ARTICLE

Decreased nitric oxide levels in the hippocampus may play a role in learning and memory deficits in ovariectomized rats treated by a high dose of estradiol

Níveis diminuídos de óxido nítrico no hipocampo podem ser importantes nos déficits de aprendizado e de memória em ratas ooforectomizadas tratadas com alta dose de estradiol

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ABSTRACT

The effects of a high estradiol dose on memory and on nitric oxide metabolites in hippocampal tissues were investigated. Sham-Est and OVX-Est Groups were treated with 4 mg/kg of estradiol valerate for 12 weeks. Time latency and path length were significantly higher in the Sham-Est and OVX-Est Groups than in the Sham and OVX Groups, respectively ($p<0.001$). The animals in the Sham-Est and OVX-Est Groups spent lower time in the target quadrant (Q1) than those of the Sham and OVX Groups during the probe trial test ($p<0.05$ and <0.001, respectively). Significantly lower nitric oxide metabolite levels in the hippocampi of the Sham-Est and OVX-Est Groups were observed than in the Sham and OVX ones ($p<0.001$). These results suggest that decreased nitric oxide levels in the hippocampus may play a role in the learning and memory deficits observed after treatment with a high dose of estradiol, although the precise underlying mechanisms remain to be elucidated.

Key words: estradiol, rat, hippocampus, nitric oxide.

RESUMO

Os efeitos de uma alta dose de estradiol na memória e nos metabólitos do óxido nítrico de tecidos hipocampais foram estudados. Os Grupos Sham-Est e OVX-Est foram tratados com 4 mg/kg de valerato de estradiol por 12 semanas. O tempo de latência e o comprimento do caminho foram significativamente maiores nos Grupos Sham-Est e OVX-Est em relação aos Grupos Sham e OVX, respectivamente ($p<0,001$). Os animais dos Grupos Sham-Est e OVX-Est passaram menos tempo na meta do quadrante (Q1) do que aqueles dos Grupos Sham e OVX durante o teste inicial ($p<0,05$ e $<0,001$, respectivamente). Níveis significativamente menores de metabólitos do óxido nítrico foram observados nos hipocampos dos Grupos Sham-Est e OVX-Est em relação aos Grupos Sham e OVX ($p<0,001$). Esses resultados sugerem que os níveis diminuídos de óxido nítrico no hipocampo podem ter um papel nos déficits de aprendizado e de memória, que são observados após tratamento com alta dose de estradiol, embora os mecanismos específicos envolvidos nestes achados ainda precisam ser elucidados.

Palavras-Chave: estradiol, rato, hipocampo, óxido nítrico.

Cognitive impairments occur in both men and women as the age increases. However, the decline in cognitive ability, for example Alzheimer's disease, is more sever in women during menopause[1,2]. Therefore, it seems that changing the levels of steroid hormones after menopause, and particularly the loss of estradiol, has a role in senile cognitive worsening[3]. The main source of sex hormones is the gonads, which reach the brain via the blood circulation. However, estrogens and androgens are synthesized in mammalian brain areas such as the hippocampus[4]. Neuromodulatory actions of gonadal sex hormones in the hippocampus, as an important center involved in learning and memory, are also attractive[5].

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There are controversial reports regarding the effect of estrogen on learning and on memory. The positive effect[6], no effect[7,8], or even negative effect[9] of estrogen on learning and memory have been reported. For example, numerous studies have shown that estrogens attenuate memory dysfunction due to a surgical or natural menopause in women[7] as well as in adult ovariectomized (OVX) rats and mice[10]. Likewise, treatment of young female mice by estradiol improved foot shock avoidance tasks in comparison with estrogen plus progesterone[11]. However, treatment of OVX rats by estradiol, such that physiological concentrations of hormone in plasma are achieved, performance in hippocampally mediated tasks such as the water maze, radial arm maze, elevated plus maze, and passive avoidance tests are improved[5,10,12,13]. Results of studies conducted in animals and in humans suggest that the initiation of estrogen therapy at the time of menopause, or soon after ovariectomy, provides an opportunity to prevent memory loss in females, whereas administration of the hormone with a considerable delay has little or even no valuable effects[7]. In contrast, it has been shown that the acute treatment of young female rats by estradiol and progesterone impairs spatial memory in the Morris water maze[9].

