Ferulic Acid Inhibits Catamenial Epilepsy Through Modulation of Female Hormones

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Research Article

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Abstract

Approximately 40% of women with epilepsy experience perimenstrual seizure exacerbation, referred to as catamenial epilepsy. These seizures result from cyclic changes in circulating progesterone and estradiol levels and there is no effective treatment for this form of intractable epilepsy. We artificially increased progesterone levels and neurosteroid levels (pseudo-pregnancy) in adult Swiss albino female mice (19-23 g) by injecting them with pregnant mares' serum gonadotropin (5 IU s.c.), followed by human chorionic gonadotropin (5 IU s.c.) after 46 hours. After this, ferulic acid (25, 50, 100 mg/kg i.p.) treatment was observed for 10 days. During treatment, progesterone, estradiol, and corticosterone levels were estimated in blood on days 1, 5, and 10. Neurosteroid withdrawal was induced by finasteride (50 mg/kg, i.p.), a 5 α-reductase inhibitor on treatment day 9. Twenty-four hours after finasteride administration (day 10 of treatment), seizure susceptibility was evaluated with the sub-convulsant pentylenetetrazol (PTZ) dose (40 mg/kg i.p.). Four to six hours after PTZ, animals were assessed for depression like phenotypes using tail-suspension test (TST). Four to six hours following TST, animals were euthanized, and discrete brain parts (cortex and hippocampus) were separated for estimation of norepinephrine, serotonin, and dopamine as well as glutamic acid decarboxylase enzyme activity. PMSG and HCG treatment elevated progesterone and estradiol levels, assessed on days 1, 5, and 10 causing a state of pseudo-pregnancy. Treatment with finasteride increases seizure susceptibility and depression-like characteristics possibly due to decreased progesterone levels and elevated estrogen levels coupled with decreased monoamine and elevated corticosterone levels. Ferulic acid treatment, on the other hand, significantly decreased seizure susceptibility and depression like behaviours, possibly as a result of increased progesterone, restored estradiol, corticosterone, monoamine, and glutamic acid decarboxylase enzyme activity. We concluded that ferulic acid exhibited antiepileptic effects in a mouse model of catamenial epilepsy and comorbid depression due to its restorative effects on circulating hormones and cerebral monoamine and glutamic acid decarboxylase enzyme activity.

Introduction

Catamenial epilepsy, affecting around 40% of women with epilepsy, describes a condition in which seizures worsen during the menstrual cycle (Maguire and Nevitt 2019]. Seizures mostly occur during follicular phase (type I seizures), peri-menstrual phase (type II seizures), and luteal phase (type III seizures). Type III seizures in the luteal phase are typically observed in non-ovulatory menstrual cycles (Joshi and Kapur 2019). Current clinical practices involve the use of non-hormonal (e.g. clobazam or acetazolamide) and hormonal (progesterone) treatment in women with normal menses. However, in women with irregular menses, treatment involves synthetic hormones such as medroxyprogesterone, triptorelin and goserelin which ceases the menstruation and defends against catamenial seizures (Maguire and Nevitt 2019). However, a double-bind, multicenter, phase III trial showed limited efficacy for suppressing these seizures (Herzog et al. 2012). In line with this, Nestrone, a progesterone receptor agonist, also reported to increase seizure frequency in epileptic animals (Shiono et al. 2019). Thus, it would be inappropriate to focus therapies solely on the development of progesterone receptor

modulators, since development and progression of catamenial seizures involve the complex interplay of multiple hormones, including progesterone and estrogen (Reddy 2004; 2013).

In addition to that, antiepileptic drugs (AEDs) (e.g., carbamazepine, phenytoin, topiramate, phenobarbital) have also been reported to stimulate the enzyme induction via CYP3A4 which leads to the enhanced metabolism of endogenous steroidal hormones. This may account for the pharmacoresistance to AEDs associated with catamenial epilepsy (Isojarvi et al. 2005; Brodie et al. 2013). The affected females additionally tend to have epilepsy-associated depression due to the interaction between female hormones and their central nervous system (CNS). Approximately 1–7 percent of women with epilepsy suffer from mood disorders related to their menstrual cycle (Guille et al. 2008). There is no specific drug therapy available to treat catamenial epilepsy as well as comorbid depression, thus worsening the disease prognosis (Reddy 2004).

