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PTZ KINDLING MODEL: EVALUATION OF EEG FACTOR AND BIOCHEMISTRY PARAMETERS UNDER THE INFLUENCE OF RAMELTEON

PTZ kindling modeli: Ramelteon'un etkisi altında EEG faktörü ve biyokimya parametrelerinin değerlendirilmesi

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ABSTRACT

Many selective synthetic melatonin receptor agonists have anticonvulsant/anti-epileptogenic properties. These agonists bind to melatonin receptor 1 (MT1) and receptor 2 (MT2), causing their activation. Therefore, we evaluated the anticonvulsant effect of Ramelteon (RMLT) as a melatonin agonist in the PTZ (Pentylenetetrazol)-kindling model. In the study, 36 male Wistar Albino rats were assessed in 6 groups (Sham, PTZ, dimethylsulphoxide (DMSO), Valproic acid (VPA) (150 mg/kg) + PTZ, RMLT (30 mg/kg)+PTZ, VPA+RMLT+PTZ). Cortical electroencephalography (EEG) data were recorded for all groups. Seizures were scored according to the Racine scale. Seizure scores and onset times of the first myoclonic movements were compared in EEG traces. Total antioxidant status (TAS), total oxidant status (TOS), catalase, myeloperoxidase (MPO), and Thiol levels were measured in serum samples. Also, Calcineurin (CaN), Neuropeptide-Y (NPY), Neuron Specific Enolase (NSE), and S100B levels were measured in brain tissue samples. There was a significant difference between the PTZ and PTZ+Valproic acid+RMLT groups for the onset of the first myoclonic movements and the rate of spikes in the EEG traces in Racine's convulsion stages ($P<0.001$). Biochemical parameters were not significant between the groups ($P > 0.05$). RMLT has anticonvulsant properties. Additionally, the receptor preference of RMLT can be investigated.

Key words: Anticonvulsant effect, Electroencephalography, Epilepsy, Pentylenetetrazole, Ramelteon.

ÖZ

Pek çok seçici sentetik melatonin reseptörü agonisti, antikonvülsan/anti-epileptojenik özelliklere sahiptir. Bu agonistler melatonin reseptörü 1 (MT1) ve melatonin reseptörü 2'ye (MT2) bağlanarak onların aktivasyonlarına neden olmaktadır. Bu nedenle, bir melatonin agonisti olarak Ramelteon'un (RMLT) antikonvülsan etkisini PTZ (Pentilenetetrazol)-kindling modelinde değerlendirdik. Çalışmada 36 adet erkek Wistar Albino sıçan grupta (Sham, PTZ, dimetilsülfoksit (DMSO), Valproik asit (VPA) (150 mg/kg) + PTZ, RMLT (30 mg/kg)+PTZ, VPA+RMLT+PTZ) değerlendirildi. Tüm gruplar için kortikal elektroensefalografi (EEG) verileri kaydedildi. Nöbetler Racine skalasına göre skorlandı. EEG traselerinde nöbet skorları ve ilk miyoklonik hareketlerin başlangıç zamanları karşılaştırıldı. Serum örneklerinde toplam antioksidan durum (TAS), toplam oksidan durum (TOS), katalaz, miyeloperoksidaz (MPO) ve Tiyol seviyeleri ölçüldü. Ayrıca beyin dokusu örneklerinde Kalsinörin (CaN), Nöropeptid-Y (NPY), Nöron Spesifik Enolaz (NSE) ve S100B seviyeleri ölçüldü. PTZ ve PTZ+Valproik asit+RMLT grupları arasında Racine'in konvülsiyon evrelerinde ilk miyoklonik hareketlerin başlangıcı ve EEG traselerindeki ani artış oranları açısından anlamlı bir fark vardı ($P<0.001$). Biyokimyasal parametreler, gruplar arasında anlamlı değildi ($P > 0.05$). RMLT antikonvülsan özelliklere sahiptir**.** Ek olarak RMLT'nin reseptör tercihi de araştırılabilir.

Anahtar kelimeler: Antikonvülsan etki, Elektroensefalografi, Epilepsi, Pentilentetrazol, Ramelteon.

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INTRODUCTION

Epilepsy is a disease caused by the disruption of the brain's stimulatory and inhibitory balance due to functional and structural changes (Scharfman, 2007). There are striking developments in the treatment of epilepsy today. However, there is a significant rate of drug resistance in treatment (Löscher, 2002a; Rao, 2022). Therefore, there is a need to develop anti-epileptic therapies to elucidate the neurobiological mechanisms of epilepsy, suppress the progression of epilepsy and reduce severe drug resistance.

Valproic acid (VPA) is an antiepileptic drug. GABA-A receptors are thought to be responsible for the anti-epileptic effect of VPA. Additionally, VPA increases GABA production, decreases GABA transaminase, and inhibits excitatory neurotransmission (Johannessen, 2003; Löscher, 2002b). VPA limits absence, myoclonic, and tonic-clonic seizures in primary generalized epilepsies (Rogawski, 2004). Studies have also shown that VPA has an anticonvulsive effect on the PTZ-kindled model (Mori, 1992).