It has been well documented that estrogen influences the nitric oxide (NO) system in both peripheral and nervous tissues[14,15]. Additionally, estrogen increases endothelial nitric oxide synthase (eNOS) and neuronal and nitric oxide synthase (nNOS) activities, expression and production of NO in tissues including the brain, therefore, some actions of estradiol are mediated by NO[15,16]. It was previously shown that removal of ovaries impairs Morris water maze tasks which is reversible by $_{L}$-arginine, the precursor of NO[17]. It was also shown that administration of 2 mg/kg estradiol valerate improved learning of OVX, but not sham-oprated rats using Morris water maze[18,19]. In the present study, the chronic effects of a higher dose of estradiol on learning, memory and NO metabolites in hippocampal tissues of OVX rats were investigated.

METHODS

Animals and drugs

Thirty-two female Wistar rats (20-week old and weighing 250±20 g) were obtained from the Razi Vaccine and Serum Research Institute (Mashhad, Khorasan province, in Iran), and four were housed per standard cage at 22±2°C in a room with a 12-hour light/dark cycle (light on at 7:00 am), with free access to water and food *ad libitum*.

Rats were given one week for adaptation to the new environment before any procedure was initiated. Animal handling and related procedures were approved by the Mashhad Medical University Committee on Animal Research. Ketamin and xylazine were purchased from Alfasan Company (Holland). Estradiol valerate was kindly provided by Iran Hormone Pharm (Tehran, Iran).

Surgery

Rats were anesthetized with ketamine (150 mg/kg) and xylazine (0.1 mg/kg)[20]. Anesthesia was confirmed by reduced respiratory rate and no response to gentle pinching of footpad. Ventral incision was performed through the rat flank skin and ovaries and ovarian fats were removed. Ovaries were isolated by connection of the most proximal portion of the oviduct before removal. The animals were reversed to their cages in order to recover from surgery. Two groups of rats were subjected to sham. The above-mentioned procedure was also performed on the sham rats, although after laparotomy, the wound was closed without removing the ovaries[21].

Groups and treatments

After recovery, animals were randomly divided into four groups: Sham, Ovariectomy, (OVX), Sham-Estradiol (Sham-Est), and OVX-Estradiol (OVX-Est). The animals of Sham-Est and OVX-Est Groups received a weekly injection of estradiol valerate (4 mg/kg, subcutaneous) for 12 weeks. The animals of Sham and OVX Groups were injected with 1 mL/kg saline instead of estradiol valerate. All treatments were performed from the day after ovariectomy until the beginning of the behavioral study.

Morris water maze apparatus and procedures

A circular black pool (136 cm diameter, 60 cm high, 30 cm deep) was filled with water (23–25°C). A circular platform (10 cm diameter, 28 cm high) was placed within the pool and was submerged approximately 2 cm below the surface of the water in the center of the Northwest quadrant. Outside the maze, fixed visual cues were present at several locations around the room (i.e., computer, hardware, and posters). An infrared camera was mounted above the center of the maze and an infrared LED was attached to each rat for motion tracking. Before each experiment, each rat was handled in a three-day basis and habituated to the water maze for 30 seconds without a platform. The animals performed four trials on each of the five consecutive days, and each trial began with the rat being placed inside the pool and released facing the side wall at one of four positions. The boundaries of the four quadrants were labeled as North (N), East (E), South (S), and West (W). Release positions were randomly predetermined.

For each trial, the rat was allowed to swim until it found and remained on the platform for 20 seconds. If 60 seconds had passed and the animal had not found the platform, it was guided to the platform by the experimenter and allowed to stay on it for 20 seconds. Then, it was removed from the pool, dried and placed in its holding bin for another 20 seconds. The time latency to reach the platform and the length of the swimming path were recorded by a video tracking system[17-19].

On the sixth day, the platform was removed, and the animals were allowed to swim for 60 seconds. The time spent in the target quadrant (Q1) and the traveled path were compared between groups. All measurements were performed during the first of the light cycle.

Biochemical assessment

After the last session of the Morris water maze test, blood samples were taken from all rats to determine the NO metabolites NO_2 and NO_3 (Griess reagent method). The animals were then sacrificed, and their hippocampi were removed and submitted to NO metabolite measurements in the tissue. The Griess reaction was adapted to assay nitrates as previously described. Briefly, standard curves for nitrates (Sigma, St. Louis, Missouri, USA) were prepared, and samples (50 µL serum and 100 µL tissue suspension) were added to the Griess reagent. The proteins were subsequently precipitated by the addition of 50 µL of 10% trichloroacetic acid (Sigma). The contents were then vortex-mixed and centrifuged, and the supernatants were transferred to a 96-well flat-bottomed microplate. Absorbance was read at 520 nm using a microplate reader, and final values were calculated from standard calibration plots[22].