Ferulic acid, one of the major bioactive phytoconstituent of *Ferula Asafoetida*, is considered useful in the treatment of several fertility related complications concerning women such as sterility, unwanted abortion, pre-mature labor, painful, difficult, and excessive menstruation and leucorrhoea (Mahendra and Bisht, 2012). It has also been reported to increase the secretion of progesterone hormone (Keshr et al. 1999; Mahendra and Bisht 2012; Zia-Ul-Haq et al. 2012). Recently, Thapliyal and colleagues reviewed anticonvulsant effects of ferulic acid in various animal models of epilepsy, but no study was reported/mentioned in view of catamenial epilepsy (Thapliyal et al. 2021). Thus, based on available data, ferulic acid appears to be extremely valuable in treating catamenial epilepsy through its ability to normalize female reproductive hormone levels (Keshr et al. 1999; Mahendra and Bisht 2012; Zia-Ul-Haq et al. 2012) as well as restoring altered monoamine levels (Thapliyal et al. 2021; Singh et al. 2017). Furthermore, considering the safety of naturally derived phytoconstituents, present study was envisaged to elucidate anticonvulsant effect of ferulic acid in an animal model of catamenial epilepsy (Thapliyal et al. 2021; Singh et al. 2017).

Material And Methods

Animals

All studies were performed according to protocols approved by the Institutional Animal Ethical Committee (Approval no. 107/99/CPCSEA -2016-08), Punjabi University, India. Adult female Swiss albino mice (19-23g, 10-12 weeks old) were used for these studies, four to five mice were kept in a cage, mice had ad libitium access to food and water, and they were maintained on a 12h light/dark cycle.

Drugs and chemicals

Pregnant mare's serum gonadotropin (PMSG), human chorionic gonadotropin (HCG), finasteride, β-Cyclodextrin, estradiol, progesterone, pentylenetetrazole, ferulic acid, dopamine, serotonin and methanol (HPLC grade) were obtained from Sigma (USA), Heptane sulfonic acid (Merck, India), perchloric acid and

tartaric acid (Spectrochem, Mumbai), norepinephrine (Troikaa Pharmaceuticals, India). Sodium-L-glutamate (S D Fine-Chem limited, India), trichloroacetic acid and pyridoxal-5-phosphate (Sigma Chemical Co., St. Louis MO, USA), ninhydrin and gamma-aminobutyric acid (GABA) (Sigma Chemical Co., St. Louis MO, USA), triton X-100 (scintillation grade) (Loba Chemie) were also used in the study. All other chemical reagents were of analytical grade.

Neurosteroid withdrawal model of catamenial epilepsy and seizure susceptibility

A state of prolonged high serum progesterone level (pseudopregnancy) was induced in mice by sequential injection of pregnant mares' serum gonadotropin PMSG (5 IU s.c.) followed 46 h later by human chorionic gonadotropin HCG (5 IU s.c.) (Reddy et al. 2001). The day of the second gonadotropin injection was considered day 0. Neurosteroid withdrawal was induced by treatment with finasteride (50 mg/kg, i.p.), a 5α-reductase inhibitor on day 9 that blocks the conversion of progesterone to allopregnanolone, decreased neurosteroid levels, mimicking perimenstrual changes in women.

Animals were randomly divided into six cohorts (n=6 each). Cohort I (Naïve group), cohort II to VI were administered PMSG followed by HCG 46 h after, followed by finasteride (50 mg/kg, i.p.) on day 9. Cohort II and III were labelled as pseudo pregnant (positive control) and finasteride (negative control) groups, respectively. Following PMSG and HCG, cohort IV to VI also received ferulic acid (25, 50 and 100 mg/kg i.p.) for 10 days. The cohort II to VI received finasteride (40 mg/kg, i.p.) on day 9 of treatment and to assess seizure severity subconvulsant PTZ dose (40 mg/kg, i.p.) was given on day 10 (24h after finasteride injection) (Figure 1) (12). Latency to clonic seizures, latency to generalized tonic-clonic seizures (GTCS) as well as seizure severity was observed according to modified Racine's scale, Stage 0: no response, Stage 1: hyperactivity, restlessness and vibrissae twitching, Stage 2: head nodding, head clonus and myoclonic jerks, stage 3: unilateral or bilateral limb clonus, Stage 4: forelimb clonic seizures, Stage 5: generalized tonic–clonic seizures with falling, Stage 6: hind limb extensor and death was considered as Stage 7. Animals failing to show clonic spasms which are characterized by the rapid involuntary rhythmic contraction and relaxation of limbs lasting longer than 5 s were scored as protected. Animals failing to show clonic spasms (rapid involuntary rhythmic contraction and relaxation of limbs) lasting longer than 5 sec were considered protected and scored 0. Seizure scoring was performed live by one of the authors (TS) in a blinded manner. Four to six hours after PTZ injection (animals restore normal behavior two hours following PTZ injection), animals were evaluated for depression-like phenotypes using a tail suspension test (only once on treatment day 10). Four hours following TST, animals were anesthetized, chest cavity was cut opened to collect blood directly from heart and brain sub regions (cortex and hippocampus) were harvested for estimation of monoamines. We have been using PTZ model (acute as well as chronic) in our lab for over a decade to study various phenomenon related to epileptogenesis, and we are pioneers in using post kindled animals for depression and cognitive comorbidities of epilepsy. Our pilot studies have shown that 4h after tail

suspension test, circulating hormones and cerebral monoamine levels return to baseline, which explained animal euthanasia on these time intervals.

