



Comparative study on the effect of the *pimpinella anisum* and estradiol on the hippocampus and dentate gyrus of ovariectomized rats



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Abstract

Objectives: Reproductive aging is accompanied by many health problems. In experimental study, it was represented by the ovariectomized (OVX) rat. The aim of this work is to compare, for the first time, the effects of estradiol and aniseed on the hippocampal and dentate gyrus of OVX rats.

Methods: Forty adult female rats were used and divided into four groups; Group I: included twenty five rats and was further subdivided into five subgroups: control, sham vehicle, sham operated, estradiol and aniseed subgroup, Group II: (OVX group) included five rats that were subjected to the ovariectomy operation. Group III: (Estradiol treated group) included five ovariectomized rats that received oral treatment of *Estradiol Valerate* in a dose of 0.3 mg/kg dissolved in olive oil once daily for one month by gastric tube two weeks after the ovariectomy operation. Group IV: (Aniseed treated group) included five ovariectomized rats that received oral treatment of *Aniseed* crude powder in a dose of 500 mg/kg dissolved in distilled water once daily for one month by gastric tube two weeks after the ovariectomy operation

Results: Both estradiol and aniseed improved the damaging effects in hippocampus and dentate gyrus resulting from ovariectomy. There was significant down regulation of GFAP and caspase3 immune reaction with increased expression of alpha estrogen receptor when compared to OVX.

Conclusion: Aniseed could be considered as an important natural source of estrogen. It has a similar effect as estradiol or even more effective.

Keywords: Ovariectomy; aniseed; hippocampus; dentate

Abbreviation: OVX: ovariectomy; H & E: Haematoxylin and Eosin; TB: Toluidine Blue; GFAP: Glial Fibrillary Acidic Protein

Introduction

The postmenopausal women have a considerable low level of estrogen hormone. The ovariectomized (OVX) rat is a good model to study the effects of estrogen decline that may cause interruption of the function of female organs, including the brain [1]. The estrogen's effects on the brain are mediated by interaction of estrogen with two estrogen receptors α & β (ER α & β) [2]. Estrogen has neuroprotective and beneficial effects on the cognitive function of the brain. The hippocampus and dentate gyrus are considered recently as the extra reproductive brain areas for the action of estrogen and the target for the neuromodulatory effects of estrogen [3]. At the same time the hippocampus and the dentate gyrus are implicated in learning and memory processes [4]. Alzheimer's disease is a neurodegenerative cognitive disorder with defect in learning

and recalling, so estrogen deprivation is considered as a risk factor for Alzheimer's disease [5].

Pimpinella anisum L. (Anise) is the plant with white flowers and small green to yellow seeds that grows in India, Egypt, Turkey, Iran, and many other warm countries of the world [6]. The fruits of Anise plant that commercially called "seeds" are known as aniseed andyansoon. In folk medicine, aniseed has been used for the treatment of nausea, abdominal colic, insomnia and epilepsy. The characteristic constituent of aniseedis transanethole which is responsible for its taste and smell and it is considered as an active estrogenic agent. Other constituents include coumarins, lipids flavonoids, protein, carbohydrate and minerals as calcium and phosphorus [7].

Materials and Methods

Animals

Forty adult, twelve weeks aged, wistar female albino rats were used in this experiment, each weighing 150-200 grams. Food and water were provided ad libitum and the rats were left for 7 days for acclimatization before use in the experiment that was held in Anatomy and Embryology Department, Faculty of medicine, Menoufia University. All aspects of animal care and treatment were carried out according to the local guidelines of the ethical committee for animal research. Ovariectomy (OVX) surgery was done into the OVX and treated groups. The rats of these groups underwent a bilateral ovariectomy according to a standardized protocol [8].

Surgery

Firstly, the rats were anesthetized with an intra-peritoneal injection of 8% chloral hydrate solution (0.4 ml/100g then a midline abdominal skin incision was performed. By the artery forceps, the pelvic pad of fat was gently grasped and the ovaries were exposed. After ligation and crushing the bilateral ovaries, the fat pad was repositioned into the abdomen. The incision closure was performed in two layers, the muscle layer and the skin layer with single stitch sutures. All rats were given meloxicam (5mg in 250ml drinking water, Metacam, Boehringer Ingelheim, Germany) for 5 days postoperatively for pain relief.

