Original Article

Investigation of the Effect of Icariin on Pentylenetetrazole-induced Seizures in Male Mice With Gonadoctomy

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ABSTRACT

Background: In epileptic patients and humans treated with antiepileptic drugs, androgen concentration is significantly reduced, leading to an increase in seizures, followed by the destruction of hippocampal neurons and memory and learning disorders.

Objectives: This study aims to examine the effect of icariin extract on epileptic rats following gonadectomy.

Methods: A total of 84 male mice were used. Mice were randomly divided into 12 groups: Control, pentylenetetrazole (PTZ), dimethyl sulfoxide (DMSO), icariin (E), gonadectomy (GO), PTZ+E, PTZ+GO, DMSO+E, DMSO+GO, E+GO, PTZ+E+GO and DMSO+E+GO. After inducing epilepsy using PTZ, the shuttle box test and tissue parameters were evaluated.

Results: This study showed that both epilepsy-induced seizures and gonadectomy decreased neuronal density in the hippocampus, decreased Nestin expression, increased degenerated cells and inflammation, increased the number of monocytes and decreased memory and learning ability. The icariin extract improved this condition to a certain extent.

Conclusion: The use of herbal medicines, such as icariin, in the treatment of epilepsy by increasing testosterone levels and its neuroprotective properties may open up new horizons in the treatment of patients with epilepsy.

Keywords: Epilepsy, Gonadectomy, Icarin, Pentylenetetrazole (PTZ), Seizure

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Introduction

Epilepsy is one of the most crucial neurological disorders, affecting approximately 1% of the world's population suffers from this disease. Seizures are thought to be the main feature of epilepsy and occur randomly (Karoly et al., 2021). The International League Against Epilepsy (ILAE) defines epilepsy as at least two unprovoked seizures more than 24 h apart, one unprovoked seizure, and the possibility of further seizures, corresponding to a generalized risk of relapse (Fisher et al., 2014). Seizures that occur in people with epilepsy are the result of the abnormal function of ion channels with voltage that can make neurons electrically hyperactive (Fisher et al., 2017). The crucial aspect of epileptic seizures is that they are variable and can be partial or severe, short or long, frequent, or infrequent (Anwar et al., 2020). The main symptoms of this disease are headache, speech problems, memory and concentration problems, and cognitive disorders (Novak et al., 2022). Cognitive disorders in epilepsy are due to the loss of neurons in the hippocampus or entorhinal cortex (Holmes., 2015). The CA1 region of the hippocampus and dentate gyrus are involved in developing seizures and hippocampal sclerosis (Pires et al., 2021). Men and women of all ages also develop epilepsy. However, most clinical studies have focused on men (Bahramnejad et al., 2023), experimental models of susceptibility to seizures and epilepsy show sex differences and seizures and epilepsy are more common in men than in women (Scharfman et al., 2014), which may be related to factors, such as body weight, steroid hormones, cytochrome P450 activity, and biological differences in the neuronal network (Samba Reddy, 2015). In men, removal of the testicles (gonadectomy), which is the main source of androgens, leads to an increase in seizures. In contrast, the occurrence of inflammatory reactions followed by apoptosis leading to astrogliosis can effectively influence the severity of the disease. (Fisher et al., 2005), antiepileptic drugs cause changes in plasma levels of sex hormones, and this complication is particularly evident in men and the change in their androgen levels. Among androgens, the decrease in testosterone in epileptic patients causes an increase in hippocampal neuron disorders and seizures (Andréen et al., 2005); thus, considering that antiepileptic drugs can cause changes in plasma levels of sex hormones, this study aims to use icariin (E) in the treatment of epilepsy caused by injection of pentylenetetrazole (PTZ). PTZ is a convulsion-inducing substance that can cause convulsions by injecting a certain concentration of this drug, and even a single dose of PTZ can cause memory and learning impairment (Alachkar et al., 2020). E is a flavonol found in several species of plants of the Epidemium family, commonly known as bugleweed or yin yang hu. E powder is an extract from the stems and leaves of Epimedium plants. This product is a light blue to light yellow crystalline powder with a molecular weight 676.65. E powder can increase blood flow to the heart, blood vessels, and bone marrow and E can also increase testosterone production and estrogen levels, especially in postmenopausal women (Nian et al., 2009). Therefore, as an antiepileptic, E can help reduce plasma levels of sex hormones and improve brain structures by increasing blood flow to the heart and brain.

