Original Article

Neuroapoptosis Signaling Pathways in Hippocampus Following Ovariectomy and Its Inhibition by Systemic Exogenous Estradiol Replacement Therapy

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Abstract

Background: The potential neuroprotective role of estradiol in neurodegenerative diseases (NDs), suggests the role of hormone replacement therapy (HRT) in NDs. The present study investigated the possible beneficial implications of the exogenous β estradiol (ES) in the neuro-apoptosis signaling pathways in the hippocampus following long-term ovariectomy (OVX).

Materials and Methods: Thirty Wistar adult female rats were randomly assigned to 5 identical groups (n=6): 1-control (intact rats), 2-OVX (ovariectomized rats), 3-OVX+estradiol (eight weeks after ovariectomy, then intramuscular injection of 20µg/rat β estradiol for 30 days), 4-surgical sham (underwent only surgical incision), and 5-vehicle sham (eight weeks after ovariectomy, received sesame oil for 30 days). Three months following the assignment (two months post-OVX plus one month of estradiol injection for the intervention group), animals were perfused, and the hippocampus was obtained from all rats for molecular and histological studies. Nissl staining for neuronal cell counting and western blot for expression of cleaved caspase-3 and cytochrome-c were performed.

Results: Hippocampal neural density decreased in the OVX group ($P<0.01$ compared to the control), while it was restored in the OVX+ES group ($P<0.01$ compared to both OVX and sham vehicle groups). Furthermore, the cytochrome-c and cleaved caspase-3 expression increased in the OVX group in comparison to the control ($P<0.01$), whereas that of the OVX+ES decreased compared to the OVX group ($P<0.01$). In conclusion, diminished hippocampal neural density and overexpression of apoptotic proteins were observed in the OVX group.

Conclusion: Estradiol could preclude neural loss and reduce apoptotic protein expression, providing an important estrogen-induced neuroprotection mechanism via the apoptosis signaling pathway inhibition, this needs to be confirmed in further studies.

Keywords: Exogenous Estradiol, Ovariectomy, Apoptosis, Hippocampus, Caspase-3, Cytochrome-C

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Introduction

Neurodegeneration is a complex multifactorial phenomenon resulting in neurodegenerative diseases (NDs) and imposing a negative impact on individuals and healthcare systems (1, 2). Alzheimer's and Parkinson's disease are two major NDs with noticeable growth in recent decades (3). In the course of such diseases, the hippocampus undergoes extensive neuronal loss through cellular and molecular alterations with known or unknown mechanisms. Owing to the high-level expression of estrogen receptors in the hippocampus, studies have suggested the hippocampus as a potent target for hormone replacement therapy (HRT) with estradiol (4). Estradiol as the major regulator of the female reproductive system easily diffuses across biological membranes such as the blood-brain barrier and dramatically influences the development and protection of various tissues, including the heart (5), the immune system (6), and the central nervous systems (7). estradiol exerts its neurotrophic and neuroprotective effects via binding to specific receptors of the limbic structures, such as the hippocampus, especially in the CA1 and CA3 pyramidal neurons (8).

