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RESEARCH

The potential neuroprotection efficacy of Atractylenolide III on kainic-acid derived temporal lobe epilepsy in male rats

Atractylenolide III'ün erkek sıçanlarda kainik asit kaynaklı temporal lob epilepsisi üzerindeki potansiyel nörokoruyucu etkinliği

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Abstract

Purpose: Atractylenolide III (ATR III) is known for its anti-inflammatory and neuroprotective activities. In this study, we aimed to investigate the effects of ATR III on neuronal damage in temporal epileptic rats caused by kainic acid.

Materials and Methods: 16-week-old Wistar Albino rats were divided into three groups; control (C, n=8), kainic acid (KA, n=8), ATR III+ kainic acid (KA+ATR, n=8). After 21 days of injections of kainic acid, learning, and memory behavior, anxiety, and locomotor activity were evaluated. Neuron morphologies in the hippocampus were examined, the total number of neurons, and the number of degenerated neurons were determined, and the thickness of these regions was also measured. Changes in biochemical parameters such as MDA, SOD, GSHPx, AChE, and CAT were investigated to detect oxidative stress in the brain.

Results: There was no significant difference in the learning and memory function and locomotor activity. However, KA increased anxiety behavior without any effect of ATR III (closed arm duration, 244.90±25.17). There was no degeneration in the neurons of the control group. In the KA group, there was an increase in the number of degenerated neurons. In this group, the thicknesses in CA1, CA3, and DG regions were 37.39±1.90, 45.64±6.26 and 46.02±5.72 μm, respectively. In the ATR III+KA group, there were fewer degenerated neurons, less thinning of the hippocampus, and a higher number of normal neurons compared to the KA group. In this group, CA1, CA3, and DG thicknesses were calculated as 36.05±4.13, 47.09±7.09 and 43.07±5.91 μm, respectively.

Öz

Amaç: Atractilenolide III'ün (ATR III) antiinflamatuar ve nöroprotektif aktiviteleri olduğu bilinmektedir. Bu çalışmada ATR III' ün temporal epileptik sıçanlarda kainik asidin neden olduğu nöronal hasar üzerine etkilerini araştırmayı amaçladık.

Gereç ve Yöntem: 16 haftalık Wistar Albino sıçanları; kontrol (C, n=8), kainik asit (KA, n=8), ATR III+ kainik asit (KA+ATR, n=8) olacak şekilde üç gruba ayrıldı. 21 günlük kainik asit enjeksiyonundan sonra öğrenme ve hafıza davranışı, anksiyete ve lokomotor aktivite değerlendirildi. Hipokampustaki nöron morfolojileri incelendi, toplam nöron sayısı, dejenere nöron sayısı belirlendi ve bu bölgelerin kalınlıkları da ölçüldü. Beyindeki oksidatif stresi tespit etmek amacıyla MDA, SOD, GSHPx, AChE ve CAT gibi biyokimyasal parametrelerdeki değişiklikler araştırıldı.

Bulgular: Öğrenme ve hafıza fonksiyonu ile lokomotor aktivitede anlamlı bir fark yoktu. Ancak KA, ATR III' ün herhangi bir etkisi olmadan kaygı davranışını artırdı (Kapalı kolda geçirilen süre, 244.90±25.17). Kontrol grubunun nöronlarında herhangi bir dejenerasyon görülmedi. KA grubunda dejenere nöron sayısında artış görüldü. Bu grupta CA1, CA3 ve DG bölgelerindeki kalınlıklar sırasıyla 37,39±1,90, 45,64±6,26 ve 46,02±5,72 μm idi. ATR III+kainik asit grubunda, KA grubuna kıyasla daha az dejenere nöron, hipokampusta daha az incelme ve daha fazla sayıda normal nöron vardı. Bu grupta CA1, CA3 ve DG kalınlıkları sırasıyla 36,05±4,13, 47,09±7,09 ve 43,07±5,91 μm olarak hesaplandı.

Sonuç: Bu bulgular ATR III'ün temporal epilepside nöronal hasarı azaltmak için terapötik bir ajan olarak bir potansiyele sahip olduğunu göstermektedir. Altta yatan mekanizmaları araştırmak ve ATR III'ün epilepsi

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Conclusion: These findings suggest that ATR III may have the potential as a therapeutic agent for reducing neuronal damage in temporal epilepsy. Further research is warranted to explore the underlying mechanisms and evaluate the clinical implications of ATR III in the treatment of epilepsy.
Keywords: Hippocampus, epilepsy, atractylenoloide iii, kainic acid, oxidative stress.

tedavisindeki klinik etkilerini değerlendirmek için daha fazla sayıda araştırmaya ihtiyaç duyulmaktadır.

Anahtar kelimeler: Hipokampus, epilepsi, atractylenoloide iii, kainik asit, oksidatif stres.

INTRODUCTION

Epilepsy is a neurological condition associated with cognitive impairments such as memory, attention, and executive function deficits, but still the impact on cognitive and behavioral comorbidities mechanism debate[1]. According to the meta-analysis of Fiest et al., the lifetime prevalence of epilepsy worldwide is 7.6 per 1000 population[2]. Temporal lobe epilepsies constitute the most common and drug-resistant focal epilepsy group in adults[2,3]. Considering the location of the hippocampus in the temporal region, learning and memory are the leading cognitive functions most affected by temporal lobe epilepsy. For instance, Aldenkamp and collegues found that patients with epilepsy and subtle seizures have lower cognitive test scores than controls[4]. Since epilepsy is a common and important neurological disease with many comorbidities and cognitive problems that reduce the patient's quality of life, it becomes necessary to introduce new therapeutic agents[5]. Most of the antiepileptic drugs that are currently used in the treatment of epilepsy can provide symptomatic relief and cannot cure epileptogenesis[6].