Materials and Methods

In this study, 84 mice weighing 30-35 g were acclimatized the animals to the environment for 1 week in the Animal House of the Para Veterinary Faculty of Ilam City, Iran, at a temperature of 23 °C and a cycle of 12 h of light and 12 h of darkness. The mice were then randomly divided into 12 groups of 7 animals each. First group: Control (intact); second group: PTZ); third group: Dimethyl sulfoxide (DMSO); fourth group: Ikarin (E); fifth group: Gonadectomy (GO); sixth group: PTZ+E; seventh group: PTZ+GO; eighth group: DMSO+E; ninth group: DMSO+GO; tenth group: E+GO; eleventh group: PTZ+E+GO; twelfth group: DMSO+E+GO.

In the GO groups, the male mouse was placed in the prone position and a 1 cm median incision was made in the scrotum and the skin was pulled back to expose the tunica. The tunica was punctured to remove the testes. The testicles were lifted to expose the underlying spermatic cord. This was clamped and ligated at the confluence of the blood vessels and the epididymis. The same procedures were performed in sham-operated mice, except that the testicles were not removed. The skin incision was sutured using self-adhesive staples.

Two weeks after GO, the experiments were performed on mice, and all injections were administered intraperitoneally. Half an hour before the injection of PTZ (70 mg/kg), E (50 mg/kg) was administered. Immediately after the injection of PTZ, the duration of the different seizure stages was examined and recorded for 20 minutes.

Examination of seizure stages

The seizure stages are:

1) Stage zero: Threshold stage or onset of seizure (no response); 2) Stage one: Contraction of the facial and ear muscles; 3) Stage two: Spread of the contraction wave over the whole body; 4) Stage three: Myoclonic jumping while standing on two feet; 5) Stage four: Falling on the side; 6) Stage five: Falling on the back and generalized tonic and clonic seizures

Behavior test

A shuttle box device was used to measure adult rats' learning and memory capacity. This device, manufactured in Iran, consists of two light-colored rooms measuring 20×20 cm and made of transparent and dark plastic, which are covered with opaque plastic. A sliding door (8×8 cm) between the two rooms is opened and closed with a wire. The floors of both rooms is covered with stainless-steel bars. The bars are 2 mm thick and spaced one centimeter apart. Electricity can be supplied to the darkroom floor by connecting it to a power supply. The amount of current received and time can be set, and the test is performed on two consecutive days. The first day is regarded as the training day, and the second is the test day.

At the end of the work, the mice were euthanized with ketamine-xylazine. After the separation of the brain, the tissue passage was performed in the usual way, and the tissue was stained with cresyl violet to examine the division of neurons in the hippocampus and damaged areas. In addition, hematoxylin-eosin (H&E) staining was used to examine monocyte and tissue inflammation. Cresyl violet staining was used to identify and count Nissl bodies and Nestin staining was used as a marker for stem cells and neurogenesis.

Statistical analysis

Statistical analysis of the data obtained from the behavioral tests was performed using repeated-measures analysis of variance. The microscopic parameters were evaluated using a two-way analysis of variance and Tukey's complementary test, and P≤0.05 was considered a significant difference.

Results

The results of this study showed that, in the shuttle box test, the duration for which the mice entered the darkroom was shorter in the PTZ and PTZ+E+GO groups than in the other groups and was statistically significant (Figure 1), indicating memory impairment and hippocampal involvement in these groups. The duration of mice staying in the dark environment in the PTZ and PTZ+E+GO groups was significantly longer than in the other groups (Figure 2). Nestin staining showed that the expression of Nestin decreased dramatically in the PTZ tetrazole+GO groups and the endogenous cells and neural stem cells were less divided (Figures 2 and 3). In the other groups, Nestin expression was normal. In the Chryzl purple staining, dark and pyknotic cells with decreased cytoplasmic levels were examined, and cell viability and survival were examined in the pentylene groups. In the PTZ and PTZ+E+GO groups, the number