Tail suspension test

The tail suspension test was conducted as previously described (Singh et al. 2017), with some modifications on day 10. Briefly, mice were individually suspended by tail with a clamp (1 cm from the tip of the end). A mouse was suspended for a total of 6 min, and the duration of immobility was recorded during the final 4 min interval of the test. Mice were considered immobile only when they hung passively and completely motionless.

Determination of serum neurosteroids levels

Neurosteroids were estimated in serum samples using previously reported HLPC-UV method (Wei et al. 1990) with slight modifications. To prepare sample, serum (10 µl) was digested with ethyl ether (1 ml), vortexed for 3 min and then centrifuged at 3500 rpm for 5 min. The organic layer was transferred to a test tube and evaporated to dryness at 50 °C. Before injection, 50 µl mixture of methanol and water (60: 40 v/v) was added, vortexed and vibrated ultrasonically for 60 s. After centrifugation for 2 min at 4000 rpm, 20 µl of supernatant was injected into the system.

Waters HPLC system (Milford, USA) consisted of 515 binary pumps (Waters, USA), 2489 ultraviolet detector (Waters, USA) and rheodyne manual injector (20µl) was used. The chromatographic separation performed at room temperature was achieved using Zorbex SB-C18, reversed phase column (4.6 mm x 150 mm x 5 µm) (Agilent, USA) at 254 nm. The mobile phase consisted of a mixture of methanol: tetrahydrofuran: water (26:18:56 v/v/v) with a flow rate of 1 ml/min at room temperature, filtered using 0.45 µm membrane (Millipore, USA) and degassed using Transonic T 570/H, Elma, Germany. The data was acquired and processed in Empower Pro® Operating System (Waters®, Milford, USA). A stock solution of corticosterone, estradiol and progesterone were prepared in 1 mg/mL methanol and standard curve was plotted. Corticosterone, $y = 44261x + 13104$, $R^2 = 0.996$, estradiol, $y = 0.735 x + 0.905$, $R^2 = 0.987$, and progesterone, $y = 63171x - 38604$, $R^2 = 0.999$). The data were acquired and processed in Empower Pro Operating System (Waters, Milford, USA).

Neurochemical estimations

Monoamines (norepinephrine, dopamine, and serotonin) were estimated using HPLC-ECD as reported previously by our lab (Singh et al. 2016) and another half was used for estimation of total nitrite levels using microplate reader, also reported previously (Singh et al. 2015).

Glutamic acid decarboxylase (GAD) Activity

GAD activity was assayed according to the method reported (Wolf and Klemisch 1991). Enzyme activity was expressed as micro-gram GABA/ mg protein of wet tissue.

Statistical Analysis

The statistical analysis was performed using Graphpad prism® version 8 (Graph-Pad Software Inc., San Diego, CA, USA). Statistical significance was calculated using one-way ANOVA followed by Student-Newman-Keuls test. Each value was expressed as mean ± SEM, and statistical significance was considered at $p < 0.05$.

Results

Ferulic acid decreased seizure susceptibility following PTZ injection

The mean seizure severity score was significantly ($P<0.05$) increased in finasteride control animals in comparison to pseudopregnant control animals. However, ferulic acid (50 and 100 mg/kg) treatment significantly ($P<0.05$) reduced mean seizure severity score as compared to finasteride control group (Figure 2A).

Ferulic acid increased mean latency to clonic seizures and generalized tonic-clonic seizures (GTCSs) following PTZ injection

The mean latency time to the onset of clonic convulsions (seizure stage 3) was significantly ($P<0.05$) decreased in finasteride control group as compared to pseudopregnant control group. The mean latency to onset of clonic convulsions (seizure stage 3) remains unaffected in ferulic acid (25, and 50 mg/kg) treated animals as compared to finasteride control group. However, ferulic acid (100 mg/kg) significantly ($P<0.05$) increased mean latency time to onset of clonic seizures (seizure stage 3) as compared to finasteride control group (Figure 2B).

