

## Effect of 3 $\alpha$ -Anderostanediol and Indomethacin on Acquisition, Consolidation and Retrieval Stage of Spatial Memory in Adult Male Rats

Somayeh Assadian Narenji<sup>1</sup>, Nasser Naghdi<sup>\*2</sup>, Shahrbanoo Oryan<sup>1</sup> and Kayhan Azadmanesh<sup>3</sup>

<sup>1</sup>*Dept. of Biology, Science and Research Branch, Islamic Azad University, Tehran;* <sup>2</sup>*Dept. of Physiology and Pharmacology, Pasteur Institute of Iran, Tehran 13164;* <sup>3</sup>*Dept. of Virology, Pasteur Institute of Iran, Tehran 13164, Iran*

Received 15 March 2012; revised 20 May 2012; accepted 26 May 2012

### ABSTRACT

**Background:** Testosterone and its metabolites have important roles in learning and memory. The current study has conducted to assess the effect of pre-training, post-training and pre-probe trial intrahippocampal CA1 administration of 3 $\alpha$ -androstenediol (one of the metabolites of testosterone) and indomethacin (as 3 $\alpha$ -hydroxysteroid dehydrogenase enzyme blocker) on acquisition, consolidation and retrieval in Morris water maze (MWM) task. **Methods:** Adult male rats were bilaterally cannulated into CA1 region of hippocampus and then received 3 $\alpha$ -diol (0.2, 1, 3 and 6  $\mu$ g/0.5  $\mu$ l/side), indomethacin (1.5, 3 and 6  $\mu$ g/0.5  $\mu$ l/side), indomethacin (3  $\mu$ g/0.5  $\mu$ l/side) + 3 $\alpha$ -diol (1  $\mu$ g/0.5  $\mu$ l/side), 25-35 min before training, immediately after training and 25-35 min before probe trial in MWM task. **Results:** Our results showed that injection of 3 $\alpha$ -diol and indomethacin significantly increased the escape latency and traveled distance to find hidden platform in acquisition and consolidation stage, but did not have any effect on retrieval of spatial learning as compared with the control group. **Conclusion:** It is concluded that intra-CA1 administration of 3 $\alpha$ -diol and indomethacin could impair spatial learning and memory in acquisition and consolidation stage. Also, intrahippocampal injection of indomethacin + 3 $\alpha$ -diol could not change spatial learning and memory impairment effect of indomethacin or 3 $\alpha$ -diol in MWM task. *Iran. Biomed. J. 16 (3): 145-155, 2012*

*Keywords:* Androgens, Spatial memory, Indomethacin, 3 $\alpha$ -diol, GABA<sub>A</sub> receptor

### INTRODUCTION

Steroid hormones are synthesized in the gonads and reach the brain via the blood circulation [1, 2]. In addition, the local endogenous synthesis of estrogens and androgens from cholesterol occurs in glial cells, astrocytes and neurons in the central and peripheral nervous system by the mediation of cytochrome p450, but not p450 enzymes [1-3]. Steroids not only affect the sexually behavioral responses, but also affect the ability of the brain to process, store and retrieve sensory information. Neuromodulatory function of steroid hormones has been investigated in the hippocampus, because the hippocampus is attractive as a center of learning and memory [2]. Androgens can enhance neural excitability in the hippocampus of male rats and increase dendritic spine density in the CA1 and CA3 regions of the dorsal hippocampus [4]. Changes in gonadal

steroid levels over the lifetime, in addition to inducing cognitive deficits which causes the variations in cognitive function, make some types of neurodegenerative disorders such as Alzheimer's disease [5, 6].

Androgen receptors are densely expressed in rat hippocampal cells [1, 7] and much of the work on steroid-induced learning has focused on the effects of androgens in hippocampal spatial memory. The literature of androgen effects on spatial memory is complex and contradictory [1, 4, 7]. The evidence suggests that androgens can impair memory in animals [8]. It has been shown that injection of testosterone in the CA1 region of hippocampus impaired spatial memory in adult male rats [1, 7, 9]. A report also indicated that chronic treatment with androgens impaired spatial learning and retention of spatial information in young and middle-aged animals [10]. In contrast, some studies showed a positive correlation

\*Corresponding Author; Tel/Fax: (+98-21) 6646 5132; E-mail: naghdi@pasteur.ac.ir or nnaghdiir@yahoo.com

between testosterone and its metabolites and spatial ability [11, 12]. For instance, men with lower levels of testosterone due to hypogonadism or aging demonstrate poorer cognitive performance, and these deficits can be reduced through androgen-replacement therapy [13]. Also in animal models, it has been reported that gonadectomized or removal of the primary source of male androgens, the testes, decreases cognitive performance in adult male rats [1, 4, 7, 14].

One of the complexities in understanding the effects of androgens is the broad spectrum of steroid activity. One reason is that androgens represent substrates for the synthesis of several biologically active metabolites [5].

Our previous studies showed that testosterone impaired learning and memory [1, 4, 7]. An important question is how testosterone may have its effects on spatial memory. Indeed, testosterone, other routes of metabolism, should be more closely examined to determine its effects and mechanisms on spatial memory. In brain, testosterone is readily metabolized by the  $5\alpha$ -reductase enzyme to dihydrotestosterone (DHT), which is subsequently converted by the  $3\alpha$ -hydroxysteroid dehydrogenase enzyme ( $3\alpha$ -HSD) to non-aromatizable metabolite  $5\alpha$ -androstane- $3\alpha$ ,  $17\beta$ -diol ( $3\alpha$ -diol) [11, 15]. Systemically administration of  $3\alpha$ -diol to gonadectomized rats enhances cognitive performance in the inhibitory avoidance, place learning and object recognition tasks [15], but there is no information about the effect of direct intrahippocampal injection of  $3\alpha$ -diol on spatial learning and memory.

Indomethacin is a poorly water-soluble, non-steroidal anti-inflammatory drug [16] and a prostaglandin blocker that powerfully inhibits  $3\alpha$ -HSD reduction [17]. Indomethacin can act as a  $3\alpha$ -HSD inhibitor, block testosterone and DHT metabolism to  $3\alpha$ -diol and affect memory process. Some Studies showed that indomethacin as a  $3\alpha$ -HSD blocker may affect testosterone and  $3\alpha$ -diol metabolism in spatial learning and memory procedure [11, 15]. In addition, indomethacin is known as a non-selective COX (cyclooxygenase) inhibitor. COX enzymes catalyze the first two committed steps in the biosynthesis of prostanoids [17, 18]. Indomethacin as a non-selective COX inhibitor can affect learning and memory. Several lines of evidence indicate a potential role for COX in the physiological mechanisms underlying memory function [16, 17]. For example, non-specific COX inhibitors such as indomethacin impair passive avoidance memory in chick and prevent the learning-induced increase in prostaglandin release, which occurs 2 h after training [18].

Regarding the literature, the study was designed to assess the effect of intrahippocampal administration of  $3\alpha$ -diol, indomethacin and indomethacin + $3\alpha$ -diol on acquisition, consolidation and retrieval stage of spatial

learning and memory in Morris water maze (MWM) performance.

## MATERIALS AND METHODS

**Subject.** Male Albino Wistar rats (200-250 g) were obtained from Pasteur Institute of Iran and were housed five per big cage before surgery and individually in small cages after surgery. The animals were maintained at room temperature of  $23 \pm 2^\circ\text{C}$  under standard 12:12 h light-dark cycle with lights on at 07:00 A.M. Food and water were available ad libitum. Experimental procedures were carried out in accordance with recommendations from the declaration of Helsinki and internationally accepted principles for the use of experimental animals.

**Surgery.** Rats were anesthetized with a mixture of ketamine hydrochloride (Sigma, Germany) and xylazine (100 and 25 mg/kg i.p., respectively) and mounted in a stereotaxic frame (Stoelting, USA). Bilateral guide cannulae were inserted into the right and left CA1 and attached to the skull surface using dental cement and jeweler's screws. Stereotaxic coordinates based on Paxinos and Watson's Atlas of the rat brain [19] was: anterior-posterior, -3.8 mm from bregma; medial-lateral,  $\pm 2.2$  mm from midline; and dorsal-ventral, -2.7 mm from the skull surface. After surgery, at least a seven-day recovery period was supposed before any other intervention.

