Inducible nitric oxide synthase inhibitor aminoguanidine, differently affects Morris water maze tasks of ovariectomized and naïve female rats

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The role of ovarian hormones, nitric oxide, and their interaction on learning and memory has been widely investigated. The objective of present study was to investigate different effects of chronic administration of inducible nitric oxide synthase inhibitor, aminoguanidine (AM) on learning and memory of ovariectomized (OVX) and naïve (Sham) female rats. Thirty-two rats were divided into four groups: 1) Sham, 2) OVX, 3) Sham-AM and 4) OVX-AM. The animals of Sham-AM and OVX-AM chronically received 100 mg/kg/day of aminoguanidine during 8 weeks before 5 test days. The animals in Sham and OVX groups received 1 ml/kg saline instead of aminoguanidine. The animals were tested in Morris water maze and the escape latency and traveled path to reach the platform were compared between groups. On the fifth day, the platform was removed, and the animals were allowed to swim for 60 s (prob trial). The time spent in the target quadrant (Q1) was compared between groups.

Results showed that the escape latency and traveled path in OVX group were significantly higher than in the Sham group (p<0.01). Both escape latency and traveled path in the Sham-AM group was significantly higher than in the Sham group (p<0.01) however, there was no significant difference between OVX-AM and OVX groups.

The time spent by the animals of OVX group in the target quadrant (Q1) during the probe trial was significantly lower than that in the Sham group (p<0.01). The animals of the Sham-AM group spent shorter times in the target quadrant in comparison with the Sham group (p<0.01). There was no significant difference between the OVX and OVX-AM groups in the time spent in the target quadrant. It is concluded that the effect of aminoguanidine on learning and memory is different in the presence or absence of ovarian hormones but it needs further investigation.

Keywords: aminoguanidine, learning, ovariectomy, Morris water maze

Nitric oxide (NO) is a free radical gas which plays important physiologic roles in biologic systems. It acts as a diffusible intercellular signaling molecule in the central nervous system (6, 52). This gaseous messenger is synthesized by the enzyme NO synthase (NOS) when it catalyzes the conversion of L-arginine to L-citrulline, producing NO as a coproduct (6, 19) in neurons, endothelial cells, and macrophages by a family of three isoenzymes: a neuronal nitric oxide synthase (nNOS), which is constitutively expressed in neurons and is regulated by Ca²⁺; a constitutive and Ca²⁺-dependent type of NOS, which is present in the endothelial cells (eNOS) of the vasculature ; and an inducible Ca²⁺-independent NOS (iNOS), which is expressed upon induction by cytokines in macrophages (41). The hippocampus expresses both eNOS and nNOS, but data regarding the expression of iNOS in nonpathological situation in the hippocampus are conflicting (38). NO modulates neurotransmitter release in the brain (51, 52) and plays an important role in both synaptic plasticity and learning and
memory (4, 25). NOS inhibitors administration before acquisition or memory consolidation, impairs the memory of various tasks in rodents (3, 4, 8, 10, 15, 34, 61, 63). Under certain conditions, NOS inhibitors also prevent the induction of hippocampal long-term potentiation (LTP) (7, 55, 59) and cerebellar long-term depression (LTD) (11), both two forms of synaptic plasticity thought to play a role in learning and memory (5, 40).

It has been shown that nitric oxide which is produced via the inducible nitric oxide synthase (iNOS) may cause in impairment of learning behavior and hippocampal long-term potentiation (LTP) following pathological conditions such as ischemia and aminoguanidine, a relatively selective iNOS inhibitor, it ameliorates the impairment induced by ischemia (44). It also has been shown that aminoguanidine prevents memory impairment induced by amyloid β-peptide (Aβ), too (57).

Along with numerous studies on NO contribution to learning and memory, there are strong evidence approving the effects of ovarian steroid hormones on learning and memory ability (12, 37). Several experiments approve that estradiol replacement after ovariectomy enhances the memory in ovariectomized (OVX) rats (12, 20, 21, 32). Though the responsible mechanism is still unknown the enhancement might be due to activation of some cholinergic and aminergic systems (14, 20, 39).

