resulting in higher calcium influx that leads to mitochondrial dysfunction and a subsequent increase in oxidative stress [6].

Among multiple therapeutics targeted for the treatment of epilepsy, the use of antiepileptic drugs (AEDs) is the most common. These medications act on sodium ionic channels, GABAergic, and glutamatergic systems. The most common medications are sodium valproate, carbamazepine, and acetazolamide [7]. Current treatment involves the use of multiple drugs to attenuate seizures. However, if the dose is not appropriate [8], this approach can result in neurotoxic effects. Long-term use of AEDs can cause cognitive impairment and oxidative damage to the brain, such as modulation of antioxidant enzymes, stimulation of reactive oxygen species (ROS) production, and promotion of toxicity to essential macromolecules in the body [9]. It has been estimated that 30% of patients do not respond to treatment and furthermore, pharmacological resistance necessitates the search for alternative treatments to improve the quality of life in these patients [10, 11]. The need to find alternative treatments and drugs is growing as treatment-resistant cases are increasing.

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous hormone with numerous properties including immunological, anti-inflammatory, reproductive, hormonal, and aging regulation [12]. Due to its amphiphilic properties, melatonin can easily cross the blood brain barrier, assisting in neuronal processes [13]. Melatonin attenuates cellular death resulting from oxidative damage by capturing free radicals and neutralizing, and turning some of the most dangerous ROS, such as H$_2$O$_2$, O$_2$–, HO–, NO, ONOO, and HOCl, less reactive [14, 15]. These properties of melatonin provide an interesting opportunity to investigate its effectiveness in neurological diseases.

Considering the translational neuroscience perspective, zebrafish is a suitable animal model for research on the mechanisms underlying seizures and epilepsy [16, 17]. Behavioral and neurochemical screens in zebrafish offer an intriguing alternative preclinical approach to CNS drug discovery [18]. Furthermore, the glutamatergic system expresses excitatory amino acid transporters that facilitate maintenance of the neurotransmitter levels and uptake in the brain [19, 20]. Studies have shown that an injection of kainic acid (KA) can generate seizure-like behavior and changes in glutamate transport, resulting in encephalic damage [16, 21].

Considering the cognitive, neuronal, psychological, and social impact of epilepsy and the percentage of patients refractory to treatment, the use of alternative neuroprotective molecules such as melatonin is relevant for treatment. Thus, we investigated the possible influence of melatonin pretreatment on KA-induced behavioral and neurochemical changes in adult zebrafish.

**Materials and Methods**

**Animals and Housing Conditions**

Adult wild-type Zebrafish (*Danio rerio*; 4–6-month-old, 50:50 male:female ratio and weighing 0.5 g each) from a genetically uncontrolled and heterogeneous wild-type stock (striped pattern and short-fin phenotype) were obtained from the department of Biochemistry at the Federal University of Rio Grande do Sul (UFRGS). Zebrafish were conditioned at the Translational Psychiatry laboratory of UNESC and maintained according to standard husbandry procedures. The tanks were kept with water from reverse osmosis reconstituted with marine salt (Instant Ocean, Blacksburg, VA, USA) at 28 ± 2 °C, pH of 7.4, ammonia < 0.02 mg.L–1, nitrite < 0.01 mg.L–1 and a conductivity of 500 μS under light/dark 14/10 photoperiod (lights on at 8:00 a.m.) and fed twice a day with artemia and commercial flake fish food. All procedures presented in this study were approved by the Ethics Committee of University of Southern Santa Catarina (UNESC) protocol number 030/2019–1.