Materials

The fruits of *pimpinella anisum* L. plant (Aniseed) were purchased from the Chemistry Department, Agricultural Research Center, Cairo, Egypt.

Experimental plan

Forty rats were divided into four groups as the following:

Group I: included twenty five rats and was further subdivided into five equal subgroups:

I. Subgroup Ia (control subgroup) was kept without any treatment throughout the experimental period.

II. Subgroup Ib (sham vehicle subgroup) received olive oil, 2 ml by gastric tube once daily

III. Subgroup Ic (sham operated subgroup) the ovaries were exposed without ligation nor crushing.

IV. Subgroup Id (Estradiol subgroup) that received oral treatment of *estradiol Valerate* in a dose of 0.3 mg/kg dissolved in olive oil once daily for one month by gastric tube two weeks after the ovariectomy operation.

V. Subgroup Ie (Aniseed subgroup) received oral treatment of aniseed crude powder in a dose of 500 mg/kg dissolved in distilled water, once daily for one month by gastric tube.

Group II: (OVX group) included five rats that were subjected to the ovariectomy operation.

Group III: (Estradiol treated group) included five ovariectomized rats that received oral treatment of *Estradiol Valerate* in a dose of 0.3 mg/kg dissolved in olive oil once daily for one month by gastric tube two weeks after the ovariectomy operation to ensure withdrawal of natural estrogen.

Group IV: (Aniseed treated group) included five ovariectomized rats that received oral treatment of *aniseed* crude powder in a dose of 500 mg/kg dissolved in distilled water once daily for one month by gastric tube two weeks after the ovariectomy operation.

Histological and immunohistochemical studies

After rat scarification, both cerebral hemispheres were carefully dissected out. Coronal section was done in each hemisphere and brain samples were fixed in 10% for mol saline and processed to prepare 5 μ m-thick paraffin sections for use in the following histological techniques: (a) histological study (Haematoxylin & Eosin (H&E) and Toluidine Blue (TB) stains). Sections were also used in (b) immunohistochemical study for detection of glial fibrillary acidic protein (GFAP), estrogen receptor α as well as caspase3 immunoreactivity. Briefly, sections were deparaffinized, rehydrated, and after antigen retrieval with 10m mol/l citrate acid solution (pH 6), specimens were preincubated with goat serum for 5 min and were then incubated overnight at 4°C with polyclonal anti GFAP (Abcam, Cambridge, UK), anti estrogen receptor α (Dako Company, Wiesentheid/Bavaria) and anti caspase 3 (Dako Company, Wiesentheid/Bavaria) (Working dilution 1:500). Binding was detected using biotinylated secondary antibody (goat anti-mouse IgG; Sigma Aldrich) for 10 min. The specimens were then incubated with streptavidin-peroxidase complex for 5 min, followed by incubation with 3, 3'-diaminobenzidinetetrahydrochloride (DAB; Sigma Aldrich) for 3 min. Slides were counterstained with hematoxylin and mounted.

Morphometric study

Data were obtained from five different sections from each rat of all subgroups were examined using image J analyzer software program to determine:

- i. The thickness of hippocampus including the dentate gyrus in H & E stained sections (X4 magnification).
- ii. Color intensity in toluidine blue stained sections
- iii. Area % in immunohistochemical stained sections

Statistical analysis

Data obtained from morphometric study was subjected to statistical analysis using SPSS software version 20 (SPSS, Inc., Chicago, IL, USA). Data were presented as mean \pm standard deviation. Differences among the study groups were detected

by using U mannwhitney-test. The results were considered statistically significant with $p < 0.05$ [9].