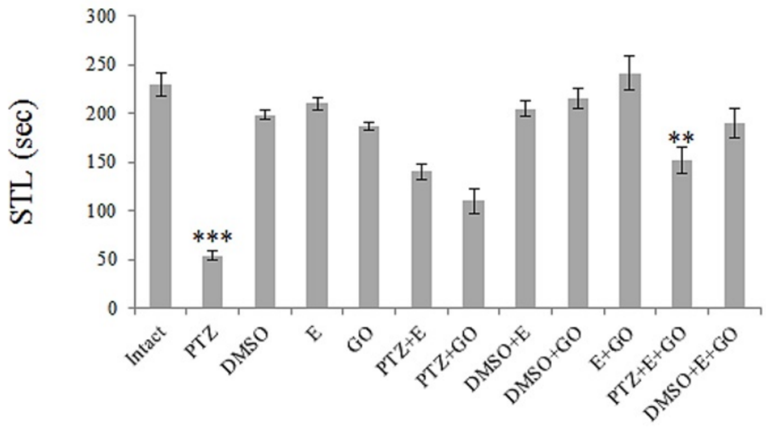

Figure 1. Shuttle box test

Note: This test measured the duration of the animal's entry into the darkroom (step-through latency). The results showed a significant difference between the group receiving PTZ and the other groups; ***(P<0.001), and between the group receiving PTZ+E+GO and the other groups; **(P<0.01).

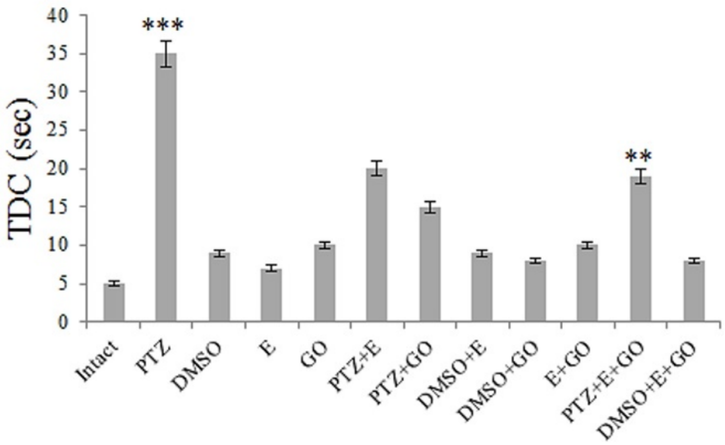

Figure 2. Shuttle box test

Note: In this test, the survival time of mice was measured in the darkroom (TDC) and the longest survival time was observed in the PTZ and PTZ+GO+E groups, which showed a statistically significant difference compared to the other groups; ***(P<0.001); **(P<0.01).