Melatonin is a hormone secreted from the pineal gland. RMLT is a specific melatonin agonist as new class of sleep agent (https://go.drugbank.com/drugs/DB00980) and acts like melatonin by binding to MT1 and MT2 melatonin receptors (Zammit, 2007). The anticonvulsant effects of melatonin have been shown in recent studies (Fenoglio-Simeone et al., 2009). In this study involving an experimental mouse model, it was shown that it reduced seizure period and frequency by reversing hippocampal excitability and provided rhythmic improvement in circadian activity. While studies including RMLT, which affects the melatonergic signaling pathway, reveal its relationship with epileptic seizures and cycles, there are still many mechanisms that need to be understood. It is not possible to find a single responsible agent in metabolic diseases such as epilepsy, but interacting molecules and receptor responses will show the relationship between intracellular, extracellular and circulation and will enable us to reach targets that can change the decision processes that govern the brain.

Determining the phenotypic reflections of biochemical changes in the most measurable parameters of the disease as a result of exposure to this agent may help in determining the nature and extent of the effect. Considering the effect of melatonin on the biological clock (insomnia, jet-lag, osteoporosis, cancer, and neurodegenerative diseases), it is not a coincidence that it has a wide spectrum. Because it is a rigid receptor, it is a serious target for specific therapies. RMLT is also the subject of phase studies. It is clinically studied in children with Dravet syndrome. The research report (Report Code: GDHCDR8829LOA-MP) states that it is in the development phase to reduce the frequency of seizures. While studies have focused on targets, it is important

for bioavailability to know which biological outcomes change the results of drug administration. In this model, the anticonvulsant effect of RMLT, a selective receptor agonist, can be evaluated by the improvement of seizures. One measure of this is the analysis of measurements as a result of preventing imbalance in EEG oscillations. Evaluation of cerebral hypoxia with serum markers may explain the processes in recovery.

We aimed to evaluate the melatonin-like anticonvulsant effect of RMLT in the pentylenetetrazol (PTZ) model epilepsy, which is similar to generalized-tonic clonic epilepsy in humans. Thus, the potential of melatonin receptors as targets for new-generation anticonvulsants will be revealed. To our knowledge, there is no study investigating the effect of RMLT on the effectiveness of VPA in the PTZ-kindled model. Therefore, we investigated the combination of VPA with RMLT in PTZ-kindled rats.

MATERIAL AND METHOD

Experimental subjects and ethics

Male Wistar albino rats (200-230 g) (n=36) used in our study were obtained from Bezmialem Vakif University Experimental Animal Production and Research Center. Study permission was obtained from Bezmialem Vakif University Experimental Animals Local Ethics Committee (2011/51). The experimental protocol was created during the planning of this study submitted to the ethics committee and recorded. After obtaining approval, the same protocol was followed until the end of the experiment. The rats used in the study were kept in standard housing cages until the day of the experiment (7 to 7.5 inches). Drinking water was changed daily, and cages were cleaned every other day. The rats were housed in rooms with room temperature between 22-24 °C, ventilation conditions provided, and 12 hours of light and 12 hours of darkness. Rats were fed an ad-libitum standard pellet diet throughout the study and randomly divided into six groups. Each animal cage was labeled with row and group numbers. The cages were placed on the shelves in the room reserved for this study. A daily process chart was created and hung on the table at the room's entrance so that the groups could be applied. Transactions are marked. From the selection of the animals to the end of the experiment, the same researcher handled the animals and used the same surgical gown and cap.

Experimental design and PTZ-kindling model

In the design of the experiment, VPA was used as a positive anticonvulsant. PTZ (P6500, Sigma) and VPA (1069-66-5, Sigma) were dissolved in distilled water (50 mg/ml) and

administered intraperitoneal injection (i.p) (150 mg/kg). RMLT (SML2262, Sigma) was dissolved in DMSO (2 mg/ml) and given intracerebroventricular injection (i.c.v) (30 mg/kg).

Electrodes were placed on the skull of each animal with permanent screws via stereotaxic surgery. Rats were weighed before surgery, and ketamine/xylazine (60 mg/kg-6 mg/kg) was injected i.p. at a dose of 1 ml/kg. Under anesthesia, the hair on the animals' scalps was shaved. Teramycine was applied to prevent dry eyes. The head was fixed to the stereotaxic frame, and the surgical field was cleaned with 70% ethyl alcohol. It was wiped with betadine, and an incision was made on the scalp with a scalpel up to the back of the ear. A 3 cm long incision was performed on the scalp along the rostrocaudal axis, and the scalp was cleaned of fascia and tendons. Then, four holes of 1 mm diameter and 1 mm depth were opened with the help of a drill. Electrodes were placed in these holes according to the coordinates in the rat brain atlas (Paxinos & Watson, 2009): Positive electrode, 4 mm anterior to Bregma, 3 mm left of midline; negative electrode, 4 mm posterior to Bregma, 3 mm left lateral to the midline; ground electrode, 4 mm posterior to Bregma, 3 mm right lateral to the midline; The fourth electrode was placed 4 mm anterior to Bregma and 3 mm right lateral to the midline.