Statistical analysis

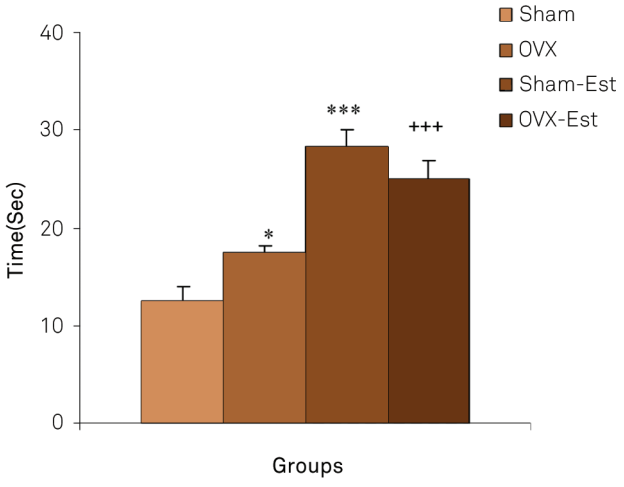
All data were expressed as mean±standard error of mean (mean±SEM). Swim time latency and length of the traveled path over the five training days were analyzed by repeated measures from the analysis of variance (ANOVA). The time spent in the target quadrant (Q1) was compared using one-way ANOVA. Comparison of serum and hippocampal tissues NO metabolites levels was also carried out using one ANOVA. The criterion for statistical significance was $p<0.05$.

RESULTS

Time latency and length of the swimming path over the five training days were significantly higher in the OVX Group in comparison with the Sham one ($p<0.05$ and $p<0.01$; Figs 1 and 2). The animals from both Sham-Est and OVX-Est Groups had significantly higher time latency and travelled length to reach platform in comparison with those of Sham and OVX Groups, respectively ($p<0.001$), as can be seen in Figs 1 and 2.

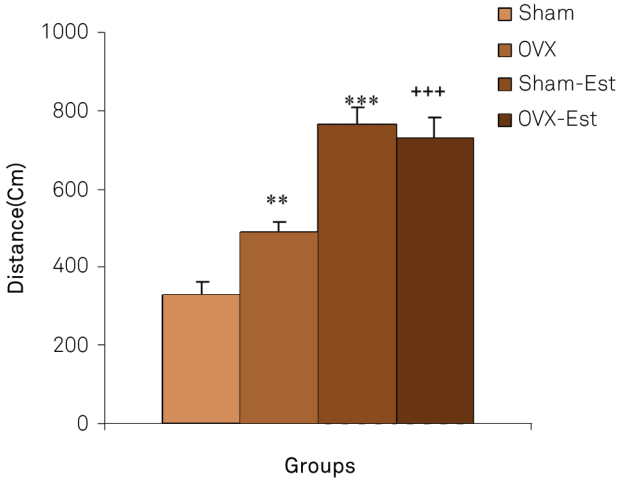
In the probe trial, the time spent in the target quadrant (Q1) by the animals of Sham-Est and OVX-Est Groups was significantly lower than Sham and OVX Groups, respectively ($p<0.05$ and <0.001), as seen in Fig 3. The animals from Sham-Est Group spent more time in nontarget quadrants (Q3 and Q4) when compared with the Sham Group ($p<0.01$ and <0.05) (Fig 3). There were no significant differences in the time spent in the Q2 between the four groups.

NO metabolite concentrations, NO_2 or NO_3, in the serum of the OVX Group were lower than that of Sham Group ($p<0.001$), as seen in Fig 4. The serum concentrations of NO_2 or NO_3 in both Sham-Est and OVX-Est Groups were significantly lower than those of Sham and OVX Groups, respectively ($p<0.001$ and <0.05) (Fig 4). The concentrations of NO



*p<0.05 and ***p<0.001 compared to Sham Group, $^{+++}$p<0.001 compared to OVX Group.

Fig 1. Comparison of swim time latency (seconds) to find the platform among Sham, OVX, Sham-Est and OVX-Est Groups. The latency was significantly higher in the OVX Group compared to the Sham one ($p<0.05$). The animals of both Sham-Est and OVX-Est Groups had significantly higher time latency to reach platform in comparison with those of Sham and OVX Groups ($p<0.001$ and <0.001 respectively). Data are shown as mean±SEM of eight animals per group.



