

# Different effects of scopolamine on learning, memory, and nitric oxide metabolite levels in hippocampal tissues of ovariectomized and Sham-operated rats

Diferentes efeitos da escopolamina no aprendizado, na memória e nos níveis dos metabólitos do óxido nítrico no tecido hipocampal de ratas ooforectomizadas e controles operadas sem ooforectomia

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## ABSTRACT

Different effects of scopolamine on learning, memory, and nitric oxide (NO) metabolites in hippocampal tissues of ovariectomized (OVX) and sham-operated rats were investigated. The animals in the Sham-Scopolamine (Sham-Sco) and OVX-Scopolamine (OVX-Sco) Groups were treated with 2 mg/kg scopolamine before undergoing the Morris water maze, while the animals in the Sham and OVX Groups received saline. The time latency and path length were significantly higher in both the Sham-Sco and the OVX-Sco Groups, in comparison with the Sham and OVX Groups, respectively ( $p < 0.001$ ). Significantly lower NO metabolite levels in the hippocampi of the Sham-Sco Group were observed, compared with the Sham Group ( $p < 0.001$ ), while there was no significant difference between the OVX-Sco and OVX Groups. The decreased NO level in the hippocampus may play a role in the learning and memory deficits induced by scopolamine. However, it seems that the effect of scopolamine on hippocampal NO differs between situations of presence and absence of ovarian hormones.

**Key words:** Morris water maze, scopolamine, ovariectomized, rat, hippocampus, nitric oxide.

## RESUMO

Diferentes efeitos da escopolamina no aprendizado, na memória e nos níveis dos metabólitos do óxido nítrico (ON) no tecido hipocampal de ratas ovariectomizadas (OVX) e controles com cirurgia sem ooforectomia (Grupo Sham) foram investigados. Os animais dos grupos Sham-Escopolamina (Sham-Sco) e OVX-Escopolamina (OVX-Sco) foram tratados com escopolamina 2 mg/kg antes de entrar no labirinto aquático de Morris, enquanto aqueles dos grupos Sham e OVX receberam solução salina. A latência de tempo e o comprimento do caminho foram significativamente maiores nos Grupos Sham-Sco e OVX-Sco em comparação com os grupos Sham e OVX, respectivamente ( $p < 0,001$ ). Foram observados níveis significativamente mais baixos de metabólitos do ON nos hipocampus do Grupo Sham-Sco em comparação aos níveis do Sham ( $p < 0,001$ ), enquanto não foi observada diferença significativa entre os Grupos OVX-Sco e OVX. A diminuição do nível de ON no hipocampo pode ter um papel no aprendizado e nos déficits de memória induzidos pela escopolamina. No entanto, parece que este efeito da escopolamina no ON hipocampal é diferente em situações de presença ou ausência de hormônios ovarianos.

**Palavras-Chave:** labirinto aquático de Morris, escopolamina, ooforectomia, rato, hipocampo, óxido nítrico.

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There is evidence that the change in the levels of steroid hormones, particularly estradiol, has a critical role in senile cognitive impairment. The source of sex hormones, which reach the brain, is the gonads. However, estrogens and androgens are synthesized in brain areas such as the hippocampus and they modulate the function of the hippocampus as an important center, which is involved in learning and memory<sup>1,2</sup>.

The positive effect<sup>3</sup>, no effect<sup>4,5</sup>, or even negative effect<sup>6</sup> of estrogen on learning and memory has been reported. The interaction of sex hormones with the neurotransmitter systems such as glutamatergic, serotonergic, adrenergic, dopaminergic, and cholinergic, which are involved in cognition, memory and learning, has been reported<sup>7-11</sup>.

The central cholinergic system is the most important neurotransmitter system, which is involved in several aspects of cognition and memory, and the cholinergic basis of memory dysfunction and cognitive decline in diseases such as Alzheimer's has also been well-documented<sup>12,13</sup>. The interaction of estrogen with the central cholinergic system has also been suggested. On one hand, estrogen has been reported to stimulate choline acetyltransferase expression and activity, the activity of acetylcholine esterase and potassium-stimulated acetylcholine release in rat hippocampus<sup>14-16</sup>. On the other hand, interaction of cholinergic system and nitric oxide (NO) for manipulation of learning and memory has been suggested<sup>17</sup>.