### *Behavioral assessment:*

**Apparatus.** The MWM is one of the most frequently used laboratory tools in behavioral neuroscience [20] that relies on distal cues to navigate from start locations around the perimeter of an open swimming area to locate a submerged escape platform [21]. The water maze is a black circular tank 140 cm in diameter and 60 cm in height. The tank was filled with water ( $21 \pm 1^\circ\text{C}$ ) to a depth of 25 cm. The maze was located in a room containing many extra maze cues (e.g. bookshelves, refrigerator and posters). The maze was divided geographically into four quadrants (northeast, northwest, southeast, southwest) and starting positions (north, south, east, west) that were equally spaced around the perimeter of the tank. A hidden circular platform (diameter: 10 cm) was located in the center of the southwest quadrant, submerged 1 cm below the surface of the water. A video camera above the water maze was connected to a video monitor and computer software (EthoVision XT, version 7) was used to track the animals' path and to calculate the escape latency, traveled distance and swimming speed.

**Procedure.** Each subject received four trials of one block per day in 4 consecutive days. Each rat was placed in the water facing the wall of the tank at one of the four designated starting points and allowed to swim and find the hidden platform located in southwest quadrant of the maze on every trial. Starting points were varied in a quasi-random fashion so that in each trial, the subject started from each location once and never started from the same place on any day. During each trial, each rat was given 90 s to find the hidden platform. If rat found the platform, it was allowed to remain on it for 20 s. If rat failed to find the platform within 90 s, it was placed on platform for another 20 s. During the first 4 days, the platform position remained constant. After completion of training, to test probe trial on day 5, the hidden platform was removed and the animal was released from the north location and allowed to swim freely for 60 s. After the probe trial, the platform was elevated above the water surface and placed in different positions (southeast quadrant). This stage assessed Visio-motor coordination toward a visible platform. All testing began at 8:00 A.M.

**Microinjection procedure.** Intrahippocampal injections were administered through a guide cannula (23 gauges) using injection needles (30 gauges) connected by polyethylene tubing to 0.5- $\mu$ l Hamilton microsyringes. The injection needle was inserted 0.5 mm beyond the tip of the cannula. DMSO (0.5  $\mu$ l) and different doses of 3 $\alpha$ -diol (Sigma, USA) and indomethacin (Darou Pakhsh Manufacturing Co., Iran) were injected into each side of CA1 region during 2 min and the needles were left in place for an additional 60 s to allow for diffusion of solution away from the needle tip. In all experiments, DMSO was used as vehicle that has no significant effect on learning and memory in MWM [1, 7]. In acquisition stage, drugs and DMSO were administrated 25-35 min before training in four consecutive days. In consolidation stage, rats were trained with one block of four trials within 4 days. Immediately after the final trial in each day, microinjection was performed. Memory retrieval for the location of the platform was tested 24 h later using a 60-s free-swim probe trial. In retrieval stage, rats were trained within 4 consecutive days. On day 5, 25-35 min before probe trial, they received intra-CA1 injection of drugs and DMSO.

**Histology.** Following behavioral testing, animals were sacrificed by decapitation and brains were removed. For histological examination of cannulae and needle placement in CA1 region, 100- $\mu$ m thick sections were taken, mounted on slides, stained with cresyl violet and the cannulae track was examined for each rat. Only those animals whose cannulae were

exactly placed in the CA1 region, were used for data analysis.

#### **Experimental protocol:**

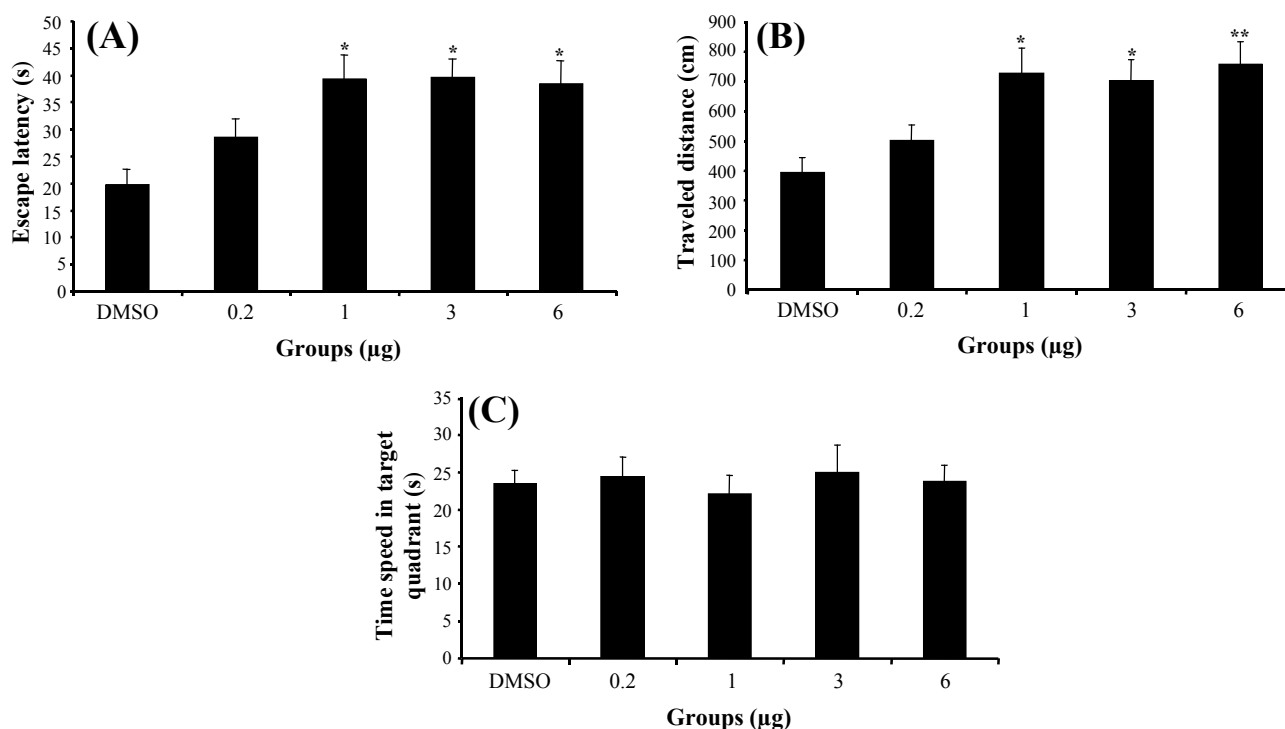
**Experiment 1.** The first experimental group consisting of intact (without cannula) and sham-operated rats was received 0.5  $\mu$ l vehicle (DMSO or 0.5  $\mu$ l DMSO + 0.5  $\mu$ l DMSO) into CA1 region of hippocampus. The aim of this experiment was to compare the effects of intrahippocampal vehicle injection with intact group of rats on MWM performance. A total of 27 rats were divided into three groups as mentioned above.

**Experiment 2.** The aim of this experiment was to determine the effect of bilateral pre-training, post-training and pre-probe trial administration of 3 $\alpha$ -diol into CA1 region, respectively on acquisition, consolidation and retrieval stages of spatial memory. A total of 130 rats were used, randomly divided into five groups in each stage and received different doses of 3 $\alpha$ -diol (0.2, 1, 3 and 6  $\mu$ g/0.5  $\mu$ l/side) or DMSO as vehicle.

**Experiment 3.** The aim of this experiment was to determine the effect of bilateral pre-training, post-training and pre-probe trial administration of indomethacin into CA1 region, respectively on acquisition, consolidation and retrieval stages of spatial memory. A total of 105 rats were used, divided into four groups in each stage and received different doses of indomethacin (1.5, 3 and 6  $\mu$ g/0.5  $\mu$ l/side) or DMSO as vehicle.