The mean latency time onset of GTCSs was also significantly ($P<0.05$) decreased in finasteride control group as compared to pseudopregnant control group. The onset of GTCSs remain unaffected in ferulic acid (25 mg/kg) as compared to finasteride control group. However, ferulic acid (50 and 100 mg/kg) significant ($P<0.05$) increased mean latency time to the onset of GTCSs as compared to finasteride control group (Figure 2C).

Ferulic acid treatment decreased immobility duration

The duration of immobility was significantly (P<0.05) increased in finasteride control group as compared to pseudopregnant control groups. However, ferulic acid (100 mg/kg) treatment significantly (P<0.05) reduced immobility time as compared to finasteride control group (Figure 2D).

Ferulic acid treatment restored progesterone, estradiol, and corticosterone levels

A significant (P<0.05) increase in the progesterone level was observed in the pseudopregnant and finasteride control groups as compared to naïve animals on days 1, 5 and 10. However, ferulic acid (25, 50 and 100 mg/kg) treatment significant (P<0.05) enhanced progesterone level as compared to finasteride control group on day 10 (Figure 3B, Supplementary Figure 1).

A significant (P<0.05) increase in the estradiol level was observed in the pseudopregnant and finasteride control animals as compared to naïve animals on days 1, 5 and 10. However, ferulic acid (100 mg/kg) significantly (P<0.05) reduced estradiol levels as compared to finasteride control group on day 10 (Figure 3C, Supplementary Figure 1).

The corticosterone level was significantly different ($F_{(5, 30)}$ = 22.55, P< 0.001) between different treatment cohorts. A significantly (P<0.05) enhanced levels of corticosterone was observed in finasteride control group as compared to pseudopregnant control group and naïve group on days 1, 5 and 10. However, the level observed in the ferulic acid (25, 50, and100 mg/kg) and was significantly (P<0.05) reduced as compared to finasteride control group on days 5 and 10 (Figure 3A, Supplementary Figure 1).

Ferulic acid treatment increased GAD enzyme activity

The GAD enzyme activity was significantly (P<0.05) was significantly increased in pseudopregnant as compared to finasteride control group. The ferulic acid (25, 50 and 100 mg/kg) significantly (P< 0.05) enhanced the GAD enzyme activity as compared to finasteride control group (Figure 3D).

Ferulic acid treatment restored norepinephrine levels

The cortical ($F_{(5, 30)}$ = 16.11, P < 0.0001) and hippocampal ($F_{(5, 30)}$ = 13.31, P < 0.0001) levels of norepinephrine was significantly different treatment cohorts. A significantly (P<0.05) reduced cortical and hippocampal level of norepinephrine was observed in finasteride control group as compared with pseudopregnant control group and naïve group. However, the cortical and hippocampal norepinephrine levels were significantly (P<0.05) enhanced in ferulic acid (25, 50, and 100 mg/kg) as compared to finasteride control group (Figure 4A, 4B).

Ferulic acid treatment restored dopamine levels

The level of dopamine was significantly ($F_{(5, 30)}$ = 6.21, P < 0.0005) different between different treatment cohorts. A significantly (P<0.05) reduced cortical and hippocampal dopamine level was observed in finasteride control group as compared with pseudopregnant control group and naïve group. However, the hippocampal dopamine level was significantly (P<0.05) enhanced in ferulic acid (25, 50, and 100 mg/kg) as compared to finasteride control group (Figure 4C, 4D).

Ferulic acid treatment restored serotonin levels

The cortical ($F_{(5, 30)}$ = 14.35, P < 0.0001) and hippocampal ($F_{(5, 30)}$ = 9.295, P< 0.0001) serotonin level was significantly different between different treatment cohorts. A significantly (P<0.05) reduced cortical and hippocampal level of serotonin was observed in finasteride control group as compared with pseudopregnant control group. However, the cortical and hippocampal serotonin was significantly (P<0.05) enhanced in ferulic acid (25, 50, and 100 mg/kg) as compared to finasteride control group (Figure 4C, 4D).

Discussion

In summary, we revealed the anticonvulsant effect of ferulic acid in a mouse model of catamenial epilepsy, evidenced by favourable seizure attenuation and curative effect on circulating progesterone, estradiol, and corticosterone levels along with restorative effect on GAD enzyme activity and monoamine levels. The study outcome and justification of methodology used are discussed herein.