It has been well-documented that estrogen influences the NO system in both peripheral and nervous tissues. Additionally, estrogen increases endothelial NO synthase (eNOS) and neuronal NO synthase (nNOS) activity and expression and production of NO in tissues including the brain. Therefore, some actions of estradiol are mediated by the NO<sup>18-20</sup>. It was previously shown that removal of ovaries impairs Morris water maze tasks, which are reversible by L-arginine, the precursor of NO<sup>21</sup>. It was also shown that the administration of 2 mg/kg of estradiol valerate improved learning of ovariectomized (OVX), but not sham-operated rats using Morris water maze<sup>22,23</sup>.

In the present study, different effects of scopolamine on learning, memory, and NO metabolites levels in hippocampal tissues of OVX and sham operated rats were investigated.

## METHODS

### Animals and drugs

Thirty-two female Wistar rats (20 weeks-old and weighing 250±20 g) were obtained from the Razi Vaccine and Serum Research Institute (Mashhad, Khorasan province, in Iran). They were housed four per standard cage at 22±2°C in a room with a 12-hour light/dark cycle (light on at 7:00 A.M.), with free access to water and food *ad libitum*. Rats were given one week to get adapted to the new environment before any procedure was initiated. Animal handling and all related

procedures were approved by the Mashhad Medical University Committee on Animal Research. Ketamine and xylazine were purchased from Alfasan Company (Holland). Scopolamine was purchased from Sigma Aldrich (St. Louis MO, USA).

### Surgery

Rats were anesthetized with ketamine (150 mg/kg) and xylazine (0.1 mg/kg). Anesthesia was confirmed by reduced respiratory rate and no response to gentle pinching of footpad. Ventral incision was made through the skin of the flank of the rat, and ovaries and ovarian fats were removed. Ovaries were isolated by ligation of the most proximal portion of the oviduct before removal. The animals were reversed to their cages to recover from surgery. Two groups of the rats were subjected to sham. The same procedure was almost as ovariectomy, except that the ovaries were not removed<sup>21-23</sup>.

### Groups and treatments

Twelve weeks after surgery, the animals were randomly divided into four groups: Sham; OVX; Sham-Scopolamine (Sham-Sco); and OVX-Scopolamine (OVX-Sco). Animals of Sham-Sco and OVX-Sco Groups received scopolamine (2 mg/kg, ip) before testing in Morris water maze. The animals of Sham and OVX Groups were injected by 1 mL/kg saline instead of scopolamine.

### Morris water maze apparatus and procedures

A circular black pool (136 cm diameter, 60 cm high, 30 cm deep) was filled with water (23 to 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 water in the center of the Northwest quadrant. Outside the maze, fixed visual cues were present at various locations around the room (i.e., computer, hardware, and posters). Before every experiment, each rat was daily handled for three days 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 in the pool and released facing the side wall at one of four positions (the boundaries of the four quadrants, labeled 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 there for 20 seconds. The rat was then removed from the pool, dried and placed in its holding bin for 20 seconds. The time latency to reach the platform and the length of the swimming path were recorded by a video tracking system<sup>21-24</sup>. 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) was compared between groups. All measurements were performed during the first of the light cycle.