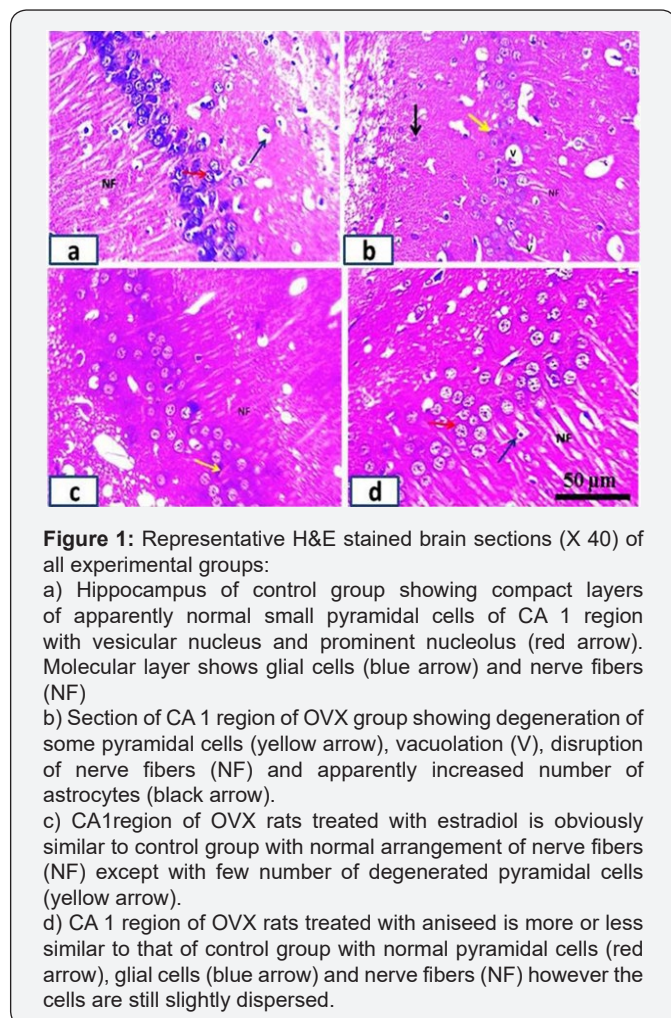
Results

There was no significant difference between the subgroups Ia-e in all results; therefore, the whole group I was considered as a control group.

Histological study

Haematoxylin and Eosin (H&E) stain

Hippocampus (CA1 region) of control group consists of compact layers of small pyramidal cells with regularly arranged nerve fibers and molecular layer containing glial cells. Disorganization of general architecture, degeneration of some pyramidal cells with vacuolated cytoplasm and disrupted nerve fibers were revealed in ovariectomized group. These findings are highly ameliorated in estradiol and aniseed treated groups (Figure 1).



Dentate gyrus of control group consisted of compact arranged granular cell layers with dark nuclei and normal hilar cells. Degenerative changes of the cells and absent hilar cells were obviously noticed in dentate gyrus of ovariectomized rats.

However dentate gyrus of estradiol and aniseed treated groups were highly protected in comparison to ovariectomized non treated group (Figure 2).

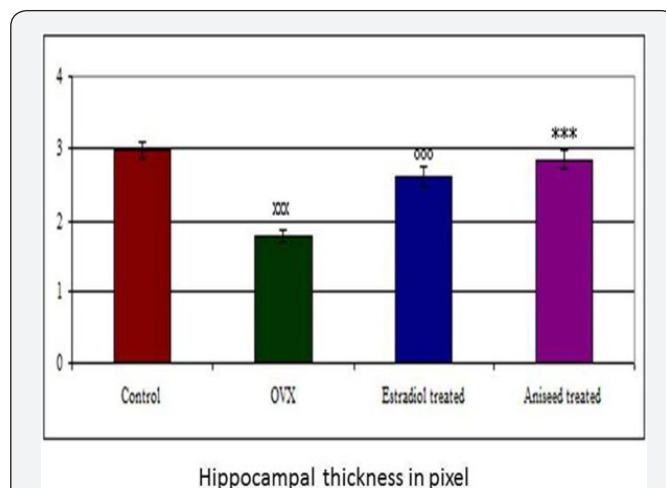
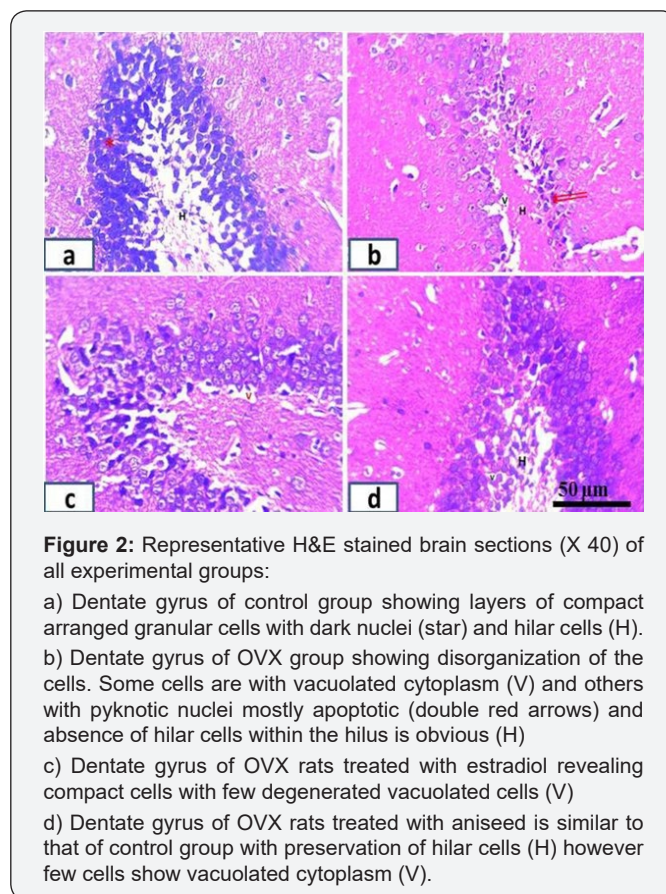


