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# Effects of Exogenous Estrogen Treatment on Hippocampal Neurogenesis of Diabetic Ovariectomized Rats

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## ABSTRACT

**Background:** Mellitus Diabetes (DM) is the most important metabolic diseases. The incidence of DM is prone to increase. Vasculopathy, retinopathy, central and peripheral neuropathy are the most important reported side effects of DM. Cognitive dysfunction following DM reported in both sexes. Hippocampus is a major part of brain involving in cognitive function, its cells are able to neurogenesis, so it is possible that DM affects the hippocampus. In addition, neuroprotective effects of female sex steroids are reported elsewhere. In order to answer the question of whether female sex steroid are able to suppress the effects of DM on neurogenesis of dentate gyrus (DG) in diabetic ovariectomized rat the present study designed.

**Methods:** Sprague-Dawley adult female rats were used in this study. The animals randomly divided in 8 groups including; control, diabetic (Diab), ovariectomy (OVX), Diab+OVX, estrogen treated (E2; Diab+OVX+E2), surgical and vehicle sham. Intrapritoneal injection of STZ, subcutaneous injection of E2 and routine bilateral surgery were used respectively to induce diabetes, estrogen treatment and OVX. Nissl staining, Brdu immunohistochemistry (IHC) and western blotting were used in this study. Statistical analysis was done and the results presented in mean ± SD, $P_v < 0.05$ considered significant.

**Results:** Brdu IHC showed that the neurogenesis significantly decreased in OVX, Diab and OVX-Diab groups ($P_v < 0.05$) in comparison with control and sham groups. Western blotting showed significant increase of Bax and decrease of Bcl2 proteins of trial groups comparing to control. Estrogen treatment significantly improved neurogenesis in animals of Diab+OVX+E2 group. The neurogenesis impairment was more sever in OVX + Diab animals than OVX and Diab ones merely.

**Conclusion:** Based on our data, cognitive dysfunction caused by DM is related to hippocampal neurogenesis reduction and might improve under the influence of ovarian steroidal hormone therapy.

**Keywords:** Mellitus diabetes; Ovarian hormones; Cognitive function; Neurogenesis

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## INTRODUCTION

Mellitus Diabetes (DM) is a metabolic disorder that is mainly defined by hyperglycemia resulting from insulin secretion deficiency, insulin resistance or both [1]. DM leads to some serious clinical complications including neuropathy, nephropathy and retinopathy [2]. Diabetic