Even if there are suitable drugs for epilepsy patients, side effects such as memory loss, dizziness, and headaches occur with the treatment process. Faced with such limitations, researchers began to seek natural remedies to treat or alleviate the symptoms of epilepsy[7]. Traditional Chinese medicine (TCM) has been used extensively for the treatment of epilepsy for more than 2000 years[8]. With the development of phytochemistry, more anticonvulsant compounds will be isolated from plants for use in the treatment of epilepsy[9]. One of the TCM drugs -Atractylenolide III- is defined as a sesquiterpenoid compound obtained from the rhizomes of A. macrocephala and well established with its anti-inflammatory, anticancer, and neuroprotective potential and can be used as a possible therapeutic agent10. ATR-III exhibits several pharmacological properties including anti-inflammatory, anticancer activity, and neuroprotective effects[10]. For instance, Ji and colleagues reported the anti-inflammatory effect of ATR-III on mouse macrophages[11]. In addition, a neuroprotective effect was observed in rats with learning and memory impairments through inhibition of ATR-III, ROS production, and protein kinase C levels[12]. In the study of Izumi, ATR-III administration significantly restored the impaired memory function via Ca2+/calmodulin-dependent protein kinase II (CaMKII) autophosphorylation[13]. In Liu's study, atractylenolide III exhibited a significant neuroprotective effect against glutamate-induced neuronal apoptosis[14].

In addition to their cognitive effects, the impacts of ATR-III on anxiety are also being investigated in the literature[10,15]. Some preclinical studies have shown that Atractylenolide III promising anxiolytic properties, meaning it has the potential to reduce anxiety symptoms. For instance, Zhou showed Atractylenolide III produces antidepressant- and anxiolytic-like effects in rats. Moreover, they also stated that these effects appear to be mediated by inhibition of hippocampal neuronal inflammation[15]. In another study, that conducted with corticosterone-injured rat phaeochromocytoma (PC12) cells, it was indicated that Atractylenolide III may serve as a therapeutic agent in the treatment of depression[10]. Remarkably, there has been a notable absence of research exploring the connection between the epilepsy model, anxiety, and ATR-III, leaving a captivating void in our comprehension of their intricate relationship.

This study hypothesizes that ATR III will mitigate the cognitive dysfunctions induced by temporal lobe epilepsy (TLE) in a well-characterized animal model. The specific mechanism of action of ATR III is not addressed in the literature and animal studies are insufficient in epilepsy models. It addresses the specific mechanism of action of ATR III, which has not been explored in previous studies, and evaluates the potential side effects of combining ATR III with other drugs, thus providing valuable insights into its medicinal value and safety profile. In conclusion,

atractylenolides possess significant medicinal properties, particularly in the areas of cognitive function, and anxiety-related outcomes.

MATERIALS AND METHODS

Experimental animals

Experimental procedures were performed following the Mersin University of Health Guide for Care and Use of Laboratory Animals and were approved by the University of Mersin Institutional Animal Care and Use Committee (approval no. 2019/11, dated 07. 01/2019). We used 24, 16-week-adult male Wistar Albino rats (250-300g).

The rats were housed in plastic cages (8/per cage, 20x35x45 cm). Animals were housed in a room (22-25 ^{o}C) with a 12-h light/dark cycle (05:00–17:00 light, 17:00– 05:00 dark). Rats had free access to food and water. They were handled during cage cleaning. All cages were subjected to weekly cage cleaning during an experiment.

Experimental design

Experiments were executed with three groups; control (C), 4 mg/kg kainic acid (KA), 30 mg/kg atractylenolides + 4 mg/kg kainic acid (KA+ATR). 4 mg/kg kainic acid (Sigma) that dissolved in 0.9% NaCl solution was injected by the intraperitoneal administration as a single dose. Convulsive phases were observed 4-6 hours after the injection of KA. The experimental animals in the control group were injected with 10 mg/kg saline (0.9% NaCl) at one-week intervals. Experimental animals in the ATR III + KA group were injected with ATR III (Sigma) dissolved in 0.9% NaCl solution and injected one week apart (10 mg/kg) for a total of 3 doses. Rats were injected once with kainic acid (4 mg/kg) 30 minutes after the first ATR III administration.

After the injection, we observed mental symptoms such as absent-mindedness, staring, and stagnation; visceral with masticatory movement, chewing, increased salivation, oral automatism; extremity automatism, myoclonic jerk, shaking, tonic-clonic movement, somatomotor seizures ending with status epilepticus for 21 days. The frequency and duration of seizures were evaluated by considering the seizure behavior scale according to their clinical characteristics.

Behavioral tests

Testing anxiety level using the elevated plus-maze test

The elevated plus maze test (EPMT) is used extensively in the discovery of novel anxiolytic agents and is considered to be the gold standard in the development of novel anxiolytic agents[16]. The elevated plus-maze apparatus was a black opaque plexiglass labyrinth that was 40 cm above the ground and consisted of four arms (two were closed and two were open). The number of entries to the open or closed arm (frequency) and the duration on both arms during the 5 min were measured with the Noldus Ethovision XT recording system[17]. At the end of the test, the animal was returned to the home cage, and the maze was thoroughly cleaned with 20 % ethanol.

Testing locomotor activity using the open-field test

The open field test (OFT) was carried out with the apparatus in the behavioral physiology laboratory of the department of physiology. The open-field apparatus (100×100×40 cm) was a black opaque plexiglass open area that was divided into the central zone and the peripheral zone using the software (Noldus Ethovision XT). Each rat was left to the apparatus to explore the area for 10 minutes. The total distance moved (cm), entries to the center, and duration in the center were measured with the Noldus Ethovision XT recording system. At the end of the test, the animal was returned to the home cage, and the maze was cleaned with 20% ethanol.

Testing spatial learning and memory using the Morris water maze test

To evaluate the learning and memory performances of rats with the Morris water maze test (MWMT), we used a 150 cm diameter, 60 cm deep, stainless steel, temperature-controlled water tank in the behavioral laboratory of the physiology department. The tank was filled with water at a temperature of 50 cm and 22 ± 1°C. Visual cues consisting of various geometric shapes were placed around the pool to show three different directions. The labyrinth was divided into four quadrants with the Noldus ethovision tracking system. The experiments were carried out every day between 09:00 and 13:00 for 5 days.

We used learning and memory tests based on the method defined by Morris[18]. For spatial learning, rats were trained for 5 trials per day for 4 days (learning

trials) and allowed to search the tank for 60 s. When they found the platform, they were allowed to stay on it for 10 seconds. The time to find the platform (sec), the total distance moved (cm) and swimming speed (cm/sec) of the rats during 4 days were recorded with the Noldus Ethovision XT recording system. On the 5th day, which is the last day of the experiment (memory test), the platform was removed and a single shot was made from the north direction and the recording was 60 seconds. At the end of this period, the average time spent by the rats in the southwest quadrant, where the platform was previously located, was recorded[17].