**p<0.01 and ***p<0.001 compared to Sham Group; $^{+++}$p<0.001 compared to OVX Group.

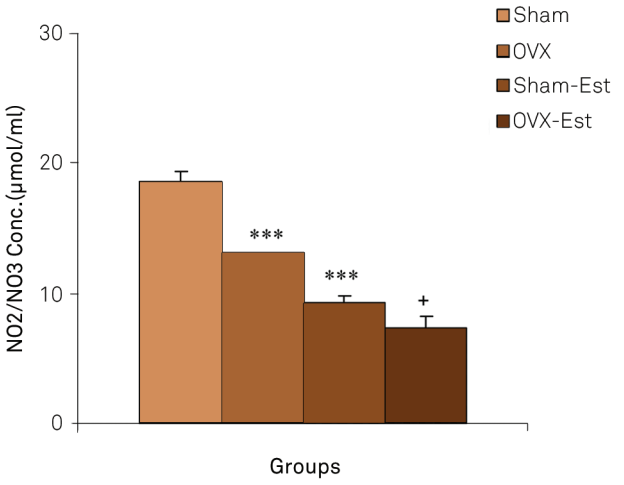
Fig 2. Comparison of the length of the swimming path (cm) to find the platform among Sham, OVX, Sham-Est, and OVX-Est Groups. The latency was significantly higher in the OVX compared to the Sham Group ($p<0.01$). The animals of both Sham-Est and OVX-Est groups had significantly higher travelled length to reach platform in comparison with those of Sham and OVX Groups respectively ($p<0.001$). Data are shown as mean±SEM of eight animals per group.

metabolites in the hippocampal tissues of the OVX Group were lower than those from the Sham Group ($p<0.01$), as shown in Fig 5. The concentration of NO metabolites in the hippocampi of Sham-Est and OVX-Est Groups was lower than that of the Sham and OVX Groups, both $p<0.001$ (Fig 5).

DISCUSSION

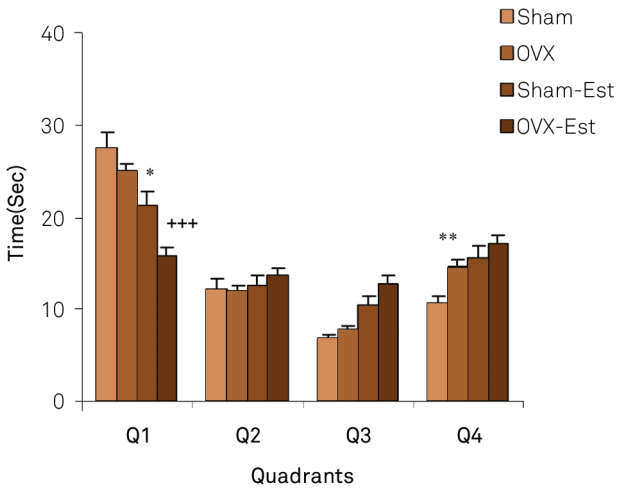
In the present study, the chronic effects of a high estradiol valerate dose on learning and spatial memory as well as NO metabolites in serum and hippocampal tissues of OVX and sham-operated female rats were investigated. OVX rats have been frequently used as a model of hormone deprivation to study postmenopausal changes in adult females[17-19]. The results showed that deletion of ovarian hormones impaired learning and memory in Morris water maze. The OVX rats spent more time and travelled longer distances to reach platform during five days. The results also showed that the animals of the OVX Group remembered the location of platform better than sham-operated ones. These results were in agreement with the previous studies[17,19]. In contrast with these findings, Herlitz et al. showed that there were no considerable differences in cognitive performance between premenopausal and postmenopausal women[23].

We have previously shown that administration of 2 mg/kg estradiol for eight weeks improved learning and memory in ovariectomized rats, but it had deleterious effect in sham-operated ones[18,19]. Therefore, the effect of chronically administering a higher dose of estradiol was examined in the present study. The results indicated that estrogen therapy by a high dose has deleterious effects on spatial memory retention in both OVX and sham-operated rats. The time latency and traveled path length to find the hidden platform in animals of OVX-Est Group were significantly lower than in the OVX Group. Other researchers have also reported that estrogen has negative effects[9] or no effect[8,13] on learning and memory. It has also been reported that the OVX rats had a better performance that intact rats at the spatial version of the water maze and, therefore, it seems that



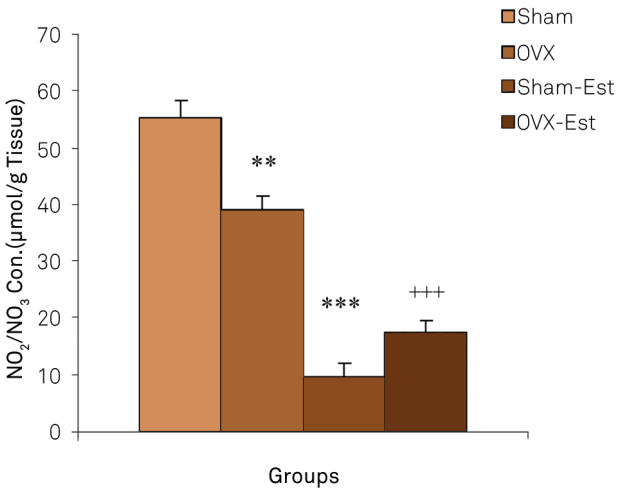
***p<0.001 compared to Sham Group; $^{+}$:p<0.05 compared to the OVX Group.