**Melatonin Exposure and Seizure Induction**

The molecule was administered directly into the aquariums half an hour before the lights went off in the zebrafish facility at a final concentration of 100 nM, as previously described [22]. Fish were exposed to melatonin in two different time periods, being: melatonin added for 3 nights, and 7 nights treatment in which seizures were induced the morning after each period (Fig. 1A). To ensure the same concentration of melatonin daily, the water in the tanks was completely exchanged every day. After melatonin treatment, fish were immersed in 160 μg/mL of tricaine and then inoculated (10 µL) intraperitoneally (i.p.) with KA at 5 mg/kg [21]. The fish groups that were not induced were similarly anaesthetized and injected with PBS. A total of 72 animals were divided in three groups: CTL + KA, Mel 3 days + KA and Mel 7 days + KA. After seizure induction, fish were observed individually for a period of 60 min. The seizures present classic behaviors and abnormal brain activity, which can be observed through a numerical system of scores, varying from 1 to 7, being: (1) immobility and hyperventilation; (2) whirlpool-like swimming; (3) rapid movements from right to left; (4) abnormal and...
spasmodic muscular contractions, (5) rapid whole-body clonus-like convulsions; (6) sinking to the bottom of the tank and spasms for several minutes; (7) death [16]. Seizure intensity, latency, and SE as duration of 5 and 6 score time were evaluated.

After the preliminary test verifying the effect of different times of melatonin exposure, we established 7 days of pretreatment for biochemical analyzes (Fig. 2A). We also adopted this exposure time and melatonin concentration because it is not able to alter the zebrafish locomotor profile (data not shown). For this, fish were divided into four groups according to each treatment: CTL + PBS, Mel + PBS, CTL + KA and Mel + KA. Control groups underwent the same procedures without the administration of any molecule into the water. Seizure-induced by KA generates neurochemical consequences after different periods, including glutamate transport and microglial markers [21]. In order to verify whether melatonin is able to influence the changes caused by KA-induced glutamatergic hyperstimulation, glutamate uptake and oxidative stress parameters were evaluated. Thus, after 12-h seizure induction, the animals were anesthetized by immersing them in 160 mg/ml of tricaine (4 °C), suffered euthanasia and their brain contents were dissected for neurochemical analysis.

Evaluation Oxidative Stress Parameters

Tissue Preparation

To evaluate the melatonin and KA exposure on oxidative stress, we divided 120 animals (five brain per n; n = 6) in four different groups: CTL + PBS, Mel + PBS, CTL + KA and Mel + KA. Tissues homogenized in 1 mL of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 750×g for 10 min at 4 °C.
to discard nuclei and cell debris [23]. The pellet was discarded, and the supernatant was collected and used for the evaluation of oxidative stress parameters.

**Determination of Oxidation of DCFH**

The production of reactive oxygen species (EROS) was measured from the oxidation of 2',7'-dihydrodichlorofluorescein diacetate forming DCF, where the intensity of fluorescence is in accordance with the formation of EROS [24]. A calibration curve was performed with standard DCF (0.25–10 μM) and the levels of reactive species were expressed as nmol DCF formed.mg of protein$^{-1}$.

**Determination of TBA-RS Levels**

The levels of substances reactive to thiobarbituric acid (TBA-RS) evaluate the lipid peroxidation produced by EROS. This technique is based on the principle of species reactive to thiobarbituric acid forming a pink color in the samples [25]. A calibration curve was established using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as supernatants. TBA-RS values were measured and expressed as nmol of TBA-RS.mg protein$^{-1}$.

**Antioxidant Enzyme Activities Determination**

**Determination of SOD and CAT Activities**

Superoxide Dismutase (SOD) was carried out using methods already established, based on the oxidation of adrenaline by the enzyme SOD, disabling the dismutation of the radical O2$^\cdot$ in H$_2$O$_2$ [26]. The reaction medium consisted of 50 mM glycine buffer, pH 10.2, 0.1 mM catalase and 1 mM epinephrine. Absorbance was measured at 480 nm. SOD specific activity is expressed as nmol.min$^{-1}$mg protein$^{-1}$. Catalase (CAT) activity assay was performed through the measuring of absorbance decrease at 240 nm in a reaction medium containing 20-mM H$_2$O$_2$, 0.1% Triton X-100, 10-mM potassium phosphate buffer, pH 7.0, and the supernatants containing 0.1–0.3 mg protein.mL$^{-1}$ [27]. The specific activity was represented as nmol.min$^{-1}$mg protein$^{-1}$. The set of two enzymatic activities was expressed using the SOD/CAT ratio.