Histological analysis

The right brain hemispheres were taken for light microscopic examinations, placed in a 4% buffered neutral formaldehyde solution, and left for 48 hours. After the tissues were fixed, they were washed with running tap water and then followed according to the tissue tracking protocol[18]. Sections stained with hematoxylin-eosin were examined with a light microscope (Olympus BX50 Olympus GmbH, Germany) and images were taken with a digital camera integrated into the same microscope (Olympus LC30 Olympus Soft Imaging Solutions GmbH, Germany).

In six sections from different levels of each subject, the Cornu Ammonis 1 (CA1), Cornu ammonia 3 (CA3), and Gyrus Dentatus (DG; Gyrus Dentatus Granular Layer, DG-GL and Gyrus Dentatus Subgranular Layer, DG-SGL) regions of the hippocampus were examined. For this aim, the following evaluations have been made: Total number of neurons (TNN) and number of degenerated neurons (DNN) were determined in an area of 10000 μm2. Moreover, the thickness of the neuron layers was measured in CA1, CA3, and DG. In each region, the average of the thickness measured in 3 different areas was calculated (Figure 2).

Immunohistochemistry

The expression density of GRP 78 protein, one of the endoplasmic reticulum stress markers, in hippocampal neurons, was demonstrated by immunohistochemical staining. For immunohistochemistry, sections taken on adhesive slides were deparaffinized and stained according to the following immunohistochemical staining protocol[19,20]. The sections were incubated in antigen retrieval solution (Tris-EDTA,100X, Abcam, Catalog no: ab93684) then endogenous peroxidase activity was blocked with 3% H2O2. After blocking (Abcam, Catalog no: ab93697), sections incubated with rabbit polyclonal anti-GRP 78 primary antibody (1:100, BosterBio, Catalog no: PB9640) in phosphate buffered saline-bovine serum albumin (PBS-BSA) (containing 0.1% Triton X-100) overnight at 4 °C. Negative control sections were incubated with a PBS-BSA instead of the primary antibody. The next day, sections were treated with biotinylated goat anti-rabbit secondary antibody (1:200, Invitrogen, Catalog no: 65-6140). After the streptavidin peroxidase solution, a 3,3- diaminobenzidine substrate kit (Abcam, Catalog no: ab94665) was used to detect enzymatic activity. Lastly, sections were counterstained with hematoxylin (Merck, Catolog no:109252). After the immunohistochemical staining process, images of the areas examined in the sections were taken. In sections taken from different levels of each subject; GRP 78 protein expression of all neurons in the 10000 μm2 area in CA1, CA3, and DG (DG-GL and DG-SGL) regions of the hippocampus was determined as "mean pixel density" by ImageJ (National Institutes of Health-NIH, USA) image analysis program[21].

Transmission electron microscopy

For the electron microscopic examinations, the hippocampus region of the isolated right brain hemispheres was cut in a size of 1 mm3 and placed in 2.5% glutaraldehyde solution, and incubated at 4°C for 4-6 hours. The fixed tissues were washed 2-3 times with PBS and followed according to the tissue processing protocol. Ultrathin sections (70 nm thick) taken from the obtained resin blocks via ultramicrotome (Leica UCT125, Leica GmbH, Germany) were contrasted with uranyl acetate and lead citrate solutions. The contrasted sections were examined with an electron microscope (JEOL JEM1011, JEOL Corp., Tokyo, Japan) and images were taken with a digital camera (Megaview III, Olympus GmbH, Germany) integrated into the same microscope.

Biochemical analysis

Lipid peroxidation measurement

Malondialdehyde (MDA), a lipid peroxidation product was measured using commercially available TBARS kits (Cayman) according to the manufacturer's instructions. The method is based on the principle of spectrophotometric measurement of

the pink color formed during the reaction of MDA and thiobarbituric.

Glutathione peroxidase

Glutathione peroxidase (GSHPx) enzyme activity was measured using a commercially available GSHPx test kit(Cayman) according to the manufacturer's instructions. The method is based on the principle of measuring NADPH formed during the conversion of reduced glutathione to oxidized glutathione at a wavelength of 340 nm.

Superoxide dismutase

Superoxide dismutase (SOD) activity was measured using a commercially available SOD kit (Cayman) according to the manufacturer's instructions. Measurement of SOD activity is based on the principle of determining the superoxide radical produced by xanthine oxidase and hypoxanthine. The radicals formed form the red-colored formazone dye with the tetrazolium salt. SOD activity is measured by inhibition of this reaction. The 50% inhibition produced by the enzyme corresponds to one unit of enzyme activity.

Catalase

Catalase activity (CAT) was measured using a commercially available CAT kit (Cayman) according to the manufacturer's instructions. Catalase breaks down hydrogen peroxide (H2O2) into water and molecular oxygen. The experiment is based on the principle that catalase reacts with methanol in the presence of H2O2.

Acetylcholinesterase activity

Acetylcholinesterase activity (AChE) was measured using a commercially available AChE kit (Cayman) according to the manufacturer's instructions. Acetylcholinesterase is an enzyme that catalyzes the breakdown of acetylthiocholine with thiocholine to acetate. AChE activity is determined by measuring the intensity of the yellow color given by 5-thio-2-nitrobenzoic acid, which is formed as a result of the reaction between thiocholine and DTNB, in a spectrophotometer at a wavelength of 412 nm.

Statistical analysis

As a result of the power analysis, type 1 error 0.05, power of the test 80% and effect size 72% were obtained. It was decided that the number of subjects should be 6. However, considering the possibility of ex in the groups, the number of animals should be increased by 20%. Thus, a total of 21 rats, 7 for each experimental group, were used. Shapiro Wilk and Kolmogrov Smirnov ($p > 0.05$) test examined conformity for normal distribution in each group. The homogeneity of variance was confirmed with the Levene test. The data are expressed as mean values ± SD. For normally distributed data, a one-way analysis of variance (ANOVA) was conducted for the probe trial of the MWMT, open field and elevated plus maze test, and biochemical test, followed by LSD post hoc comparisons (significance determined as $P<0.05$). Within-subject measurements, such as escape latencies, distance move, and velocity across trials (1., 2., 3., 4., days) in the MWM, were analyzed using repeated-measures ANOVA. For each day in water maze testing, to test the difference between groups for variables (distance move, latency, and velocity), a one-way analysis of variance (ANOVA) was conducted followed by LSD post hoc comparisons.