encephalopathy with certain cognitive dysfunction also reported in diabetic patients [3]. Definite molecular and cellular changes were shown in different types of neuropathy in DM, it is believed that these changes might lead to cerebrovascular diseases, AD, dementia, depression, cognitive disorders and etc. [4]. Among the cognitive dysfunction in patients with DM memory and learning impairment reported [5]. The role of hippocampus in memory processing and learning has been shown by many studies. Thus it seems that any cognitive dysfunction seen in diabetic patients might come from cellular and molecular changes in certain parts of hippocampus, such as damage to presynaptic and postsynaptic structures, deregulation of calcium homeostasis, neuronal and dendritic loss, reduced expression of insulin growth factors and their receptors, and decreased neurogenesis [6]. Neurogenesis in certain area of hippocampus including dentate gyrus (DG) and subventericular zone (SVZ) is the most recently reported feature of hippocampus, it is shown that neurogenesis might be influenced by many factors such as exercise, sensory deprivation, stressors, hormonal and metabolic imbalance [7]. Newly generated neurons in the SVZ migrate to the olfactory bulb, [8,9], where they differentiate into functional cellular units such as interneurons, granular cells and periglomerular neurons [8,10]. It is also shown that the newly generated cells of the DG layer, in the subgranular zone (SGZ), migrate to the granular layer. Following migration, they differentiate to the neurons that make new synapses and circuits with pyramidal neurons in CA1 and CA3 layers through their new formed projections [11]. It is accepted that neurogenesis is associated with neuronal plasticity particularly for hippocampus, so that the new neurons that enter into the hippocampal circuitry give rise to functional neurons which are associated with some types of hippocampal-dependent memory [12]. There are growing evidences that showed the certain factors such as, different types of stressors, oxidative stress and metabolic disorders like diabetes influence the neurogenesis in hippocampus. For female gonadal hormones it is shown that these hormones especially estrogen not only exert broad effects on various tissues throughout body, but also insert dramatic effects on brain. Gonadal hormones easily pass through blood brain barrier and exert their effects on certain structures of the brain [13]. Due to this feature of gonadal hormones the response of neurons to certain types of physiological or pathological conditions differs between male and female [14]. It is revealed that any fluctuation in the level of circulatory sex hormones lead to cellular and molecular changes such as neuronal loss, decrease of dendritic spines and arborization and alteration in neurotransmitter release and even gene expression [15,16]. Estrogen is also known for its neuroprotective and neurotropic roles that influence neurogenesis and prevent neuronal death induce by neurotoxins such as β-amyloid peptide toxicity, oxidative stress, diabetes, experimental autoimmune encephalomyelitis, Parkinson's disease, and forebrain ischemia [17]. In addition it is reported that hypo-estrogenic condition during menopause leads to age dependent decrease of neurogenesis which in turn resulting to cognitive behavior impairment in menopause women [18]. It is shown that circulating estradiol level effects on hippocampus morphological parameters, such as neuronal morphology, dendritic branching, density of synapses in DG and CA1 layer [19,20]. With attention to the neuroprotective effects of endogenous female sex steroid hormones and also cognitive dysfunction caused by DM, it is not clear that whether exogenous steroid could suppress the effects of DM on hippocampus, in order to answer this question the present research designed.

## MATERIALS AND METHODS

### Biological model

Female adult Sprague-Dawley rats (200-250gr) (Razi Institute, Iran) were used in this study. The animals kept under standard conditions of controlled humidity and temperature with 14/10 h light-dark cycle, food and water ad libitum. All the procedures approved by Ethic Research Committee of Iran University of Medical Sciences. The animals randomly divided into eight groups as follows: STZ-induced diabetic(Diab), STZ-induced diabetic with ovariectomy (Diab+ OVX), STZ-induced diabetic + ovariectomy treated with estrogen(Diab + OVX+ E2), ovariectomized animals (OVX), intact females (Control), surgical sham (Sur sham), vehicle of STZ (OIL), and group of vehicle of estrogen. Ovariectomy was done one week before STZ injection, the interval time for study of the animals of trial, control and sham was 9 weeks.

### Diabetes induction

To induce diabetes the animals of groups 1, 2, and 3 received a single i.p dose of 50mg/kg of streptozotocin (STZ) (Sigma-Ald) dissolved, immediately before administration, in freshly prepared in 0.1 M sodium citrate buffer. All animals received injection at same time (16:00). Age-matched control animals received an equivalent volume of citrate buffer. STZ-treated rats showed marked hyperglycemia 2 days after STZ injection. Diabetic rats were entered into the study when they were confirmed to be diabetic (i.e., blood glucose

levels ≥300 mg/dl and urine glucose levels ≥ 1,000 mg/dl). Blood glucose levels checked weekly. Eight weeks after STZ injection or vehicle, animals were weighted and decapitated or perfused.

**Surgery:** Under anesthesia (Ketamine/ Xylazine, 8/1/Kg i.p.) and sterile condition by ventral incision, the abdominal cavity opened and the ovaries with distal part of uterus removed and the remaining part ligated. Abdominal cavity washed with saline and sutured. Ovariectomy was done one week before STZ injection.

**E2 administration:** Seven week after STZ injection, 20µgr of 17β estradiol (E2) (Sigma) or vehicle was injected for 10 days in group of STZ-induce diabetic with ovariectomy.