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Fig. 2 Effects of melatonin pretreatment (3 and 7 days) on KA-induced seizures in zebrafish. A Seizure-like behavioral scores across time for 60 min represented as median±interquartile range (B), and intensity evaluated by area under the curve (AUC) for each time of pretreatment. C Representative heat maps showing the individual scores across the 60-min exposure period of each individual animal. Seizure intensity was represented as mean±S.E.M and analyzed by one-way ANOVA followed by Tukey’s test. Asterisks above bars express significant differences compared to the CTL+KA group (n=20–24 animals per group; *p<0.05)
Determination of GR and GPx Activities

Glutathione reductase (GR) is responsible for catalyzing the GSSG reduction reaction in NADPH-dependent GSH [28]. The enzyme activity was assessed in a solution containing 50-mM potassium phosphate buffer, pH 7.0, containing 1-mM EDTA, 0.2-mM NADPH and the supernatant containing 0.3–0.5 mg protein.ml⁻¹. The reaction was initiated by the addition of 1-mM oxidized glutathione and a change in absorbance was measured at 340 nm. GR activity was expressed as nmol NADPH oxidized.min⁻¹.mg protein⁻¹. The enzymes Glutathione peroxidases (GPx), are responsible for the consumption of NADPH to generate reduced glutathione (GSH) from oxidized glutathione (GSSG), acting on the metabolism of H₂O₂ [29]. This method was performed using Tert-butylhydroperoxide as a substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in 50 mM potassium phosphate buffer, pH 7.0, containing 1-mM EDTA, 0.2-mM NADPH and the supernatant containing 0.2–0.3 mg protein.ml⁻¹. GPx activity was expressed as nmol NADPH oxidized.min⁻¹.mg protein⁻¹.

Glutamate Uptake Assay

Glutamate uptake assay was performed as previously described [20, 29]. We divided 24 animals into four different groups (one brain per n; n = 6). The brains were set in microplates of 24 wells and total glutamate uptake was measured with the addition of 0.33 μCi mL⁻¹ L-[³H] glutamate (PerkinElmer, Madrid, Spain) to the incubation medium (HBSS-HEPES buffer) pH 7.2 at 37 °C for 7 min. After this period, the uptake was stopped with two subsequent washes with 1-mL ice-cold HBSS-HEPES buffer). Na⁺-independent glutamate uptake was measured using the same conditions as described above, except that N-methyl-D-glucamine was used instead of sodium. Na⁺-dependent glutamate uptake was measured as the difference of incorporated radioactivity between the total glutamate uptake and the Na⁺-independent glutamate uptake. Radioactivity was measured by liquid scintillation and uptake was expressed as nmol [³H]-Glu.min⁻¹.mg of protein⁻¹).

Protein Determination

Total protein quantification in the samples was performed by Lowry et al. (1951) [31] method using bovine serum albumin as standard.

Statistical Analysis

Statistical analyses were performed using the statistical program SPSS Statistics (Armonk, New York, USA). The construction of all the graphs present in this study was performed using the Graph Pad Prism software version 8.4. The results were found as mean ± standard error of the mean. All data were tested for normality using a Shapiro–Wilk’s test and Levene’s test to examine homogeneity of variance. Nonparametric data (seizure scores across 60 min) were expressed as median ± interquartile range.