Neurosteroid withdrawal, a well-accepted model of catamenial epilepsy, was used for this study. In consistent with the previous studies, gonadotropin regimen significantly increased progesterone levels (pseudo-pregnancy) as observed on different treatment days (Reddy et al. 2001; Reddy 2009). In order to simulate neurosteroid withdrawal, animals were additionally injected with finasteride, on treatment day 9, similar to what happens during menstruation. A neurosteroid withdrawal was associated with decreased seizure thresholds and an increase in seizure severity scores following PTZ injection, also reported previously (Reddy and Rogawski 2001; Reddy et al. 2001). Withdrawal of progesterone might have decreased its major metabolite, allopregnanolone, which exerts its anticonvulsant effects through modulation of the γ -aminobutyric acid type-A (GABA-A) receptor. Despite being ineffective clinically, animal studies have shown that biotransformation of progesterone to its neurosteroid derivative allopregnanolone produces increased GABA-A receptor mediated inhibition in the brain and prevents epilepsy (Joshi and Kapur 2019). The results were in agreement with an earlier study, in which finasteride failed to protect against convulsions after a subconvulsant PTZ challenge in control animals (Reddy and Rogawski 2001; Reddy et al. 2001). However, ferulic acid treatment increased seizure latency in comparison with finasteride control animals. Pseudo pregnant positive control animals also showed resistance to subconvulsant PTZ challenge as compared to finasteride control animals, possibly owing to elevated progesterone levels. The observed anticonvulsant effect of ferulic acid might be discussed in reference to the observed favourable hormonal changes as follows.

Progesterone plays a crucial role in the progression of catamenial epilepsy and has an anti-convulsant properties (Joshi and Kapur 2019). It is possible that medicinal plants/constituents, like ferulic acid, have fertility enhancing effects due to their progesterone elevating effects (Salih and Jaafar 2013; Keshr et al. 1999; Mahendra and Bisht 2012; Zia-Ul-Haq et al. 2012). This was also confirmed by the significantly higher progesterone levels in ferulic acid treated animals as compared to the finasteride-treated animals. Beside progesterone, estrogen also plays a promising role in the development of catamenial seizures. It generally has proconvulsant and epileptogenic properties in the rodents and humans (Logothetis et al. 1959). Among all three biologically active estrogens (i.e., estrone (E1), estradiol (E2) and estriol (E3), estradiol plays a major role in the catamenial epilepsy (Velíšková 2006; 2007). Therefore, alteration in the serum estradiol level was monitored on different days during treatment. Our results showed decreased estradiol levels in ferulic acid treated animals as compared to finasteride control animals. Hence, the effects of ferulic acid on convulsive activity were further explained by the effect of progesterone and estradiol levels along with the previously reported antiepileptic mechanisms (Thapliyal et al. 2021; Singh et al. 2017).

Studies in both experimental and clinical settings have shown that corticosterone, in addition to the two major neurosteroids, has the ability to affect seizure activity. There are multiple evidences that corticosterone triggers epileptogenesis in animals and is known for its proconvulsant effects (Kling et al. 1993; Roberts and Keith 1994; Karst et al. 1999; Hopper et al. 2018; Basu et al. 2021). There was a significant decrease in corticosterone level in ferulic acid animals, possibly due to the high level of progesterone, since progesterone decreases the serum levels of corticosterone (Basu et al. 2021). Thus, in light of the aforementioned findings, it can be stated that decreasing corticosterone levels in ferulic acid treated animals may help treating catamenial epilepsy.

GAD is a rate limiting enzyme responsible for the synthesis of the major inhibitory neurotransmitter GABA, and its down regulation is associated with increased seizure related phenotypes (Lloyd et al. 1986). There are reports that estrogen and progesterone modulate GAD enzyme activity (Wallis and Luttge 1980). Increased estrogen reduces the GAD activity whereas increased progesterone elevates GAD activity. Therefore, we also assessed GAD enzyme activity in the brain (cortex and hippocampus collectively). The present study showed elevated GAD enzyme activity in pseudopregnant animals as compared to naïve and finasteride control animals. Ferulic acid treatment increased GAD enzyme activity, and this increase could be due to increased progesterone or decrease estradiol levels, hence justifying our findings.

In addition, ferulic acid pre-treated animals also exhibited reduced immobility as compared to finasteride-treated controls. The high progesterone levels observed in ferulic acid -treated animals may contribute to its antidepressant effects, since progesterone has been reported to have similar antidepressant effects when chronically administered (Andrade et al. 2010, Li et al. 2012; 2013). The ferulic acid treatment restored monoamine levels as well as circulating corticosterone levels, also reported previously (Thapliyal et al. 2021; Singh et al. 2017). These changes are associated with depression in various clinical and preclinical studies (Thapliyal et al. 2021; Singh et al. 2017).

Conclusion

The present study concluded the antiepileptic effects of ferulic acid in a model of catamenial epilepsy pertaining to its restorative effects on circulating hormones, cerebral monoamine and GAD enzyme activity levels (Fig. 5). Therefore, herbal formulations using ferulic acid may be used as monotherapy or adjuvant therapy along with available AEDs for the treatment of epilepsy combined with comorbid depression in women with epilepsy.