It has been well documented that estrogen influences the NO system in both peripheral and central nervous tissues (17, 35, 45). Increased nNOS and eNOS expression has been associated to hippocampal function improvement by estrogens (23). A number of recent studies show that estrogen can differentially impact the expression of iNOS depending upon the concentration of hormone and the tissue being studied. For example, estrogen reduced the iNOS expression and NO concentrations in microglia in the brain and probably had an anti-inflammatory effect which improved outcome after ischemic stroke (2, 48, 58). In contrast, it has been reported that estrogen increased iNOS expression in the rat intestinal wall ischemic injury model (60). It has also been shown that low concentrations of estrogen induced resting macrophages to increase their expression of iNOS (62). However, the iNOS production was reduced when the macrophages were exposed to higher estrogen concentrations (26).

Regarding to the possible correlation between estrogen and NO systems, specifically, iNOS / NO system, the aim of present study was to compare the effects of aminoguanidine (a selective inhibitor of iNOS) in learning and memory process in ovariectomized and naïve female rats.

**Material and Methods**

**Animals and drugs**

Thirty-two 8-week virgin female Wistar rats (200±10 g) were used. All rats were housed 4 per standard cage (26.5*42*15 cm) at room temperature (24±1 °C) on a 12 h light/dark cycle. Food and water were available *ad libitum* properly. Rats were given one week to adapt with the new environment before any procedures were initiated. Animal handling and all related procedures were approved by the Mashhad Medical University Committee on Animal Research. Aminoguanidine hydrochloride was purchased from Sigma Aldrich (USA) and dissolved in saline. Ketamin and xylazine were purchased from Alfasan Company (Holland). The animal groups were 1) Sham; 2) Ovariectomy (OVX); 3) Sham-Aminoguanidine (Sham-AM) and 4) Ovariectomy-Aminoguanidine (OVX-AM). The animals in Sham-AM and OVX-AM received 100 mg/kg/day of aminoguanidine during 8 weeks before 5 test days. The animals of Sham and OVX groups received 1 ml/kg of saline instead of aminoguanidine
for 8 weeks. The volume of the aminoguanidine injection was equal to the volume of saline. The treatments were carried out from the day after surgery to the starting of the behavioral study in all animals. Finally, all animals were tested in Morris water maze.

Ovariectomy
The animals were ovariectomized under ketamine (150 mg/kg, i.p.) and xylazine (0.1 mg/kg, i.p.) anesthesia (31). Anesthesia was confirmed by reduced respiratory rate and lack of response to a gentle pinching of the foot pad. 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 same procedure was carried out for Sham groups except removing the ovaries (54).

Apparatus
To assess behavioral functions, rats were tested by Morris water maze.

Morris water maze is a black circular pool with a diameter of 150 cm and a height of 60 cm, filled with 20 ± 1°C water to a depth of 30 cm. The maze was divided geographically into four equal quadrants and release points that were designed at each quadrant as N, E, S and W. A hidden circular platform (10 cm in diameter), made of plexiglas, was located in the center of the southeast quadrant, submerged 1.5 cm beneath the surface of the water. It has been previously shown that the plexiglas is invisible for the rats (33, 54). Fixed, extra maze visual cues were present at various locations around the maze (i.e. computer, Morris water maze hard wares and posters). An infrared camera was mounted above the center of the maze. An infrared LED was attached to each rat as a probe so that the animal motion can be recorded and sent to the computer. A tracking system was used to measure the escape latency, traveled path and swimming speed (33, 54).

Behavioral assessment
Animals received a block of four trials during five daily sessions. During the 5 days, the platform, situated in the center of the southeast quadrant, was submerged 1.5 cm below the surface of water and therefore invisible, for testing spatial learning. The platform position remained stable during 5 days.

A trial was started by placing a rat into the pool, facing the wall of the tank. Each of four starting positions (north, east, south and west) was used once in a series of four trials; their order was randomized. Each trial was terminated as soon as the rat had climbed onto the escape platform or when 60 s had elapsed. A rat was allowed to stay on the platform for 15 s. Then it was taken from the platform and the next trial was started after 20 s. Rats that did not find the platform within 60 s, were put on the platform by the experimenter and were allowed to stay there for 15 s. After completion of the 4th trial kept warm for an hour and returned to their home cage (1, 33, 54). The time latency to reach the platform was compared between groups. The latency of the first trial on day one was also compared between groups. In the retention phase (in the fifth day), a 60-s probe trial was conducted to examine how well the rats had learned the exact location of the platform. During this trial, the platform was removed from the tank. The target quadrant time (the time spent in the training quadrant) was recorded during the probe trial. All tests were conducted between 16:00 to 18:00 o’clock.
Statistical analysis
All data were expressed as means ± SEM. The data of different groups over 5 days was compared using repeated measure ANOVA test with Tukeys’ post hoc between groups. The time spent in the target quadrant (Q1) was compared using unpaired t-tests. Differences were considered statistically significant when p<0.05.

