Araştırma Makalesi/Original Article
Makale Dili "İngilizce"/Article Language "English"

Türk Hijyen ve Deneysel Biyoloji Dergisi

Evaluate the effects of rosmarinic acid in ovariectomized rats: urethane-induced cortical oscillations

Ovariektomize sıçanlarda rosmarinik asidin etkilerinin değerlendirilmesi: üretan indüklü kortikal osilasyonlar

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ABSTRACT

Objective: Diminished sleep quality is one of the most common menopausal symptoms. It is well known that sleep loss also contributes to memory impairment. Sleep disturbance and cognitive deficit in menopause might be related to a cholinergic deficit during the postmenopausal period. In the present study, we examined the protective effects of rosmarinic acid (RA) on brain activity under urethane anesthesia, memory, and cholinergic markers in ovariectomized rats.

Methods: Wistar rats were randomly divided into four groups: sham (SH); RA-treated (RA); Ovariectomized (OVX); Ovariectomized+RA-treated (OVXRA). RA (50mg/kg, daily) was administered orally by gavage for four weeks after ovariectomy. After four weeks period, we tested object localization memory (OLM). After the behavioral tests, we recorded urethane-induced spontaneous brain activity as a model of sleep brain activity to assess ovariectomy-related sleep alterations. At the end of the experimental period, we measured acetylcholine (Ach) level and acetylcholinesterase (AChE) activity by biochemical methods.

ÖZET

Amaç: Azalan uyku kalitesi en yaygın gözlenen menopoz semptomlarından biridir. Uyku kaybının hafıza bozukluğuna katkıda bulunduğu iyi bilinmektedir. Menopoz sonrası dönemde gözlenen uyku ve bilişsel bozukluk kolinerjik ileti bozukluğuyla ilişkili olabilir. Bu çalışmada, ovariektomi yapılmış sıçanlarda rosmarinik asidin (RA) üretan anestezisinde beyin aktivitesi, uzaysal hafıza ve kolinerjik belirteçler üzerindeki koruyucu etkisini inceledik.

Yöntem: Wistar albino sıçanlar rastgele olarak sham (SH); RA uygulanan (RA); Ovariektomi yapılmış (OVX); Ovariektomi yapılan ve RA uygulanan (OVXRA) şeklinde dört gruba ayrıldı. RA (50 mg/kg, günlük) ovariektomi sonrası dört hafta süreyle gavaj yoluyla uygulandı. Dört haftalık sürenin ardından uzaysal hafızanın test edilmesi için obje uzaysal hafızası (OLM) testi gerçekleştirildi. Davranış testlerinden sonra, ovariektomi ile ilişkili uyku değişikliklerini değerlendirmek için uyku beyin aktivitesi modeli olarak üretan indüklü spontan beyin aktivitesi kaydedildi. Deney süresi sonunda biyokimyasal yöntemler kullanılarak asetilkolin (ACh) düzeyi ve asetilkolinesteraz (AChE) aktivitesi ölçüldü.

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Geliş Tarihi / Received : 07.06.2021
Kabul Tarihi / Accepted : 20.10.2021

DOI ID : 10.5505/TurkHijyen.2022.68815

Kantar D, Acun AD, Er H. Evaluate the effects of rosmarinic acid in ovariectomized rats: urethane-induced cortical oscillations
Turk Hij Den Biyol Derg, 2022; 79(4): 632 - 645

Results: OVX rats exhibited elevated slow-wave delta and REM delta, theta, beta power. The amplitudes of slow-wave delta and REM delta, theta, beta oscillations were increased in the OVX rats. In parallel to impaired OLM, decreased Ach level and increased AChE activity were detected in OVX rats. Four weeks RA treatment was significantly improved oscillatory power and amplitude alterations in the OVXRA group versus the OVX group. OLM index was increased in the OVXRA group compared to the OVX group. Decreased AChE activity, as well as increased Ach level, was observed in the OVXRA group versus the OVX group.

Conclusion: Thus, the present study indicates that RA might be protective against ovariectomy-induced oscillatory changes under urethane anesthesia and memory impairment by improving the cholinergic system.

Key Words: Ovariectomy, urethane-induced oscillations, memory, cholinergic system, rosmarinic acid

Bulgular: OVX sıçanlarda, artan yavaş dalga delta ve REM delta, teta, beta gücü gözlendi. Buna ek olarak, OVX sıçanlarında yavaş dalga delta ve REM delta, teta, beta salınımlarının genlikleri arttığı izlendi. Bozulmuş OLM'ye paralel olarak, OVX sıçanlarında azalmış ACh seviyesi ve artmış AChE aktivitesi tespit edildi. Dört haftalık RA tedavisi, OVX grubuna kıyasla OVXRA grubunda osilasyonlarda gözlenen güç ve genlik değişikliklerini önemli ölçüde iyileştirdi. OLM indeksinin OVXRA grubunda OVX grubuna göre arttığı görüldü. OVXRA grubunda OVX grubuna göre AChE aktivitesinde azalış ve ACh seviyesinde artış gözlendi.

Sonuç: Sonuç olarak bu çalışma, RA'nın kolinerjik sistemi iyileştirerek ovariektominin neden olduğu üretan anestezisi indüklü osilasyon değişikliklerine ve hafıza bozulmasına karşı koruyucu olabileceğini göstermektedir.

