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Original Article

Effects of long term administration of testosterone and estradiol on spatial memory *in rats*

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Abstract

There are many discrepancies around the effect of sex hormones on spatial learning and memory in rodents. The aim of the present study was to investigate the effects of chronic administration of estradiol (ES) and testosterone (TES) on spatial memory in adult castrated male rats. Cholinesterase activity of the hippocampus in treated animals was also measured to seek if hormonal treatment can change the acetylcholinesterase (AChE) activity in this region. Six groups of castrated male rats received different doses of ES valerate (1, 4, 10 mg/kg, by subcutaneous, sc) and TES enanthate (10, 20, 40 mg/kg, sc) in weekly injection intervals for 6 weeks. Morris water maze (MWM) was used to assess the spatial reference memory of the rats. The specific activity of AChE in the hippocampus was also measured. The treatment duration and the dose quantity of ES had significant (P<0.001 and P=0.048, respectively) effect on the learning ability in the rats. For TES treated rats, treatment duration showed a significant effect (P<0.001) on learning performance of the rats. The activity of AChE compared to the control group was significantly increased in ES treated rats in a dose dependent manner and it was decreased in the group that received the highest dose of TES. Our results showed that chronic high dose of ES decreased the learning ability of male castrated rats in a reference memory version of MWM test. This can be explained by the decreased AChE activity in the hippocampus.

Keywords: Spatial reference memory; Testosterone; Estradiol; Acetylcholinesterase; Morris water maze; Hippocampus

INTRODUCTION

Spatial memory is the ability of the brain to store information within a spatio-temporal frame (1). Spatial learning depends upon the integrated action of many brain regions and involves different neurotransmitter systems (2). Many studies support the notion that the information from the environment is stored in the hippocampus as a map-based representation (cognitive map) (3).

It is shown that reduced cholinergic activity in hippocampus results in spatial memory deficits in rats (4-6). In humans, hippocampus and amygdala are major end points for the cholinergic fibers originating in the subnuclei of the basal forebrain (7,8). Degeneration of these fibers is associated with Alzheimer's dementia and possibly other forms of memory deficit observed in the elderly (9,10).

Sex hormone receptors are abundant in the hippocampus (11-13) indicating their functional importance in this structure. There is a plethora of investigation regarding hormonal effects on hippocampal plasticity and spatial memory. Sex steroids differently change such aspects in hippocampal function as neurogenesis (14), cholinergic activity (15-21), hippocampal connectivity (12,22-25) and size (26-28).

Gender differences in spatial learning ability are shown both in rodents (29,30) and in humans (31-34). Most results show a male superiority in spatial tasks (30) but female advantage (35,36) or no sex difference results are also reported (37,38). These inconsistencies can be explained by some

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factors such as methodological variables, age of the animals, the estrous cycle stage of the female animals, etc. (39).

To the best of our knowledge there is less evidence on the effects of chronic administration of sex steroids, especially female steroids, on adult male rat performance in tests of spatial memory. In addition most studies have used single doses of sex hormones. The other issue is that there is a lack of evidence for the probable effects of sex hormones on acetylcholinesterase (AChE) activity in the hippocampus. The aim of our study was to investigate the effects of chronic systemic administration of two slow releasing esters of sex hormones, on spatial reference memory in adult castrated male rats. We have also measured cholinesterase activity of the hippocampus in tested animals to find if chronic sex steroids could change the cholinergic activity in this structure of the brain.

MATERIALS AND METHOD

Chemicals

Chemicals used in AChE assay and normalization such as acetylthiocholine iodide, 5,5'-dithiobis(2-nitrobenzoic acid), bovine serum albumin and Bradford reagent were purchased from Sigma Chemicals, USA. ES valerate and TES enanthate were obtained from Boditech Med Inc, Korea.

Subjects

Adult male Wistar rats (approximately 16 weeks old) were obtained from the Pasteur Institute (Tehran, Iran). The housing place was temperature controlled (22 ± 2 °C) with a 12:12 h light/dark cycle. All animal procedures were carried out in accordance with the ethical guidelines of the National Institutes of Health. All subjects were castrated bilaterally under ketamine/xylazine (100/10 mg/kg) anesthesia. A small incision was made at the posterior end of the scrotum to remove each testis. The remaining tissue was ligated with chromic gut suture (4-0) and the muscle and skin layers were stitched afterwards.