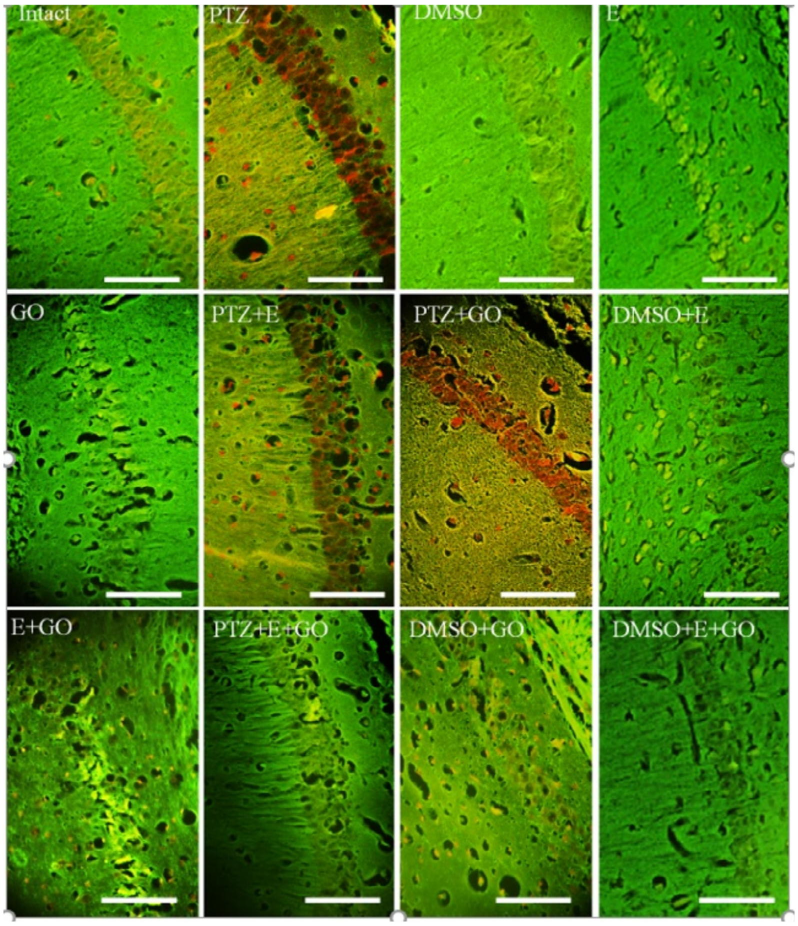

Figure 3. Nestin staining

Note: As a neuro-fundamental marker, Nestin expression in the PTZ and PTZ+GO groups decreased drastically. In contrast, in the E and PTZ groups, the expression rate increased compared to the previous two groups. In other groups, Nestin expression was normal.

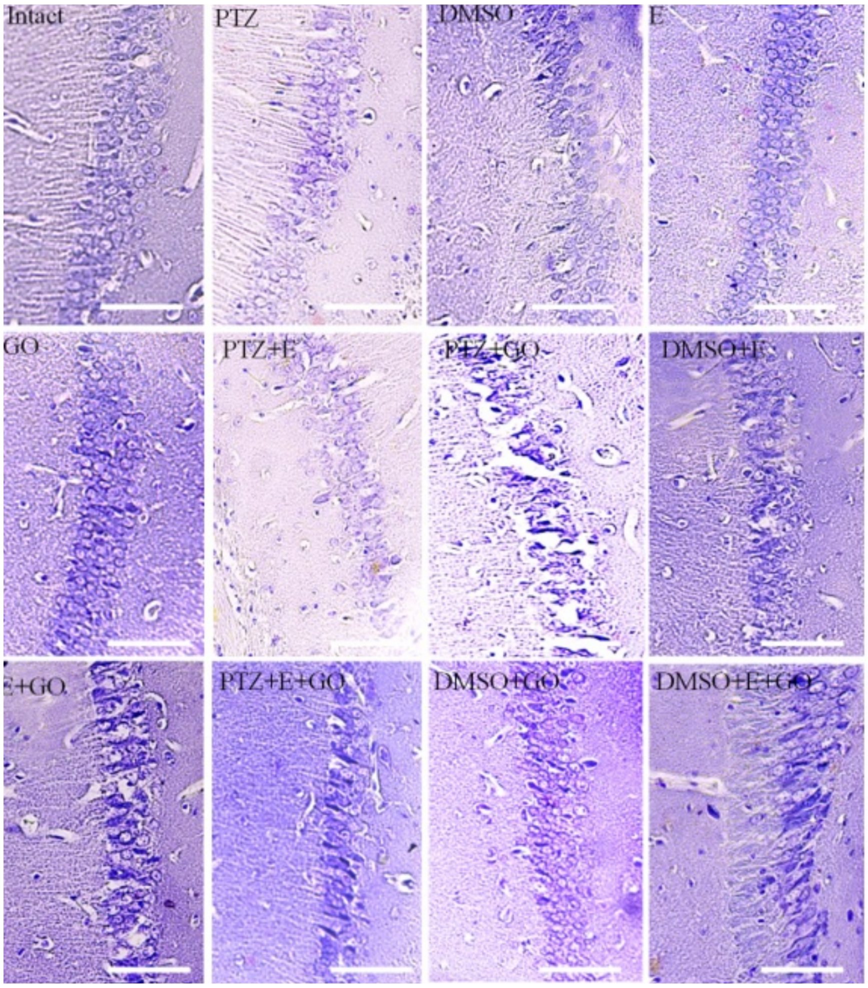

Figure 4. Chryzl violet staining

Note: In the PTZ and PTZ+GO groups, the number of pyknotic cells increased with a decrease in the cytoplasm, that is, dark cells and the survival rate of nerve cells decreased; however, in the PTZ+E groups, the number of dark cells decreased compared to the previous two groups; in the other groups, the number of dark cells and cell viability were normal.