Figure 3: The OVX group showing significant decrease in the thickness of the hippocampus including the dentate gyrus as compared to the control group. The estradiol and aniseed treated groups showing significant increase in the dentate gyrus thickness as compared to the OVX group but there is no significant difference (P value = 0.06) in the thickness between the estradiol and aniseed treated groups $P^{***} < 0.001$, compared to control group. P^{ooo} and $P^{***} < 0.001$ compared to OVX group.

Regarding the thickness of hippocampus including the dentate gyrus, the OVX group showed significant decrease in the thickness as compared to the control group. The estradiol treated and aniseed treated groups showed significant increase in the thickness as compared to the OVX group but there is no significant difference between the estradiol treated and aniseed treated groups (Figures 1-3).

Toluidine blue (TB) stain

The control hippocampus (CA1 region) and dentate gyrus showed dark blue staining of pyramidal and granular cells denoting the presence of dense nissl's granules in their cytoplasm. The OVX hippocampus (CA1 region) and dentate gyrus showed significant decrease in the color intensity. The estradiol treated sections showed significant increase in the color intensity of toluidine blue stain. The restoration of the normal color intensity appeared in the aniseed treated sections (Figure 4).

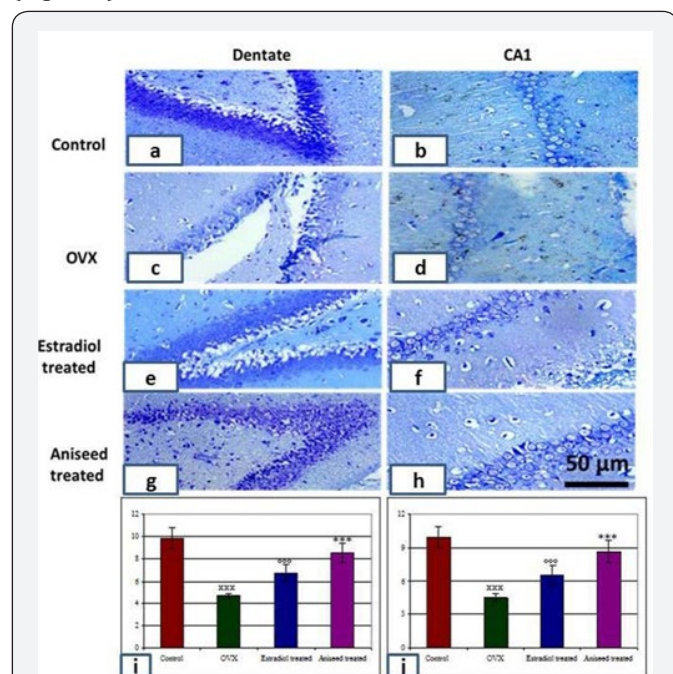