The electrodes placed for EEG recording were connected to a small (3-prong) plug with thin cables. Electrodes were fixed to the skull using dental acrylic. Each animal was placed in a separate cage and waited at least five days for recovery (Sefil, Acar, Bostancı, Bagirici & Kozan, 2015). Animals were connected to the PowerLab (AD Instruments) data acquisition system with the aid of a small plug placed on their heads. The electrical activities in the animals' brains were transferred to the computer environment using Graph 5.1.1. This software is divided into one-second segments. Thus, the number of spikes per minute was automatically calculated.

Animal groups

The first group was described as the sham group (n=6), and the rats were given no medication. All the groups except the control group received 60 mg/kg PTZ i.p. The second group was PTZ (n=6), was given 60 mg/kg PTZ i.p., the third group was DMSO+PTZ (n=6), DMSO i.p was administered 30 min before the PTZ injection, and the fourth group was VPA (150 mg/kg) + PTZ (n=6), VPA i.p. was administered 30 min before the PTZ injection, The fifth group was RMLT (30 mg/kg) + PTZ (n=6), was given Ramelteon i.p. 30 min before the PTZ injection, and the sixth group RMLT (30 mg/kg i.p.) + VPA (150 mg/kg i.p.) + PTZ (n=6), RMLT was administered 60 minutes before the PTZ injection, and valproic acid was administered 30 minutes before.

The EEG was recorded from thalamic region because generalized seizures begin the deep structures of the brain such as the thalamus, which have broad projections to all areas of the cerebral cortex (Blumenfeld, 2012; Wang et al., 2012). Thalamic EEG recordings were performed in rats while awake in a special container. An EEG recording was taken every 30 min (Souza et al., 2013). The EEG signals were amplified 10.000 times and filtered with a range of 1-60 Hz using an AD Instruments Power Lab 8/30 ML870 Data Acquisition and Amplifier System, and the spike percentages were evaluated. The EEG traces were analyzed using the Power Lab Lab Chart.

Seizures were evaluated according to Racine's Convulsion Scale (RCS) and the onset time of the first myoclonic jerk. RCS was used to evaluate the seizures as follows: 0: no convulsion; 1: twitching of the vibrissae and pinnae; 2: motor arrest with more pronounced twitching; 3: motor arrest with generalized myoclonic jerks; 4: tonic-clonic seizure while the animal-maintained posture; 5: tonic-clonic seizure with loss of the righting reflex; 6: lethal seizure. The onset times of the first myoclonic jerk (FMJ) were measured after the PTZ injections. The observation period for PTZ-induced seizures was limited to a duration of 30 min (Kaputlu & Uzbay, 1997). The Racine score was measured for the maximal seizure intensity observed at 30 min EEG traces, Racine's convulsion stages, and the time of onset of the first myoclonic jerk were compared between the groups. The animals were then euthanized.

Biochemical analysis

Tissue Preparation and Oxidative Stress Determination

Brain tissue samples were homogenized (10 times (w/v)) with ice-cold 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 3.000 rpm for 15 min, and supernatant aliquots were separated and used for biochemical experiments of Calcineurin (CaN), Neuropeptide-Y (NPY), Neuron Specific Enolase (NSE), and S100B.

Also, blood samples were taken by the cardiac puncture method. Blood samples were taken into EDTA blood collection tubes and centrifuged at 3.000 rpm for 10 min. Serum samples were separated and stored at -20 C until the analysis of TAS, TOS, catalase, MPO, and thiol.

TAS levels were determined using the fully automatic spectrophotometric method developed by Erel. Results were expressed as mM Trolox equivalents per gram (mmol Trolox equivalent). TOS levels were also determined spectrophotometrically using a method described by Erel (Erel, 2004, 2005). Results were expressed in micromolar hydrogen peroxide equivalents (μmol H_2O_2 equivalent) per liter.

Catalase activity was measured by UV spectrophotometric method, which depends on monitoring the change of 240 nm absorbance at high levels of hydrogen peroxide solution (≥30 mM) (Aebi, 1984).

The MPO assay is based on the kinetic measurement of the absorbance of the yellowish-orange complex at 460 nm wavelength, which results from the oxidation of MPO, and o-dianisidine in the presence of H_2O_2 (Bradley, Priebat, Christensen & Rothstein, 1982).

Total serum thiol concentration was measured by methods originally described by Ellman (Ellman, 1959) and modified by Hu (Hu, 1994). Thiols interact with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to form a highly colored anion with a maximum peak at 412nm.