of pyknotic cells increased with a decrease in cytoplasmic levels, indicating dark cells (Figure 4), even when cell survival was assessed in the CA1 region of the hippocampus. It was found that cell survival and survival rate decreased in the PTZ and PTZ+E+GO groups compared to the other groups, which was statistically significant (Figure 5). H&E staining was used to examine the trend of inflammation and presence of monocytes. In the PTZ group, the nuclei were highly stained due to pyknosis and cell death and the inflammation and presence of monocytes were also extremely high in this group. The PTZ+GO group also showed high rates of inflammation and cell death. This trend was slightly lower in the PTZ+E group than in the other two groups (Figure 6).

Discussion

Epilepsy is one of the most critical neurological diseases. One of the vital features of epilepsy is seizures (Milligan., 2015). In men with epilepsy, a decrease in plasma testosterone levels is observed and a decrease in testosterone levels leads to an increase in seizures (Kim et al., 2023). Many synthetijc antiepileptic drugs cause a decrease in plasma testosterone levels (Moazzami et al., 2013); after this disorder, memory and learning are impaired due to the destruction of neurons in the hippocampus region (Zaitsev et al., 2021). The results of this study in the shuttle box test showed that in the swallowing group (PTZ) and the swallowing and GO and E-receiving groups (PTZ+E+GO), the time the animal entered the dark area (selective laser trabeculoplasty [SLT]) decreased, indicating impairment. However, in the E group, E+G and the E+PTZ, this period was normal. This finding suggests that E may prevent

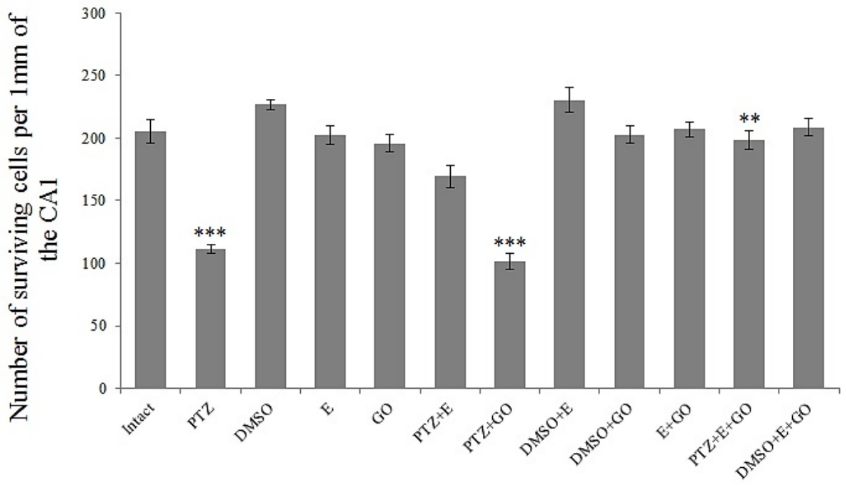

Figure 5. Cell viability count in the CA1 region of the hippocampus

Note: The number of viable cells in the PTZ, PTZ+GO and PTZ+GO+E groups was significantly lower than in other groups; ***(P<0.001); **(P<0.01).