The potential neuroprotective role of estradiol has been described in a variety of mechanisms, including axonal and synaptic promotion (9, 10), antioxidant and anti-inflammatory properties (11), β-amyloid peptide deposition (12), and expression of anti-apoptotic factors in neurons (13). Moreover, estradiol enhances synaptic and astrocyte density in the hippocampal CA1 region and also increases synaptic protein expression, such as synaptophysin, spinophilin, and postsynaptic density 95 (PSD-95) (14). Several neural synaptogenesis signaling pathways, including MAPK/ERK and CREB, PI3K/Akt, and CamKII could be initiated by estradiol, resulting in structural and functional plasticity in various areas of the hippocampus (15). In addition, estradiol enhances learning and memory behavior through excitatory N-Methyl-D-Aspartate (NMDA), inhibitory GABAergic neurotransmission, and activating the cholinergic system (16). The involvement of estradiol in antiapoptotic actions is through several intracellular signaling pathways, the intrinsic and extrinsic pathways, resulting in the inactivation of caspase-3, cytochrome C (Cyt-c) translocation (17), and enhanced Bcl-2 and Bad (Bcl-2-antagonist of cell death) expression (18). It has been revealed that Bcl-2 and Bcl-XL block the translocation of Cyt-c from mitochondria to the cytosol which, in turn, inhibits caspase activation and cell apoptosis. The activation of these two anti-apoptotic genes in the neurons of the hippocampus is regulated by estradiol, and their expression diminishes following ovariectomy (OVX) (19). Moreover, the inhibitory role of estradiol on hippocampal neuron injury is through preventing TRP melastatin 2 (TRPM2) and TRP vanilloid 1 (TRPV1) activation (20). In addition to sexual dimorphism in certain parts of the nervous system that are reported previously, it has been shown that sex differences also exist in the neuroprotective effects of estradiol, suggesting that estradiol triggers a sexually dimorphic pattern in neurogenesis (9, 21).

NDs have become a growing concern and a severe health problem in many societies, which limits patients' quality of life and impose a significant global economic burden on individuals as well as healthcare systems. Currently, there is no promising therapeutic agent for NDs, but recent studies investigated the potential effect of systemic estradiol therapy on cognition improvement in postmenopausal women (22-24). However, the exact effect of estradiol replacement therapy following OVX on memory and cognition function within the brain remained to be better explored. So the present study aimed to examine the possible effects of systemic estradiol replacement therapy on the expression of apoptotic proteins in rat hippocampus following bilateral OVX in an in vivo model system.

Methods

Ethical publication statement: All protocols were confirmed by the Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1399.426).

Animals: Animals: A total of 30 adult female Wistar rats (200-220g body weight, 7-8 weeks old) were provided from the Laboratory Animal Center of Iran University of Medical Sciences (Tehran, Iran). All

animals were kept in the individual polycarbonate cage with unrestricted access to water and food, under standard conditions (12h/12h light/dark cycle, 23±2°C, humidity 50–60%) following the Ethics Committee, Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1399.426). Subsequently, all animals were randomized into five identical experimental groups (n=6): 1. Control group (intact animals) 2. OVX group, 3- OVX+ ES group (eight weeks following OVX, this group received an intramuscular injection of β ES (20µg/rat) for 30 days) 4. Surgical sham (underwent only the surgical incision for OVX) and 5. Vehicle sham (eight weeks following OVX, this group received sesame oil for 30 days). Animals were sacrificed after three months (equivalent to eight weeks post-OVX and 30 days of ES administration for the intervention group), and samples were obtained for histological (n=3) and molecular (n=3) assessments.

OVX surgery: Animals were anesthetized by a combination of intraperitoneal (IP) ketamine (20 mg/kg) and xylazine (10 mg/kg). Following induction of anesthesia, an abdominal incision was made, and both ovaries got dissected ventrally. Subsequently, the incision was sutured. Tetracycline ointment was used to prevent surgical site infection.

Blood estrogen level measurement: Three months after the intervention assignment, one cc blood sample was taken from the rats' hearts using a microcapillair and centrifuged (3000rpm for 10 minutes). Subsequently, serum was separated and exposed to the Mouse/Rat Estradiol ELISA kit (Sigma-Aldrich, USA) to measure the estrogen level.

Perfusion, fixation, and sectioning: At the end of the experiment, three rats in each experimental group were deeply anesthetized (10 mg/kg diazepam and 80 mg/kg ketamine, IP), and then saline washed and fixed transcardially by aldehyde solution (paraformaldehyde 4%), followed by 10% sucrose solution in phosphate buffer saline (PBS) 0.1M, PH 7.4. Subsequently, rats' brains were removed immediately and kept in graded sucrose solution (10, 20, and 30 %) at 4 ◦C for three days overnight.

The rats' hippocampus was identified using the Paxinos atlas. The cryostat provided serial 20 µm coronal sections. Subsequently, gelatin-coated slides were prepared for staining. Serial coronal sections of 20 µm were prepared using a cryostat.