RESULTS

In the KA and ATR+KA groups, the open arm duration was decreased compared to the control although these differences were not significant ($P>0.05$). Open arm frequency increased in the KA group compared to the other two groups and in the ATR+KA group compared to the control group, but these increases were not significant ($P>0.05$). In KA and ATR+KA groups, closed arm duration increased significantly compared to control ($P<0.05$, Table 1). Closed arm frequency in the ATR+KA group was increased significantly compared to the control group ($P<0.05$, Table 1).

While a decrease was observed in the center duration and frequency in the KA group compared to the control group, and an increase in the ATR+KA group compared to the other groups, but these differences were not significant ($P>0.05$, Table 2). Total distance move increased in the KA and ATR+KA groups compared to the control, and in the ATR+KA group compared to the KA group, but these increases were not significant ($P>0.05$).

On the 1st and 2nd days, the latency in the KA group increased compared to the control and decreased on the 3rd and 4th days, but these differences were not significant ($P>0.05$, Figure 1B). In the KA+ATR group, the latency on the 1st, 2nd, and 4th days decreased compared to the other two groups and increased on the 3rd day compared to the other two

groups, but all these differences were not significant (P>0.05). The swimming speed was decreased significantly in the 2nd and 4th days compared to the 1st day for all groups (P<0.05, Figure 1A). During the 4 days, the swimming speed in the KA and KA+ATR groups increased compared to the control groups, but it was not significant (P>0.05). In the C, KA, and KA+ATR groups, total distance move on the 2nd, 3rd, and 4th days decreased significantly compared to the 1st day (P<0.05, Figure 1C). On the 1st and 2nd days, the total distance move in the KA group increased compared to the control and decreased in the KA+ATR group compared to the other two groups, but these differences were not significant (P>0.05). On the 3rd day, the total distance in the KA group decreased compared to the control and increased in the KA+ATR group compared to the other groups, but also these differences were not significant (P>0.05). On the 4th day, the total distance in the KA group decreased compared to the control and decreased in the KA+ATR group compared to the other groups, but these decreases were not significant (P>0.05). During the memory performance on the last day of MWMT, there was no significant difference in target quadrant duration between groups (p>0.05, Figure 1D).

Table 1. Elevated plus maze parameters.

Groups	Open arm duration*	Open arm entries*	Closed arm duration*	Closed arm entries*
Control	49.20±34.41 [a]	8.63±6.48 [a]	161.50±106.35 [a]	10.13±5.28 [a]
Kainic Acid	23.38±20.22 [a]	11.00±2.93 [a]	244.90±25.17 [b]	17.13±6.47 [a]
Kainic Acid + ATR	32.93±28.35 [a]	9.38±6.48 [a]	244,38±23.66 [b]	21.38±5.13 [b]

Data was given as mean ± standard deviation. *: a and b are used to distinguish between groups. There is a difference of p<0.05 between the data indicated with different letters. ATR: Atractylenolide III

Table 2. Open field test parameters

Groups	Total distance move *	Duration in the center *	Entries to the center*
Control	1809.31±572.84 [a]	52.58±37.11 [a]	7.63±4.37 [a]
Kainic Acid	2163.18±252.69 [a]	24.48±16.18 [a]	6.38±3.96 [a]
Kainik Acid + ATR	2381.45±634.21 [a]	60.68±41.49 [a]	7.88±3.72 [a]

Data was given as mean ± standard deviation. *:a is used to indicate the distinction between groups.
There is no distinction at the p<0.05 level between the data shown with the same letters. ATR: Atractylenolide III

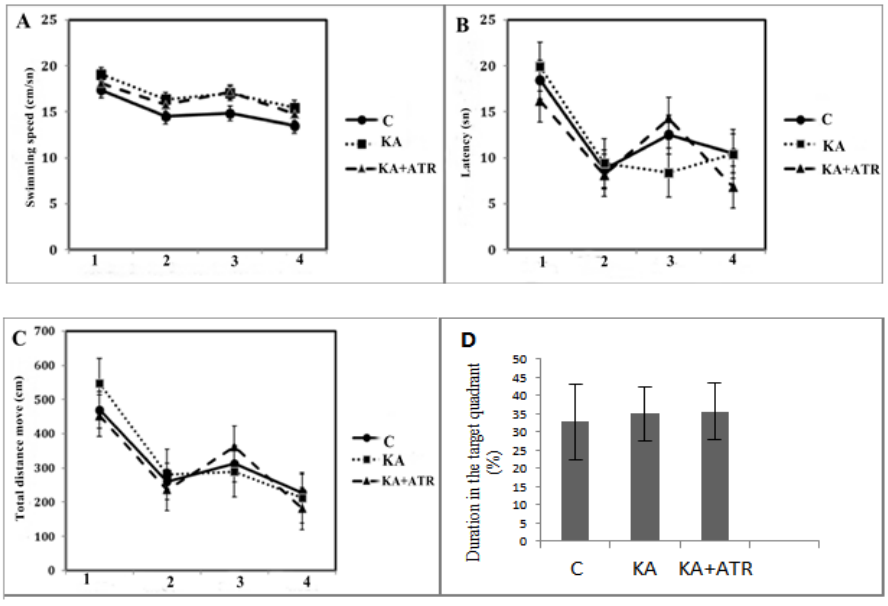

Figure 1. A) Swimming speed B) Latency, C) Total distance move during four learning days, D) Duration in the target quadrant (memory test). C: Control, KA: Kainic acid, KA+ATR: Kainic acid+ Atractylenolide III.

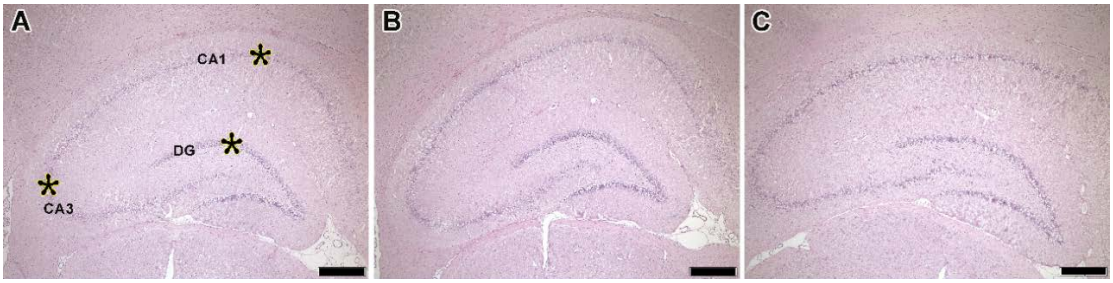

Figure 2. Morphology of hippocampus in C (A), KA (B) and KA+ATR (C) groups (CA1; cornu ammonis 1, CA3; cornu ammonis 3, DG; gyrus dentatus), the neuron layers measured in CA1, CA3 and DG of the hippocampus (black asterisks) (H&E, X40), Scale bar: 200 µm.