Seizure intensity evaluated by the area under curve (AUC), latency to score 5 across, time of SE (duration of 5 and 6 scores) and biochemical parameters (parametric data) were expressed as means ± standard error of mean (S.E.M) and further evaluated by one- or two-way analysis of variance (ANOVA) in which the degree of significance was evaluated, where p < 0.05. As a result, where there was a difference, the Tukey test was performed as a post hoc test when necessary. Individual seizure-like behavioral scores across time for 60 min for each group was obtained using n = 20–24 animals. Because of the total fish per biochemical experiment, our strategy was to carry out different independent rounds (1 round per n) until completing the sample number previously described.

Results

The seizure-related behavioral phenotypes were assessed and all fish injected with KA presented with at least one rapid whole-body clonus-like convulsion, classified as a score of 5. To establish the standard conditions for melatonin exposure, zebrafish were initially pretreated for 3 and 7 days. Figure 2 shows seizure intensity over 60 min. The data profile shows that the pretreated groups had decreased seizure intensities compared to KA-exposed fish (Fig. 2A). Melatonin pretreatment for 7 days significantly decreased the total seizure score as estimated by the area under the curve (F(4,109) = 13.78, p < 0.05) (Fig. 2B). Figure 2C shows the representative heat maps of individual scores of zebrafish for 60 min after KA injection. The distribution of the individual score values is indicative of changes in the latency time to 5 and the SE duration. The control KA group presented a latency time of 8.5 ± 1.18 min to reach a score of 5 (Fig. 3A). Pretreatment of melatonin for 7 days significantly increased the latency to score 5 to 22 ± 3 min in comparison to the CTL + KA group (F(2, 68) = 6.061, p < 0.01). Three days of exposure did not significantly alter this parameter. In addition to reducing the time to reach stage 5, melatonin also affected the duration, F(2, 89) = 3.102, p < 0.01. CTL + KA group exhibited 41 ± 3.72 min of SE duration, spending 68% of the time in seizure-like behavior, transitioning between
scores 5 and 6 (Fig. 3B). SE duration showed a significant decrease for Mel 3 days + KA and Mel 7 days + KA with $28.6 \pm 4.29$ (31%, $p < 0.05$) and $29.6 \pm 4.37$ (29%, $p < 0.05$) min, respectively.

Considering that zebrafish pretreated with melatonin for 7 days showed a significant decrease in latency to reach score 5, this time period was adopted to investigate glutamate neurotransmission and oxidative stress. To investigate whether melatonin modulated glutamate transport, we assessed glutamate uptake in the brain tissue of animals pretreated with melatonin for 7 days (Fig. 4). Two-way ANOVA revealed significant differences between the tested groups. The CTL + PBS and Mel + PBS groups showed similar values for glutamate uptake, indicating that melatonin in isolation did not influence glutamate transport ($F(4,54) = 36.18$, $p < 0.05$). CTL + KA showed a significant decrease (37.7%) compared to CTL + PBS ($p < 0.05$). Interestingly, the Mel + KA group showed recovery in glutamate uptake, displaying a significant difference in comparison with CTL + KA ($p < 0.05$), similar to CTL + PBS.