memory impairment by increasing androgen levels and improving hippocampal neurons by reducing inflammation in the hippocampus. Epileptic seizures cause memory impairment by damaging the hippocampal neurons (Solati et al., 2019). Agarwal et al. (2011) showed that PTZ-induced epilepsy in rats impaired passive avoidance memory and significantly reduced the latency to enter the dark compartment in the shuttle box test, which is consistent with our results. In addition, studies have reported that PTZ-induced epileptic seizures impair spatial memory, cause learning impairment and impair passive avoidance behaviour (Agarwal et al., 2011). Our study showed that memory and learning performance were lower in the GO group than in the GO+E group. This shows that testosterone levels are related to memory and learning performance, which is consistent with the results of the present study by Roshanaei et al. (2013) showing that a decrease in testosterone levels in gonadectomized male rats leads to a decrease in memory and learning performance (Roshanaei et al., 2013). Ghahramani et al. (2018) investigated the memory of gonadectomized rats using the shuttle box test and reported that gonadectomy of rats increases the persistence of this group of rats in the darkroom (time in dark compartment [TDC]), indicating a decrease in memory and learning of rats. Gonadectomy is currently being pursued (Ghahramani et al., 2018). The absence or lack of sex hormones has been shown to have crucial effects on the hippocampus, which is one of the vital structures playing a role in memory and learning (Mosleh., 2013); it has also been shown that testosterone affects the growth of dendritic spines and increases synaptic density. In pyramidal neurons of the hippocampus, it increases memory and learning performance. Gonadectomy causes a decrease in dendritic spines and hippocampal neurons, especially in the CA1 area of the hippocampus, which is due to these interactions that decrease memory and learning performance (Leranth et al., 2003). In recent years, it has been reported that a decrease in testosterone levels increases the level of beta-amyloid protein, and the increase of beta-amyloid is more evident in patients with Alzheimer's disease suffering from memory loss (Gillett et al., 2003). Erasmus et al. reported that E increases testosterone levels (Erasmus et al., 2021). Thus, assuming that antiepileptic drugs decrease testosterone levels, E may increase testosterone levels and enhance learning and memory performance. Asgharzade et al. (2020) reported that epileptic rats with PTZ showed memory impairment in the shuttle box test compared to other groups, which is consistent with this study. They believed that these memory impairments were due to the death of the hippocampal neurons (Asgharzade et al., 2020). The histological results of this study showed that the survival rate of neurons in the CA1 region decreased dramatically in the castration, gonadectomy, castration-gonadectomy and E groups; however, in the other groups, especially in the groups that received E, this proportion was normal and increased. Lopim et al. (2016) after counting neurons in the hippocampal area on consecutive days in epileptic rats, showed that the number of neurons decreased drastically in epileptic rats, which is consistent with the present study (Lopim et al., 2016). Several animal model studies have shown that epilepsy is characterized by various changes in the hippocampus, including the destruction of neurons in the CA1 and CA3 regions of the hippocampus, a decrease in gamma-aminobutyric neurons and the disappearance of