Anahtar Kelimeler: Ovariektomi, ürethan-indüklü osilasyonlar, hafıza, kolinerjik sistem, rosmarinik asit

INTRODUCTION

Menopause is defined as the end of menstruation due to the loss of follicular activity in the ovaries. In menopause, a sharp decrease of estradiol and progesterone production occurs in ovaries which is mainly related to the depletion of the ovarian reserves (1). Given that estrogen is a critical signaling molecule within the brain (2), this permanent fall in circulating estradiol levels affects multiple brain functions. Reports have emphasized the estrogen hormone in sleep regulation and circadian rhythm (3). One of the most common menopausal symptoms in women is diminished sleep quality (4-6). Given that sleep loss ultimately impairs memory (7, 8), improving sleep quality can be a good way to promote cognitive functions in aging women. Studies indicated that post-menopausal women had higher slow-wave sleep percentages and elevated beta EEG power that accompanies the disturbed sleep quality (9). In parallel, ovariectomized (OVX) rats spent much time in spontaneous slow-wave sleep (SWS) (non-rapid eye movement, NREM) and/or rapid eye movement (REM) sleep compared to OVX rats treated with estradiol and/or progesterone (10). Findings showing an increment in delta EEG activity following sleep deprivation in NREM sleep and a decrement in the night following a daytime nap indicate that delta EEG power reflects the differences in homeostatic sleep regulation (11, 12). Furthermore, elevated beta power is found in some insomniacs and may indicate a higher arousal level related to less satisfactory sleep (13, 14). Besides, both rats and humans exhibit cortical theta wave activity during REM sleep. And

increased sleepiness is associated with increased theta wave power (15, 16). So spectral analysis of the EEG may provide objective sleep measures that change with estrogen deficiency and may be informative to unravel the sleep disturbance problem during menopause.

It has been shown that urethane anesthesia is a good model to study sleep-related EEG changes in rats (17). Urethane anesthesia has sleep like cyclic fluctuations between active REM and quiet SWS stages that closely mimic the full spectrum of natural sleep. So we used this model to examine the oscillatory changes in sleep at OVX rats.

As mentioned above, sleep disturbance in the postmenopausal period may contribute to cognitive decline might be related to the cognitive decline seen in women. It is well known that sleep plays an important role in memory consolidation (18). Both REM and SWS periods, which have specific characteristics of brain oscillations, are associated with the formation of memory. Thus, we analyzed object localization memory in OVX rats in relation to the oscillatory changes during urethane anesthesia.

Both sleep disturbance and cognitive deficit in menopause might be related to a cholinergic deficit in aged women (19). It is known that loss of ovarian activity decreases cholinergic transmission (20, 21). In rodents, ovariectomy reduces performance on learning and memory tasks and this decline in performance parallels a decline in cholinergic activity and choline acetyltransferase (ChAT) levels in several brain regions (22). In addition to its role in cognition, the cholinergic system is also a known modulator of sleep (23). During REM sleep, when low voltage fast activity (LVFA) is present in the cortical EEG, ACh release is elevated, while the release is reduced during SWS, when slow delta waves (<4 Hz) dominate the EEG (24, 25). Therefore, improving cholinergic transmission can be beneficial in the postmenopausal period for both sleep and memory functions. A known strategy to overcome the disorders related to estrogen deficiency is hormone replacement therapy. But it has been associated with an increased risk of breast cancer and cardiovascular disease for some women (26). Nowadays, there has been a growing interest in natural products (27). A natural compound, rosmarinic acid, has been proposed as a beneficial agent with its multiple bioactivities. In addition to its well-known antioxidant properties, recent studies indicated that RA can improve cholinergic transmission (28, 29). Therefore we suggest that RA may have a potential for the treatment of menopausal symptoms. However, no enough studies are investigating the effects of RA on sleep and cognitive alterations seen in menopause. We also aimed to investigate the protective effects of RA on sleep EEG and memory in OVX rats.

MATERIAL and METHOD

Animals

Thirty two healthy female Wistar rats, aged five months, weighing 300 to 350 g were used. Animals were housed in stainless steel cages in groups of 4 rats per cage at standard conditions (24 ± 2°C and 50 ± 5% humidity) with a 12 h light-dark cycle and given food and water ad libitum. The experiments were performed between 9:00 and 17:00. Rats were randomly divided into four groups (n = 8 per group): Group 1: rats were sham-operated and treated with saline (SH); Group 2: rats were sham-operated and treated with rosmarinic acid (RA); Group 3: rats were ovariectomized treated with saline (OVX); Group 4: rats were ovariectomized and treated with rosmarinic acid (OVXRA). The surgical procedures of ovariectomy were as follows: the rats were intraperitoneally anesthetized with a combination of ketamine (80 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) A vertical incision was made inferior to the abdomen. The uterine tubes were clamped, and the ovaries were excised. The skin was closed with wound clips. RA (Carbosynth, San Diego, CA, USA; 50 mg/kg/day, 50 mg of RA dissolved in 0.9% saline solution, to a total volume of 1 mL) was given via gavage for 30 days. After the experimental period, all rats were anesthetized under urethane and

brains were perfused transcardially with heparinized saline. Brain tissues were collected and stored frozen at −80°C until assay determinations.