**Experiment 4.** The aim of this experiment was to determine the effect of bilateral pre-training, post-training and pre-probe trial administration of indomethacin + 3 $\alpha$ -diol into CA1 region, respectively on acquisition, consolidation and retrieval stages of spatial memory. A total of 50 rats were used, randomly divided into two groups in each stage and received bilateral microinjections of 3  $\mu$ g/0.5  $\mu$ l/side indomethacin + 1  $\mu$ g/0.5  $\mu$ l/side 3 $\alpha$ -diol or DMSO + DMSO as a vehicle with a similar volume.

**Statistical analysis.** Statistical evaluation was carried out by Kolmogorov-Smirnov test to examine the normal distribution. Data were analyzed by student's *t*-test for comparison between the two groups and one-way analysis of variance (ANOVA) was followed by Tukey's test for multiple comparisons. All results have been shown as means  $\pm$  S.E.M. In all statistical comparisons, *P*<0.05 considered as significant difference.



**Fig. 1.** The effect of intra-CA1 administration of 3 $\alpha$ -diol and DMSO on acquisition of spatial memory in MWM task. The columns represent the mean  $\pm$  S.E.M. Average escape latency (A) and traveled distance (B) across all training days and time spent in target quadrant (C) in probe trial. \* $P$ <0.05 and \*\* $P$ <0.01 indicate significant difference vs. DMSO group.

## RESULTS

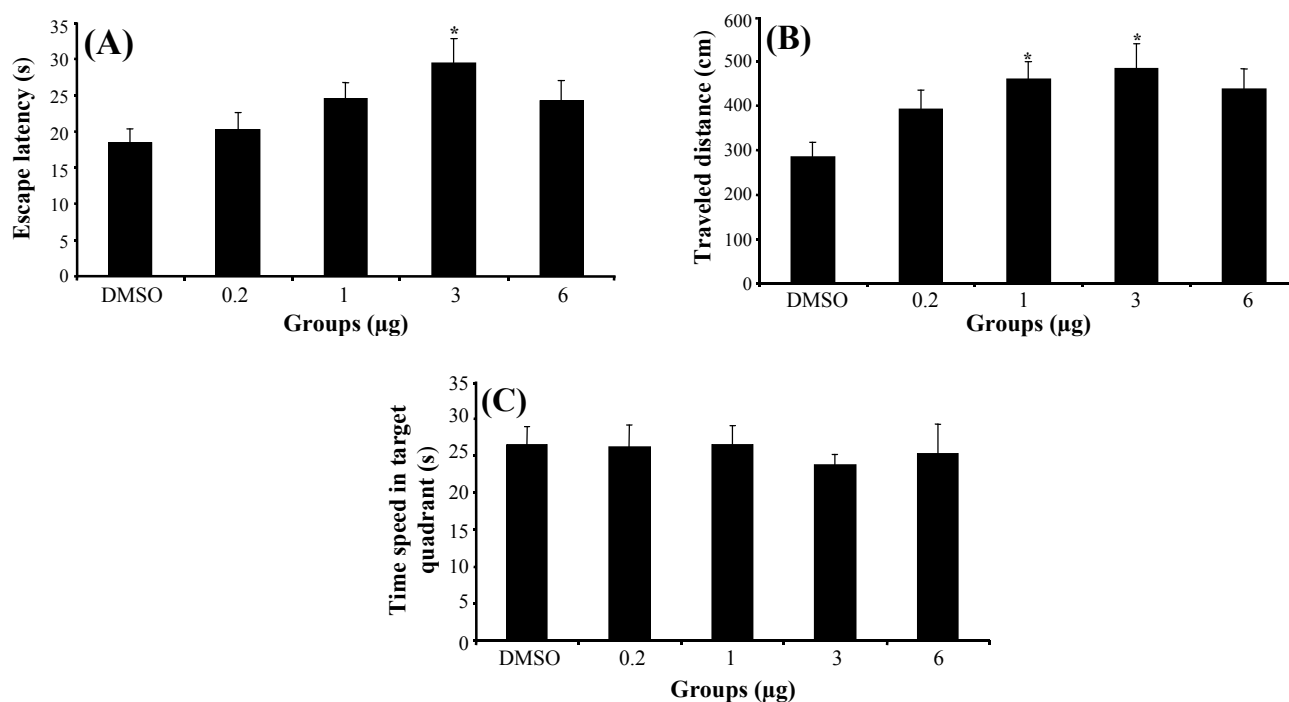
**Experiment 1. The effects of vehicles.** The results obtained from intact and vehicle (DMSO and DMSO + DMSO) indicate no significant difference in escape latency ( $F_{2,80} = 0.211$ ,  $P = 0.810$ ), traveled distance ( $F_{2,80} = 1.197$ ,  $P = 0.307$ ) and swimming speed ( $F_{2,80} = 1.838$ ,  $P = 0.166$ ) among the groups. These results indicated that injection of DMSO or injection of DMSO + DMSO together had no effect on spatial learning and swimming ability in behavioral studies.

**Experiment 2. The effect of 3 $\alpha$ -diol on acquisition, consolidation and retrieval of MWM task.** In acquisition stage, a significant difference was generally found in escape latency ( $F_{5,166} = 5.713$ ,  $P < 0.001$ ) and traveled distance ( $F_{5,166} = 5.281$ ,  $P < 0.001$ ) (Fig. 1A and 1B) among the groups in hidden platform trials (days 1-4), but no significant differences were found in swimming speed ( $F_{5,166} = 1.825$ ,  $P > 0.05$ ). Post hoc multiple comparisons showed that 3 $\alpha$ -diol at doses 1, 3 and 6  $\mu$ g had significantly impaired acquisition of spatial learning compared to control group. One way ANOVA in probe test revealed no significant differences in escape latency ( $F_{5,41} = 0.753$ ,  $P = 0.563$ ) (Fig. 1C), traveled distance ( $F_{5,41} = 1.047$ ,  $P = 0.397$ ) and swimming speed ( $F_{5,41} = 2.16$ ,  $P = 0.094$ ) among the groups. Also, there was no significant difference of

performance among the groups on visible platform day for escape latency ( $F_{5,41} = 0.741$ ,  $P = 0.597$ ) or for traveled distance ( $F_{5,41} = 1.173$ ,  $P = 0.339$ ).

In consolidation stage, a significant difference was generally found in escape latency ( $F_{4,159} = 2.511$ ,  $P < 0.05$ ) and in traveled distance ( $F_{4,159} = 3.196$ ,  $P < 0.05$ ) (Fig. 2A and 2B) among the groups in hidden platform trials, but no significant differences were found in swimming speed ( $F_{4,159} = 2.29$ ,  $P = 0.062$ ). Post hoc multiple comparisons showed that 3 $\alpha$ -diol at doses 1 and 3  $\mu$ g had a significantly impaired consolidation stage in training trials compared to control group. One way ANOVA in probe test revealed no significant differences in escape latency ( $F_{4,37} = 0.169$ ,  $P = 0.953$ ) (Fig. 2C), traveled distance ( $F_{4,37} = 0.381$ ,  $P = 0.821$ ) and swimming speed ( $F_{4,37} = 1.85$ ,  $P = 0.139$ ) among the groups. Also, there was no significant difference of performance among the groups on visible platform day for escape latency ( $F_{4,37} = 0.988$ ,  $P = 0.426$ ) or for traveled distance ( $F_{4,37} = 2.22$ ,  $P = 0.085$ ).