Reduced glutamate uptake is associated with excitotoxicity and oxidative damage. Furthermore, a return to basal levels of glutamate uptake in fish pretreated with melatonin could show up as antioxidant neuroprotection. In order to evaluate oxidative damage, we assessed the free radical formation through TBA-RS and DCFH oxidation (Fig. 5). Regarding DCFH oxidation, two-way ANOVA using treatment and KA as variable factors showed significant effects of treatment ($F(4,54) = 18.82$, $p < 0.001$). No alterations were observed in DCFH levels in CTL + PBS, compared to Mel + PBS pretreated zebrafish, indicating that melatonin in isolation does not modulate the biomarkers of oxidative damage (Fig. 5A). Additionally, CTL + KA significantly increased DCFH oxidation by 71.1% compared to CTL + PBS ($p < 0.05$), demonstrating that KA administration causes oxidative damage to the zebrafish brain. Furthermore, Mel + KA showed a significant decrease of 30% in DCFH oxidation compared to CTL + KA ($p < 0.05$).
Lipid peroxidation was evaluated by measuring the levels of TBA-RS after melatonin and KA-induced seizures, which showed a significant effect \((F(4,54)=92.14, p<0.0001)\) (Fig. 5B). TBA-RS levels showed a significant increase of 62.7% in the CTL + KA group, compared to the CTL + PBS group \((p<0.05)\). These results demonstrate that seizures themselves generate oxidative damage and increase free radical formation in the brain. A decrease in TBA-RS levels (17.7%) was observed in the Mel + KA group compared to that in the control + KA group. To evaluate the underlying antioxidant properties of melatonin, we assayed antioxidant enzymes such as, SOD, CAT, GR, and GPx (Fig. 6). SOD activity was significantly lower in the Mel + PBS group than in the CTL + PBS group (36.5%; \(p<0.05)\) (Fig. 6A). Furthermore, enzyme activity also decreased significantly for Mel + KA compared to CTL + PBS (26.5%; \(p<0.05)\). Melatonin pretreatment and KA-induced seizures altered the CAT activity \((F(3, 20)=4.552; p<0.05)\) (Fig. 6B). A decrease in CAT activity was observed for Mel + PBS (43.1%; \(p<0.01)\) and Mel + KA (38.46%; \(p<0.05)\), respectively. We evaluated the activities of CAT and SOD in the decomposition of superoxide radical anions and dismutation of hydrogen peroxide, respectively, using the SOD/CAT ratio. Pretreatment with Mel reversed the KA-induced imbalance in CAT/SOD to the basal level (Fig. 6C). No alterations were observed in GR and GPx activity in any of the groups tested (Fig. 6D, E).

**Discussion**

This is the first study to investigate the neuroprotective effects of the pretreatment with melatonin against SE induced by KA in zebrafish. Melatonin is an important natural hormone responsible for the regulation of essential physiological processes in the organism. It is able to easily cross the blood–brain barrier and act as a potent antioxidant, with clinical uses in numerous diseases, and most recently in epilepsy studies [13, 32].

In this study, we first addressed the intensity of seizure behavior. Classic seizure movements were recorded and characterized for a period of 60 min through well-established assays in literature [16]. Our findings show that control KA fish spent an average of 41 min in SE, with extremely intense seizures, and a short latency time. These findings implicate that injection of KA in the zebrafish brain results in a hyperexcitability model, presenting cellular changes, and classic seizure-like behavior, which is consistent with previous studies in the zebrafish brain [16, 21].

Zebrafish pretreated with melatonin exhibited a decrease in seizure intensity and SE duration, and an increase in latency to reach score 5. These findings corroborate with previous studies, in which melatonin elicited neuroprotective responses through retardation of seizures, cognitive function, and electroencephalographic brain activity in a mammalian kainate model of temporal lobe epilepsy [33, 34]. To establish the conditions for melatonin pretreatment, we investigated different periods of exposure. We found that 7 days of melatonin exposure showed a significantly higher protective response in the seizure score profile, compared to 3 days of pretreatment. Furthermore, the significant increase in latency to achieve tonic–clonic seizure, duration of scores 5 and 6, and individual heat maps of scores confirmed the reduction of seizure profile. Establishment of these suitable conditions including period and concentration can be useful for understanding and exploring the neurophysiological and neurochemical properties of melatonin from the translational perspective.
The glutamatergic system plays a key role in the imbalance of excitatory and inhibitory synapses during SE. As an endogenous neuroendocrine neuromodulator, melatonin exerts a positive control on glutamate excitotoxicity that leads to receptor desensitization by decreasing glutamate uptake, resulting in excessive Ca²⁺ influx into neurons, causing oxidative stress and cellular damage which are the underlying mechanisms of neuronal impairment [35]. We found a decrease in Na⁺-dependent glutamate transporters 12 h after KA-induced zebrafish seizures. These data are in accordance with this time window, in which 12 h post-seizure, there is disruption of glutamate uptake and alteration of astrocytic markers such as GFAP and S100 in the zebrafish brain [21]. In this context, we investigated whether melatonin treatment could counteract glutamatergic excitotoxicity in zebrafish in accordance with the reduction of seizure profiles and in the amelioration of episodes of seizures in animal models [36] and human [37]. Melatonin pretreatment alone did not modify the glutamate uptake. In contrast, zebrafish subjected to KA-induced seizures and previously treated with melatonin recovered Na⁺-dependent glutamate uptake similar to that in the untreated PBS group. These results suggest that melatonin can influence the disruption of this parameter, which is responsible for controlling glutamate homeostasis in the brain tissue. These pieces of evidence can be associated with the control of oxidative balance and the possible antioxidant properties of melatonin in zebrafish due to its ease in crossing the blood–brain barrier, resulting in facilitated access to neurons.