Experimental groups

Animals were divided into 7 groups of 8 rats each. Three groups received doses of ES valerate (1, 4, 10 mg/kg, sc) and three groups received doses of TES enanthate (10, 20, 40 mg/kg, sc) in weekly injection intervals for 6 consecutive weeks. Doses and interval of injection were chosen according to previous studies (40-43). A control group received injections of sesame oil (0.5 ml) as the vehicle for the hormones. Behavioral testing began the day following the last injection.

Water maze apparatus

The spatial memory was assessed in the Morris water maze (MWM) (44). The maze was a black circular pool, measuring 150 cm in diameter and a height of 80 cm. The water level was 40 cm and its temperature was set at 22 ± 2 ºC. The pool was divided geographically into four quadrants named Northeast (NE), Northwest (NW), Southeast (SE) and Southwest (SW). A hidden circular platform (17 cm in diameter) was adjusted in the center of the south-west quadrant so that its surface was 2 cm below water level. The pool was surrounded by a black curtain on which spatial clues were pinned. Animal behavior was recorded by a video camera and analyzed by a computerized video-tracking system EthoVision XT6 (Noldus Information Technology, Netherlands)

Behavioral assessment

The test was done according to Pourmotabbed and coworkers with slight modifications (45). Before the training days rats went through two days of habituation sessions. On the first day the platform was positioned in the center of the empty pool and rats were put on the platform for 60 s. The second day the tank was full of water and the rats were put on the platform in the same position for another 60 s. When the rats jumped off the platform they were guided back onto it.

Training (acquisition) trials started the day after habituation days. Training took five consecutive days and each day consisted of four trials. In each trial the rat was placed in the pool from one of the randomly determined points of NE, NW, SE and SW. If the rat could find the platform within a time span of 60 s it was allowed to stay on it for 15 s, otherwise it

was guided to the platform and remained there for 15 s. The rats were then towel dried and kept in a heated cage. The inter-trial time was 2 min for each rat. On the sixth day a probe trial was performed to test the spatial memory of the rats. In this trial the rats were allowed to swim in the pool for 60 s while the platform was removed.

The total distance to the target platform, center of the previous position of the platform, was recorded during this period. This total distance is called search error which is a good reflection for spatial learning in MWM test (46). On the seventh day a similar trial to the training trials was done but with a visible cue, a Ping-Pong ball affixed to the top of a rod, on the platform. All distal cues were removed in the visible phase.

This trial was done to assess any sensory defects or motivational factors that may interfere with the rat's ability to escape (45). Other behavioral parameters such as speed of the animals and the distance moved within the 30 cm outermost edge of the pool were also recorded. Animal speed was an indicator of locomotor activity. Thigmotaxis behavior was represented by the distance each animal moved near the wall of the pool. Trials were conducted each afternoon (13.00–18.00 h), and the order in which rats were tested was randomized each day.

Assessment of serum hormone levels

ES and TES were determined in serum by radioimmunoassay. Blood samples were collected at the end of behavioral testing.

AChE assay

The specific activity of AChE in the hippocampus was measured according to the spectrophotometric method of Ellman and colleagues (47) with slight modifications. AChE assay was performed after behavioral tests were finished. Briefly the whole brain was taken out from each rat skull and the hippocampus was removed according to Li (48). The dissected hippocampus was homogenized with a Silent Crusher homogenizer (Heidolph, Schwabach, Germany) in sodium phosphate buffer (30 mM, pH=7.0) to make a 10% (w/v) homogenate.

All the homogenates were centrifuged at 12000 rpm at -4 ºC in a Hettich centrifuge (universal 320R, Germany) using a fixed angle rotor (1420 A) for 45 min. Supernatant was stored at -20 ºC. Aliquots of this supernatant were diluted in the ratio of 1:10 and used as a source of enzymes for the spectrophotometric assay. The protein content of each sample was also determined with the Bradford protein assay for normalization of the results.

Statistical analysis

Significant differences of the distance to the platform in the MWM test were evaluated by two-way analysis of variance (ANOVA) with repeated measures. Analysis of the probe trial results were performed by one way ANOVA. Tukey's significant difference test was used for post hoc comparisons. Data for the AChE assay was compared to the control with student's *t-test*. Differences in serum TES and ES concentrations among the treatment groups were also analyzed using a *t-test*.

RESULTS

Serum hormonal levels

The results of serum TES and ES levels are shown in Table 1. Hormonal levels of serum were compared in drug treated rats and the control group. Student's *t-test* showed that in both TES and ES treated rats the serum concentrations of the hormone increased in a dose dependent manner compared to the control group.