## Biochemical assessment

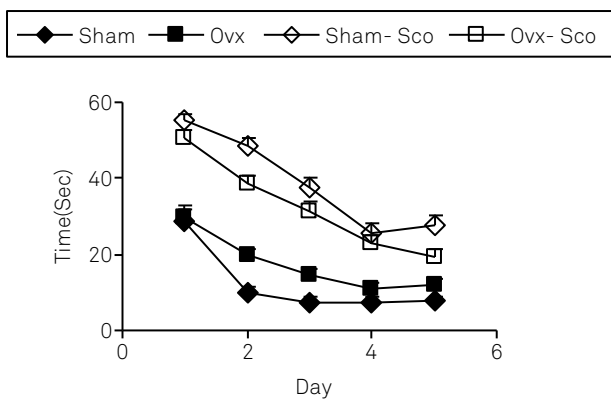
After the last session of the Morris water maze test, blood samples were taken from all rats to determine the NO metabolites NO<sub>2</sub> and NO<sub>3</sub> (Griess reagent method). The animals were then sacrificed, and hippocampi were removed and submitted to NO metabolite measurements in the tissue. 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  $\mu$ L serum and 100  $\mu$ L tissue suspension) were added to the Griess reagent. Proteins were subsequently precipitated by the addition of 50  $\mu$ L of 10% trichloroacetic acid (Sigma). The contents were 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<sup>24</sup>.

## Statistical analysis

All data are expressed as mean  $\pm$  standard error of mean (SEM). Swim time latency and length of the traveled path over the five training days were analyzed by repeated analysis of variance (ANOVA) measures. 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  $p < 0.05$ .

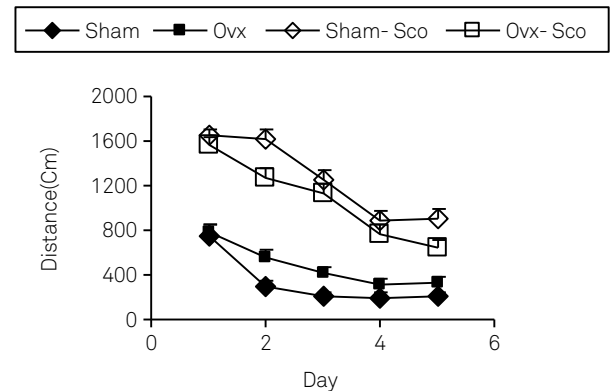
## RESULTS

The time latency and the length of the swimming path over the five training days were significantly higher in OVX Group in comparison with the Sham Group ( $p < 0.05$  and

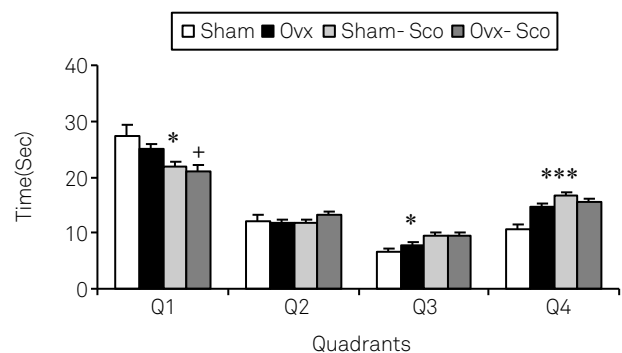


**Fig 1.** Comparison of swim time latency (seconds) to find the platform among Sham, OVX, Sham-Sco and OVX-Sco Groups. The latency was significantly higher in the OVX Group compared to Sham Group ( $p < 0.05$ ). The animals of both Sham-Sco and OVX-Sco Groups had significantly higher time latency to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

$p < 0.01$ ; Figs 1 and 2, respectively). The animals of both Sham-Sco and OVX-Sco groups had significantly higher time latency and travelled length to reach platform in comparison with those of Sham and OVX groups, respectively (both  $p < 0.001$ ), as seen in Figs 1 and 2. The time latency and the length of the swimming path to reach the platform in the Sham-Sco Group was higher than in the OVX-Sco Group ( $p < 0.001$ ). In the probe trial, animals of the Sham-Sco Group spent lower time in Q1 ( $p < 0.5$ ) and more times in nontarget quadrants (Q3 and Q4), with  $p < 0.05$  and  $p < 0.001$ , in comparison with the Sham Group (Fig 3). Animals of OVX-Sco Group also spent lower time in



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



\* $p < 0.05$ ; \*\*\* $p < 0.001$  compared to Sham Group; + $p < 0.05$  compared to the OVX Group.