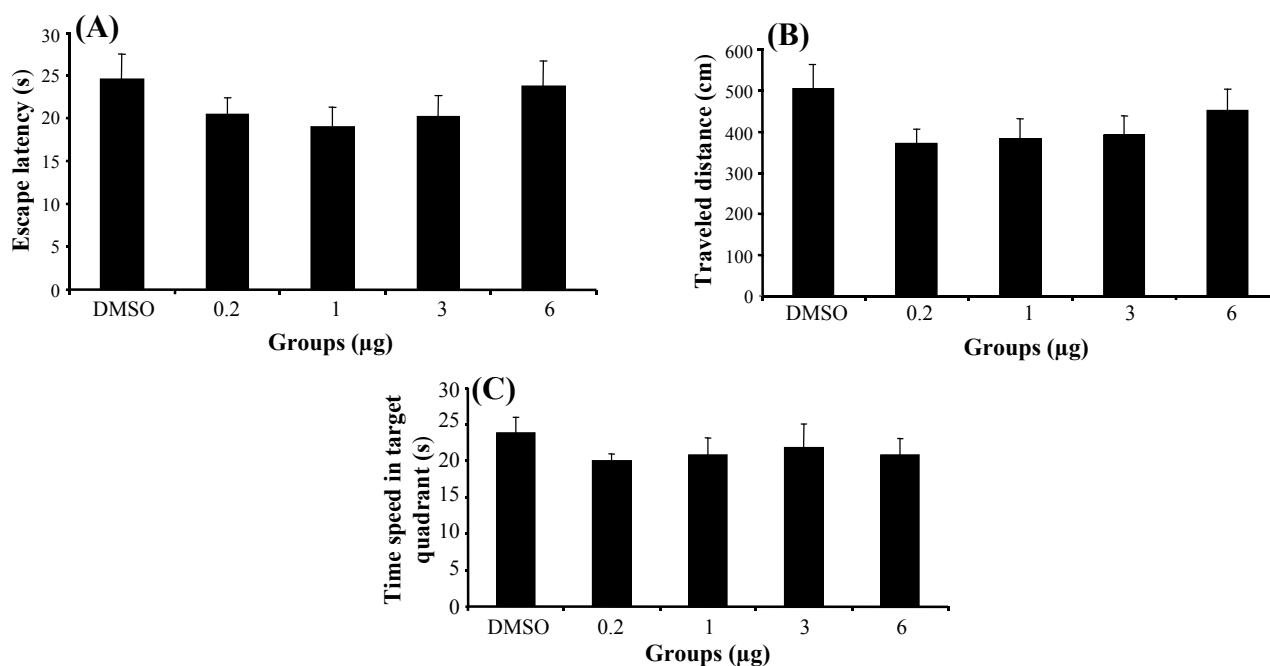
In retrieval stage, no significant difference was generally found in escape latency ( $F_{4,155} = 0.891$ ,  $P = 0.471$ ), traveled distance ( $F_{4,155} = 1.264$ ,  $P = 0.287$ ) (Fig. 3A and 3B) and swimming speed ( $F_{4,155} = 1.56$ ,  $P = 0.188$ ) among the groups in hidden platform trials. One way ANOVA of probe test revealed no significant differences in escape latency ( $F_{4,36} = 0.382$ ,  $P = 0.820$ )



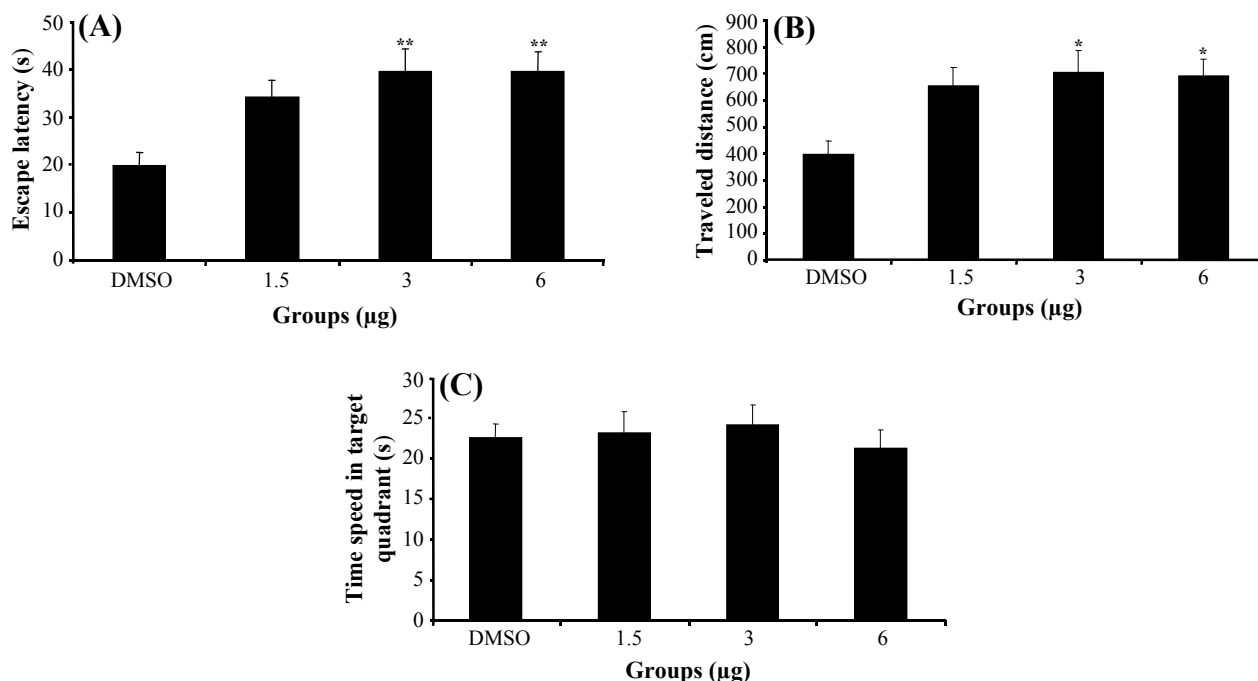
**Fig. 2.** The effect of 3 $\alpha$ -diol and DMSO on consolidation. The columns represent the mean  $\pm$  S.E.M. Average escape latency (A) and traveled distance (B) across all training days and time spent in target quadrant (C) in probe trial. \* $P < 0.05$  indicates significant difference vs. DMSO group.

(Fig. 3C), traveled distance ( $F_{4,36} = 0.460$ ,  $P = 0.764$ ) and swimming speed ( $F_{4,36} = 0.871$ ,  $P = 0.491$ ) among the groups. Also, there was no significant difference of performance among the groups on visible platform day for escape latency ( $F_{4,36} = 1.728$ ,  $P = 0.165$ ) or for traveled distance ( $F_{4,36} = 0.691$ ,  $P = 0.603$ ).

**Experiment 3. The effect of indomethacin on acquisition, consolidation and retrieval of MWM task.** In acquisition stage, a significant difference was generally found in escape latency ( $F_{3,112} = 4.974$ ,  $P < 0.01$ ) and traveled distance ( $F_{3,112} = 3.761$ ,  $P = 0.013$ ) (Fig. 4A and 4B) among the groups in hidden



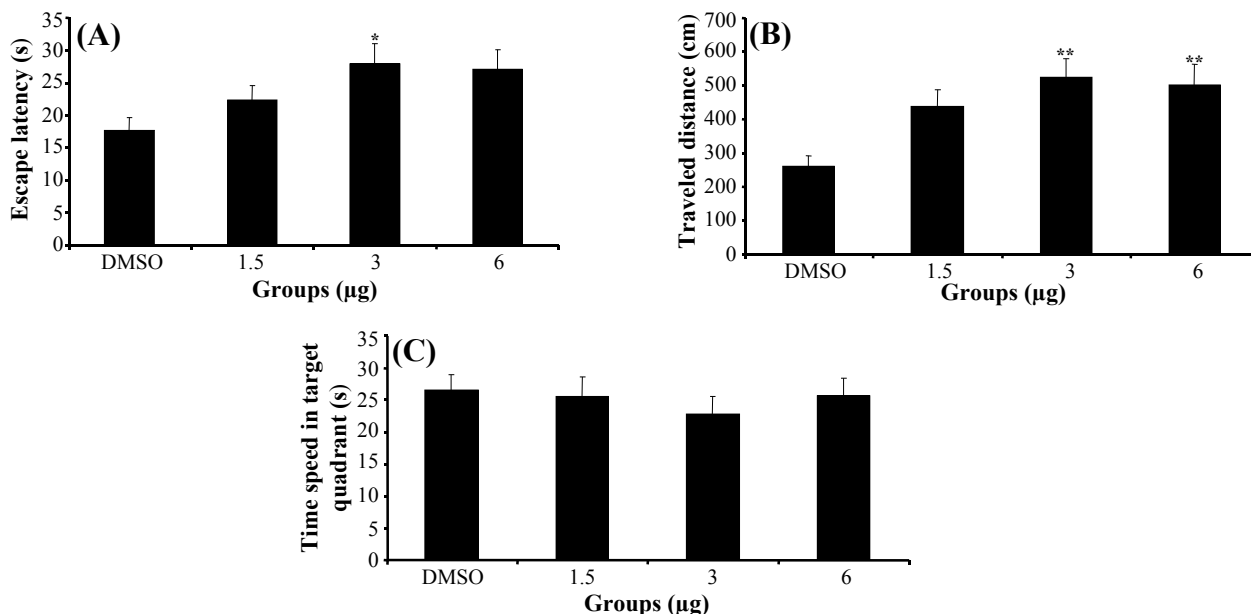
**Fig. 3.** The effect of 3 $\alpha$ -diol and DMSO on retrieval. The columns represent the mean  $\pm$  S.E.M. Average escape latency (A) and traveled distance (B) across all training days and time spent in target quadrant (C) in probe trial.