**Perfusion and fixation**

For light microscopic histological study perfusion and fixation was done. To do this the animals of the groups that were supposed to study by Nissl staining, were deeply anesthetized by lethal dose of anesthetic, perfused and fixed intracardially with saline, followed by 4% paraformaldehyde in 0.1% M phosphate buffer (PB) PH: 7.2. The brains were removed and kept in the same fixation overnight. By using rotary microtome serial coronal sections of 10 µm of forebrain that containing entire length of hippocampus (-2.12 to -5.30 of Bregma according to Paxinos Atlas) prepared and mounted on gelatin coated slides. DG area of hippocampus was studied for morphometric parameters by Nissl staining.

**Nissl staining**

To count neurons and study the neuronal density of DG area Nissl staining was used. The selected mounted sections were subjected to staining according routine procedure for Nissl. DG area of hippocampus located between Bregma -2.12 to -5.30 was studied. By using Analysis Imaging Software (Soft Imaging System) at magnification of 400X the neurons of DG that showed distinct cytoplasm, clear nuclear outline and visible nucleolus were counted. The mean for neuronal number for each group computed and compared among different groups.

**Brdu (5-bromo-2′-deoxyuridine) immunocytochemistry**

To study neurogenesis in DG, Brdu immunohistochemistry was used. The animals received a single i.p injection of Brdu (Sigma) of 300µgr/kg, scarified 2 h later. Subsequent to perfusion and preparing brain tissue slides, Brdu-labelled cells were observed in 9-10 sections from each animal in the sections containing DG [21]. By using florescent Microscope the Brdu positive cells observed and counted.

**Western Blotting**

In order to study apoptosis, expression of Bax and Bcl2 proteins were detected using western blot technique. Frozen hippocampus tissue homogenized in 0.5 ml of RIPA buffer 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA,1% Triton X-100, 0.1%ripa buffer sodium dodecyl sulfate, and 0.5% sodium deoxycholate) containing protease and phosphatase cocktails (Sigma). After centrifugation at 13,000 g for 20 min at 4ºC, the supernatant was collected. Protein concentrations were determined by Bradford protein assay, and equivalent amounts of total cellular protein were separated by 10% SDS–PAGE. The gels were then electro blotted onto nitrocellulose membranes. Subsequently, membranes were blocked 1 h with 5% nonfat skim milk in TBS containing 0.1% (v/v) Tween-20, and probed with specific primary antibodies overnight at 4ºC. After three washes in TBS-T, membranes were incubated with secondary antibodies -conjugated by alkaline phosphatase Proteins were then visualized with NBT/BCIP Tab. By using UVtec Soft wear western blot bundle measured according to amount of protein staining and quantifies.

**Data analysis**

The results are presented as mean ± SEM. Statistical analysis of data was done by using SPSS 19. Data were compared among groups with two-way analysis of variance (ANOVA) followed by the Tukey post hoc test. The $Pv < 0.05$ considered as significant.

## RESULTS

**STZ induced hyperglycemia and loss of body weight**

The STZ-treated animals showed diabetic signs 48 hours after the injection, and hyperglycemia condition remained for 8 weeks (to 300 mg/dl). During the experimental period, diabetic rats showed significant body weight decrease compared with the control group.

**Result of Nissl staining**

Cell counting was done in all groups for both right and left DG according to Paxinos (-2.12 to -5.30 of Bregma). No significant difference was found among control, surgical sham and vehicle-treated ($P_V>0.05$)