Nissl staining: Nissl staining was performed as described previously elsewhere (25). By light microscopy, the number of neurons in all the subfields of rat hippocampus (CA1, CA2, CA3, DG) was assessed using Image j software (version 1.48).

Western blotting technique: Three rats were sacrificed from each group, and their hippocampus was rapidly removed. Western blotting evaluated the expression of Cyt-c and cleaved caspase-3 protein. The Radioimmunoprecipitation Assay (RIPA) buffer was used to extract the whole hippocampus tissue protein. This buffer helps to break the tissues and cellular components, causing the proteins to separate from other cellular parts. The suspension was centrifuged for 15 minutes at 14000 rpm and 4°C and deposited at -20 °C until use. Protein concentration (Cyt-c, cleaved caspase-3, β-actin) from the homogenized hippocampus was measured with the Bradford assay (Bio-Rad Laboratories, Germany). Next, a similar amount of loading buffer was added to the samples (15 µg) and boiled for 5 min. Sample proteins were treated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then moved to polyvinylidene difluoride (PVDF) membranes (Millipore). PVDF membrane was kept overnight (4◦C) in 2% non-fat powdered milk and Tris-buffered saline contains (Tris-buffered saline with Tween 20, TBS-T). Then, the PVDF membranes were washed to remove the extra blocking solution and incubated with Cyt-c, cleaved caspase-3, and β-actin (room temperature for 3 hours). After being washed with TBS-T, samples were treated with horseradish peroxidase-conjugated secondary antibody (room temperature for 60 minutes). The Electrochemiluminescence (ECL) assay was used to detect antibody-antigen complexes. Finally, the images of the bands recorded on the film were checked for protein density using the Lab Work analyzing software.

Statistical Analysis: Descriptive data are reported as mean ± SEM. Protein density values were measured by band densitometry and calculated based on the ratio of protein/beta-actin expression. One-way analysis of variance (ANOVA) and post hoc test (Newman-Keuls) were performed for multiple comparisons between experimental groups (25). $P<0.05$ was considered the minimum level of significance.

Results

Validation of the menopause model by ovariectomy: Removed ovaries were stained by H&E staining to confirm the complete removal of ovaries and histological study. In addition, all animals whose ovaries were removed (the OVX and sham vehicle groups) showed marked atrophy of the external genital system eight weeks after the surgery. Figure 1-A shows the removal of complete ovaries. Circulating estrogen level was significantly lower among the OVX and vehicle sham groups compared to the control (both $P<0.01$). Further, there was a significant increase in the circulating estrogen level of the OVX+ ES in comparison to the OVX group ($P<0.001$, Fig. 1B). No significant difference was observed between the OVX+ES group and the control group.

Hippocampal neuronal number by Nissl staining: Figure 2A. and 2B. demonstrate the Nissl-stained neurons. There was a significant decrease in the number of hippocampal neurons in the OVX and vehicle sham group compared to their control counterparts (both $P<0.001$). The absence of a significant difference between the surgical sham and control indicated that this decrease could be attributed to the OVX surgery (not the surgical incision)**.**

In contrast, there was a significant restoration in the neuronal number in the OVX+ES group than in both OVX and sham vehicle groups (both $P<0.01$). The absence of a significant difference in the neuronal count between the sham vehicle and OVX group can be accredited to ES administration (and not the sesame oil). Further, neuronal numbers did not differ significantly between the OVX+ES and control groups.

Hippocampal Cyt-c and cleaved caspase-3 expression by Western blotting: Fig. 3 illustrates the Cyt-c and cleaved caspase-3 protein expression in the rat hippocampus. According to the Nissl staining results, in which there was no evidence in favor of neuronal reduction or apoptosis in the control group, this group was excluded from western blotting to comply with the responsibility for reducing the number of animals in ethical guidelines. Apoptotic protein expression was significantly lower in the surgical sham compared to the OVX group ($P<0.01$), implying that the OVX surgery was to blame for this overexpression. The expression did not differ significantly between the vehicle sham and OVX group, indicating the lack of sesame oil influence. We found a significant decrease in Cyt-c and cleaved caspase-3 expression in the OVX+estradiol group compared to the OVX group (both $P<0.01$). At the same time, there was no significant difference compared to the vehicle sham, indicating that this phenomenon attributes to the administration of β estradiol and not the sesame oil.