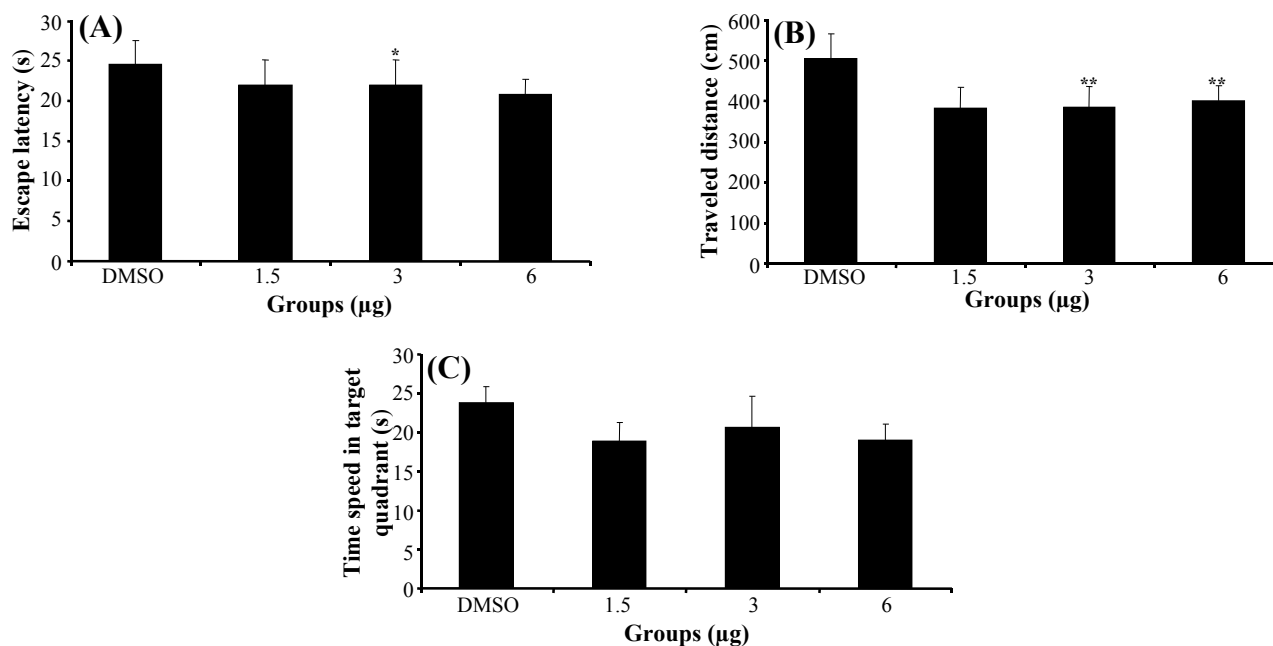
**Fig. 4.** The effect of indomethacin and DMSO on acquisition. The columns represent the mean  $\pm$  S.E.M. Average escape latency (A) and traveled distance (B) across all training days and time spent in target quadrant (C) in probe trial. \* $P < 0.05$  and \*\* $P < 0.01$  indicate significant difference vs. DMSO group.

platform trials (days 1-4), but no significant differences were found in swimming speed ( $F_{3,112} = 1.530$ ,  $P = 0.211$ ). Post hoc multiple comparisons showed that the rats treated with 3 and 6  $\mu\text{g}$  indomethacin had significantly impaired acquisition of spatial learning compared to the control group. One way ANOVA of probe test revealed no significant differences in escape

latency ( $F_{3,30} = 0.779$ ,  $P = 0.515$ ) (Fig. 4C), traveled distance ( $F_{3,30} = 0.329$ ,  $P = 0.804$ ) and swimming speed ( $F_{3,30} = 1.083$ ,  $P = 0.371$ ) among the groups. Also, there was no significant difference of performance among the groups on visible platform day for escape latency ( $F_{3,30} = 0.790$ ,  $P = 0.510$ ) or for traveled distance ( $F_{3,30} = 2.625$ ,  $P = 0.075$ ).



**Fig. 5.** The effect of indomethacin and DMSO on consolidation. The columns represent the mean  $\pm$  S.E.M. Average escape latency (A) and traveled distance (B) across all training days and time spent in the target quadrant (C) in probe trial. \* $P < 0.05$  and \*\* $P < 0.01$  indicate significant difference vs. DMSO group.



**Fig. 6.** The effect of indomethacin and DMSO on retrieval. The columns represent the mean  $\pm$  S.E.M. Average escape latency (A) and traveled distance (B) across all training days and time spent in the target quadrant (C) in probe trial. \* $P < 0.05$  and \*\*  $P < 0.01$  indicate significant difference vs. DMSO group.

In retrieval stage, no significant difference was generally found in escape latency ( $F_{3,120} = 0.301$ ,  $P = 0.825$ ), traveled distance ( $F_{3,120} = 1.272$ ,  $P = 0.287$ ) (Fig. 6A and 6B) and swimming speed ( $F_{3,120} = 1.01$ ,  $P = 0.391$ ) among the groups in hidden platform trials. One way ANOVA of probe test revealed no significant differences in escape latency ( $F_{3,27} = 0.845$ ,  $P = 0.482$ ) (Fig. 6C), traveled distance ( $F_{3,27} = 1.187$ ,  $P = 0.334$ ) and swimming speed ( $F_{3,27} = 0.644$ ,  $P = 0.594$ ) among the groups. Also, there was no significant difference of performance among the groups on visible platform day for escape latency ( $F_{3,27} = 1.323$ ,  $P = 0.287$ ) or for traveled distance ( $F_{3,27} = 0.1678$ ,  $P = 0.195$ ).

**Experiment 4. The effect of indomethacin + 3 $\alpha$ -diol on acquisition, consolidation and retrieval of MWM task.** In acquisition stage, a significant difference was generally found in escape latency ( $T_{62} = 4.65$ ,  $P = 0.029$ ) (Fig. 7A) and traveled distance ( $T_{62} = 3.265$ ,  $P = 0.02$ ) among the groups in hidden platform trials (days 1-4), but no significant differences were found in swimming speed ( $T_{62} = 10.885$ ,  $P = 0.521$ ). T-test probe of trial revealed no significant differences in escape latency ( $T_{14} = 0.872$ ,  $P = 0.686$ ) (Fig. 7B), traveled distance ( $T_{14} = 0.537$ ,  $P = 0.500$ ) and swimming speed ( $T_{14} = 0.163$ ,  $P = 0.790$ ) among the groups. Also, there was no significant difference of performance among the groups on visible platform day for escape latency ( $T_{14} = 2.912$ ,  $P = 0.697$ ) or for traveled distance ( $T_{14} = 3.204$ ,  $P = 0.421$ ).