**Fig 3.** Results of the time (seconds) spent in target quadrant during the probe trial on day 6 (24 hours after the last session of learning). Data are shown as mean standard error of mean of eight animals per group. The platform was removed, and the time spent in the target quadrant (Q1) was compared between the groups. The time spent in the target quadrant (Q1) by the animals of Sham-Sco and OVX-Sco groups was significantly lower than Sham and OVX Groups, respectively ( $p < 0.05$ ).

the Q1 compared to OVX Group ( $p < 0.05$ ). There were no significant differences in the time spent in the Q2 between the four groups.

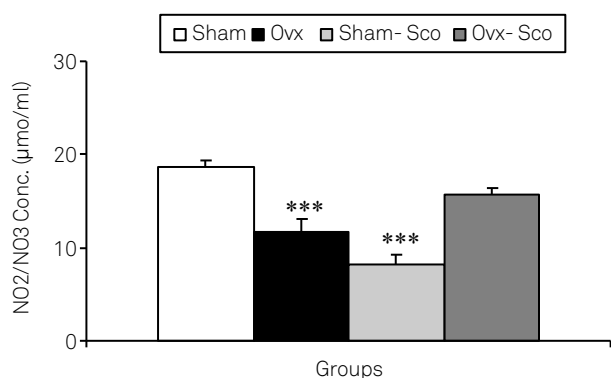
NO metabolites concentrations, NO<sub>2</sub> or NO<sub>3</sub>, in the serum of OVX Group, were lower than those of the Sham Group ( $p < 0.001$ , Fig 4). The serum concentrations of NO<sub>2</sub> or NO<sub>3</sub> in the Sham-Sco Group were significantly lower than those of the Sham Group ( $p < 0.001$ , Fig 4). The concentrations of NO metabolites in the hippocampal tissues of the OVX Group were lower than those of the Sham Group ( $p < 0.01$ , Fig 5). Concentration of NO metabolites in the hippocampi of the Sham-Sco Group was lower than that of the Sham one ( $p < 0.001$ , Fig 5). There was no significant difference between NO<sub>2</sub> or NO<sub>3</sub> con-

centrations in the serum and hippocampal tissues of the OVX-Sco Group compared with the OVX one.

## DISCUSSION

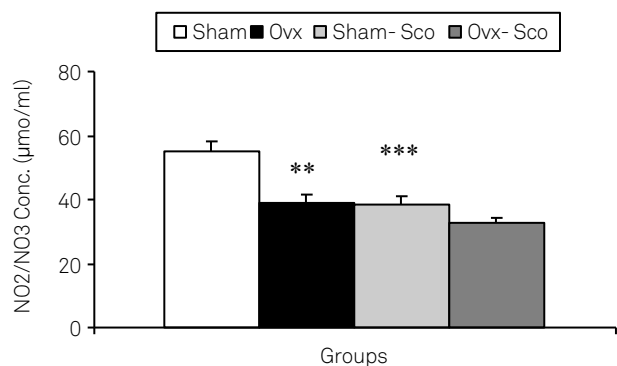
In the present study different effects of scopolamine on learning, memory, and NO metabolites levels in hippocampal tissues of ovariectomized and sham operated rats were investigated. The ovariectomized rats spent more times and travelled longer distance to reach platform during five days. The results also showed that the sham-operated rats remembered the location of the platform better than ovariectomized rats. Deletion of ovarian hormones impaired learning and memory in Morris water maze. These results were in agreement with the previous studies<sup>21-23</sup>. In contrast with these findings, Herlitz, Thilers and Habib showed that there were no considerable differences in cognitive performance between premenopausal and postmenopausal women<sup>25</sup>. Other researchers have also reported that estrogen has negative effects<sup>6</sup> or no effect<sup>4</sup> on learning and memory. It has also been reported that the ovariectomized rats performed better than intact rats at the spatial version of the water maze and, therefore, it seems that the ovarian steroids impair performance on hippocampal-dependent versions of this task<sup>26</sup>. 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, gamma-Aminobutyric acid (GABA), acetylcholine, and their receptors. Direct or indirect effects of estradiol on hippocampal neurons are also attractive as possible mechanisms<sup>27,28</sup>.