Tissue NSE levels were performed immunofluorimetric assay (Kryptor®, Brahms and Modular® E170, Roche Diagnostics). S100B protein levels were measured with BioVendor Human S100B ELISA (Enzyme Linked Immunosorbent Assay) kit. Tissue levels of NPY were measured according to the kit instructions using commercially available high-sensitivity ELISA plates (USCN Life Sciences Inc.). Tissue levels of CaN (SEB323Hu, cloudclone corp. Houston, USA) were quantified by using an Enzyme-linked Immunosorbent Assay Kit (ELISA).

RESULTS

EEG evaluation for experimental groups

EEG recordings and time domain are shown in Figure 1.

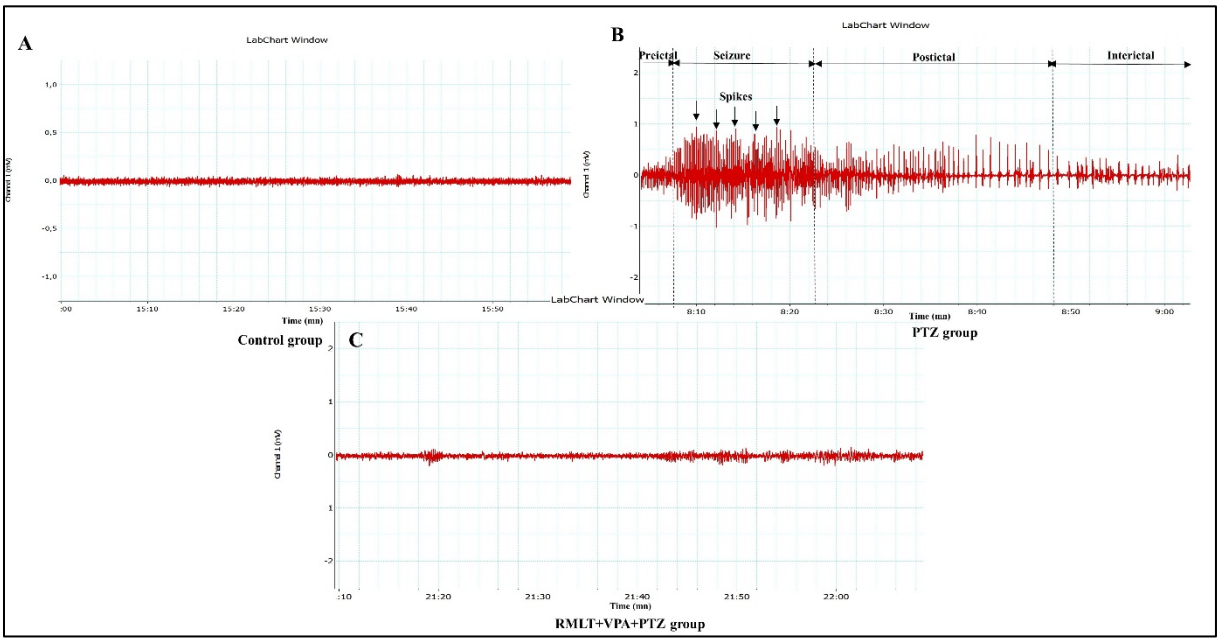

Figure 1. EEG Waves of Rats in Three Groups. Data Represent Groups. **A.** Control Group; Fast Waves of Normal Number and Short Duration, **B.** PTZ Group; Long-Lasting, Frequent, and Fast Waves (Alpha and Beta Waves), **C.** Recovery of Waves After RMLT Treatment.

When differences due to invasive electrode placement between seizures, during and after seizures were excluded, the amplitude was between -1 and 1 mV (0.5 increments) in the control group and between -2 and 2 mV (1 increments) in the PTZ and RMLT+VPA+PTZ groups. While rhythmic spikes are observed in the control group, serious spike waves are observed in the PTZ group. Active waves during the seizure were recorded for approximately 10 minutes. Within approximately 30 minutes after the seizure, the width of the spikes increased and became shorter. During the 10-minute period between seizures, the spikes continued but became shorter. In the RMLT group, normal waves were recovered, and a significant decrease in the number and height of spikes was observed. The results were evaluated as improvement in epileptic seizure.

Seizure scoring analysis

Significant differences were found between the PTZ and RMLT+VPA+PTZ groups in Racine's convulsion stages, the onset of the first myoclonic movements, and the rate of spikes in the EEG traces (P<0.001) (Figure 2). The scores show that seizures, which were high in PTZ, decreased dramatically after RMLT treatment.