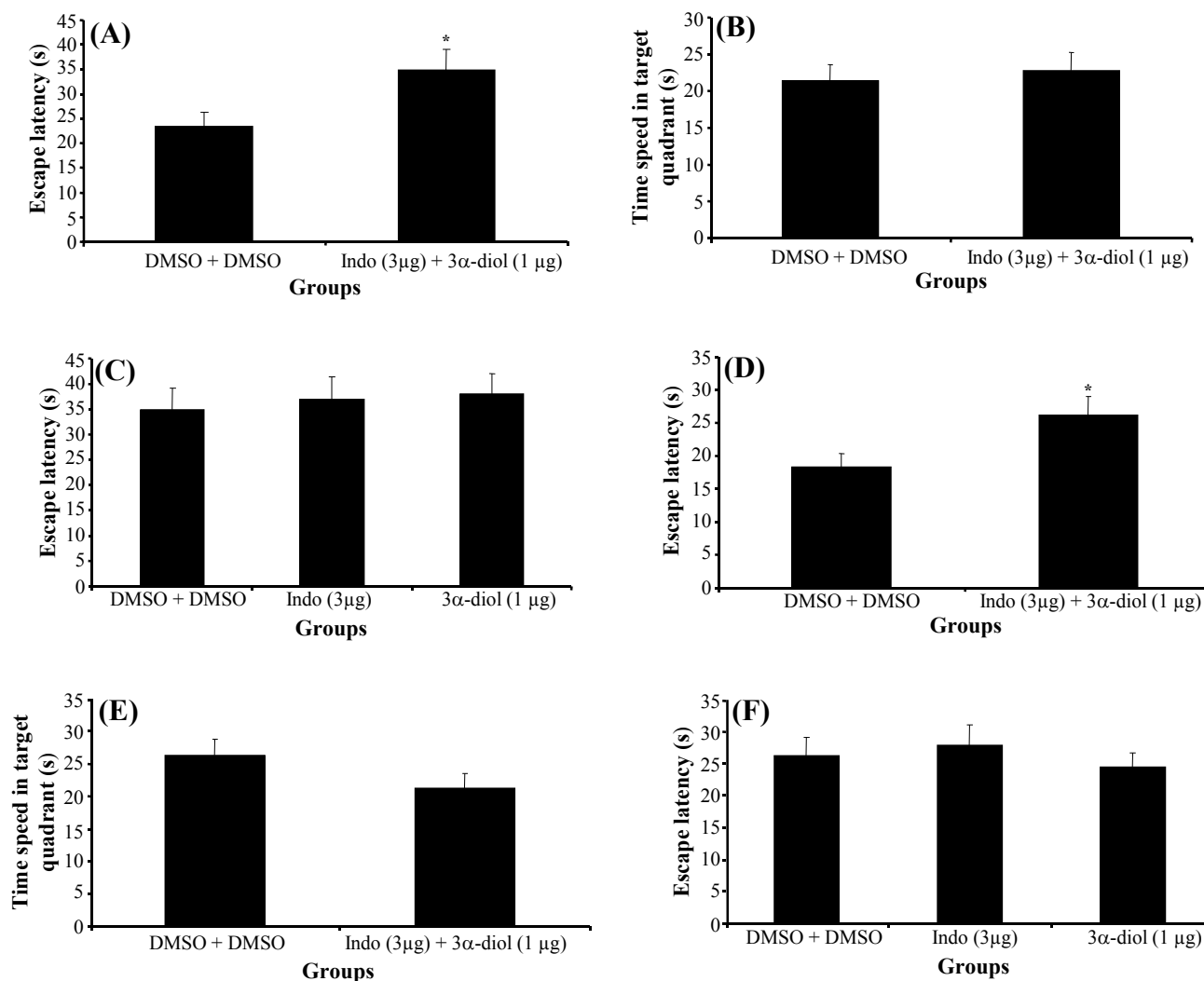
There were no significant differences in escape latency ( $F_{2,97} = 0.139$ ,  $P = 0.870$ ) (Fig. 7C), traveled

distance ( $F_{2,97} = 0.170$ ,  $P = 0.844$ ) and swimming speed ( $F_{2,97} = 1.054$ ,  $P = 0.353$ ) between indomethacin 3  $\mu\text{g}$  + 3 $\alpha$ -diol 1  $\mu\text{g}$ , indomethacin 3  $\mu\text{g}$  and 3 $\alpha$ -diol 1  $\mu\text{g}$  group in acquisition stage. These data indicated that presence of indomethacin cannot inhibit impairing effect of 3 $\alpha$ -diol on acquisition stage of spatial memory.

In consolidation stage, a significant difference was generally found in escape latency ( $T_{54} = 2.42$ ,  $P < 0.05$ ) (Fig. 7D) and traveled distance ( $T_{54} = 8.79$ ,  $P < 0.01$ ) among the groups in hidden platform trials, but no significant differences were found in swimming speed ( $T_{54} = 0.002$ ,  $P = 0.102$ ). T-test probe trial revealed no significant differences in escape latency ( $T_{14} = 0.061$ ,  $P = 0.146$ ) (Fig. 7E), traveled distance ( $T_{14} = 0.070$ ,  $P = 0.798$ ) and swimming speed ( $T_{14} = 0.436$ ,  $P = 0.098$ ) among the groups. Also, there was no significant difference of performance among the groups on visible platform day for escape latency ( $T_{14} = 0.1$ ,  $P = 0.591$ ) or for traveled distance ( $T_{14} = 0.780$ ,  $P = 0.280$ ).

There were no significant differences in escape latency ( $F_{2,93} = 0.413$ ,  $P = 0.663$ ) (Fig. 7F), traveled distance ( $F_{2,93} = 0.994$ ,  $P = 0.374$ ) and swimming speed ( $F_{2,93} = 1.628$ ,  $P = 0.202$ ) between indomethacin 3  $\mu\text{g}$  + 3 $\alpha$ -diol 1  $\mu\text{g}$ , indomethacin 3  $\mu\text{g}$  and 3 $\alpha$ -diol 1  $\mu\text{g}$  group. These data indicated that presence of indomethacin cannot inhibit impairing effect of 3 $\alpha$ -diol on consolidation stage of spatial memory.

In retrieval stage, no significant difference was generally found in escape latency ( $T_{54} = 0.00$ ,  $P = 0.734$ ), traveled distance ( $T_{54} = 0.943$ ,  $P = 0.255$ ) and swimming speed ( $T_{54} = 1.84$ ,  $P = 0.104$ ) among the



**Fig. 7.** The effect of indomethacin + 3α-diol and DMSO + DMSO on acquisition and consolidation. The columns represent the mean ± S.E.M. Average escape latency (A) and time spent in the target quadrant (B) in acquisition and consolidation stage (D and E). One way ANOVA between indomethacin + 3α-diol, indomethacin and 3α-diol in acquisition and consolidation have been shown in the Figure (C and F, respectively). \* $P < 0.05$  indicates significant difference vs. DMSO group. Indo, indomethacin.

groups in hidden platform trials. T-test probe trial revealed no significant differences in escape latency ( $T_{15} = 0.951$ ,  $P = 0.290$ ), traveled distance ( $T_{15} = 2.28$ ,  $P = 0.368$ ) and swimming speed ( $T_{15} = 1.11$ ,  $P = 0.817$ ) among the groups. Also, there was no significant difference of performance among the groups on visible platform day for escape latency ( $T_{15} = 0.395$ ,  $P = 0.657$ ) or for traveled distance ( $T_{15} = 1.25$ ,  $P = 0.586$ ).

## DISCUSSION

In the present study, the role of 3α-diol and indomethacin on acquisition, consolidation and retrieval of learning and memory has been investigated. The findings of the present study includes: (1)

Bilaterally pre-training microinjection of 1, 3 and 6 μg/0.5 μl 3α-diol and 3 and 6 μg/0.5 μl indomethacin into CA1 region of the adult male rats impaired acquisition and consolidation; however, they had no effect on retrieval in 5-day protocol of MWM task. (2) Bilaterally microinjection of 3 μg/0.5 μl indomethacin + 1 μg/0.5 μl 3α-diol impaired acquisition and consolidation, but not retrieval of spatial learning and memory in MWM task. (3) There were no differences in the swimming speed of different doses in treated groups. (4) Intrahippocampal microinjection of 3α-diol and indomethacin had no effect on escape latency, traveled distance and swimming speed to find the visible platform in non-spatial visual discrimination task.

DMSO was used as vehicle in other investigations with similar conditions [1, 7]. In this experiment, we



can see double injections of vehicle (DMSO + DMSO) had no significant effect on learning and memory. This finding is in consistent with some other reports [1, 4, 7].