Fig 4. Comparison of serum nitric oxide metabolite levels between the four groups. Data are shown as mean±SEM of eight animals per group. Nitric oxide metabolite concentrations in the serum of OVX Group was lower than that of the Sham Group ($p<0.001$). The serum concentrations of NO_2 or NO_3 in both Sham-Est and OVX-Est groups were significantly lower than those of Sham and OVX Groups respectively ($p<0.001$ and <0.05).



*p<0.05 and **p<0.01 compared to Sham Group, $^{+++}$p<0.001 compared to OVX Group.

Fig 3. The results of the time (second) spent in target quadrant during the probe trial on day 96 (24 hours after the last secession of learning). Data are shown as mean±SEM of eight animals per group. The platform was removed, and the time spent in the target quadrant (Q1) was compared between the groups.



**p<0.01, ***p<0.001 compared to Sham Group, $^{+++}$p<0.001 compared to the OVX Group.

Fig 5. Comparison of the concentrations of nitric oxide metabolite in hippocampal tissues between groups. Data are shown as mean±SEM of eight animals per group. The concentrations of nitric oxide metabolites in the hippocampal tissues of the OVX Group were lower than those of the Sham Group ($p<0.01$). The concentration of nitric oxide metabolites in the hippocampi of Sham-Est and OVX-Est groups was lower than that of the Sham and OVX groups ($p<0.001$).

the ovarian steroids impair performance on hippocampal-dependent versions of this task[24]. The exact mechanisms by which estrogen regulates the functions of spatial memory have been widely investigated. It has been shown that estrogen affects neurotransmitters such as glutamate, gaba, acetylcholine, and their receptors[25,26]. Direct or indirect effects of estradiol on hippocampal neurons are also attractive as possible mechanisms[27].

NO contributes in hippocampal dependent learning and memory tasks including spatial memory[22,28]. On the other hand, it has been suggested that NO mediates the effects of estradiol in many organs[29]. Considering the fact that estradiol affects the release of NO and the NOS isoforms activity in the brain, the interaction of estrogen and NO in nervous system functions is conceivable[14-16]. It has been previously shown that NO contributes in sex hormone-dependent changes of behavior[21-30]. Previous studies also implied that some functions of estrogen in the central nervous system are related to increased NO production[15]. It was previously shown that $_{L}$-arginine, the precursor of NO, improves learning and memory impairments in OVX rats, however, it did not have an effect on sham-operated ones[17]. It was also suggested that regarding the presence of an interaction between estrogen and NO, estrogen deprivation causes to diminish NO in OVX rats and $_{L}$-arginine could improve spatial memory impairment. Attenuation of the improving effects of estradiol on learning and memory by NO inhibitor $_{L}$-NAME confirmed this hypothesis[19].

In the present study, learning and memory impairments in OVX rats were accompanied by lower levels of NO_2/NO_3 in serum and hippocampal tissues in comparison with sham-operated rats. This result confirms the involvement of NO in improvement effect of estradiol in physiologic conditions. With regard the finding that administration of estradiol improves learning and memory in OVX rats with deleterious effect in sham-operated rats in previous study[18], the effect of chronic administered effect of a higher dose of estradiol was examined in the present study. Negative effect of estradiol was shown on both OVX and Sham Groups. As mentioned, some actions of sex hormones including estradiol are mediated by NO. Therefore, we assumed that NO may have a role in deleterious effect of estradiol on learning and memory. The results showed that NO metabolites in serum and hippocampal tissues in both OVX and Sham animals treated with 4 mg/kg estradiol valerate were lower than the nontreated ones. The results obtained from other studies also show that memory impairment is accompanied with low level of NO[31]. The results confirm the role of NO as a critical mediator in synaptic plasticity, long-term potentiation (LTP), and consolidation of long-term memory[32]. Some researchers also believe that NO production decreases with age and may be involved in Alzheimer's disease[31,33], its neuronal damage has been found to result in an impairment of NO synthesis and in a decrease in NO containing neurons in the hippocampus[34].

In conclusion, the results presented in this study show that a high estradiol dose impairs spatial learning and memory. They also indicate that the low level of NO in hippocampal tissues might take part in spatial learning and memory impairments due to high levels of estradiol, however further investigations need to be carried out.

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