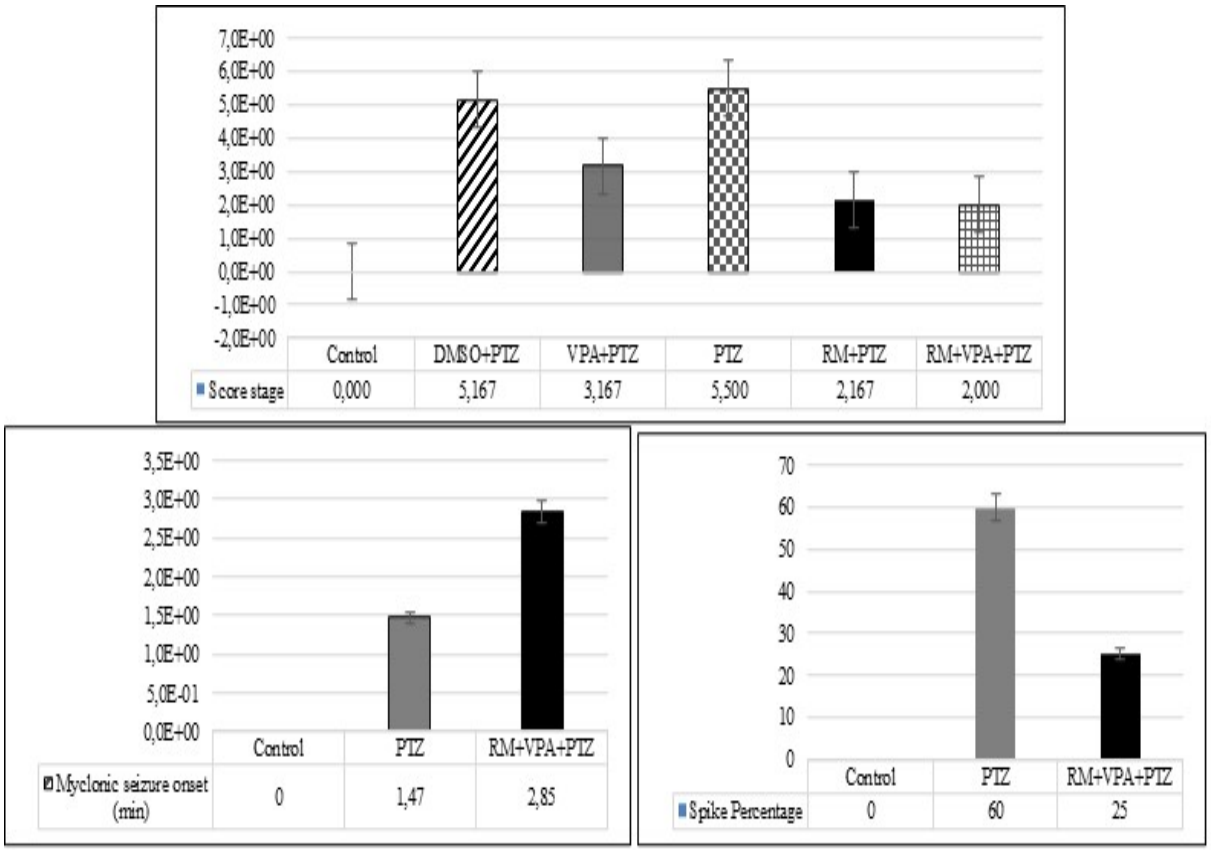

Figure 2. Racine's Convulsion Stages. 0: No Convulsion; 1: Twitching of the Vibrissae and Pinnae; 2: Motor Arrest With More Pronounced Twitching; 3: Motor Arrest With Generalized Myoclonic Jerks; 4: Tonic-Clonic Seizure While the Animal-Maintained Posture; 5: Tonic-Clonic Seizure with Loss of the Righting Reflex; 6: Lethal Seizure. P<0.001

Oxidative Stress Parameters Analysis

In the analysis of five serum parameters (TAS, TOS, catalase, MPO, THIOL), and four brain tissue parameters (CaN, NPY, NSE, and S100B) of all groups, the results were not significant ($P > 0.005$) except S100B ($P<0.005$). Accordingly, the results are expressed respectively (SS; sum of squares, MS; mean square). **TAS**; SS=33.975, df (5,30), MS=6.795, F=1.405, P=0.251, **TOS**; SS=0.14, df (5,30), MS=0.003, F=1.456, P=0.234, **Catalase**; SS=82.698, df (5,30), MS=16.538, F=1.731, P=0.158, **MPO**; SS=360575.915, df (5,30), MS=72115.183, F=1.332, P=0.278, **THIOL**; SS=0.000385, df (5,30), MS=0.000077, F=1.845, P=0.134, **CaN**; SS=0.037, df (5,30), MS=0.007, F=0.811, P=0.551, **NPY**; SS=14382.006, df (5,30), MS=2876.401, F=0.505, P=0.770, **NSE**; SS=3.164, df (5,30), MS=0.633, F=1.144, P=0.359, **S100B**; SS=3.893, df (5,30), MS=0.779, F=2.679, P=0.041 (P<0.005). No difference was detected between groups in post hoc tests for S100B (Table 1).

Table 1. Multiple Comparisons of Experimental Groups for S100B.