Object training and testing procedures

Object location memory (OLM) was tested in a experimental apparatus was an open-field box (40x40x40 cm) made of light brown-painted wood, as previously described (30). The floor was covered with sawdust, and the box was placed in a dimly illuminated room. The objects to be discriminated were white glass light bulbs (5 cm diameter, 10 cm length). On the training trial, the rat was placed in the box and allowed to explore two identical objects, placed 5 cm away from the corners of the apparatus for 5 min. To avoid the presence of olfactory trails, sawdust was stirred and the objects were cleaned with 70% ethanol between rats. Retention was tested 24 h after the training trial. For OLM testing, one copy of the familiar object was placed in the middle of the box, and the other familiar object was placed in the same location as during the training trial. All combinations and locations of objects were randomly used in a balanced manner to reduce potential biases due to preference for particular locations. The rat was placed in the experimental apparatus for 5 min, and the time spent exploring each object and the total time spent exploring both objects were recorded. Exploration of an object was defined as pointing the nose to the object at a distance of <1 cm and/or touching it with the nose. The place discrimination index was calculated by using the formula, the time spent with the object moved to a novel place/the total time spent in exploring both the object moved to a novel place and the object remaining in the familiar place × 100.

EEG recordings and analyses

EEG was recorded between 09:00 am and 02:00 p.m. Rats were anesthetized (24 g/100 ml) with intraperitoneal injections of urethane (1.2 g/kg, Sigma-Aldrich, St Louis, MO, USA). The head of the anesthetized animal was attached to the standard stereotaxic frame and four small holes (1.5 mm diameter) were drilled for the placement of the stainless steel electrodes. Recording electrodes were placed bilaterally on temporal and frontal cortices and reference and ground electrodes were placed on the cerebellar skull. The anesthetized animal was moved into a sound-attenuated recording room. The mean background noise level of the recording room measured 46 dB with a sound level meter (Testo 816 Sound Level Meter, Germany). The EEG signal was amplified (Brainamp EEG/EP Amplifier, Brain Products, Munich, Germany), band-pass filtered (0.1-300 Hz), and digitized at a 1000 Hz sampling rate (Brainvision Recorder, Brain Products, Munich, Germany). EEG signal was recorded for 10 minutes. The EEG data were filtered (0.1-150 Hz) and segmented into SWS and REM periods. Then each period was segmented into 2 s epochs. Frequency analysis was performed using a fast Fourier transform (FFT) algorithm with a 10% Hanning window and spectral EEG powers computed. The EEG variables chosen were absolute power in three frequency bands, delta (0.5–4 Hz), theta (4–8 Hz), beta (13–28 Hz). The epochs were digitally filtered in the delta, theta, and beta frequency ranges. Subsequently, we measured the maximum peak-to-peak amplitudes for each rat's averaged response in terms of microvolts. All analyses were performed with the BrainVision Analyzer program (Brain Products GmBH).

Acetylcholine level

The ACh level was measured using an Amplex® Red Acetylcholine/Acetylcholinesterase Assay Kit (A-12217; Invitrogen, USA). The isolated brain tissues were homogenized for ACh (20 mM sodium phosphate buffer, pH 7.4). According to the manufacturer's instructions, reactions were initiated by adding 100 µl of the working solution, containing 400 µM Amplex Red reagent, 2 U/ml horseradish peroxidase (HRP), 0.2 U/ml choline oxidase, and 1 U/ml acetylcholinesterase, to each microplate well containing 100 µl of the standard or test sample. Each reaction was incubated for 1 h at room temperature with plate agitation and

protection from light. Absorbance was then measured using a microplate reader (Molecular Devices, USA) at a wavelength of 563 nm. Acetylcholine levels were calculated from a standard curve and expressed as mmol/g protein.

Acetylcholinesterase activity

The AChE activity was also measured using an Amplex® Red Acetylcholine/Acetylcholinesterase Assay Kit (A-12217; Invitrogen). The isolated brain tissues were homogenized for AChE (20 mM sodium phosphate buffer, pH 7.4). A working solution, containing 400 µM Amplex Red reagent, 2 U/ml HRP, 0.2 U/ml choline oxidase, and 100 µM acetylcholine, was used for AChE activity measurement. Absorbance was measured using a microplate reader (Molecular Devices, USA) at a wavelength of 590 nm.

Statistical analysis

The statistical analysis of the obtained data was performed by SPSS (SPSS 18.0, SPSS Inc., Chicago, IL) software for Windows. Statistical comparisons between groups were performed by using the One-way ANOVA test and post hoc Bonferroni test.

The study was approved by the Akdeniz University Animal Experiments Local Ethics Committee (Date: 08.02.2021 and Number: 28).

RESULTS

The mean values of the OLM index for the testing period are given in Figure 1. There was no statistically significant difference in the OLM index between groups during the training period ($p > 0.05$). In the test period, there was a statistically significant difference in OLM index between groups [$F_{3.28} = 11.81$, $p < 0.01$]. In posthoc comparisons, it was found that the OVX group had a significantly lower OLM index compared with the other groups ($p < 0.01$). OLM index was significantly higher in the OVXRA group compared to the OVX group ($p < 0.01$).