To evaluate the underlying processes of SE behavioral profile and glutamate uptake, we assessed the oxidative stress parameters. Here, KA-induced seizures increased TBA-RS and DCFH in the zebrafish brain, resulting in free radical formation and higher levels of biomarkers of oxidative damage, similar to the findings in rat epilepsy models [38]. Furthermore, these phenomena corroborate previous studies, which suggest that epilepsy patients show higher levels of oxidative stress biomarkers, elevated ROS formation, reduced antioxidant enzyme activity, and vulnerability to oxidative brain damage [39, 40]. Our results show that control KA-injected zebrafish have increased lipoperoxidation and ROS formation and release in the brain, suggesting that oxidative stress could be related to glutamatergic disruption. Additionally, 7 days of melatonin pretreatment followed by seizure induction reduced the DCFH and TBA-RS levels, implying that melatonin is able to attenuate ROS formation in the zebrafish brain. Indeed, melatonin is a highly potent natural antioxidant with free radical scavenging properties, due to its electron-rich chemical structure, which makes these molecules less reactive [41]. Therefore, melatonin has a promising neuroprotective response against oxidation in the cerebral tissue of zebrafish, which can be associated with the neutralization of increased ROS formation in this SE glutamatergic model.

Fig. 6 Effect of melatonin pretreatment on KA-induced seizures on the activity of superoxide dismutase (SOD) (A), catalase (CAT) (B), SOD/CAT ratio (C) glutathione reductase (GR) (D) and glutathione peroxidase (GPx) (E). Results are expressed as mean±S.E.M (n=6), each performed in triplicate. The data were analyzed by two-way ANOVA followed by Tukey’s post hoc test. *p<0.05; **p<0.01.
To further understand the mechanisms underlying the antioxidant defense of melatonin, we assessed the enzyme activities of SOD, CAT, GR, and GPx. Our findings showed an inhibition of SOD and CAT activities when melatonin was tested without SE induction and KA-induced seizures, compared to the control. A reduction in antioxidant enzyme activity or an imbalance in the redox system leads to oxidative stress [42, 43]. SOD and CAT enzyme activities of SOD, CAT, GR, and GPx. Our findings of this study are available within the article. The raw data are available from Eduardo Pacheco Rico upon reasonable request. The study protocol was approved by the Ethics Committee of University of Southern Santa Catarina (UNESC), Criciúma, Brazil, number 030/2019–1.

**Declarations**

**Consent to Participate** All participants gave written informed consent and signed by them. All consents are available upon request.