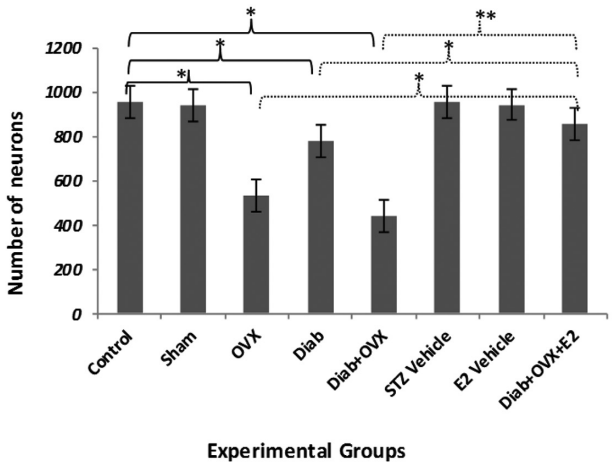


**Figure 1.** The number of positive Nissl stained neurons in DG area of the hippocampus in trial and control groups.
The number of Nissl positive neurons in DG is significantly lower in OVX, Diab & Diab+OVX as compared with control and sham groups (*$P_v$ < 0.05). E2 treatment in Diab+OVX+E2 significantly restored the number of Nissl positive neurons as compared with OVX, Diab & Diab+OVX groups (*$P_v$ < 0.05, **$P_v$ < 0.001). The difference between Diab+OVX+E2 as compared with control is not significant ($P_V$>0.05).

(Figure 1 & 2). Comparing trial groups including Diab, OVX, Diab+OVX with control showed significant reduction in the number of Nissl positive neurons ($P_V$ <0.05) (Figure 1 & 2). The number of Nissl positive neurons in Diab+ovx+E2 comparing to Diab, OVX and Diab+OVX significantly restored ($P_V$ <0.05) (Figure 1 & 2). However, it is not significant comparing with control group ($P_V$ >0.05) (Figure 1 & 2).

## Result of Brdu IHC

To study cell proliferation, quantitative analysis of the Brdu-labelled cells was performed in the DG region of hippocampus. Cell counting in this area showed a mean of 184±6.80 in control, 177.00±12.20 in sham surgery, 174±15.40 in STZ vehicle, 178.20±16.93 in E2 vehicle with no significant difference among them (Pv>0.05). For groups of Diab, OVX and OVX + Diab the mean of Brdu positive cells in DG was as follows respectively; 150.80±9.52, 102.40±7.50 and 69.20±15.57 that show significant decrease in neurogenesis comparing with control group (Pv<0.05). In Diab+OVX+E2 group the mean of Brdu positive cells was (171.60± 11.50) that showed a few but not significant reduction comparing control group (Figure 3 & 4). Although this difference was not significant with control group, comparing this group with Diab, OVX and Diab+OVX showed significant increase of neurogenesis following E2 treatment (Figure 3 & 4).