Discussion

Based on many previous studies, long-term ovarian steroid depletion following OVX or during the menopause period dramatically affects various body systems, including the heart, immune and nervous systems (26). Our findings confirmed the neuroprotective effects of estradiol on the hippocampus region of adult female menopausal model rats, as mentioned in our previous works (10, 25). Following the OVX and the decrease in circulating estrogen level, hippocampal neuronal death and increased apoptotic protein expressions were observed. Subsequently, treatment with estradiol prevented apoptotic events and neuronal death by regulating the expression of cleaved caspase-3 and cyt-c proteins. The molecular and cellular neuroprotective mechanisms of estradiol have been previously depicted in experimental and clinical research (8, 24, 25). It has been shown that sex steroid hormones, due to their size

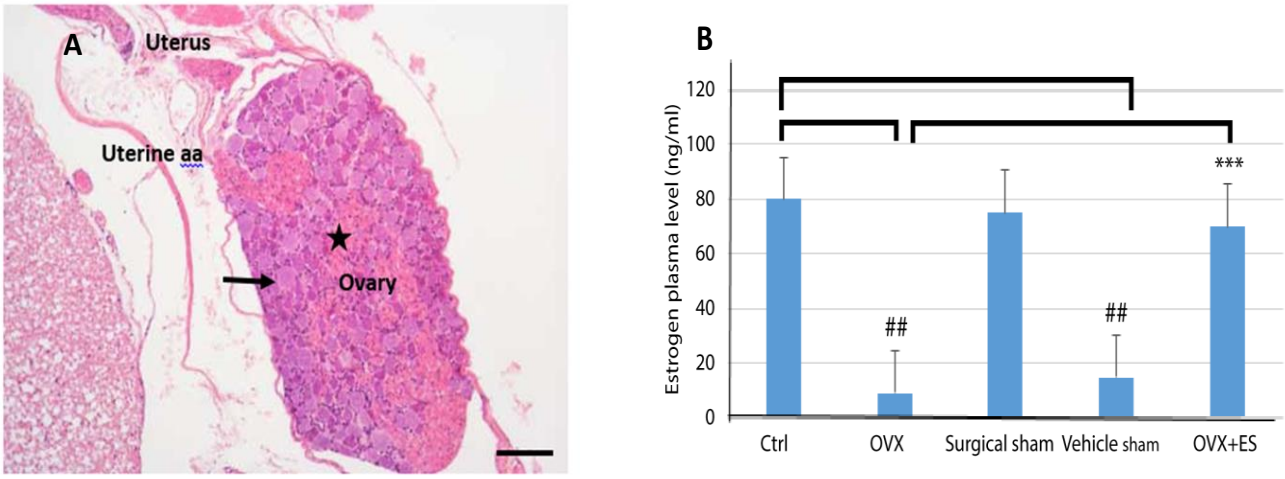

Figure 1. (A) H & E of ovarian tissue in the OVX group. (*: blood vessels, arrow: follicle). Scale bar: 50 µm. (B). The estrogen level of the experimental groups (n=6). Data are presented as mean±SEM.. ## P<0.01 for OVX/sham vehicle vs. control; ***P<0.001 for OVX+β Estradiol vs. OVX.