Fig. 1. The comparison of escape latency (A), path length (B) and swimming speed (C) between Sham and OVX groups. Data are presented as mean ± SEM. Repeated measure ANOVA analysis showed that escape latency and path length of the OVX group was significantly higher than that in the Sham group (p<0.01 and p<0.05, respectively). There was no significant difference between two groups in swimming speed.
Fig. 2. The comparison of escape latency (A), path length (B) and swimming speed (C) between Sham and Sham-AM groups. Data are presented as mean ± SEM. Repeated measure ANOVA analysis showed that escape latency and path length of the Sham-Am group was significantly higher than that of the Sham group (both $p<0.01$). The swimming speed in the Sham-AM group was significantly higher than that of the Sham group ($p<0.01$).
Fig. 3. The comparison of escape latency (A), path length (B) and swimming speed (C) between OVX and OVX-AM groups. Data are presented as mean ± SEM. Repeated measure ANOVA analysis showed no significant difference between two groups in escape latency, path length. The animals of the OVX-AM group had higher swimming speed in comparison with the OVX group (p<0.01)
Results

Escape latency and traveled path in the OVX group were significantly higher than in the sham group ($p<0.01$) (Figs 1A and 1B). There were no significant differences between the Sham and OVX group in swimming speed (Fig. 1C). The animals of the Sham-AM group had significantly higher time latency to reach the platform compared to the Sham group ($p<0.01$) (Fig. 2A). Traveled path length to reach the platform in the Sham-AM group was also longer than that in the Sham group ($p<0.01$) (Fig. 2B). The swimming speed in the Sham-AM group was significantly higher than that of the Sham group ($p<0.01$) (Fig. 2C). There was no significant difference either in time latency or in the traveled path length in the OVX-AM group in comparison with the OVX group (Figs 3A and 3B). The animals of the OVX-AM group had a higher swimming speed in comparison with the Sham group ($p<0.01$) (Fig. 3C). Figures 1A, 2A and 3A also show the learning pattern of the animals of all 4 groups. This figure shows that there is a negative linear correlation between escape latency and the training sessions in all the groups. This means that all groups have learnt the platform location.

In the probe trial, the time spent in the target quadrant (Q1) by the animals of the OVX group was significantly lower compared to Sham group ($P<0.01$; Fig. 4). The time spent Q1 by the animals of the Sham-AM group was shorter than for those in the Sham group ($P<0.01$; Fig. 4). There was no significant difference between OVX-AM and OVX groups in this criterion ($P<0.01$) compared to the Sham group.

Fig. 4. The comparison of the time spent in the target quadrant by the animals of 4 groups. Data are presented as mean ± SEM. One-way ANOVA analysis showed that the time spent in the target quadrant (Q1) by the animals of the OVX group was significantly lower compared to the Sham group ($p<0.01$). The time spent in Q1 by animals of the Sham-AM group was shorter than for those in the Sham group ($P<0.01$). There was no significant difference between the OVX-AM and OVX groups in this criterion. **$p<0.01$ compared to the Sham group.
Fig. 5. The latency to find the platform in the first trial on day one between groups.
Data are presented as mean ± SEM. There was no significant difference between groups.