Declarations

Authors' contributions HKD and TS performed experiments. TS and RKG conceived the idea, edited, and wrote the manuscript.

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Data availability: Data will be available on reasonable request

Compliance with ethical standards

Conflicts of interest/Competing interests None

Ethical approval The animal experiments were obtained the approval from the Animal Ethics Committee of Punjabi University, India.

Consent for publication: All authors have their consents for publication

Code availability: Not applicable

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Figures

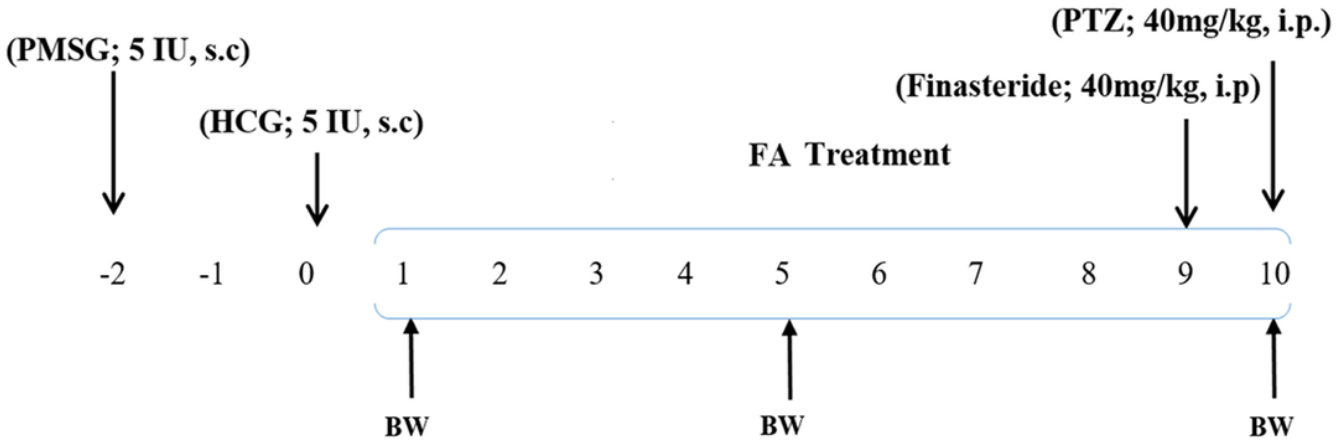

Figure 1

Schematic presentation of experimental study design

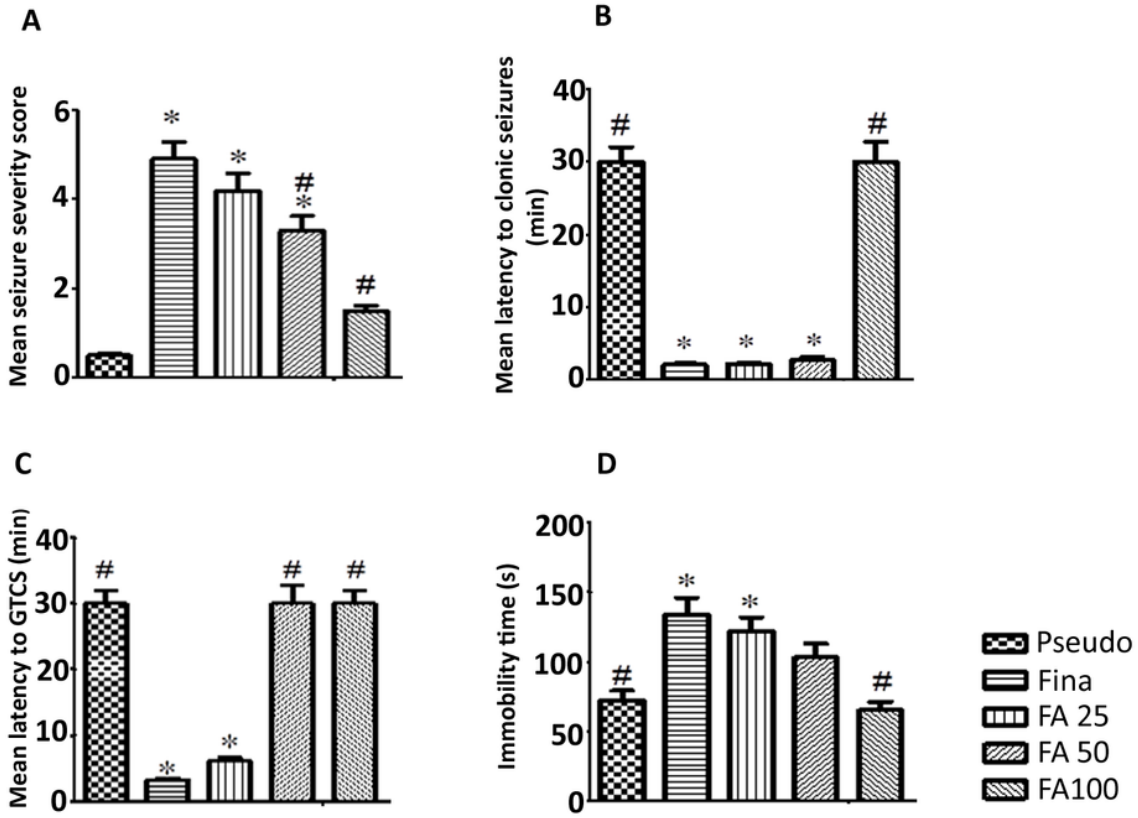

Figure 2