Parameter	Groups	Comparisons	MD	SE	95% CI		P-value
S-100B (pg/mL)	PTZ	VPA+PTZ	0.753	0.311	-0.239	1.746	0.327
		SHAM	0.719	0.311	-0.273	1.712	0.419
		RMLT+PTZ	0.376	0.311	-0.617	1.368	1.000
		DMSO+PTZ	0.150	0.311	-0.842	1.142	1.000
		RMLT+VPA+PTZ	0.888	0.311	-0.104	1.881	0.116
	VPA+PTZ	PTZ	-0.753	0.311	-1.745	0.239	0.327
		SHAM	-0.034	0.311	-1.026	0.958	1.000
		RMLT+PTZ	-0.377	0.311	-1.370	0.615	1.000
		DMSO+PTZ	-0.603	0.311	-1.596	0.389	0.931
		RMLT+VPA+PTZ	0.135	0.311	-0.857	1.127	1.000
	SHAM	PTZ	-0.719	0.311	-1.712	0.273	0.419
		VPA+PTZ	0.034	0.311	-0.958	1.027	1.000
		RMLT+PTZ	-0.343	0.311	-1.336	0.649	1.000
		DMSO+PTZ	-0.569	0.311	-1.562	0.423	1.000
		RMLT+VPA+PTZ	0.169	0.311	-0.823	1.162	1.000
	RMLT+PTZ	PTZ	-0.376	0.311	-1.368	0.617	1.000
		VPA+PTZ	0.377	0.311	-0.615	1.370	1.000
		SHAM	0.343	0.311	-0.649	1.336	1.000
		DMSO+PTZ	-0.226	0.311	-1.218	0.767	1.000
		RMLT+VPA+PTZ	0.512	0.311	-0.480	1.505	1.000
	DMSO+PTZ	PTZ	-0.150	0.311	-1.142	0.842	1.000
		VPA+PTZ	0.603	0.311	-0.389	1.596	0.931
		SHAM	0.569	0.311	-0.423	1.562	1.000
		RMLT+PTZ	0.226	0.311	-0.767	1.218	1.000
		RMLT+VPA+PTZ	0.738	0.311	-0.254	1.731	0.364
	RMLT+VPA+PTZ	PTZ	-0.888	0.311	-1.881	0.104	0.116
		VPA+PTZ	-0.135	0.311	-1.127	0.857	1.000
		SHAM	-0.169	0.311	-1.162	0.823	1.000
		RMLT+PTZ	-0.512	0.311	-1.505	0.480	1.000
		DMSO+PTZ	-0.738	0.311	-1.731	0.254	0.364

MD; mean differences, SE; standard error, CI; confidence interval

DISCUSSION

Ramelteon (RMLT), a selective melatonin agonist, acts on melatonin receptors. It plays a role in regulating the sleep-wake cycle as a chemical messenger in the brain. Its potential therapeutic use as it targets melatonin receptors in sleep disorders suggests the role of melatonin in epilepsy. Therefore, it raises the possibility that RMLT could be a powerful treatment source for epilepsy. The detection of the seizure-regulating effect of melatonin in epilepsy supports this idea (Khan, Khurana, Vyas & Vohora, 2021). When looking at the research, there is no comprehensive study linking the parameters included in this study with melatonin treatment, and it is the first evaluation to investigate the effect of RMLT.

In addition, in a study conducted on rats, melatonin treatment in the universe-induced epilepsy model increased the delay in the occurrence of spontaneous recurrent seizures and improved conditions such as hyperactivity, light-phase depression-like behavior, and hippocampal memory deficit. In the model, the decrease in neuronal damage and the increase in serotonin levels under the influence of melatonin showed that it could be an effective treatment (Tchekalarova et al., 2013).

The relationship between sleep and epilepsy may be bidirectional. While epilepsy predisposes to the development of sleep disorders, sleep deprivation can also change the existing epilepsy in terms of seizure quality and frequency. In short, while treating the seizure and treating the sleep, the seizure can get out of control. In a study conducted to solve this situation with melatonin, its treatment potential in seizure frequency, EEG and sleep was demonstrated (Dell'Isola et al., 2023). The improvement in sleep quality and t2 EEG after treatment revealed the effect of melatonin. It has been shown that EEG traces returned to normal in 3 out of 21 children after melatonin treatment in refractory epilepsy patients (Millichap, 2010).

Epilepsy models are important for research markers. The advantage of this study is that the PTZ kindling model allows the analysis of oxidative biomarkers because it induces oxidative stress, as in epilepsy. Since the oxidant-antioxidant nature of treatment agents regulates many processes in diseases, it is very important to determine these qualities in their use for therapeutic purposes. The changes observed after convulsions caused by PTZ are important for investigating both the whole brain and regional areas. Therefore, in this model, it will be possible to determine the effectiveness of RMLT treatment by monitoring pre-ictal and post-ictal EEG changes. EEG traces and frequency, which are one of the indicators of epileptic seizures, are frequently used in the monitoring of seizure changes and management of treatment

processes. The studies offer a new approach to providing a bridge between EEG and clinic, with the seizure severity score guiding treatment (Pattnaik et al., 2023). However, it is also expressed that Racine scores cannot predict EEG events in a kainic acid mouse model (Bergstrom et al., 2013). Although the score of 2 in the RMLT group in our study and the decrease in spikes in the EEG recordings of the group indicate that there may be a relationship between them, more comprehensive data are needed for definitive results. Since EEG measurements have different standards, especially in animal models, depending on the chemicals used and the animal species, they should be evaluated in detail. However, the results show that RMLT has a clear effect on the discharges subject to EEG.