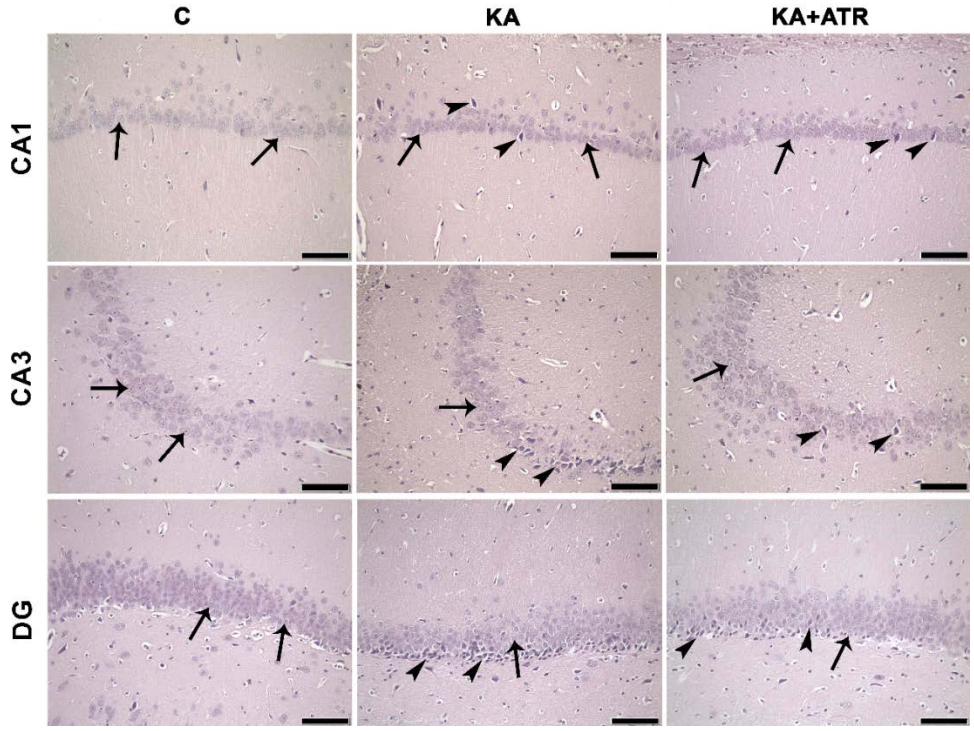

Figure 3. Normal (arrows) and degenerated neurons (arrowheads) in CA1, CA3 and DG regions of the hippocampus. In KA and KA+ATR groups, degenerated neurons are seen in CA1, CA3 and DG. (H&E, X200). Scale bar: 50 µm.

In the control group, the general structure of the hippocampus, the neuron distributions, and morphologies in the CA1, CA3, and DG regions were normal (Figure 2A and Figure 3). The thicknesses of CA1, CA3, and DG regions of the hippocampus were measured as 42.20±1.61, 52.60±1.90 and 45.60±4.13 µm, respectively (Figure 5A). TNN and DNN were found to be 203.54±6.22 and 2.03±0.83 in CA1, respectively, and 159.43±15.95 and 7.70±4.56 in CA3. In DG-GL and DG-SGL, the TNN was 552.10±50.63 and 76.67±6.61, respectively, while

their DNN was 2.10±0.50 and 7.20±3.85, respectively (Figure 5B, C).

The distribution of neurons in the KA group was similar to the control group, but there was an increase in the number of degenerated neurons in some areas (Figure 2B and Figure 3). In this group, the thicknesses in CA1, CA3, and DG regions were 37.39±1.90, 45.64±6.26 and 46.02±5.72 μm, respectively (Figure 5A). We observed that the thickness of the CA1 region for the KA group decreased significantly compared to the control group (P<0.05). Although the thickness of the CA3 region decreased compared to the control group, this decrease was not significant (P>0.05). TNN and DNN in CA1 were 183.88±14.80 and 4.46±2.38, respectively, while in CA3 they were 132.96±21.49 and 20.33±14.01. In DG-GL and DG-SGL, TNN was 531.92±3.70 and 75.62±5.90, respectively, while their DNN was calculated as 18.96±11.40 and 24.67±10.28, respectively (Figure 5B, C). TNN in CA1 and CA3 regions were significantly decreased in the KA group compared to the control groups (P<0.05). However, TNN in DG-GL and DG-SGL were decreased compared to the control group, but these decreases were not significant (P>0.05) (Figure 4, Figure 5B). DNN increased significantly in the DG-GL and DG-SGL compared to the control group (P<0.05). Although there was an increase in the CA1 and CA3 of DNN compared to the control group, these increases were not significant (P>0.05) (Figure 4, Figure 5C).