(25-50 KD), could easily pass the blood-brain barrier and insert their effects on certain structures of the brain. These effects include genomic (classical or structural) and non-genomic (non-structural) implications that finally lead to sexual dimorphism in the structure and function of the nervous system in all vertebrates, including mammals (27). In addition to brain sexual dimorphism**,** the sex steroid hormones, mainly female estradiol hormone, have well-known neuroprotective, anti-apoptotic, and antioxidant properties (28). Accordingly, any decrease in sex steroid hormones circulating level, mainly estradiol, leads to cellular and molecular changes in neural structures, such as neuronal death, and decreased neuronal, synaptic, and dendritic spines density. The expression of pro-apoptotic proteins has been reported to increase following ovariectomy. In addition to gonadal sex steroids, there are also neurosteroids (synthesized by certain neurons), steroids from adrenal glands (29, 30), and steroids with external sources (exogenous steroids), all of which almost play similar roles in the nervous system (31, 32). All of these neuroactive steroids easily cross the blood-brain barrier and use their factors via binding to classical and nonclassical receptors on different sections of the Central Nervous System includes corpus callosum (33) and anterior commissure (34), the bulbocavernosus spinal nucleus (35), spinal motor neurons (36), Purkinje neurons (37), hippocampal pyramidal neurons (38), the bed nucleus of the stria terminalis (BNST) (39), substantia nigra (40), raphe nuclei (41) and nigrostriatal dopaminergic neurons (42-44). Within these structures, estradiol mediates neuronal survival through several mechanisms, such as antioxidant activity (45), synaptic formation (46), and DNA repair of the central nervous system (47). In this regard, studies have indicated a neural loss in the dendritic spine of dorsal and median raphe nuclei following deprivation of ovarian steroid hormones after OVX (41, 48). The current result regarding apoptotic cell death and DNA fragmentation in the hippocampus following ovariectomy is in line with a previous study that emphasized the neuroprotective function of estradiol on the association between steroid hormone deprivation and neuronal viability in hippocampal cells of CA1, CA2, and CA3 areas (19). Although the exact molecular and signaling mechanisms of these effects are still unknown, there are some possible suggested mechanisms. As reported previously, brain-derived neurotrophic factor (BDNF) and its receptor TrkB could be upregulated by estradiol which as result promotes neurogenesis and differentiation of hippocampal neurons as well as modulates apoptosis (49). Furthermore, estradiol could activate the ERK/MAPK pathway that is responsible