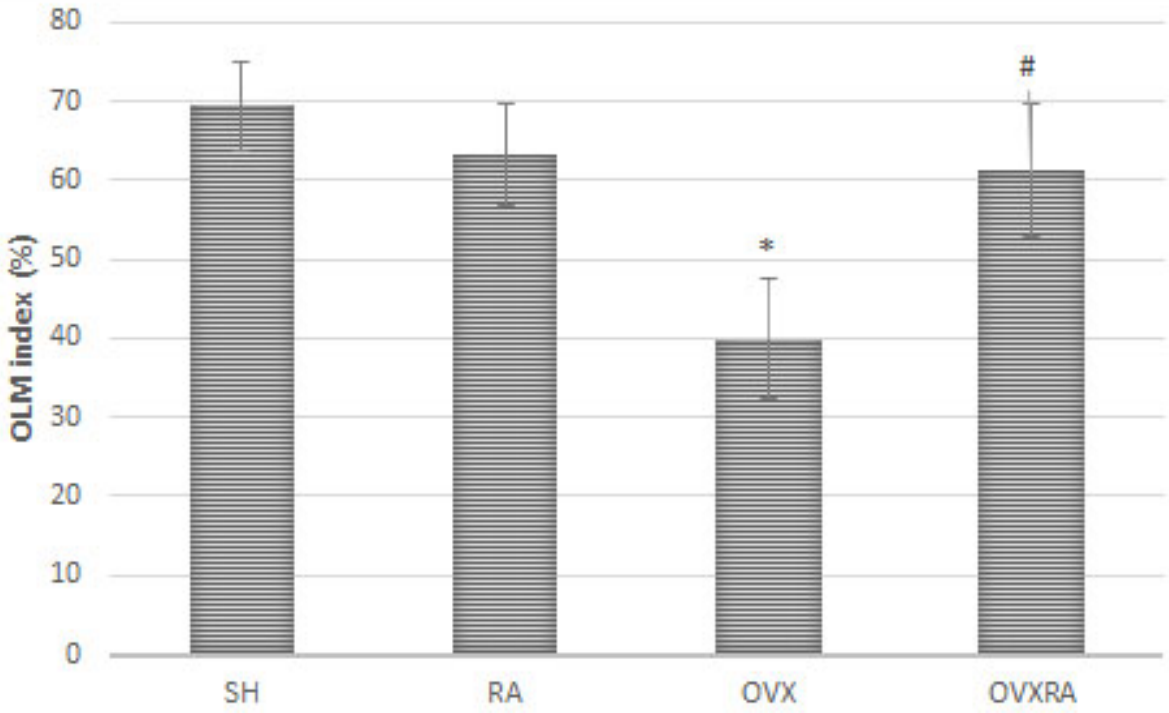

Figure 1. OLM index values of all experimental groups. The results are presented as mean±SEM, n=8 for each group. (*significant vs. SH group; #significant vs. OVX group)

Slow-wave delta-band power spectrum and mean values of all groups are given in Figure 2A and 2B. Figure 2C shows the slow-wave delta oscillations and Figure 2D shows the mean amplitude values of peak-to-peak delta oscillations of all groups. There was a significant difference between group in terms of delta-band power [$F_{3.28}$= 50.21, p < 0.01] and peak-to-peak delta oscillations [$F_{3.28}$= 16.80, p < 0.01]. In post hoc comparisons, it was found that the OVX group had significantly higher SW delta oscillatory responses and delta-band power compared with the other groups over frontal locations (p < 0.01). The SW delta power and amplitudes were significantly lower in the OVXRA group than the OVX group (p < 0.01).