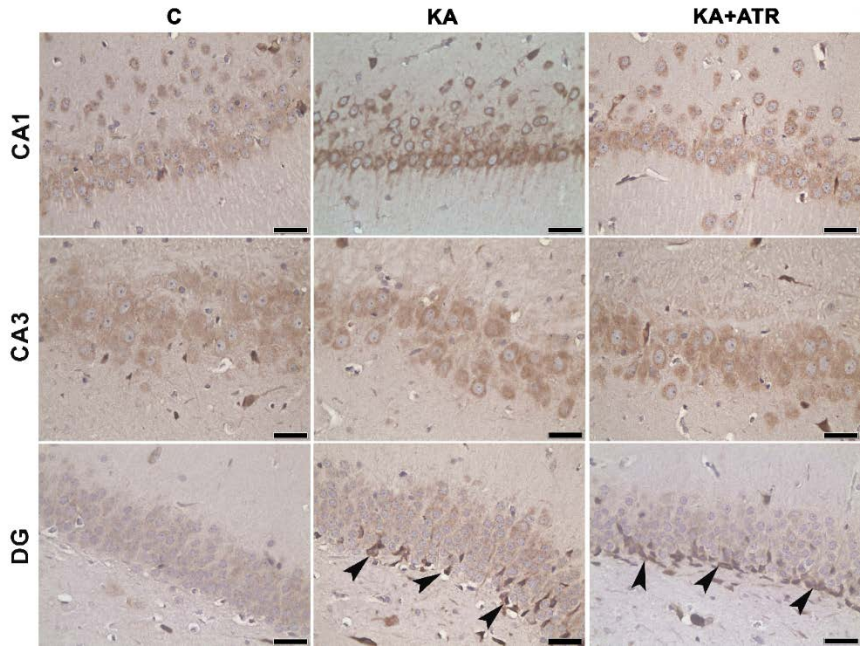

Figure 4. GRP78 immune labeling in CA1, CA3 and DG regions of the hippocampus. Intense GRP78 immune--labeled neurons are seen in DG-SGL of KA and KA+ATR groups (arrowheads) (Indirect peroxidase, X400) Scale bar: 20 μm.

In the ATR+KA group, hippocampal neuron distributions were similar to the other groups, and degenerated neurons were found in some places (Figure 2C and Figure 3). In this group, CA1, CA3, and DG thicknesses were calculated as 36.05±4.13, 47.09±7.09 and 43.07±5.91 μm, respectively. It was determined that the CA1 thickness in this group decreased significantly compared to the control and kainic acid groups (P<0.05). It was observed that CA3 thickness decreased compared to the control group and increased compared to the KA group, but these differences were not significant (P>0.05). Although DG thickness decreased compared to the other two groups, it was determined that these decreases were not significant (P>0.05) (Figure 2C, Figure 5A). TNN and DNN were found to be

175.33±6.04 and 2.69±0.79 respectively in CA1, and 111.00±14.26 and 6.95±3.80 in CA3. In DG-GL and DG-SGL, the TNN was 527.07±38.75 and 82.10±4.25, respectively, while their DNN was 8.03±8.22 and 12.23±6.23, respectively (Figure 5B, C). TNN was significantly decreased in CA1 and CA3 areas in this group compared to other groups (P<0.05). Compared to other groups, TNN decreased in DG-GL and increased in DG-SGL, but these differences were not significant (P>0.05) (Figure 2, Figure 5B). It was determined that DNN levels decreased significantly in DG-GL and DG-SGL compared to KA groups and increased significantly compared to control groups (P<0.05). It was observed that DNN levels decreased in CA3 compared to other groups, but these decreases were not significant (P>0.05). However, DNN decreased in CA1 compared to the KA group and increased compared to the control group, but these differences were not significant (P>0.05) (Figure 2, Figure 5C).

GRP78 immunolabeling of neurons in CA1 and CA3 regions in the control group was 17.87±6.42 and 60.60±9.66, respectively, while it was 56.00±18.74 and 62.80±11.16 in GL and SGL of DG (Figure 5D). GRP 78 labeled neurons in CA1, CA3, DG-GL, and DG-SGL in the KA group were calculated as 36.45±8.60, 87.40±14.31, 120.71±20.26 and 110.25±35.17, respectively. It was observed that GRP 78 immune labeling was significantly increased in neurons in CA1, CA3, DG-GL, and DG-SGL in this group compared to control groups (P<0.05) (Figure 4, Figure 5D). In the ATR+KA group, GRP 78 immune labeling was calculated as 21.29±8.92, 80.23±18.14, 111.52±27.70 and 117.62±19.94 in CA1, CA3, and DG-GL and DG-SGL neurons, respectively. In this group, it was observed that GRP 78 immunolabeling was significantly increased in all studied areas except CA3, compared to the control groups, and decreased significantly compared to the KA group (P<0.05). In CA3, GRP 78 expression was increased significantly compared to the control group (P<0.05) and decreased compared to the kainic acid group, but this decrease was not significant (P>0.05) (Figure 4, Figure 5D).

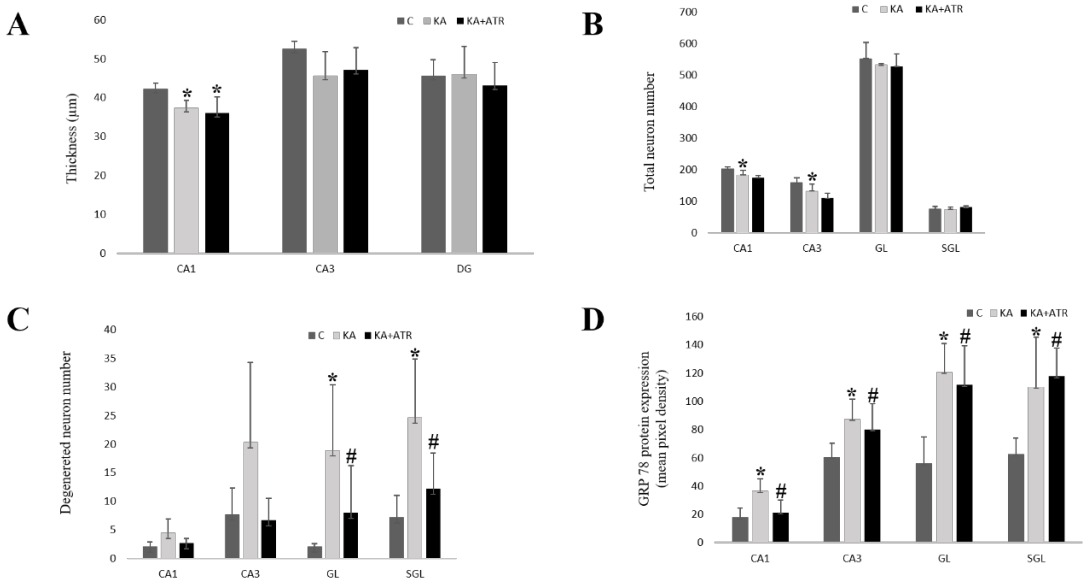

Figure 5. Thicknesses (µm) (A), total number of neurons (TNN) (B), number of degenerated neurons (DNN) (C), and GRP78 protein expression levels (D) of CA1, CA3 and DG (GL and SGL) regions of hippocampus. Data was given as mean ± standard deviation. * There is a difference of p<0.05 compared to control group and # is a difference of p<0.05 compared to kainic acid group.