Discussion

In the present study, the chronic effects of aminoguanidine, a selective inhibitor of iNOS, on learning and spatial memory of OVX and Sham-operated female rats were investigated. The results of the present study indicated that depletion of ovarian hormones impaired both learning and memory in rats. Both escape latency and traveled path length to find the platform in OVX group was higher than that of the Sham group. In the retention phase (in fifth day), when a 60-s probe trial was conducted to examine how well the rats had learned the exact location of the platform, the animals of the OVX group spent shorter time in the target quadrant when compared with Sham-operated rats. It is improbable that the results which were seen in ovariectomized rats can be attributed changing of motor activity because there was no significant difference in swimming speed between the two groups. These results were in good agreement with those of Monteiro and colleagues showing that hormone deprivation in ovariectomized rats impaired spatial memory measured in Morris water maze (43). Treatment with estrogen significantly improved spatial learning performance, both in low-estrogen (OVx) and in normal-estrogen (Sham-operated) rats (50). In contrast, a study on premenopausal and postmenopausal women showed no differences in cognitive performance between them (28). It has also been described that estrogen has a negative effect (9, 18) or no effect (16, 27) on learning and memory. The results of the present study cannot explain the mechanism of ovarian hormones or how ovarian hormone deprivation impairs learning and memory. It is demonstrated that the deleterious effect of ovariectomy on memory could be related to its capacity to induce oxidative stress (24), cholinergic (22, 39, 50) and monoaminergic dysfunction (30) in the brain. Previous studies imply that some functions of estrogen in the central nervous system are related to increased nitric oxide production (35). The results of our previous study confirmed this hypothesis. In our previous study we showed that treatment of ovariectomized rats by $l$-arginine improved spatial learning and memory impairment (54). To the best of our knowledge the effect of iNOS inhibitors on learning and memory in normal conditions has not been elucidated. It has been reported that nitric oxide (NO) production via inducible nitric oxide synthase (iNOS) is involved in the impairment of learning behavior and hippocampal long-term potentiation (LTP) following transient ischemia. It has also been shown that aminoguanidine, a selective iNOS inhibitor, ameliorates the memory impairment induced by ischemia (13). In the present study we showed
that aminoguanidine had deleterious effects on Sham-operated but not ovariectomized rats. The results showed that aminoguanidine impaired spatial learning as well as memory of Sham rats both time and length criterion in AM-treated Sham rats was higher than in non-treated ones. The lower time spending in target quadrants where the platform was located during 5 training blocks confirms that AM affects spatial memory in the presence of ovarian hormones. However, aminoguanidine, had no significant effects on learning and spatial memory in OVX animals; there was no significant difference between OVX and OVX-AM groups in escape latency and swimming path in 5 training days. There was no significant difference between this two groups either in the time spent in the target quadrant in retention phase. It is also suggested that the effect of aminoguanidine is not due to its effect on motor activity because its effect on swimming speed was positive in both OVX and Sham-operated rats but it has a negative effects on learning and memory criteria in Sham at the same time no effect in ovariectomized rats. On the other hand, the pattern learning shows that the animals of all four the groups learned to find the platform. It is necessary to consider that treatment of the animals was stopped when behavioral study was started. Comparing of the time to reach the platform in the first trial on the first day may also confirm that pretreatment of the animals by AM did not affect motor conditions. It seems that aminoguanidine has an inhibitory effect on spatial learning and memory in the presence of estrogen. This results showed for the first time that iNOS/NO system contributes to the modulatory effect of estrogen on learning and memory. One possibility for estrogen to improve spatial learning and memory through iNOS and increase its mRNA expression, consequently, an inhibition of iNOS activity by aminoguanidine reduces the positive effect of estrogen on spatial learning and memory (42). In contrast to this hypothesis there are evidence that estrogen inhibits iNOS activity (49). It has also been reported that amyloid β-peptide increases nitric oxide production in the hippocampus of rats which is reversible by iNOS inhibition (57). Another explanation is that estrogen and iNOS act on spatial learning and memory and improve it through two different pathways on the one hand, both of these pathways are required for learning and memory formation, on the other hand, these pathways cooperate to form spatial learning and memory. In this case, the treatment with aminoguanidine and the inhibition of iNOS activity results impairment of spatial learning and memory in the presence of estrogen. Treatment with aminoguanidine has no effect on spatial learning and memory formation in the absence of estrogen.

Finally, there are documents showing that aminoguanidine is a relatively selective inhibitor for iNOS. It has been shown that aminoguanidine reduces nNOS and eNOS activities (36). It has been shown that estrogen increases eNOS activity and expression (53, 56) and production of nitric oxide in endothelial cells (29). There are also evidences showing that estrogen change nNOS mRNA and the number of nNOS neurons in brain regions and influences NO production in the brain (23, 47). It has recently been reported that deletion of both neuronal and endothelial forms of NOS is required to prevent hippocampal LTP induction in vitro (46). It is possible for aminoguanidine to inhibit nNOS and eNOS activities and disrupt learning and memory formation in the presence of estrogen. However, further studies are necessary for determination of mRNA levels all NOS isoforms to clarify the paradoxical effects of aminoguanidine on spatial learning and memory, and the mechanisms by which this compound impairs memory in Sham rats.
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Aminoguanidine affects learning of rats

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