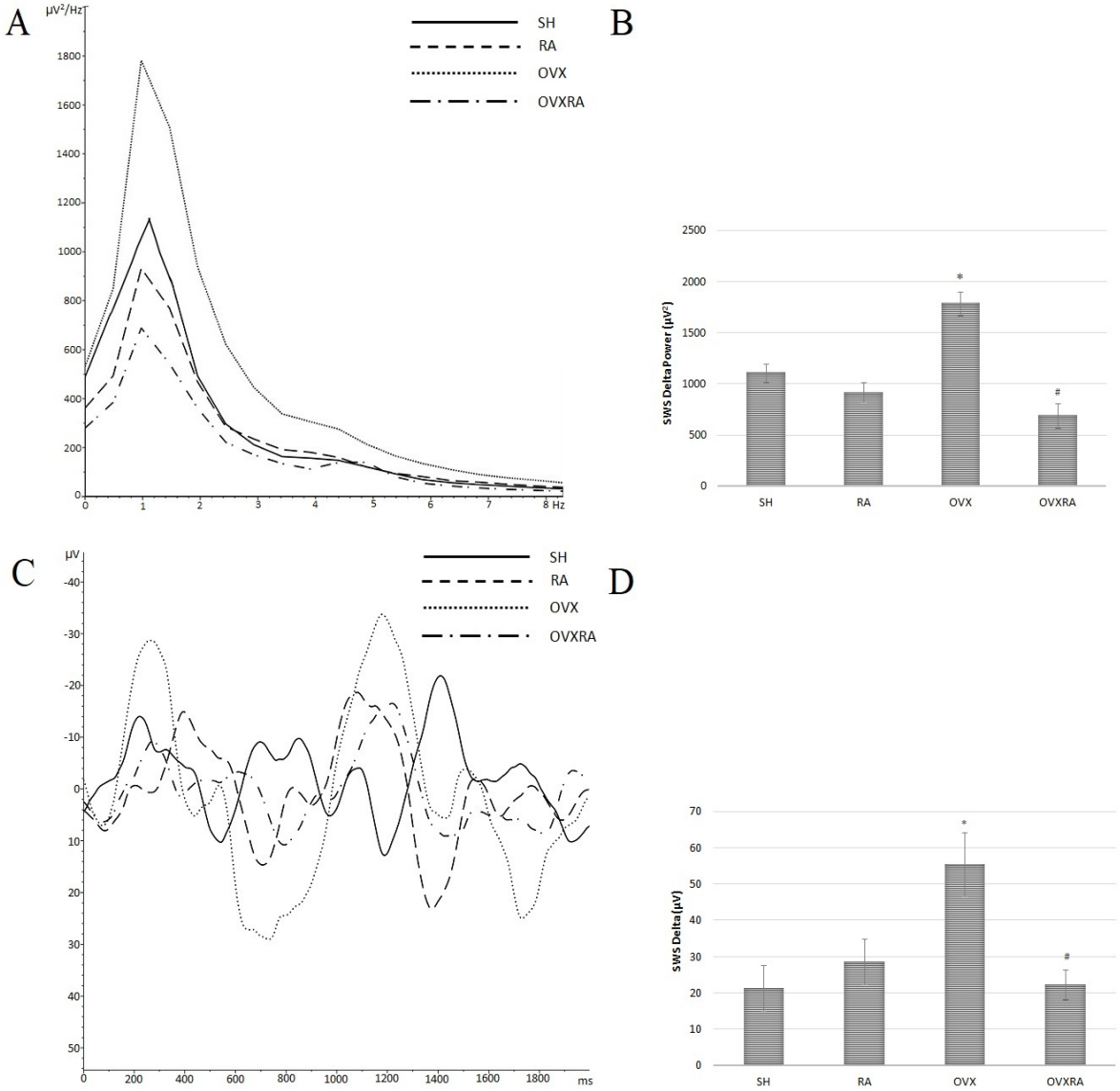

Figure 2. OLM index values of all experimental groups. The results are presented as mean±SEM, n=8 for each group. (*significant vs. SH group; #significant vs. OVX group)

REM delta/teta band power spectrum and mean values of all groups are shown in Figure 3A and 3B, respectively. Figure 3C shows the REM beta band power spectrum and Figure 3D shows the mean values. REM delta, theta, beta oscillations and peak-to-peak amplitude values of oscillations for all groups are presented in Figure 4A, 4B, and 4C, respectively. There was a significant difference between group in terms of delta, theta and beta band powers [Delta: $F_{3.28} = 22.34$, $p < 0.01$; Theta: $F_{3.28} = 11.95$, $p < 0.01$; Beta: $F_{3.28} = 24.09$, $p < 0.01$] and peak-to-peak delta, theta and beta amplitudes [Delta: $F_{3.28} = 22.71$, $p < 0.01$; Theta: $F_{3.28} = 27.18$, $p < 0.01$; Beta: $F_{3.28} = 14.29$, $p < 0.01$]. In post-hoc comparisons, it was found that the OVX group had significantly higher oscillatory responses and power compared with the other groups over frontal locations for all related bands ($p < 0.01$). The all REM band powers and amplitudes were significantly lower in the OVXRA group than the OVX group ($p < 0.01$) and all values returned to the SH group level.