Effect of different treatments on (a) seizure severity score, (b) mean latency to develop clonic seizures (stage 3 seizures), (c) mean latency to develop generalized tonic-clonic seizures (GTCS), and (d) immobility time. Each value is expressed as mean± standard error mean (n=6). The significance level was

considered at P< 0.05 (Student Newman Keuls Post hoc test). *: significant as compared to pseudo pregnant control, # significant as compared to finasteride control.

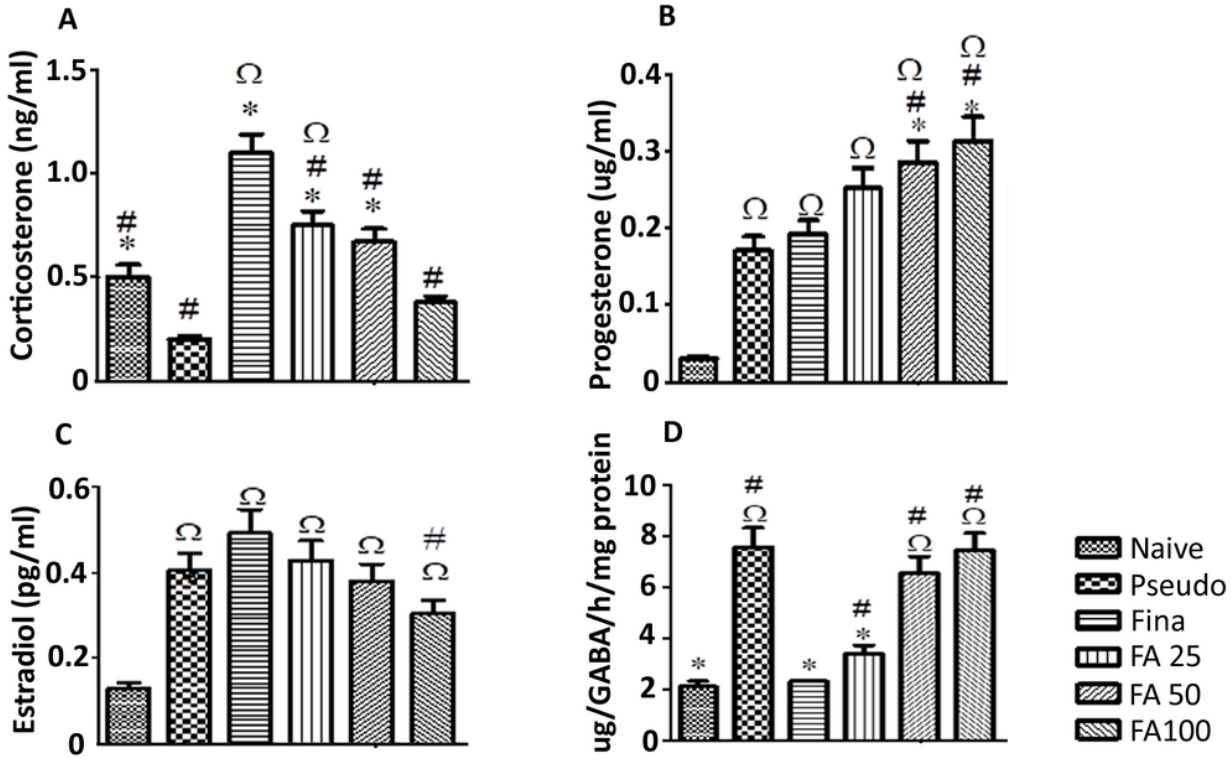

Figure 3

Effect of different treatments on corticosterone, progesterone, estradiol, and GAD enzyme activity on day 10. Each value is expressed as mean± standard error mean (n=6). The significance level was considered at P< 0.05 (Student Newman Keuls Post hoc test). *: significant as compared to pseudo pregnant control, # significant as compared to finasteride control, Ω: significant as compared to naïve.