The swimming speed in DMSO and 3 $\alpha$ -diol or indomethacin-treated groups indicates that micro-injection of 3 $\alpha$ -diol or indomethacin in CA1 area has no effect on locomotors behavior. There were no significant differences between the control and experimental groups on the 5<sup>th</sup> day of training on visible platform. Therefore, it can be inferred that the observed changes could not be attributed to alterations of non-mnemonic factors, such as motivation, motor, or sensory processes induced by the treatments.

Our finding based on 3 $\alpha$ -diol role in impairing memory processing, taken with other previous studies showing conflicting effects of androgens on cognition, suggests that cognitive-hormone interactions are quite complex. In several studies, androgens impaired spatial learning and memory [1, 4, 7, 9], but other studies reported that androgens such as testosterone enhanced spatial memory [11, 12].

Androgens exert these effects on memory through genomic and non-genomic pathways. The genomic pathway typically takes at least more than half an hour to be involved in long-term effects of androgens affecting gene expression via the intracellular androgen receptors. In addition to the classical genomic effects of steroids, many neurosteroids induce non-genomic effects, manifested within seconds to few minutes [22]. This pathway involve in modulation of neurotransmitter receptors and ion channels, ranging from activation of G-protein coupled membrane receptors or sex hormone-binding globulin receptors, stimulation of different protein kinases to direct modulation of voltage and gated ion-channels and transporters [4, 7, 22, 23].

Our previous study revealed that testosterone via both genomic and non-genomic pathways impaired long-term memory [7].

There are several possible explanations for memory impairment effect of 3 $\alpha$ -diol. The first possibility resulted from this fact that neurons and glia express enzymes able to convert testosterone to estradiol and several 5 $\alpha$ -reduced androgens such as DHT. Furthermore, metabolism of DHT results in formation of 5 $\alpha$ -androstane-3,17 $\beta$ -diol (3 $\alpha$ -diol) as well as its 3-isomer (3 $\beta$  A-diol) [24], both of which are capable of eliciting estrogen receptor-dependent responses [25, 26]. Although testosterone and DHT bind with high affinity to androgen receptors, 3 $\alpha$ -diol does not typically bind to androgen receptors. Instead, 3 $\alpha$ -diol has some effects on estrogen receptor especially estrogen receptor  $\beta$  [4]. Previous studies have shown that intrahippocampal microinjection of estrogen impaired spatial memory in rats [27]. Also, activation

of estrogen receptor  $\beta$  reduces the transcription process and impairs cognitive performance [27].

The second possibility is based on this fact that in addition to effect on estrogen receptor, 3 $\alpha$ -diol also acts similar to the analogous metabolites of progesterone and corticosterone such as allopregnanolone to enhance GABA-benzodiazepine regulated chloride channel function [25, 26]. One of the best documented examples of non-genomic actions of steroids is the ability of these hormones to activate GABA<sub>A</sub> receptors. The GABA<sub>A</sub> receptor is a member of the ligand-gated ion channel family that  $\alpha$  and  $\beta$  subunits of this receptor affect GABA<sub>A</sub> neuro-modulation by either positive or negative neurosteroid modulators [5]. Activation of the GABA<sub>A</sub> receptor complex by neuroactive steroids resulted in opening of its central Cl<sup>-</sup>-conducting pore, which led to a hyperpolarization of the plasma membrane and inhibition of neuronal fires. The major groups of neuroactive steroids and their metabolites are progesterone, dehydrocorticosterone and some metabolites of them. In general, they mediate their actions not only through classic steroid receptors, but also through other mechanisms such as ligand-gated ion-channels, including GABA<sub>A</sub>, glutamate or opioid receptors [23]. On the other hand, the biphasic effects of progesterone are consistent with a critical role for GABA-mediated responses. Progesterone such as DHT is rapidly converted in the brain to 5 $\alpha$ -reduced metabolites, some of which potentiate GABA action on the GABA-benzodiazepine-chloride channel complex [5]. Therefore, the effects of androgens as 3 $\alpha$ -diol could be mediated in much the same way as the rapid responses of progesterone, via enhancement of GABAergic neurotransmission.

Beside, some studies have shown that all hippocampal subregions are rich in GABA<sub>A</sub> receptors as well as some neurosteroids such as allopregnanolone can inhibit neural activity in the CA1 and dental gyrus areas of the hippocampus [3, 28]. Treatment with GABA<sub>A</sub> receptor active substance, benzodiazepine, can inhibit learning and memory in humans and animals [3]. Acute treatment with neurosteroids that have GABA-modulatory effects impairs learning and memory. In contrast, steroids that act as GABA<sub>A</sub> receptor antagonists enhance learning and memory [29, 30].

The third possibility is based on this fact that there is an interaction between steroids and the serotonin system in the hippocampus. Some evidence showed that steroids including estrogen and progesterone metabolites such as allopregnanolone can affect spatial learning and memory via the serotonin system [3]. Also, a direct interaction between the GABA and the serotonin systems in the hippocampus is proved, where serotonin neurons often end at inhibitory GABAergic

interneurons [3, 31]. Many studies have shown that excitation of serotonergic neurotransmission impairs learning and memory, whereas reduction of serotonin activity can improve these processes [32]. Serotonin and GABA systems may therefore interact in the hippocampus, a region which is important for cognitive functions [3, 33]. Since  $3\alpha$ -diol act similar to the analogous metabolites of progesterone, probably induce impairment in learning and memory performance via this interaction with serotonin system.

Also, steroid hormones modulate the memory processes perhaps by their relationship with other neurotransmitter systems, such as acetylcholine, dopamine, noradrenaline and glutamate, and by their relationship with the cerebral regions that participate in these phenomena [4]. Based on the evidences, testosterone and its metabolites,  $3\alpha$ -diol, can reduce acetylcholine release in the hippocampus via positive modulating hippocampal GABAergic interneurons that is proven to induce memory impairment [4, 34]. Many studies have also shown that NMDA receptors are critical for synaptic plasticity and long-term memory [1, 5, 35]. By acting as non-selective sigma ( $\sigma$ ) antagonist, testosterone may produce a tonic damping of the function of sigma receptors and consequently a decrease in NMDA receptor function [4]. Therefore, these facts may be a good and reliable proof for the effects of  $3\alpha$ -diol (as one metabolites of testosterone) on spatial memory.

We purpose in using of indomethacin resulted from the Frye *et al.* [11, 15] literature about this fact that indomethacin is the major  $3\alpha$ -HSD inhibitor. Furthermore, blocking testosterone or DHT metabolism to  $3\alpha$ -diol with indomethacin decreases cognitive performance and increases anxiety behavior of gonadally-intact and/or DHT-replaced rats. In experiments 2 and 3, we assayed the effect of  $3\alpha$ -diol and indomethacin alone on learning and memory. Our finding shows that both  $3\alpha$ -diol and indomethacin impair acquisition and consolidation learning and memory performance. In experiment 4, we used indomethacin as  $3\alpha$ -HSD inhibitor to prevent the effect of endogenous  $3\alpha$ -diol on learning and memory. Then, we studied the effect of exogenous  $3\alpha$ -diol on acquisition, consolidation and retrieval learning and memory. Our results show that exogenous  $3\alpha$ -diol has impairment effect on acquisition and consolidation memory and indomethacin could not prevent the impairment effect of  $3\alpha$ -diol. Besides, there is no significant difference between intra-CA1 administration of indomethacin +  $3\alpha$ -diol, indomethacin and  $3\alpha$ -diol on acquisition and consolidation stage using One way ANOVA. It is possible that  $3\alpha$ -diol cognitive impairment effects cannot be influenced by indomethacin. This result indicates that another mechanism may be responsible for the observed effects. It is

believed that indomethacin as a non-selective COX inhibitor [18] can affect learning and memory. Therefore, COX enzymes catalyze the first two committed steps in the biosynthesis of prostanoids. Several lines of evidence indicate a potential role for COX in physiological mechanisms underlying memory function and indomethacin as a COX inhibitor could impair these mechanisms [16-18]. For example, indomethacin as a COX inhibitor impairs passive avoidance memory in chick and prevents the learning-induced increase in prostaglandin release, which occurs 2 h after training [18].