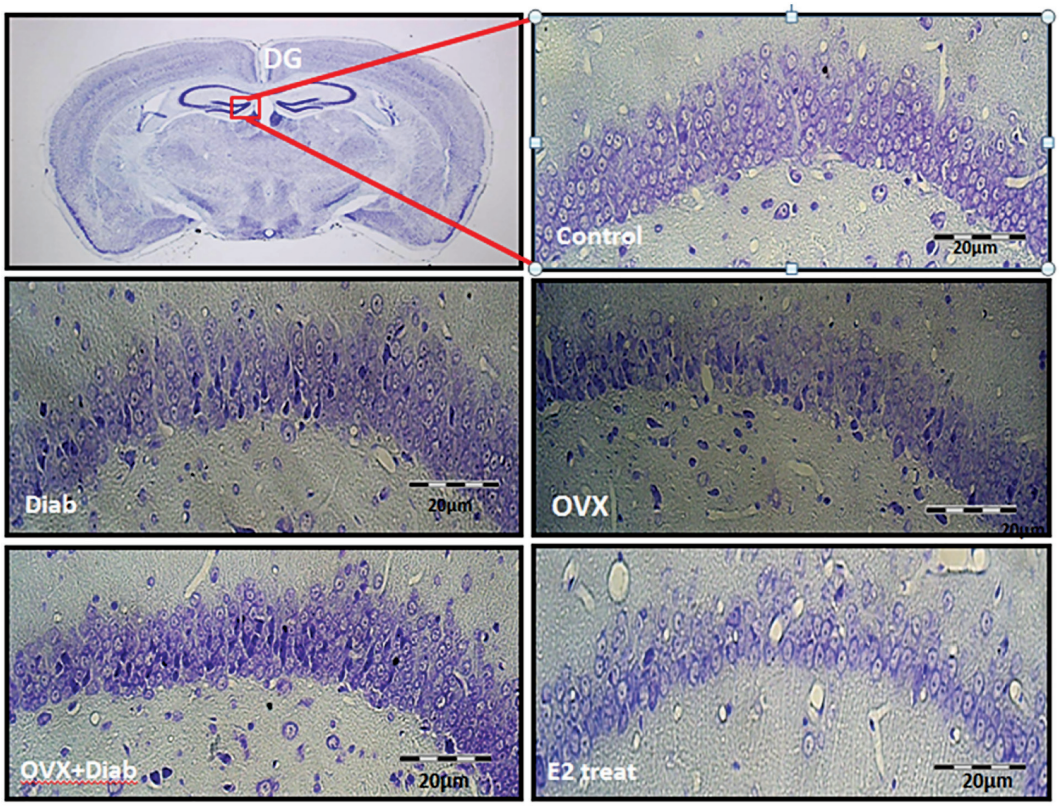


**Figure 2.** DG area of hippocampus with Nissl staining in control and trial groups, decreased number of Nissl positive neurons seen in Diab, OVX & Diab+OVX as compared with control group. E2 treat restore the number of Nissl positive neurons in DG. (Magnification: 400x).

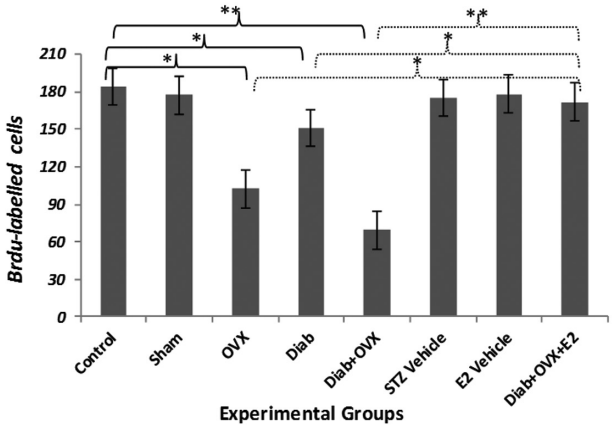


**Figure 3.** Brdu IHC for neurogenesis in DG of trial and control groups
The number of Brdu positive neurons in DG is significantly lower in OVX, Diab & Diab+OVX as compared with control and sham groups (* Pv < 0.05). E2 treatment in Diab+OVX+E2 significantly restored the number of Brdu positive neurons as compared with OVX, Diab & Diab+OVX groups (* Pv < 0.05, ** Pv < 0.001). The difference between Diab+OVX+E2 as compared with control is not significant ($P_V$>0.05).

## Results of Bax and Bcl-2 expression

Expression of the proteins involve in apoptosis including Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) was evaluated by using of western blotting. The results showed that in Diab + OVX and OVX groups there were significant decrease in Bcl-2 protein expression (with a mean of 59623.00 ±1000.00, 82117.00 ±1000.00, respectively) and significant enhance in Bax protein expression (with a mean of 64456.00 ±1000.00, 51138.00 ±1516.57, respectively) as compared to other groups (Pv <0.05). Regarding the group received E2, the result showed that E2 treatment has been able to prevent apoptosis in the Diab+OVX+E2 group (Bcl2: 125007.00 ±10000.00, Bax: 31354.00 ±1000.00) although it is not similar to control group (Bcl2:108231.00 ±10000.00, Bax: 32009.00 ±547.72), no significant difference was found (Pv >0.05) (Figure 5 & 6).