NO contributes in hippocampal dependent learning and memory tasks including spatial memory<sup>21-23</sup>. It has also been suggested that NO mediates the effects of estradiol in many organs<sup>29</sup>. Considering the fact that estradiol affects the release of NO and NOS isoforms activity in the brain, the interaction of estrogen and NO in nervous system functions is conceivable. It has been previously shown that NO contributes in sex hormone-dependent changes of behavior<sup>30</sup>. Previous studies also imply that some functions of estrogen in the central nervous system are related to increased NO production<sup>31</sup>. It was previously shown that L-arginine, the precursor of NO, improves learning and memory impairments in ovariectomized rats. However, it did not have an effect on sham operated ones<sup>21</sup>. It was 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<sup>23</sup>. In the present study, learning and memory impairments in ovariectomized rats were accompanied by lower levels of NO<sub>2</sub>/NO<sub>3</sub> in serum and hippocampal tissues in comparison with sham operated rats. These results confirm the involvement of NO in improvement effect of



\*\*\* $p < 0.001$  compared to the Sham Group.

**Fig 4.** Comparison of serum NO metabolite levels between four groups. Data are shown as mean  $\pm$  SEM of eight animals per group. NO metabolites concentrations in the serum of OVX Group was lower than that of the Sham Group ( $p < 0.001$ ). The serum concentrations of NO<sub>2</sub> or NO<sub>3</sub> in the Sham-Sco Group was significantly lower than that of the Sham Group ( $p < 0.001$ ).



\*\* $p < 0.01$ ; \*\*\* $p < 0.001$  compared to the Sham Group.

**Fig 5.** Comparison of the concentrations of NO metabolites in hippocampal tissues between groups. Data are shown as mean  $\pm$  standard error of mean of eight animals per group. The concentrations of NO metabolites in the hippocampal tissues of the OVX Group were lower than those of the Sham Group ( $p < 0.01$ ). The concentration of NO metabolites in the hippocampi of Sham-Sco Group was lower than that of the Sham one ( $p < 0.001$ ).

estradiol in physiologic conditions. The results obtained from other studies also show that memory impairment is accompanied with low level of NO<sup>32</sup>. They confirm the role of NO as a critical mediator in synaptic plasticity, long-term potentiation (LTP), and consolidation of long-term memory. Some researchers also believe that NO production decreases with age and may be involved in Alzheimer's disease<sup>32,33</sup>. Neuronal damage, which occurs in Alzheimer's disease, has been found to result in an impairment of NO synthesis and a decrease in NO containing neurons in the hippocampus<sup>34</sup>. Besides other neurotransmitters, acetylcholine has a critical role in learning and memory.

The interaction of cholinergic with NO for manipulation of learning and memory has been suggested. It was shown that co-administration of subthreshold doses of NO inhibitors and muscarinic receptor antagonist, scopolamine, has more deleterious effects on learning and memory of rats in comparison with the administration of each of them alone<sup>17</sup>. It has also been reported that NO donors inhibit acetylcholinesterase activity and, therefore, it might be suggested that NO oxide may directly inhibit it<sup>34</sup>. Results of the present study

showed for the first time that acetylcholine receptor antagonist, scopolamine, inhibits NO production and the latter may take part in learning and memory impairment due to the administration of scopolamine.

Taking into account that NO interacts with cholinergic system in memory and learning functions and each of NO cholinergic system has interaction with estradiol, we assumed that the effect of scopolamine on learning and memory and NO levels may be different in OVX and sham-operated ones. NO metabolites level in serum and hippocampal tissues of sham operated rats treated by scopolamine was lower than nontreated ones, while there was no significant difference between OVX-SCO Group compared to the OVX Group. It seems that scopolamine differently affects NO in the presence and absence of ovarian hormones.

In conclusion, NO oxide may be involved in deleterious effects of scopolamine on learning and memory. Our results also indicate that the effect of scopolamine on NO metabolites levels is different in the presence or absence of ovarian hormones, however further investigations need to be carried out.

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