Figure 4: Representative toluidine blue staining of the brain sections of all experimental groups, the control group showing dark blue staining of CA1 pyramidal cells (a) and dentate gyrus granular cells (b), the hippocampal CA1 region and dentate gyrus of OVX group (c & d) revealing significant decrease in the color intensity as compared to the control group (a & b), the estradiol treated (e & f) and the aniseed treated (g & h) CA1 region and dentate gyrus show significant increase in the color intensity as compared to the OVX group (c & d), there is significant difference (P value = 0.03) in the color intensity of CA1 and dentate gyrus between the estradiol treated (e & f) and aniseed treated groups (g & h) $P^{***} < 0.001$, compared to control group. P^{***} and $P^{***} < 0.001$ compared to OVX group. Toluidine blue stain, scale bar = 50 μ m and statistical analysis: (i & j)

Immunohistochemical study

The brain sections of OVX group showed a significant increase in area % of positive immune reaction for GFAP positive

cells in the dentate gyrus and CA1 region of hippocampus as compared to the control group. The estradiol treated as well as aniseed treated brain sections showed significant decrease in area % of positive immune reaction for GFAP positive cells in the dentate gyrus and CA1 region of hippocampus as compared to the control group however there is significant difference between estradiol treated and aniseed treated groups (Figure 5).

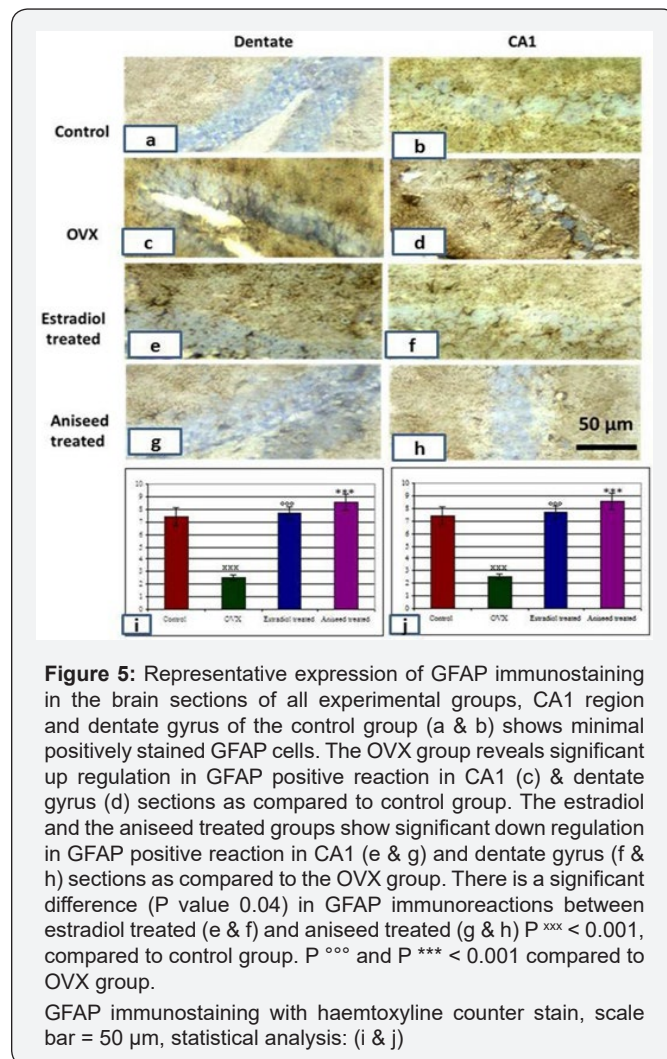
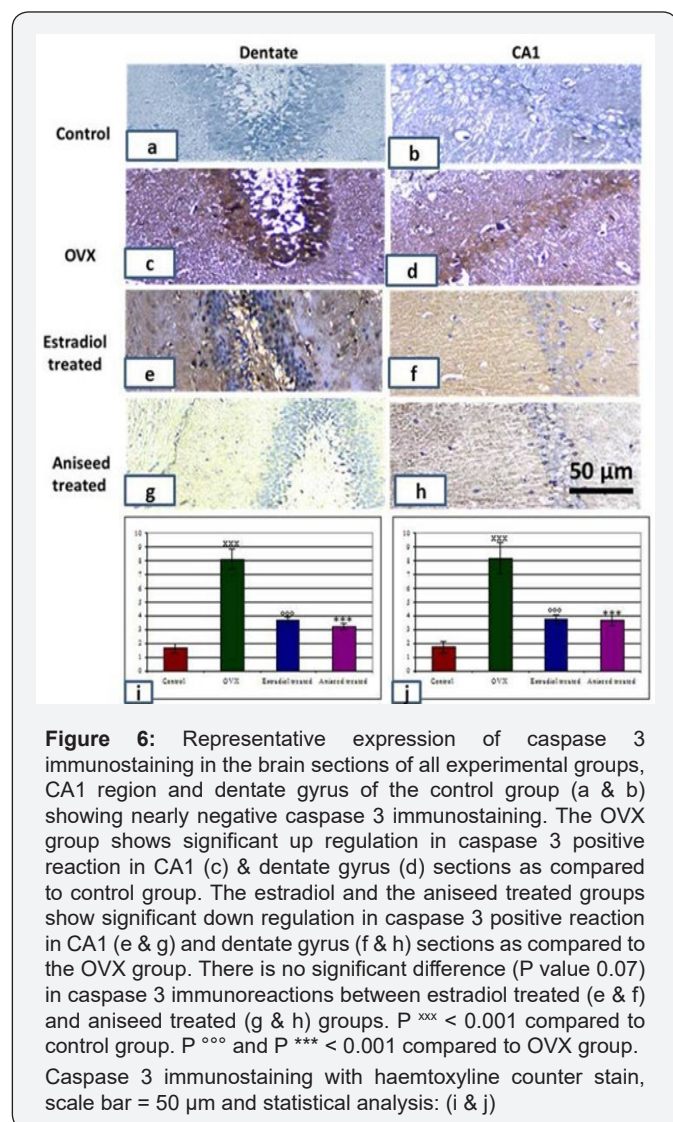