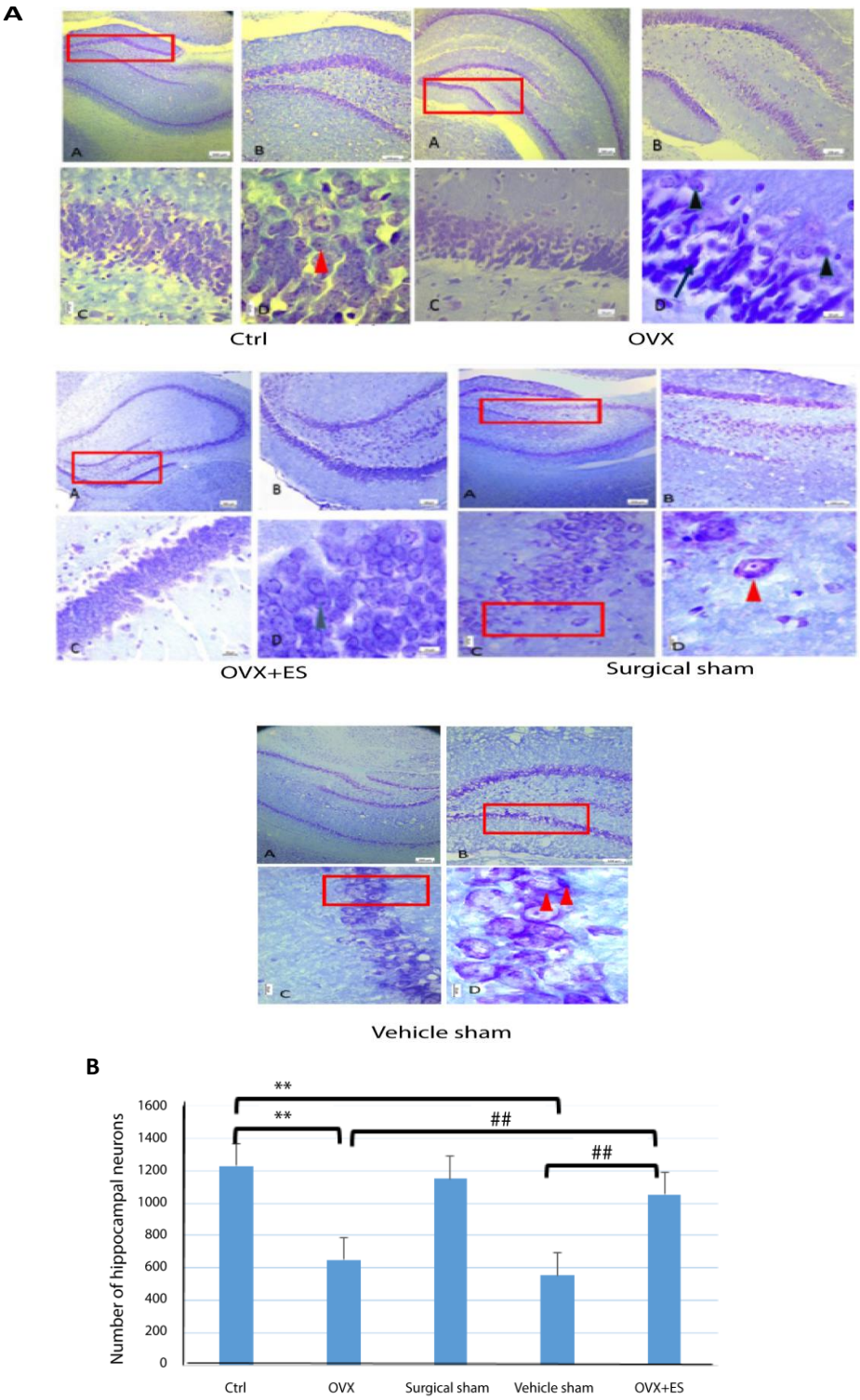

Figure 2. (A) Representative photographs of the Nissl-stained rat hippocampus. (B) The number of hippocampal neural cells in the experimental groups (n=3 for each group). Data are presented as mean±SEM**P<0.01 and ## P<0.01.

for the phosphorylation of CREB and next positive adjustment of the Bcl2 in hippocampal CA1 pyramidal cells (48). In addition, it has been observed that G-protein coupled receptors (GPR) intervene rapid effects of estradiol on synaptic transmission and have a decisive role in the activation of the MAPK and/or