The striking improving effect of RMLT on EEG, like melatonin, shows that it is effective from the seizure pre-ictal period until the end of the post-ictal period. When the reflection of the improvement in EEG on biochemical markers is examined, S100B shows borderline significance. As a matter of fact, when this significance is investigated with further analysis, the lack of a relationship between the groups. Meta-analysis studies show that the significant increase in peripheral blood levels of S-100B is associated with epilepsy (Liang et al., 2019). The fact that S-100B is high in our epilepsy model and that it decreases under the VPA effect, while it is observed at the same level with PTZ in the RMLT effect, shows that it has no effect. On the other hand, in a study conducted in children, an irregular distribution was detected in S-100B measurements made within the first 6 hours (Bai et al., 2018). Serum S-100B is a sensitivity index in the evaluation of epileptic nerve damage. Our findings show that serum S-100B value may be important in the treatment of RMLT. It is possible that S-100B delayed its reflection in the brain tissue because the process of seizure formation in PTZ modeling occurs differently between animals.

Studies investigating the effects of antiepileptic drugs on serum thiol-disulfide show that these drugs change the thiol level in patients with epilepsy (Kösem et al., 2021). In a study investigating the effect of VPA, used as an antiepileptic in epilepsy, on serum thiol-disulfide change, it was found that thiol levels decreased in the treatment group (Arhan et al., 2019). Thiol is an important antioxidant barrier and a marker of oxidative stress in the thiol/disulfide balance (Altıparmak et al., 2016). For thiol, disruption of cellular dynamics is observed as an outcome of oxidative stress-induced disease processes. The significance of this marker in epilepsy patients could not be achieved in our model. Contrary to the literature data, there was no significant difference between groups in our model for thiol levels affected by VPA treatment. The fact that RMLT treatment does not change thiol levels indicates that the RMLT effect cannot be expressed clinically with this marker. On the other hand, EEG data of RMLT

show that it can improve seizures. The metabolic model created is shorter and less effective compared to human epilepsies. Careful regimes of discontinuation of antiepileptics are partial to avoid sudden overload of electrical charges. The metabolic model created is shorter and less effective compared to human epilepsies. Careful regimes of discontinuation of antiepileptics are partial to avoid sudden overload of electrical charges. The measurements made here may not be at a level that would affect thiol levels, considering individual metabolic differences after discontinuation of the drug. Extending the deadlines may be more beneficial in terms of the accuracy of the results.

Catalase is an important antioxidant enzyme as a clinical biomarker in the conversion of hydrogen peroxide to water and molecular oxygen. It is also used for treatment of diabetic retinopathy and heart diseases (Mahomoodally & MA-L, 2022). The status of catalase in epileptic patients treated with VPA may reveal the effect of RMLT more clearly. A study conducted in epileptic children shows a decrease in catalase levels after VPA treatment (Beltrán-Sarmiento et al., 2018). No effect of VPA was observed in our findings. There was also no effect of RMLT. No effect of VPA was observed in our findings. There was also no effect of RMLT. In this case, we can say that RMLT, even though it is a melatonin agonist, does not have a metabolic action that would inhibit catalase, an enzyme important in oxidative processes. Melatonin and RMLT target receptors in the suprachiasmatic nucleus (SCN), which orchestrates the biological rhythm. According to the chemical structure of these two molecules, the S-configuration and either group in the chemical structure of RMLT give it affinity for MT1/MT2 receptors (Miyamoto, 2009). Additionally, RMLT responds more strongly than melatonin when it comes to sleep. According to our findings, this does not appear to be the case in epilepsy. However, due to the melatonin secretion rhythm of the SCN (Liu, Ding & Wang, 2022), it may have a significant corrective effect on EEG in epilepsy.

It is thought that MPO plays its role in the progression of epileptic seizures by taking part in the disruption of the blood-brain barrier through impaired matrix metalloproteinase (Zhang et al., 2016). Experimental studies point out an increase in the level of hippocampal MPO in mice with temporal epilepsy. Moreover, the inactivation of MPO resulted in a decrease in seizure severity. VPA treatment of MPO may help predict RMLT. In a study investigating the effect of VPA on MPO levels in epileptic children, plasma levels were found to be high (Zhang et al., 2011). Regarding melatonin, data regarding post-treatment MPO levels are not clear. In addition to the known effect of VPA, melatonin treatment reduces MPO levels in hypertension, indicating that RMLT does not work in the same direction. The idea that hypertension may have

a role in epilepsy indirectly indicates that RMLT may be effective in seizures by reducing MPO levels, as in our treatment groups.