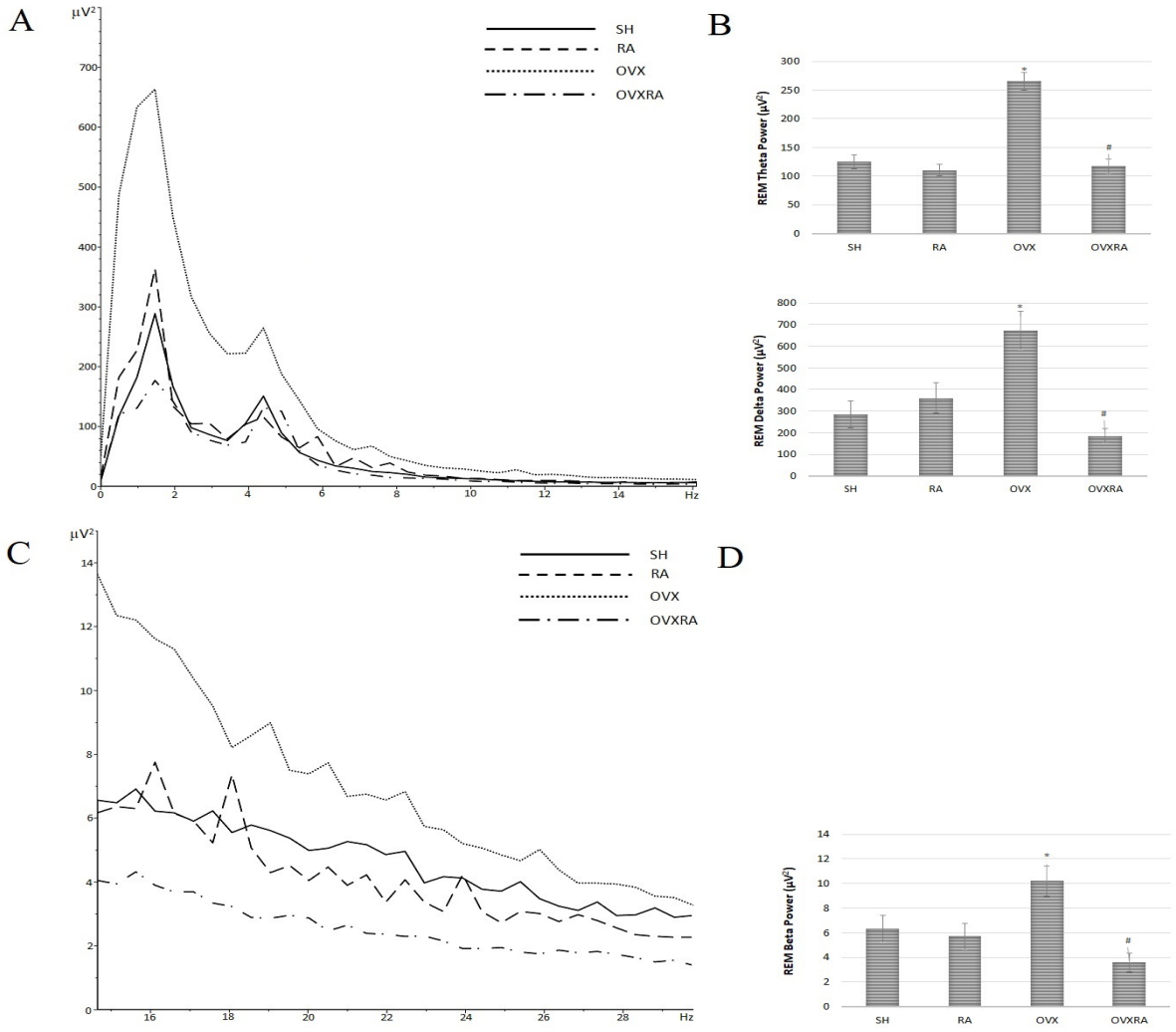

Figure 3. REM delta/theta power spectrum (A) and mean values (B); REM beta power spectrum (C) and mean values (D) of all experimental groups The results are presented as mean±SEM, n=8 for each group.
(*significant vs. SH group; #significant vs. OVX group)

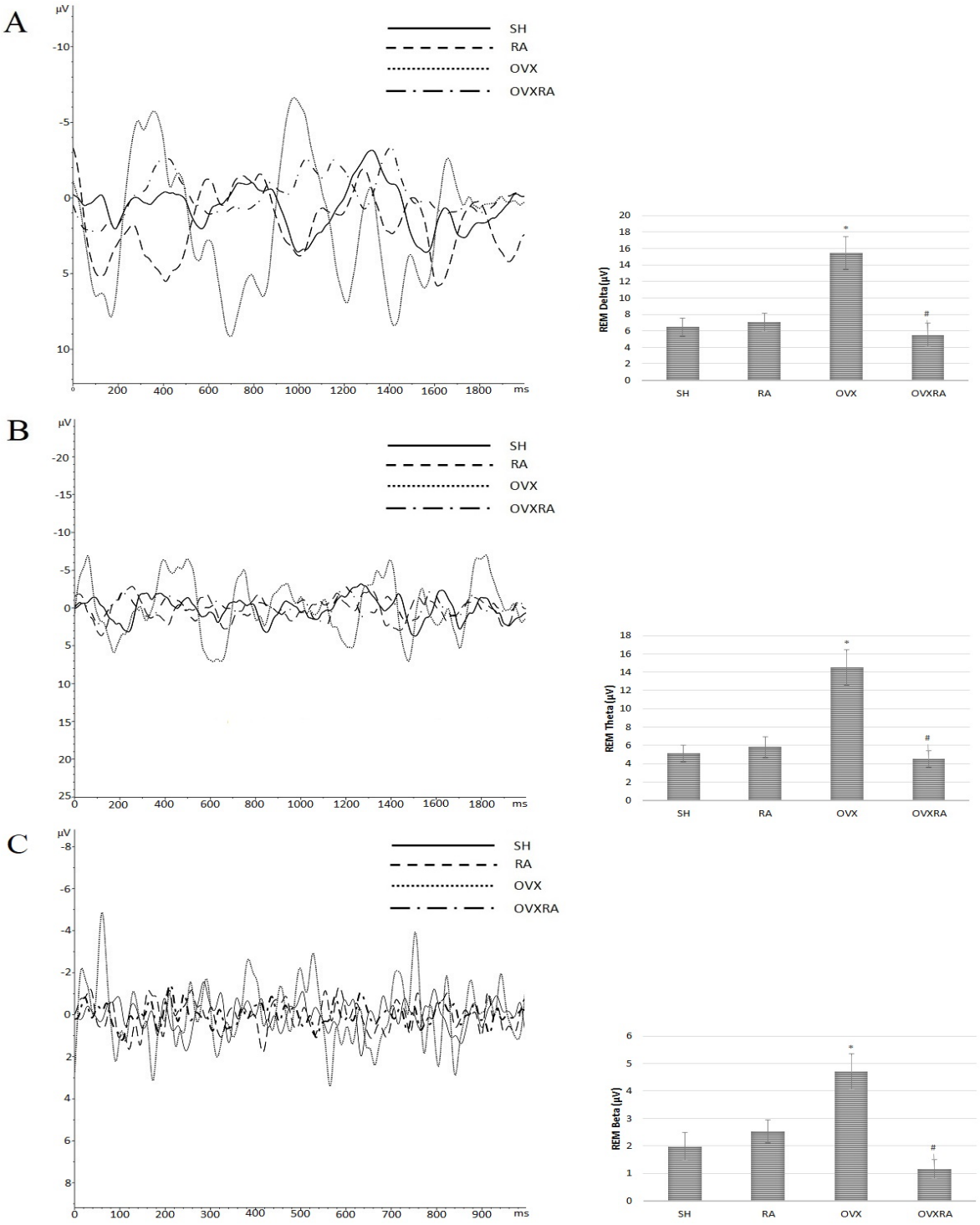

Figure 4. REM delta (A), theta (B), beta (C) oscillations and peak-to-peak amplitude values of oscillations for all experimental groups. The results are presented as mean±SEM, n=8 for each group.
(*significant vs. SH group; #significant vs. OVX group)