Figure 5: Representative expression of GFAP immunostaining in the brain sections of all experimental groups, CA1 region and dentate gyrus of the control group (a & b) shows minimal positively stained GFAP cells. The OVX group reveals significant up regulation in GFAP positive reaction in CA1 (c) & dentate gyrus (d) sections as compared to control group. The estradiol and the aniseed treated groups show significant down regulation in GFAP positive reaction in CA1 (e & g) and dentate gyrus (f & h) sections as compared to the OVX group. There is a significant difference (P value 0.04) in GFAP immunoreactions between estradiol treated (e & f) and aniseed treated (g & h) $P^{***} < 0.001$, compared to control group. P^{***} and $P^{***} < 0.001$ compared to OVX group. GFAP immunostaining with haematoxyline counter stain, scale bar = 50 μ m, statistical analysis: (i & j)

The brain sections of OVX group showed a significant increase in area % of positive immune reaction for caspase 3 positive cells in the dentate gyrus and CA1 region of hippocampus as compared to the control group. Brain sections of estradiol treated as well as aniseed treated groups showed significant decrease in area % of positive immune reaction for caspase 3 positive cells in the dentate gyrus and CA1 region of hippocampus as compared to the control group but there is no significant difference between estradiol treated and aniseed treated groups (Figure 6).