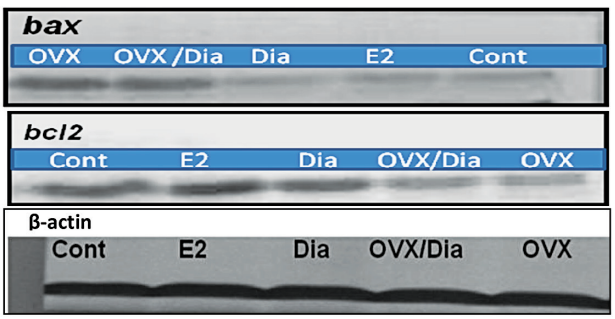


**Figure 5.** Western blot analysis of Bax and Bcl2 in control, Diab+OVX+E2, Diab+OVX, Diab, OVX
Bax expression increased in OVX, Diab+OVX compared to control, Diab+OVX+E2, Diab. Bcl2 expression decreased in OVX, Diab+OVX compared to control, Diab+OVX+E2, Diab

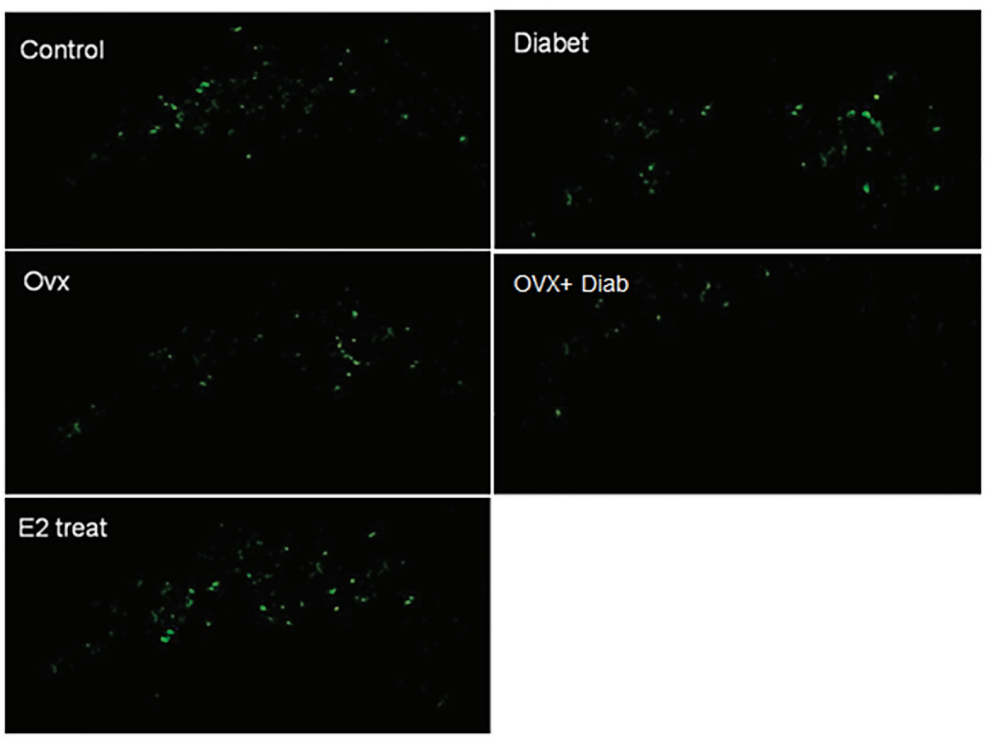


**Figure 4.** Neurogenesis decreased significantly in Diab, OVX & Diab+OVX (*: Pv < 0.05) comparing to control group. Estrogen treatment restores neurogenesis in Diab+OVX+E2 comparing to Diab, OVX & Diab+OVX ((*: Pv < 0.05, **: Pv < 0.001).