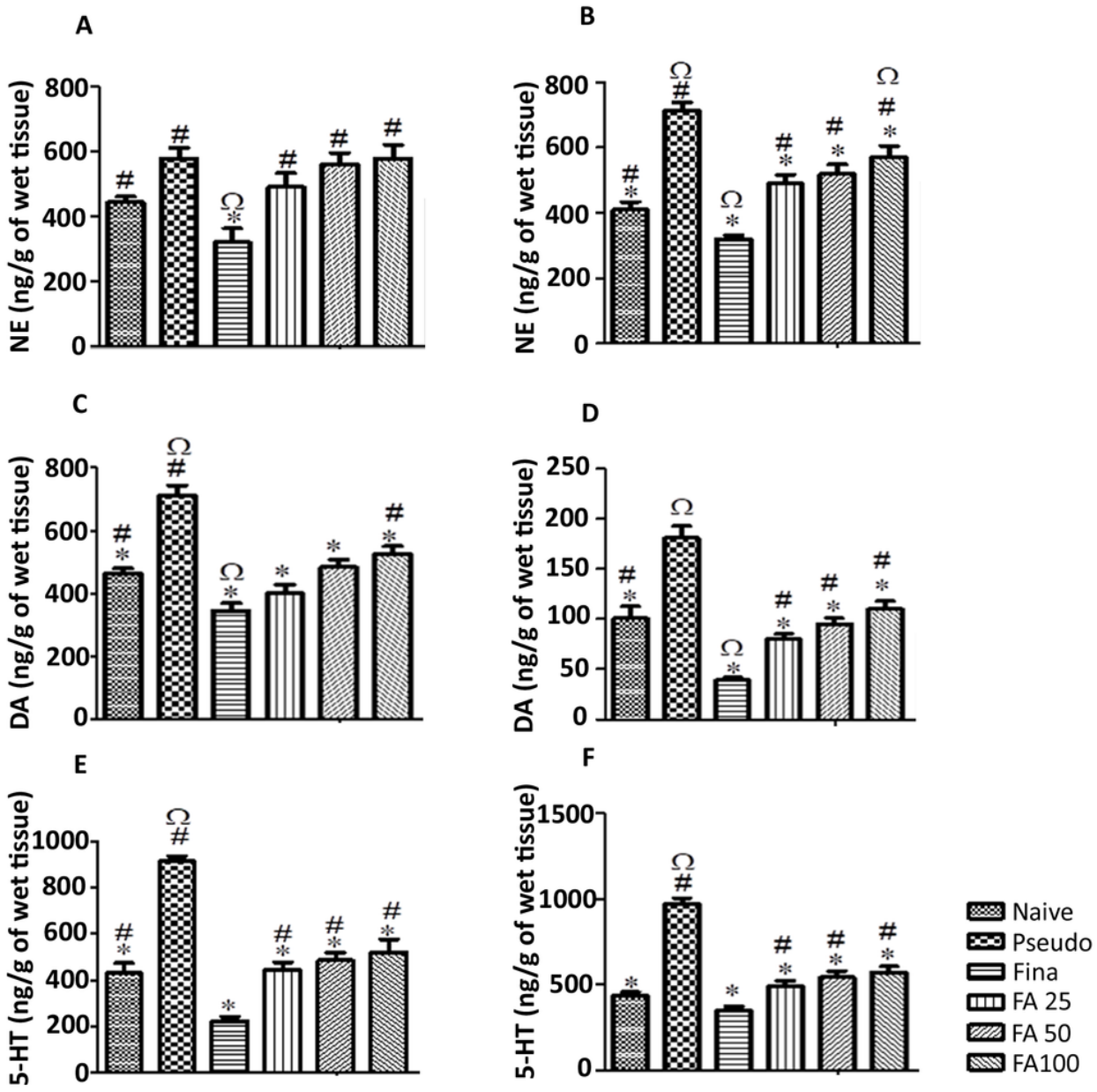

Figure 4

Effect of different treatments on cortical and hippocampal monoamine (norepinephrine, dopamine, serotonin (5-HT)) levels in mice brain. Each value is expressed as mean± standard error mean (n=6). The significance level was considered at P< 0.05 (Student Newman Keuls Post hoc test). *: significant as compared to pseudo pregnant control, # significant as compared to finasteride control, Ω: significant as compared with naïve.

Image not available with this version

Figure 5

Schematic representation of the anticonvulsant mechanism of ferulic acid

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfigure1.tif