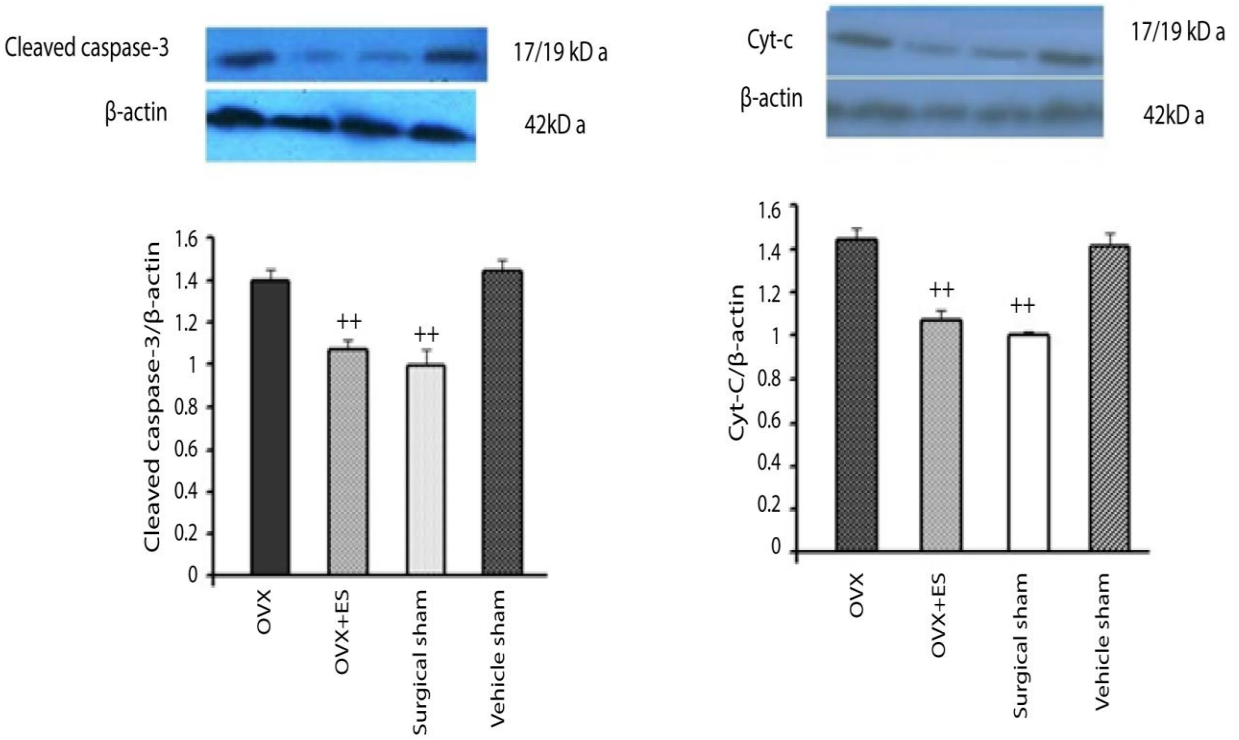

Figure 3. Protein level of A) Cleaved caspase-3 and B) Cyt-c in the hippocampus of experimental groups (n=3 for each group). Data are presented as mean±SEM ++ P<0.01 compared to the OVX group.

PI3K/Akt cellular pathways which prevent apoptotic cell death (50). Another possibility is that neuronal cell death could be the result of oxidative stress, which mediates mitochondrial dysfunction, abnormal Ca^{2}+ accumulation, and activation of death signaling cascades; and it is believed that estradiol contributes to neural protection against oxidative toxicity (51). In this respect, Sales et al. investigated the mRNA levels of Bcl-2 and Bax proteins in rat hippocampus after ovariectomy. They observed that the physiological concentration of beta-estradiol therapy maintained neuronal survival in the hippocampus and also bring on a reduction in expression of apoptotic proteins and the formation of apoptotic bodies (19). Similarly, in another study, it has been shown that long-term estrogen or tamoxifen administration in ovariectomized rats results in the modulation of Bax and Bcl-2 proteins within the hippocampus (50). Tamoxifen and beta-estradiol had similar consequences on the expression of these two proteins, and these findings not only confirmed the neuroprotective effects of beta-estradiol but also claimed that beta-estradiol and tamoxifen have therapeutic potential for neurodegenerative diseases, especially during menopause (52). Our results chime in with those of Jover et al. who showed the protective therapeutic effects of estrogen in ischemia-induced neuronal cell death and also modulated the apoptotic cascades. They reported that estrogen downregulates the activity of caspase 3 in an estrogen receptor-dependent manner within the hippocampal CA1 region (53). Nevertheless, Nunez et al. demonstrated that administration of estradiol aggravated hippocampal neuron death in a model of hypoxia-ischemia of preterm infants (54).

Moreover, estradiol has protective effects on reducing beta-amyloid plaques in postmenopausal women with AD (55). Accumulation of β-amyloid protein (Aβ) is a pivotal risk factor in AD. However, estradiol would potentially reduce Aβ neurotoxicity through upregulation of PPAR expression and reduction in APP processing by γ-secretase, which

accordingly causes a decrease in Aβ peptide formation (11).

Conclusion

In conclusion, the present study indicated that estradiol administration maintains neural viability and regulates neuroapoptosis by reducing the expression of Cyt-c and cleaved caspase-3 proteins, hence the promising role of HRT in alleviating NDs among postmenopausal women. However, the various mechanisms involved in estradiol -induced neuroprotection have yet to be explored. Future research can investigate the optimal dose and duration of estradiol therapy as well as its role in the expression of other apoptotic and anti-apoptotic protein families.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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