Mean values of brain ACh levels are given in Figure 5A. There was a statistically significant difference in ACh levels between groups [$F_{3.28}$ = 19.71, p < 0.01]. Brain ACh levels were significantly decreased in the OVX group with respect to the SH group (p < 0.01). ACh levels were significantly elevated in the OVXRA group versus the OVX group (p < 0.01). No significant difference was observed in ACh levels in the RA group versus the SH group.

AChE activities in the brain tissues of all groups are shown in Figure 5B. There was a statistically significant difference between groups [$F_{3.28}$ = 22.56, p < 0.01]. AChE activity was significantly increased in the OVX group with respect to the SH group (p < 0.01). The AChE activity was significantly decreased in the OVXRA group versus the OVX group (p < 0.001). RA treatment alone was slightly decreased the AChE activity in the RA group but this increment did not reach the significance level.

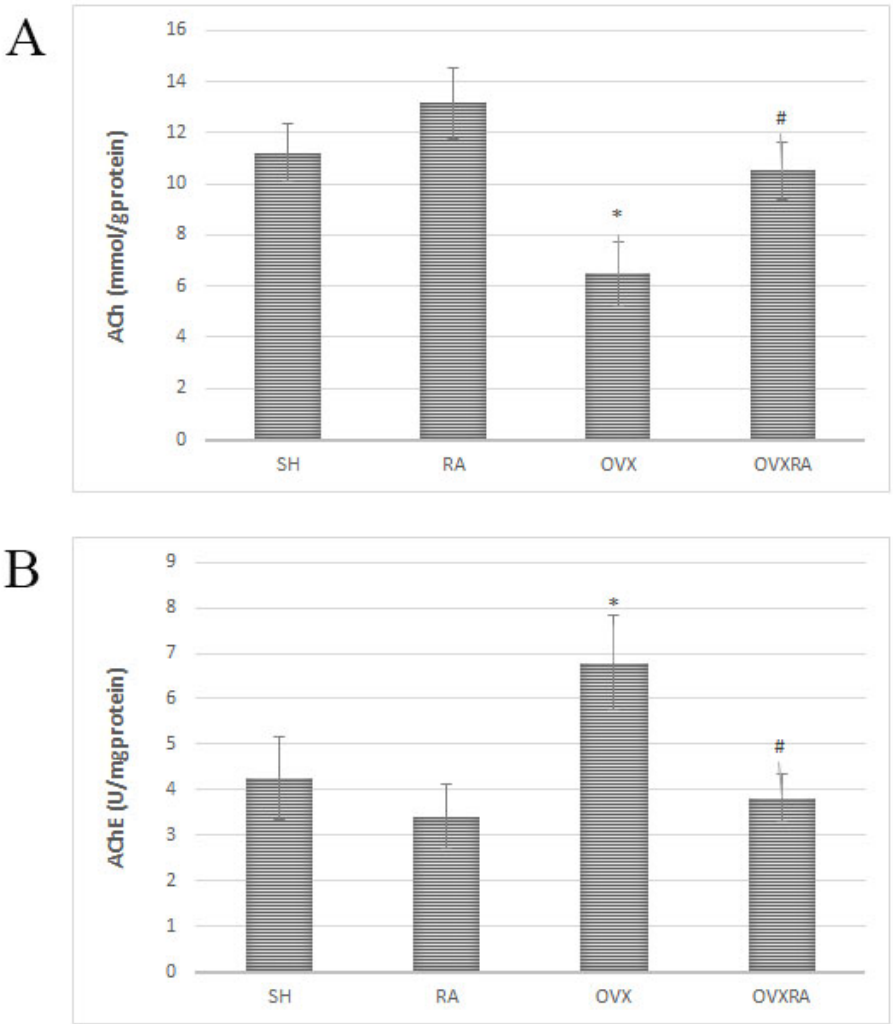

Figure 5. ACh levels and AChE activities of the brain tissues in experimental groups. The results are presented as mean±SEM, n=8 for each group.
(*significant vs. SH group; #significant vs. OVX group)

DISCUSSION

The deficiency of estrogen in the postmenopausal period is known to be related to sleep disturbance which is a common symptom seen in women. Because sleep regulation plays an essential part in many brain functions including memory formation, improving sleep quality might be a good target to reverse the cognitive decline during menopause. Estrogen replacement therapy (ERT) is a commonly known approach to unravel menopausal symptoms (31, 32). As long-term ERT has been associated with adverse effects in a postmenopausal woman (33, 34), investigating alternative phytoactive agents has gained importance currently. Thus, we examined the effects of RA on urethane-induced EEG as sleep model, spatial memory, and ACh/AChE levels in OVX rats.

Because sleep EEG is a good tool to understand the mechanism underlying the sleep disturbance related to estrogen deficit, we recorded EEG activity during urethane anesthesia as a known model for sleep (17). As natural sleep, urethane anesthesia EEG has two-phase in rats such as SWS and REM sleep. In both phases, we detected a prominent increment in both delta power and amplitude in the OVX group versus the SH group. These results contradict the findings of some human studies showing no change (9) or decrement of sleep delta power in postmenopausal women (35).