In the control group, the nucleus structure and chromatin distribution were normal in the neurons, the distribution and amount of polyribosomes in the cytoplasm, the rough endoplasmic reticulum (GER) cisterns, and other organelles were also normal morpologhy (Figure 6A).

In the KA group, while the nuclear chromatin distribution was normal in neurons, some regions of the nuclear membrane were irregular. While the distribution and amounts of polyribosomes in the cytoplasm are normal, we found electron-dense inclusions of different sizes and locally containing lipid vacuoles in some areas. In addition, significant enlargement of the GER cisterns and fragmentation of the GER membranes in some areas were detected, while the cristae deformation, swelling, and vacuolization were observed in the mitochondria (Figure 6B).

Nucleus chromatin distribution was normal in neurons in the ATR+KA group. We observed that the distribution and amount of polyribosomes in the cytoplasm were similar to those in other groups. In this group, fewer electron-dense inclusions were detected in the cytoplasm of neurons compared to those in the KA group. While normal GER cisterns were observed in neurons, significant enlargement and fragmentation of GER cisterns were also detected in some areas. In mitochondria, degenerative changes in the form of enlargement, swelling, and vacuolization were observed in the cistern (Figure 6C).

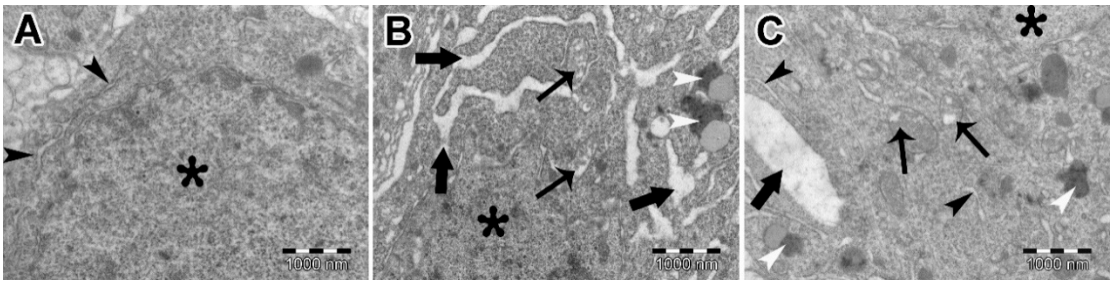

Figure 6. In group C, the neuron has a normal nucleus (asterisk) and rough endoplasmic reticulum cisternae (arrowheads). In KA group (B), neuron nucleus (asterisk), enlarged GER cisternae, and membrane disruptions (thick arrows), degenerated mitochondria (thin arrows), and electron-dense inclusions (white arrowheads) in GER cisterns in some areas. In the KA+ATR group (C), nucleus (asterisk), normal GER cisterns (black arrowheads), prominent enlargement of the GER cistern (thick arrow), degenerated mitochondria (thin arrows), electron-dense inclusions (white arrowheads), (Uranil acetate-lead citrate X25000), Scale bar: 1000nm.

Changes in biochemical parameters such as MDA, SOD, GSHPx, AChE and CAT in brain tissue were investigated in C, KA and ATR+KA groups. An increase in MDA was observed in the KA and ATR+KA groups compared to the control group and compared to the ATR+KA, KA groups. There was an increase in SOD in the ATR+KA group compared to the other two groups, and a decrease in the KA group compared to the control group. A decrease in CAT and GSHPx was observed in the KA and ATR+KA groups compared to the control group and compared to the KA, ATR+KA groups. While a decrease in AChE was observed in the KA group compared to the other groups, there was no difference between K and ATR+KA (Table 3).

Table 3. Brain tissue biochemical parameters

Groups	MDA *	SOD *	GSHPx *	AChE *	CAT*
Control	9.30 ± 0.50 [a]	12.47 ± 0.74 [a]	4.25 ± 3.32 [a]	0.54 ± 0.43 [a]	15.06 ± 3.16 [a]
Kainic acid	9.85 ± 1.03 [a]	12.42 ± 1.35 [a]	2.51 ± 0.25 [a]	0.53 ± 0.41 [a]	12.87 ± 3.03 [a]
Kainic+ATR	9.90 ± 1.03 [a]	12.64 ± 0.98 [a]	2.70 ± 0.26 [a]	0.54 ± 0.17 [a]	13.25 ± 2.17 [a]

Data was given as mean ± standard deviation. *: a is used to indicate the distinction between groups. There is no distinction at the $p<0.05$ level between the data shown with the same letters. MDA: Malondialdehyde, SOD: Superoxide dismutase, GSHPx: Glutathione peroxidase, AChE: Acetylcholinesterase activity, CAT: Catalase activity, ATR: Atractylenolide III.

DISCUSSION

The present study indicates for the neuroprotective effect of Atractylenolide - III on degenerated neurons in the dentate gyrus region of the hippocampus induced by kainic acid administration in rats. In this study, C, KA, and KA+ATR groups were compared in terms of behavior, learning, and memory, and histological and biochemical analyses. Learning and memory are the important components of cognition. We investigated the effects of kainic acid and Atractylenolide – III on learning and memory function. According to the MWMT results, in the C, KA, and KA+ATR groups, we observed that swimming speed and latency on the 2nd and 4th days were decreased compared to the 1st day. We observed that the total distance on the second, 3rd, and 4th days of the C, KA, and KA+ATR groups decreased significantly compared to the 1st day. According to these results, we can say that all groups learned the place of the platform. On the other hand, we did not find any significant difference between the groups on the memory test (last day of MWMT). According to this information, the application of the kainic acid and Atractylenolide III did not alter the memory function significantly. In plus maze results, closed arm duration in KA and KA+ATR groups, and closed arm frequency in the KA+ATR group increased compared to control groups. We can assume that kainic acid increased the anxiety behavior without the effects of ATR. However, we could not show the protective effects of ATR on anxiety behavior in epileptic rats. In addition, without any significant difference, there is a trend for increased locomotor activity for the KA and KA+ATR groups. On the other hand, Zhou and colleagues found that atractylenolide III produces antidepressant- and anxiolytic-like effects LPS-induced depression model[22]. We could say that, atractylenolide III has been insufficient to ameliorate increased anxiety levels in the kainic acid-derived epilepsy model.