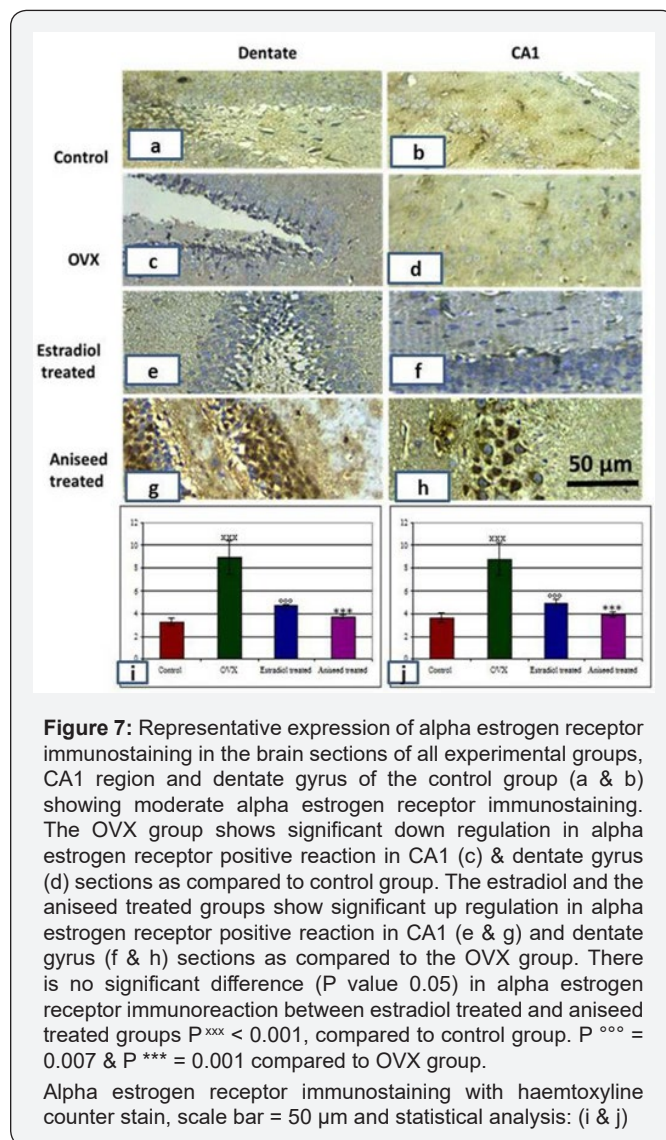
The brain sections of OVX group showed a significant decrease in area % of positive immune reaction for alpha estrogen receptor positive cells in the dentate gyrus and CA1 region of hippocampus as compared to the control group. Brain sections of estradiol treated as well as aniseed treated groups showed

significant increase in area % of positive immune reaction for alpha estrogen receptor positive cells in the dentate gyrus and CA1 region of hippocampus as compared to the control group with no significant difference between estradiol treated and aniseed treated groups (Figures 5-7).



Discussion

There was a great controversy on the effect of reproductive aging on the brain and the administration of estradiol for postmenopausal woman in improvement of the mood and relief of the postmenopausal depression [10,11]. So, we needed a natural source carrying the benefits of estrogen. Regarding the histological results, the brain sections of OVX group showed various changes including the vacuolated cytoplasm and condensed nuclei of the pyramidal cells, degeneration of the nerve fibers; decreased thickness of the hippocampus as shown in H & E stained sections together with disturbed architecture. These changes were in agreement with other authors who postulated that the ovariectomy led to aging and neurodegeneration of the brain [12]. As estrogen deprivation might lead to initiation of the inflammatory response [13].



In our study the aging of the brain was detected by the significant up regulation of GFAP which detect the active gliosis of OVX brain sections, Salmina et al. [12] found that the activated glial cells produce many inflammatory factors including prostaglandin E2, tumour necrosis factor- α , interleukin-1 and nitric oxide which facilitate the neurodegeneration [12]. Ghisletti et al. [14] revealed that the anti-inflammatory effect of estrogen is mediated by estrogen receptor alpha in microglial cells, which down regulates the inflammatory gene transcription, resulting in reducing the synthesis of inflammatory mediators [14]. The beneficial effects of the estrogen were attributed to its interaction with the cholinergic projections originating from the basal forebrain. These cholinergic projections have an important benefit in neuronal plasticity and cognitive performance [15].

Conclusion

Our result emphasis on that concept by the partial improvement of the brain sections of estradiol treated group. The expression of mitochondrial proteins that involved in

energy metabolism, oxidative stress and apoptosis in the brain is regulated by estrogen. So that brain mitochondria are the target organelles for the neuroprotective effects of estrogen, the accumulation of reactive oxygen species in the brain may be the cause of aging in postmenopausal women as referred by Chong et al. [16]. This explained the up regulation of caspase 3 immunoreactions in the brain sections of OVX group. The up regulation of alpha estrogen receptor in aniseed treated brain sections indicated the high level of estrogen in the aniseed treated group, this is in agreement with some authors who stated that the greater the immune reaction, the higher estrogen level [3]. In the aniseed treated group, the high estrogen level played antiaging role which was revealed by the down regulation of GFAP in its brain sections in addition to its anti-apoptotic role with down regulation of caspase 3 immunoreaction in its brain sections. So aniseed could be considered as an important natural source of estrogen in addition to many ingredients as antioxidants.

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