Calcineurin (CaN) has important functions in the biological system, such as cell cycle control, T cell activation, heart and muscle functions, learning, and mechanisms such as apoptosis, and is intensely expressed in the brain (Shibasaki, Hallin & Uchino, 2002). This importance of calcineurin has made it a target in epilepsy. Disrupted hippocampal actin cytoskeleton in status epilepticus results in primordial germ cell death. Studies show that various CaN inhibitors can improve actin depolymerization (Wen et al., 2017; Xiong et al., 2018). It is a fact that CaN-mediated events occur more in the epileptic brain. Additionally, evidence of its association with seizures confirms the accuracy of the CaN data we obtained. Although not statistically significant, the decrease in increased levels in epilepsy with RMLT treatment indicates that it may have an effect. The role of doses in testing the treatment effect in drug research is undeniable. At this point, increasing the doses and application times in our study may be effective for the significance of both CaN and other oxidative parameters.

Serum reflections of biomarkers, as messengers of the system, are related to seizure stimuli as well as duration. Behaviors at the same time or at different times, together or alone, are a major factor in the characteristics of epilepsy. In this mechanism, the biggest stimulus is glutamate. The remarkable neuropeptide array differentiation of interneurons in this system highlights the role of NPY (Colmers & Bahh, 2003). NPY is a modulatory neuropeptide that has the capacity to stimulate many neuronal and metabolic cell functions. NPY has an effect in many different tissues thanks to a wide range of receptors. It is stated that Y2 and Y5 receptors are responsible for the anticonvulsant effect of NPY in epilepsy. Inhibiting NPY signaling with viral treatments provides an anticonvulsant effect in animal models of epilepsy. Thanks to these properties, it is presented as a powerful therapeutic target. Excessive activation of NPY is observed in epileptic seizure models (Drexel & Sperk, 2022). The results we obtained in our model show that there is an increase in the PTZ effect in the RMLT group in response to the expression of NPY. Experimental studies investigating the melatonin-NPY mechanism have shown that NPY increases melatonin production (Vacas, Sarmiento, Pereyra, Etchegoyen & Cardinali, 1987). Studies of NPY-defective mice show that seizure activity does not terminate in these animals (Baraban, 2004). Our results indicate that both the high EEG activities under the PTZ effect and the decrease in NPY levels compared to other groups, but the increase in these levels after RMLT treatment, indicate the RMLT effect through NPY and its relationship with the improvement in EEG data.

Neuron specific enolase (NSE) is cell specific and enolase gamma is neuron specific. Its presence in the late stages of neuronal differentiation shows that it is effective in neuronal differentiation and has the potential to be an index (Isgrò, Bottoni & Scatena, 2015). NSE increases following neuronal damage in the epileptic brain and is suggested as a marker of damage. The strongest evidence is the increase in NSE levels obtained in analyzes of the cerebrospinal fluid of status epilepticus patients (Herman, 2006). Persistence of these levels throughout the disease can lead to permanent changes. In our experimental group, the decrease in NSE, which was high in PTZ, with RMLT, although not significant, indicates its effect on the epileptic brain. In a study, the potential of NSE as a biomarker was investigated by determining the status of NSE in seizures. NSE was found to be significantly high in serum samples taken during the seizure (Shaik, Reddy, Mohammed, Tandra & KSS, 2019). In another study, the effect of melatonin application on seizures and NSE levels was investigated. In another study, the effect of melatonin application on seizures and NSE levels was investigated. The decrease in the frequency and duration of seizures with the decrease in serum NSE levels was remarkable (Verma et al., 2021). These results provide support for functional RMLT on the same target as melatonin.

The corrective effect of RMLT on oxidative metabolism has been demonstrated in various studies. Methotrexate has been shown to damage the cerebral cortex through oxidative stress, apoptosis/autophagy, and inflammation. RMLT inhibits these mechanisms (Aslankoc et al., 2022). This situation can be explained by the fact that methotrexate seriously disrupts the oxidative balance. The failure to observe the antioxidant feature of RMLT may be due to the active antioxidant nature of VPA (Terzioglu Bebitoglu & Gokce, 2020) . Therefore, its effects on EEG traces could not be observed.

CONCLUSION

The lack of a monotherapy approach in epilepsy and the contribution of adverse drug effects and comorbidities make treatment difficult. Additional treatments to support antiepileptics may constitute an alternative that can be decided in combination with EEG. In this process, RMLT is at least as strong a candidate as melatonin. Although there are no strong aspects in the correlation with brain and serum parameters in the analyzes performed here, these parameters need to be investigated more deeply and with more groups in measuring RMLT treatment. The improvement of EEG spikes and the disappearance of discharges show the RMLT effect.

Limitations

A limitation of this study is that the doses and application times were evaluated in a wide range.

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Conflict Of Interest

There is no conflict of interest.

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