Glutamate-induced excitotoxicity appears to play an important role in neurological disorders. Neuroprotection against glutamate-induced excitotoxicity has been proposed as a therapeutic strategy to prevent and/or treat these excitotoxicity-mediated diseases. Atractylenolides applied as an anti-inflammatory, anti-cancer activity, neuroprotective effect, spleen, and stomach tonic, according to the Chinese Pharmacopoeia[23]. It has been reported that Atractylenolide III exhibits neuroprotective activity against ischemia/reperfusion in neurons such as mouse pheochromocytoma PC12 cells[10,24]. According to our light microscopy evaluations, we determined the general structure of the hippocampus has normal morphology in the control group, and the neuron distribution and morphology in the CA1, CA3, and DG regions were also normal. An increase in the number of degenerated neurons was observed in some areas in the kainic acid group. In this group, we observed that the thickness of the CA1 region was decreased compared to the control group. Moreover, we showed that TNN in the hippocampus, CA1, and CA3 regions of kainic acid-treated rats were significantly reduced compared to the control groups. DNN levels increased significantly in DG-GL and DG-SGL compared to control groups in the KA group. In the ATR+KA group, hippocampal neuron distributions were similar to the control group. We determined that the CA1 thickness in this group decreased significantly compared to the control and kainic acid groups. TNS was significantly decreased in CA1 and CA3 areas in the ATR+KA group compared to other groups. Furthermore, DNN levels decreased significantly in DG-GLand DG-SGL areas compared to kainic acid groups and increased significantly compared to control groups. We assumed that kainic acid has a decreasing effect on neuron numbers alone and together with ATR. ATR did not show protective effects on total neuron numbers; however, it has success in decreasing degenerated neurons in the dentate gyrus region of the hippocampus. In the CA1, CA3, DG-GL, and DG-SGL, GRP 78 immune-labeled neurons significantly increased in the KA group compared to the control groups. In the ATR+KA group, GRP 78 immune labeling was significantly increased in all areas except CA3, compared to the control groups, and significantly decreased compared to the kainic acid groups. We assumed that ATR has ameliorated the increased endoplasmic reticulum stress that derived from kainic acid. In a very recent study, Zuo and colleagues indicated that Atractylenolide can protect cardiomyocytes by regulating ROS/GRP78/caspase-12 signaling pathway to inhibit H2O2-induced endoplasmic reticulum stress and apoptosis[25]. In recent years, the increasing importance of ATR and the beginning of investigation of changes related to ER stress in diverse systems seem to be advantageous for ATR to be a promising supplement.

In the ultrastructural examination with electron microscopy, we observed that the nucleus structure

and chromatin distribution were normal in the control group's neuron, the distribution and amounts of polyribosomes in the cytoplasm, the rough endoplasmic reticulum (GER) cisterns and other organelles were also normal. In the KA group, we determined that there were irregularities in some parts of the nuclear membrane of the neurons. In the cytoplasm, inclusions of different sizes and electron-dense appearance containing lipid vacuoles were detected in some areas. Significant enlargement of the GER cisterns and fragmentation of the GER membranes in some areas were detected, while the cristae deformation, swelling, and vacuolization were observed in the mitochondria. In the ATR+KA group, the nucleus chromatin distribution was normal in neurons. We showed that the distribution and amount of polyribosomes in the cytoplasm were similar to those in other groups. While normal GER cisterns were observed in neurons, significant enlargements and fragmentation in GER cisterns were detected in some areas. In mitochondria, degenerative changes in the form of enlargement, swelling, and vacuolization were observed in the cisterns. In the control group, all examinations were normal in light microscopy, GRP 78 immunolabeling, and ultrastructural examinations with electron microscopy. The increase in the number of degenerated neurons in the kainic acid group, thinning of the hippocampus layers, inclusions, significant enlargement of the GER cisterns and fragmentation of the GER membranes in some areas, deterioration in the cristae structure in the mitochondria, swelling and vacuolization are the indicators that kainic acid causes damage to the hippocampus due to oxidative stress. In the ATR+KA group, these damages were less common and the neurons were mostly normal in appearance, similar to the control group. We showed that atractylenolide has mild neuroprotective effects in our study. Xue and colleagues indicated that the Atractylenolide III has neuroprotective effects after spinal cord injury in rats by modulating microglial/macrophage polarization[26]. However, the mechanisms of the neuroprotective effects of ATR need to be studied especially in the epilepsy model.

The production of free radicals is a normal consequence of electron transport in the mitochondrial organelle. Under normal physiological conditions, naturally occurring antioxidants lose their radical formation. Thus, the interaction between free radicals and antioxidant defense systems is part of normal brain function. High rates of free radicals mediated by natural defense mechanisms are prominent in post-traumatic neuronal degeneration[27, 28]. We investigated the changes in biochemical parameters such as MDA, SOD, GSHPx, AChE, and CAT in brain tissue. However, we could not find significant differences between groups. Wang and colleagues showed pro-oxidant and cytotoxic activities of atractylenolide I in human promyeloleukemic HL-60 cells[29]. However, in general, the studies that were conducted with ATR were executed with cell lines in the field of pro-inflammatory. In this area, we need more in vivo studies and dose-dependent responses that enlighten the mechanism of ATR on neurodegenerative diseases.

The literature has demonstrated the neuroprotective properties of atractylenolide III in a variety of animal models. Information regarding the impact of ATR on temporal lobe epilepsy is provided by the current study. To clarify the fundamental processes by which ATR III carries out its neuroprotective actions, more research is necessary. Especially potential mechanisms involving the modulation of endoplasmic reticulum stress and oxidative stress pathways should be investigated in future studies. To figure out exactly the neuroprotective effects of ATR, research that includes different types of epilepsy and long-term effects needs to be investigated.

The study suggests potential mechanisms involving the modulation of endoplasmic reticulum stress and oxidative stress pathways by ATR. However, these mechanisms were not thoroughly investigated. Further research is required to elucidate the precise molecular pathways through which ATR exerts its neuroprotective effects, particularly in the context of epilepsy. In addition, comparative studies involving different models of neurodegeneration and epilepsy could provide a more comprehensive understanding of ATR's therapeutic potential and limitations. In additionto explain the mechanism more clearly, long-term studies are needed to evaluate the sustained neuroprotective effects and safety profile of ATR, which are crucial for its potential therapeutic application.

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