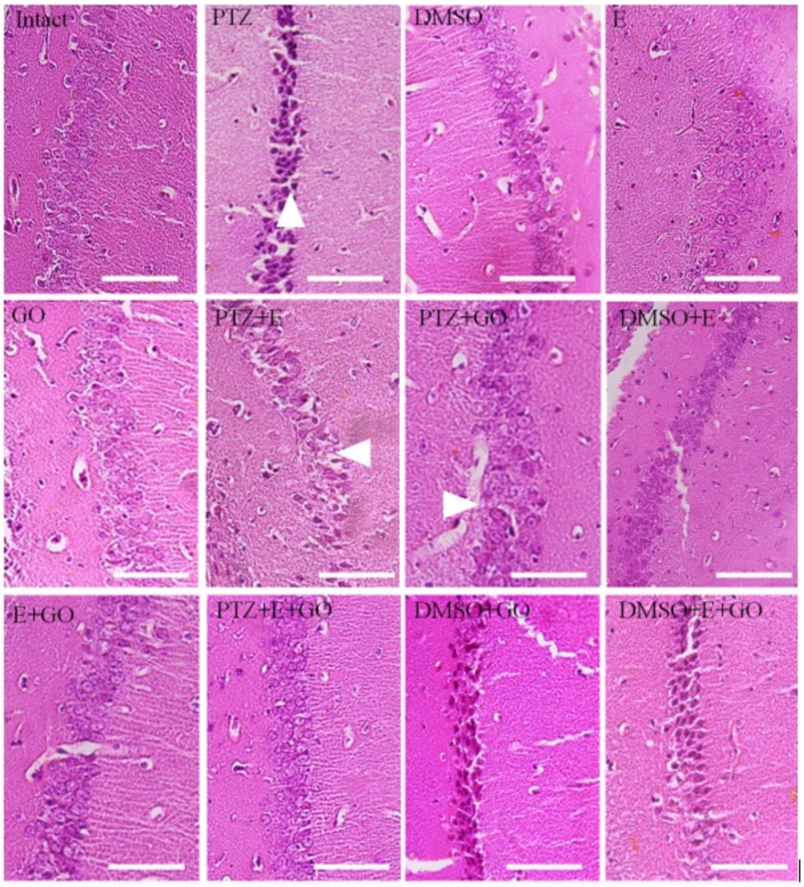

Figure 6. H&E staining

Note: In the PTZ group, the number of inflammatory monocytes and inflammation was strongly increased (white arrow). Inflammation was also observed (white arrow) in the PTZ+GO and PTZ+E groups, but the extent of inflammation was lower. No inflammation or increase in monocytes was observed in the other groups.

calcium-binding proteins in dentate granule cells (Rao et al., 2005; Shetty et al., 2007). Moghadami et al. (2016) reported that gonadectomy decreases the density of androgen receptor-immunoreactive neurons in the hippocampus and that the injection of testosterone and subsequent elevation of testosterone levels increases the density of these neurons (Moghadami et al., 2016). Androgens, particularly testosterone, have nutritional effects on the dendrites of the hippocampus, thereby enhancing neurogenesis in the hippocampal region (McMahon. 2014). Liu et al. (2020) have shown that E can improve the function of hippocampal neurons through anti-inflammation mechanisms (Liu et al., 2020). The results of our study showed that the Nestin expression decreased dramatically in the epilepsy group and in the epilepsy and gonadectomy group. Consistent with this study, Cho et al. (2015) also decreased. In this study, Nestin staining showed that the E extract enhanced neurogenesis in the E-receiving group and increased Nestin expression compared to the E group, demonstrating the neuroprotective properties of E. Liu et al. (2020) reported that E has neuroprotective effects and can also significantly increase the survival rate of hippocampal neurons treated with corticotropin. The anti-apoptotic role of E has been established in several studies. Icarin inhibits CORT-induced neuronal apoptosis in the rat hippocampus by inhibiting the p38 MAPK signaling pathway and suppressing ER stress-induced neuronal apoptosis by activating the PI3K/Akt signaling pathway. (Li et al., 2015). This study and H&E staining show an increase in the number of monocytes and inflammation in the epilepsy group, but E greatly reduced inflammation in the epilepsy group receiving E, neuroinflammation mediated by microglia has a significant contribution, and it has epilepsy in the pathophysiology (Wang et al., 2023). E prevents inflammation by suppressing pro-inflammatory signals, such as NF-kB and MAPK. In addition, E prevents inflammation by activating the anti-inflammatory signaling of glucocorticoid receptor (GR) and nuclear factor erythroid 2- related factor 2 (Nrf2) (Luo et al., 2020). Cong et al. showed that E prevents acute demyelination and regulates the number of microglia, astrocytes and oligodendrocytes (Cong et al. 2021), which is consistent with the results of this study.

Conclusion

It can be concluded that epilepsy due to frequent seizures and gonadectomy due to the decrease in testosterone levels can cause memory and learning disorders, reduce the density of hippocampal neurons, and increase inflammation in the brain structures. In contrast, the E extract, with its anti-inflammatory and neuroprotective properties, can prevent the complications of epilepsy and also has the property of increasing testosterone.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Ilam University, Ilam, Iran (Code: IR.ILAM.REC.1403.006).

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Authors' contributions

Conceptualization: Aref Nooraei, Salman Soltani, Marzihe Darvish, and Marzihe Havasi: Data analysis: Salman Soltani; Methodology: Javad Cheraghi; Writing the original draft: Aref Nooraei, Marzihe Darvish, and Marzihe Havasi: Review and editing: Javad Cheraghi and Salman Soltani,

Conflict of interest

The authors declared no conflict of interest.

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