**Consent for Publication** Not applicable.

**Conflict of Interest** The authors declare no competing interests.

**References**

1. Epilepsy (2020). World Health Organization. https://www.who.int/news-room/fact-sheets/detail/epilepsy. Accessed 15 May 2020.
2. Ngugi, AK, Bottomley C, Kleinschmidt I, Sander JW, Newton CR (2010). Estimation of the burden of active and life-time epilepsy: a meta-analytic approach. Epilepsia 51: 883–890. https://doi.org/10.1111/j.1522-1167.2009.02481.x
3. Goldberg EM, Coulter DA (2013) Mechanisms of epileptogenesis: a convergence on neural circuit dysfunction. Nat Rev Neurosci 14:337–349. https://doi.org/10.1038/nrn3482
4. Zhou Y, Danbolt NC (2014) Glutamate as a neurotransmitter in the healthy brain. J Neur Transm 121:799–817. https://doi.org/10.1007/s00702-014-1180-8
5. Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1–105. https://doi.org/10.1016/s0301-0082(00)00067-8
6. Vishnoi S, Raisuddin S, Parvez S (2016) Glutamate excitotoxicity and oxidative stress in epilepsy: modulatory role of melatonin. J Environ Pathol Toxicol Oncol 35:365–374. https://doi.org/10.1615/JEnvironPatholToxicolOncol.v2016i1.00136
7. Rogawski MA, Löscher W (2004) The neurobiology of antiepileptic drugs. Nat Rev Neurosci 5:553–564. https://doi.org/10.1038/nrn1430
8. Kwan P, Brodie MJ (2006) Combination therapy in epilepsy. Drugs 66:1817–1829. https://doi.org/10.2165/00003495-20066140-00004
9. Hamed SA, Abdellah MM (2004) Trace elements and electrolyte homeostasis and their relation to antioxidant enzyme activity in brain hyperexcitability of epileptic patients. J Pharmacol Sci 96:349–359. https://doi.org/10.1258/jpsr.04004x
10. Kwan P, Schachter SC, Brodie MJ (2011) Drug-resistant epilepsy. N Engl J Med 365:919–926. https://doi.org/10.1056/NEJMra1004418
11. Sheng J, Liu S, Qin H, Li B, Zhang X (2018) Drug-resistant epilepsy and surgery. Curr Neuropsychopharmacol 16:17–28. https://doi.org/10.2174/1570159X15666170504123316
12. Zhdanova IV, Tucci V (2003) Rhythms, and sleep. Curr Treat Options Neurol 5:225–229. https://doi.org/10.1007/s11940-003-0013-0
13. Alghamdi BS (2018) The neuroprotective role of melatonin in neurological disorders. J Neurosci Res 96:1136–1149. https://doi.org/10.1002/jnr.24220
14. Tan DX, Manchester LC, Esteban-Zubero E, Zhou Z, Reiter RJ (2015) Melatonin as a potent and inducible endogenous anti-oxidant: synthesis and metabolism. Molecules 20:18886–18906. https://doi.org/10.3390/molecules20188866
15. Tarocco A, Caroccia N, Morciano G, Wieczkowski MR, Ancora G, Garani G, Pinton P (2019) Melatonin as a master regulator of cell death and inflammation: molecular mechanisms and clinical
implications for newborn care. Cell Death Dis 10:317. https://doi.org/10.1038/s41419-019-1556-7
16. Alfaro JM, Ripoll-Gómez J, Burgos JS (2011) Kainate adminis-
terated to adult zebrafish causes seizures similar to those in rodent
models. Eur J of Neurosci 33:1252–1255. https://doi.org/
10.1111/j.1460-9586.2011.07622.x
17. Fontana BD, Ziani PR, Canzian J, Mezzomo NJ, Muller TE, dos
Santos MM, Loro VL, Barbosa NV, Mello CF, Rosenberg DB (2019)
Taurine Protects from pentylentetrazole-induced behav-
ioral and neurochemical changes in zebrafish. Mol Neurobiol
56:583–594. https://doi.org/10.1007/s12035-018-1107-8
18. Wyatt C, Bartoszek EM, Yaksi E (2015) Methods for studying
the zebrafish brain: past, present and future. Eur J Neurosci