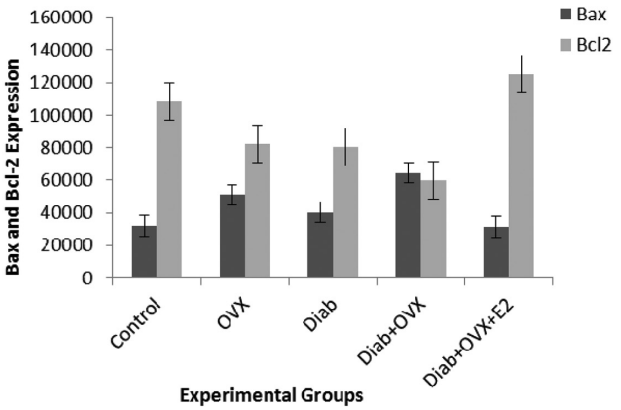


**Figure 6.** Quantitative analysis of expression levels of Bax and Bcl2 by western blotting.

## DISCUSSION

Our findings showed that DM could lead to dramatic changes on DG area of hippocampus at both level of cellular and molecular in presence or not, of female sex steroid hormones. However these changes were more sever in animals of Diab+OVX group. We also showed that exogenous E2 treatment was able to suppress or decrease these changes. Exogenous E2 treatment restore cell number, enhanced neurogenesis and Bcl-2 expression, and reduced Bax expression.

Recent studies have shown that DM causes neuropathological changes, which lead to alterations of neuronal structure, function and eventually followed by behavioral changes [22]. Ristow et al reported that more than 20 neurodegenerative diseases (NDD) are associated with diabetes mellitus in humans [23]. How diabetes can insert such broad changes in nervous system is still a matter of question. Makimattila et al claimed that such correlation between DM and NDD may reflect a direct effect of hyperglycemia on the brain, or of the diabetes-associated comorbidities of hypertension, dyslipidemia, or hyperinsulinemia [24]. In addition to these mentioned possible mechanisms, Saravia et al went further and demonstrate the role of immune reactions in DM. They showed that astrocytes become highly reactive in diabetic animals, showing increased immunoreaction for GFAP, a marker for astrocytosis [25]. Increased the number of Apo-E-immunoreactive astrocytes, that indicating glial cells developed a mechanism to offset neurodegeneration, has been shown in diabetic animals [26,27]. By attention to these findings we believe that same mechanisms might be responsible for our results in Diab and Diab+OVX animals. Additionally, hyperglycemia effects on endocrine system and cortisol level enhanced in diabetic patients [28]. It is shown that the corticosteroids influenced neuronal plasticity and cell death it may start under mechanism associated with NDD caused by diabetes. Glucocorticoids (GCs) also regulated IGF system in both animals and human [29]. Regarding the role of insulin-like growth factors (IGFs) in neuronal development, differentiation and function in the nervous system,it is possible that any changes in GCs level could lead to DM dependent neurodegeneration [30,31].

There are numerous evidences that show DM dependent changes are more evident in the hippocampus. It is reported that in normal rat hippocampus, proliferation and neuronal differentiation were upraised by IGF-I [31,32]. But in the hippocampus of diabetics adult rat, decrease of IGF-I expression and its receptor is accompanied with apoptotic neuronal loss and thus functional cognitive disorders [20]. Zhen et al showed that any reduction in IGF and defects in insulin activities promote apoptotic process [33]. TUNEL-positive neurons and neuronal damage were seen in the CA1, the region of hippocampus that expression of IGF-IR and IR was decrease, indicating a link between IGF, insulin activities and progression of apoptosis [34]. Regarding the role of CA1 area of the hippocampus in memory and learning process, any cellular changes of this region might lead to cognitive disorders [35].