But our results are in agreement with earlier reports showing the sleep delta potentiation due to the increment of metabolic needs at some conditions such as sleep deprivation. Delta EEG activity was increased in SWS sleep due to sleep deprivation and was decreased in the night following a daytime nap (11, 12). Moreover, one-night sleep deprivation induced a delta rise in sleep which was attenuated with hormone therapy in OVX rats (10). Thus, it can be concluded that the alterations in the metabolic homeostasis of the brain caused by estrogen deficiency might be responsible for the observed sleep delta increment.

Furthermore, REM theta and beta power and amplitudes were significantly increased in the OVX group versus the SH group. This is inconsistent with previous studies showing elevated beta power accompanied by lower sleep quality in postmenopausal women (9, 36). This finding could be attributed to a higher arousal level related to less satisfactory sleep in the OVX rats. Besides, in parallel with our finding showing elevated theta response in the OVX rats, increased theta power indicates increased sleepiness (16). It seems quite likely that the estrogen deficiency condition causes arousal at the neural network.

OLM test is a commonly used task that assesses cognition, specifically spatial memory and discrimination in a variety of rodent models. Therefore, in the current study, we measured long-term spatial memory in experimental groups by determining the place discrimination index. OLM index was lower in the OVX rats than the SH rats. Our result is consistent with previous findings which indicated that estrogen deficiency may cause learning and memory impairment (37). Besides, there is emerging evidence for the role of sleep disorders in exacerbating the risk for dementia (38). The observed consequences of estrogen deficiency on sleep EEG, including oscillatory changes, may partly contribute to memory impairment seen in the OVX rats.

Previous studies demonstrated that loss of ovarian activity decreases cholinergic transmission (20, 21). In accordance with these studies, brain ACh levels decreased in the OVX group. This reduction in the ACh levels is parallel to the detected disruption of the spatial memory performance. It is known that ACh transmission plays an essential role in memory performance by strengthening the new synaptic connections (39). Besides cognitive deficit, sleep EEG alterations might be associated with a cholinergic deficit in the OVX group. Because the cholinergic system is a modulator of sleep cycles by which elevation of ACh release during REM sleep and reduction during SWS sleep (24, 25). In parallel with the ACh decrement, AChE activity increased in the OVX group. AChE is the primary cholinesterase in the body that catalyzes the breakdown of ACh.

Previous studies also indicated that ovariectomy results in an increment in AChE activity in the rat hippocampus (40). Besides, many studies have clearly shown that cholinergic systems which modulate some cognitive domains are dependent on estradiol support for adequate functioning (19). These findings indicate that disturbed cholinergic functioning may mediate the deficient sleep EEG activity and spatial memory generation in OVX rats. Consequently, our findings support the idea that changes in cholinergic transmission are an important factor contributing to the development of sleep disruption that leads to cognitive decline in estrogen deficiency.

Previous studies have indicated that polyphenolic compounds such as RA with their low toxic effects might be an alternative therapeutic approach in a variety of pathological conditions. Besides its known antioxidant properties, RA potentiates the cholinergic system by increasing ACh levels (29, 41). In this context, RA supplementation may be protective against menopause-related changes in brain functions. Therefore, we investigated the protective effects of RA on OVX related changes. The current results demonstrated that RA reversed ACh decrement induced by OVX. In parallel, RA decreased the AChE level and thereby it was also damped the degradation of ACh and helped to maintain ACh level. Moreover, we showed that RA decreased the power and amplitudes of SWS delta, REM delta, theta, and beta oscillations in the OVX group compared with the SH group. Our findings are in agreement with our lab and others results which have revealed that RA effectively increased cholinergic transmission (29, 41, 42). Our results also confirm that RA can efficiently improve brain activity during sleep by decreasing the arousal level which is determined as the decrement of theta and beta activity and rebalanced metabolic homeostasis which is determined as the decrement of delta activity. It is important to mention that cholinergic mechanisms could be involved in the sleep EEG regulation induced by RA treatment in the OVX rats. Moreover, the treatment with RA prevented the decrease of spatial memory concomitant with improved sleep EEG and cholinergic transmission. Because estrogen promotes cholinergic activity (19), therefore, it could be concluded that the protective effects of RA against estrogen deficiency by reversing sleep EEG spectrum changes and related cognitive decline are probably associated with the prevention of cholinergic deficit.

In conclusion, our findings support the functional role of estrogen in sleep activity and showed that estrogen deficiency impaired sleep-related oscillations by causing an increase in homeostatic needs and arousal levels. Besides OVX-induced spatial memory deficit which is partly related to disturbed sleep EEG activity. Cholinergic transmission that plays an essential role both in sleep and memory processes was also altered in the OVX rats. In the current study, we provided evidence that RA treatment may improve sleep quality and cognition in estrogen-deficient rats. The protective effects of RA can be partly explaining by its action on cholinergic transmission. These findings strongly indicate that RA may serve as a beneficial agent to ease symptoms of menopause. Besides, the other neurotransmitter systems and molecular pathways that RA might affect estrogen deficiency remain to be determined in future researches.

ETHICS COMITTEE APPROVAL

* The study was approved by the Akdeniz University Animal Experiments Local Ethics Committee (Date: 08.02.2021 and Number: 28).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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