42:1746–1763. https://doi.org/10.1111/ejn.12932
19. Guo S (2009) Using zebrafish to assess the impact of drugs on
neural development and function. Expert Opin Drug Discov
4:715–726. https://doi.org/10.1517/1746040902988464
20. Rico EP, de Oliveira DL, Rosenberg DB, Mussulini BH, Bonan
CD, Dias RD, Wofchuck S, Souza DO, Bogo MR (2010) Expression
and functional analysis of Na+-dependent glutamate trans-
porters from zebrafish brain. Brain Res Bull 81:517–523. https://
doi.org/10.1016/j.brainresbull.2009.11.011
21. Mussulini BHM, Vizuete AFK, Braga M, Moro L, Baggio S,
Santos E, Lazzarotto G, Zenki KC, Pettenuzzo L, Rocha JBT, de
Oliveira DL, Calcagnotto ME, Zuanazzi JAS, Burgos JS, Rico EP
(2018) Forebrain glutamate uptake and behavioral parameters are
altered in adult zebrafish after the induction of Status Epilep-
ticus by kainic acid. NeuroToxicology 67:305–312. https://doi.org/
10.101
22. Pinheiro-Da-Silva J, Tran S, Luchiari AC (2018) Sleep depriva-
tion impairs cognitive performance in zebrafish: a matter of fact?
Behav Processes 157:656–663. https://doi.org/10.1016/j.
beproc.2018.04.004
23. Agostini JF, Dal Toé HCZ, Vieira KM, Baldin SM, Costa NLF,
Cruz CU, Longo L, Machado MM, da Silveira TR, Schuck
PF, Rico EP (2017) Cholinergic system and oxidative stress
changes in the brain of a zebrafish model chronically exposed
to ethanol. Neurotox Res 33:749–758. https://doi.org/10.1007/
s12640-017-9816-8
24. Lebel CP, Ischiropoulos H, Bondy SC (1992) Evaluation of the
probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen
species formation and oxidative stress. Chem Res Toxicol 5:227–
231. https://doi.org/10.1021/tr00026a012
25. Estebauer H, Cheeseman KH (1990) Determination of aldehydic
lipid peroxidation products: malonaldehyde and 4-hydroxynon-
enal. Method in Enzymol 186:407–421. https://doi.org/10.1016/
0076-6879(90)86134-h
26. Bannister JV, Calabrese L (1987) Assays for superoxide dis-
mutase. Methods Biochem Anal 32:279–312. https://doi.org/
10.1002/978047010539.ch5
27. Aebl H (1984) Catalase in vitro. Methods Enzymol. https://doi.
org/10.1016/s0076-6879(84)0106-3
28. Carlborg I, Mannervik B (1985) Glutathione reductase. Methods
Enzymol 113:484–490. https://doi.org/10.1016/s0076-6879(85)
13062-4
29. Wendel A (1981) Glutathione peroxidase. Methods Enzymol
77:325–333. https://doi.org/10.1016/s0076-6879(81)77046-0
30. Baggio S, Mussulini BH, de Oliveira DL, Zenki KC, Santos
da Silva E, Rico EP (2017) Embryonic alcohol exposure pro-
motes long-term effects on cerebral glutamate transport of adult
zebrafish. Neurosci Lett 636:265–269. https://doi.org/10.1016/j.
neulet.2016.11.016
31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Pro-
tein measurement with the Folin phenol reagent. J Biol Chem
193:265–725
32. Khan S, Khurana M, Vyas P, Vohora D (2021) The role of melato-
nin and its analogues in epilepsy. Rev Neurosci 32:49–67.
https://doi.org/10.1515/reun-2019-0088
33. Alhazza IM, Ashraf GM, Aliev G (2016) Recent updates on the
epileptogenic and neurodegenerative effects of melatonin. Mol
Neurobiol 53:423–448. https://doi.org/10.1007/s12035-016-9956-2
34. Sanchez-Barcelo EJ, Rueda N, Mediavilla MD, Martinez-Cue
C, Reiter RJ (2017) Clinical uses of melatonin in neurological
diseases and mental and behavioural disorders. Curr Med Chem
35:3851–3878. https://doi.org/10.2174/09298673246661707181
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