In addition, we also showed that exogenous E2 treatment could decreased the effects of DM. The ovarian hormone17b-estradiol is an important regulator of normal brain function that has profound effects on structure, plasticity and function of the nervous system. The neuroprotective effects of estrogen are the most well-known features reported during the recent years. Estrogen receptors (ERs) are expressed by neurons in several areas of the brain. Although the exact mechanisms of estrogen to prevent cell death are still unknown, several mechanisms of action have been proposed to explain neuroprotective and anti-inflammatory effects of estrogen. Transcriptional regulation mediate by estrogen receptors, activation of cytoplasmic signaling cascades and antioxidative process mediated by a phenolic ring of molecule are the suggested mechanisms [36-40]. Additionally, it is shown that the estrogen is able to activate cellular cascades involving growth factors, such as IGF-I [20,41]. Estrogen regulate IGF-I expression in the animals treated with exogenous estrogen so that interaction of estradiol-IGF-I influenced cell proliferation [20]. Regarding E2 effects, Picazo et al, reported that 17a-Ethynylestradiol (EE2), a major constituent of many oral contraceptives, with similar structure to17b-estradiol, has neuroprotective properties in several animal models [42]. They exactly

showed the neuroprotective effects of using EE2 against neurotoxicity induced by kainic and quinolinic acid in hippocampus of adult ovariectomized rats [42]. For the first above mentioned mechanism i.e. transcription regulation mediate by estrogen receptors, the estrogen receptors (ERα and ERβ) identified in the areas of the central nerve system involved in neurogenesis. Both receptors were recognized in progenitor cells of SVZ but the DG cells express only ERβ mRNA [43]. Based on these findings more increasing in the level of estrogen, more proliferation in DG area is expected [44]. From this point of view the sever loss of neurogenesis and Nissl positive cells in animals of Diab+OVX comparing to Diab and OVX groups are understandable. In fact, it seems that DM and OVX enhanced their each other effects in Diab+OVX animals. In functional terms, the impaired neurogenesis in dentate gyrus may lie beneath the numerous deficits in learning and memory tasks previously shown in diabetic patient especially in menopause female cases with DM [45-47].

Accordingly, the factors such as estrogen, enriched-environment living, exercise and running, considered as positive regulators of adult neurogenesis which long-lasting exposure to them has been associated with enhanced performance on hippocampal-dependent learning tasks in rodents and other cognitive tasks in humans [48-50]. Conversely, negative regulators, such as adrenal steroids and stress, has been associated with impaired performance on such tasks [51-53].

These correlations are consistent with the view that adult-generated neurons participate in hippocampal-dependent learning; however, these neuroendocrine and experiential factors also alter other aspects of brain structure and function that cannot be ruled out as alternative mechanisms for the changes in hippocampal-dependent learning.

Although the present work did not focus on mechanisms that lead to changes of neurogenesis in DG zone following DM, OVX and E2 treatment, our results confirmed that estrogens have powerful effects in altering the vulnerability of dentate granule cells to harmful mechanisms following diabetes. We believe that our findings together with same results reported by the others may open new vision for preventive strategies or therapy for diabetic women who enters menopause period and might be prone to cognitive dysfunction. Although the normal and gradual decline of estrogen subsequent to menopause differs from acute estrogen deprivation caused by ovariectomy, the increased vulnerability of gonadectomized animals to the diabetic toxicity resembles the vulnerability of postmenopausal women to cognitive disorders. Moreover, the lack of effectiveness of estrogen in totally suppressing or preventing cell death caused by DM which seen in Diab+OVX+E2 animals as compare to control group is similar to the absence of observed efficacy in definite cognitive dysfunction. Put these findings together suggest a modulatory role for estrogens for neuronal vulnerability to certain toxic such as DM, but not a classical neuroprotective effect. Liu et al emphasized on the modulatory role of estrogen for preventing apoptosis of granular neurons in adult rat hippocampus [54]. Either modulatory or classical definite role, powerful effects of estrogens on brain are concluded by most studies as same as ours.

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