In summary, it is concluded that intra-CA1 administration of  $3\alpha$ -diol and indomethacin could impair acquisition and consolidation, but not retrieval spatial learning and memory. Also, intrahippocampal injection of indomethacin +  $3\alpha$ -diol could not change impairment effect of indomethacin or  $3\alpha$ -diol alone in MWM task.

## ACKNOWLEDGMENTS

We wish to thank Department of Physiology and Pharmacology of Pasteur Institute of Iran for supporting this research.

## REFERENCES

1. Emamian S, Naghdi N, Sepehri H, Jahanshahi M, Sadeghi Y, Choopani S. Learning impairment caused by intra-CA1 microinjection of testosterone increases the number of astrocytes. *Behav Brain Res.*2010 Mar; 208(1):30-7.
2. Hojo Y, Murakami G, Mukai H, Higo S, Hatanaka Y, Ogiue-Ikeda M et al. Estrogen synthesis in the brain-Role in synaptic plasticity and memory. *Mol Cell Endocrinol.*2008 Aug;290(1-2):31-43.
3. Birzniece V, Bäckström T, Johansson IM, Lindblad C, Lundgren P, Löfgren M et al. Neuroactive steroid effects on cognitive functions with a focus on the serotonin and GABA systems. *Brain Res Rev.*2006 Aug; 51(2):212-239.
4. Harooni HE, Naghdi N, Sepehri H, Rohani AH. Intra hippocampal injection of testosterone impaired acquisition, consolidation and retrieval of inhibitory avoidance learning and memory in adult male rats. *Behav Brain Res.*2008 Mar;188(1):71-7.
5. MacLusky NJ, Hajszan T, Prange-Kiel J, Leranth C. Androgen modulation of hippocampal synaptic plasticity. *Neuroscience.*2006;138(3):957-65.
6. Moffat SD, Zonderman AB, Metter EJ, Kawas C, Blackman MR, Harman SM et al. Free testosterone and risk for Alzheimer disease in older men. *Neurology.* 2007 Jan;62(2):188-93.
7. Naghdi N, Asadollahi A. Genomic and nongenomic effects of intrahippocampal microinjection of testos-

- terone on long-term memory in male adult rats. *Behav Brain Res.*2004 Aug;153(1):1-6.
8. Brosens JJ, Tullet J, Varshochi R, Lam EW. Steroid receptor action. *Best Pract Res Clin Obstet Gynaecol.* 2004 Apr;18:265-83.
  9. Hampson E. Spatial cognition in humans: possible modulation by androgens and estrogens. *J Psychiatry Neurosci.*1995 Nov;20(5):397-404.
  10. Ogiue-Ikeda M, Tanabe N, Mukai H, Hojo Y, Murakami G, Tsurugizawa T et al. Rapid modulation of synaptic plasticity by estrogens as well as endocrine disrupters in hippocampal neurons. *Brain Res Rev.*2008 Mar;57(2):363-75.
  11. Frye CA, Edinger KL, Seliga AM, Wawrzycki JM. 5 $\alpha$ -reduced androgens may have actions in the hippocampus to enhance cognitive performance of male rats. *Psychoneuroendocrinology.*2004 Sep;29(8):1019-27.
  12. Osborne DM, Edinger K, Frye CA. Chronic administration of androgens with actions at estrogen receptor beta have anti-anxiety and cognitive-enhancing effects in male rats. *Age (Dordr).*2009 Sep;31(3):191-8.
  13. Lund BC, Bever-Stille KA, Perry PJ. Testosterone and andropause: the feasibility of testosterone replacement therapy in older men. *Pharmacotherapy.*1999;8:951-6.
  14. Vazquez-Pereyra F, Rivas-Arancibia S, Loaeza-Del Castillo A, Schneider-Rivas S. Modulation of short term and long term memory by steroid sexual hormones. *Life Sci.*1995;56(14):PL255-60.
  15. Frye CA, Edinger KL, Lephart ED, Walf AA. 3 $\alpha$ -androstenediol, but not testosterone, attenuates age-related decrements in cognitive, anxiety, and depressive behavior of male rats. *Front Aging Neurosci.*2010;2:15.
  16. Nokhodchi A, Javadzadeh Y, Siah-Shadbad MR, Barzegar-Jalali M. The effect of type and concentration of vehicles on dissolution rate of a poorly soluble drug (indomethacin) from liquisolid compacts. *J Pharm Pharm Sci.*2005 Jan;8:18-25.
  17. Sanders BK. Sex, drugs and sports: prostaglandins, epitestosterone and sexual development. *Med Hypotheses.*2007;69(4):829-35.
  18. Teather LA, Packard MG, Bazan NG. Post-Training Cyclooxygenase-2 (COX-2) inhibition impairs memory Consolidation. *Learn Mem.*2002 Jan-Feb;9(1):41-7.
  19. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 2<sup>nd</sup> ed. Academic Press, New York.
  20. D'Hooge R, De Deyn PP. Applications of the Morris water maze in the study of learning and memory. *Brain Res Brain Rev.*2001 Aug;36(1):60-90.
  21. Paul CM, Magda G, Abel S. Spatial memory: Theoretical basis and comparative review on experimental methods in rodents. *Behav Brain Res.*2009 Nov;203(2):151-64.
  22. Li J, Al-Azzawi F. Mechanism of androgen receptor action. *Maturitas.*2009 Jun;63(2):142-8.
  23. Michels G, hoppe, UC. Rapid actions of androgens. *Front Neuroendocrinol.*2008 May;29(2):182-98.
  24. Reddy DS. Testosterone modulation of seizure susceptibility is mediated by neurosteroids 3 $\alpha$ -androstenediol and 17 $\beta$ -estradiol. *Neuroscience.* 2004;129(1):195-207.
  25. Genazzani AR, Pluchino N, Freschi L, Ninni F, Luisi M. Androgens and the brain. *Maturitas.*2007 May;57(1):27-30.
  26. Pettersson H, Lundqvist J, Oliw E, Norlin M. CYP7B1-mediated metabolism of 5-androstane-3,17-diol (3-Adiol): A novel pathway for potential regulation of the cellular levels of androgens and neurosteroids. *BiochimBiophys Acta.*2009;1791:1206-15.
  27. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ et al. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science.*1997 Sep; 227(5331):1508-10.
  28. Landgren S, Wang MD, Backstrom T, Johansson S. Interaction between 3 alpha- hydroxy-5 alpha-pregnan-20-one and carbachol in the control of neuronal excitability in hippocampal slices of female rats in defined phases of the oestrus. *ActaPhysiol Scand.*1998 Jan;162(1):77-88.
  29. Mathews DB, Morrow AL, Tokunaga S, McDaniel JR. Acute ethanol administration and acute allopregnanolone administration impair spatial memory in the Morris water task. *Alcohol ClinExp Res.*2002 Nov;26(11):1747-51.
  30. Vallee M, Mayo W, Koob GF, Le Moal M. Neurosteroids in learning and memory processes. *Int Rev Neurobiol.*2001;46:273-320.
  31. Jakab RL, Goldman-Rakic PS. Segregation of serotonin 5-HT2A and 5-HT3 receptors in inhibitory circuits of the primate cerebral cortex. *J Comp Neurol.*2000 Feb;417(3):337-48.
  32. Dubrovsky, B. (2006) Neurosteroids, neuroactive steroids, and symptoms of affective disorders. *Pharmacol Biochem Behav.*2006 Aug;84(4):644-55.
  33. Sibille E, Pavlides C, Benke D, Toth M. Genetic inactivation of the serotonin (1A) receptor in mice results in down regulation of major GABA (A) receptor alpha subunits, reduction of GABA (A) receptor binding, and benzodiazepine- resistant anxiety. *J Neurosci.*2010 Jun;20:2758-2765.
  34. Moor E, DeBoer P, Westerink BH. GABA receptors and benzodiazepine binding sites modulate hippocampal acetylcholine release *in vivo.* *Eur J Pharmacol.*1998 Oct;359(2-3):119-26.
  35. Santos SD, Carvalho AL, Calderia MV, Duarte CB. Regulation of AMPA receptors and synaptic plasticity. *Neuroscience.*2009 Jan;158(1):105-25.