Effects of testosterone on learning performance on the Morris water maze

Two way repeated measures ANOVA was used to find the possible role of dose, day or their interaction on the learning ability of the rats. The test revealed a non-significant dose ($F(3,28)= 2.14$, $P=0.1$)) and a significant day ($F(4,112)=29.5$, $P<0.001$) effects for TES treated rats. There was not a significant dose-day interaction ($F(12,112)=0.85$, $P=0.59$) (Fig. 1). The probe trial results for TES treatment are shown in Fig. 2.

Table 1. Testosterone and estrogen concentrations (mean ± SEM) in serum collected after finishing behavioral tests.

Group	Testosterone (nmol/ml)	Estrogen (pg/ml)
Con	0.1 ± 0.07	26.5 ± 5.8
TES10	(15.9 ± 2.4)*	20.2 ± 4.3
TES20	(24.3 ± 3.2)**	(16.9 ± 2.2)†
TES40	(31.8 ± 2.6)***	(10.8 ± 3.6)†
ES1	0.06 ± 0.04	(103 ± 17.2)*
ES4	(0.05 ± 0.02)†	(1985 ± 106)**
ES10	0.08 ± 0.04	(3546 ± 186)***

*; Significantly increased compared to the control ($P<0.05$).
**; Significantly increased compared to the control ($P<0.01$).
***; Significantly increased compared to the control ($P<0.001$).
†; Significantly decreased compared to the control ($P<0.05$).

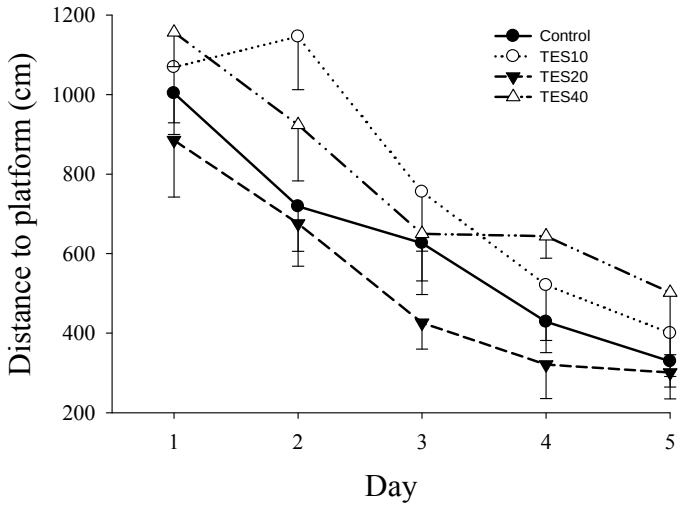

Fig. 1. Mean path length to the platform for male castrated rats (n=8) injected weekly with sesame oil (Control), testosterone enanthate 10, 20, 40 mg/kg (TES10, TES20, TES40, respectively). Results are shown as mean-SD.

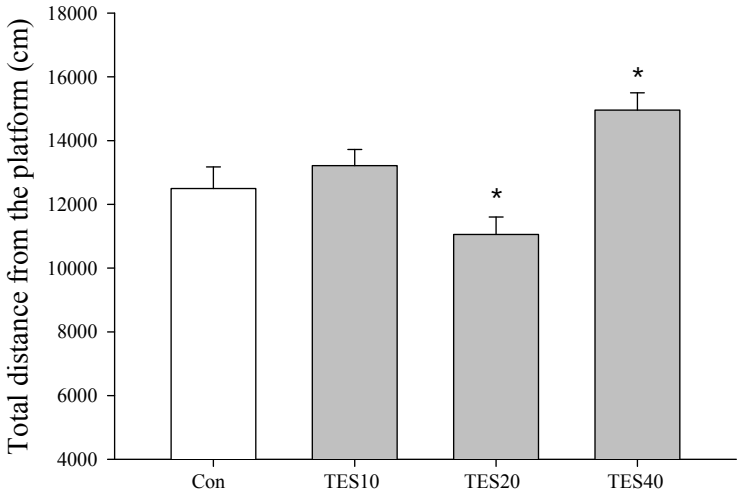

Fig. 2. Effect of testosterone treatment on performance of male castrated rats (n=8) in the Memory Recall Test. Animals are injected weekly with sesame oil (control), testosterone enanthate 10, 20, 40 mg/kg (TES10, TES20, TES40, respectively). Compared to the control group search error (total distance from the platform) is significantly reduced in TES20 and significantly increased in TES40 groups. Values are presented as mean+SD. *<0.05 versus control.

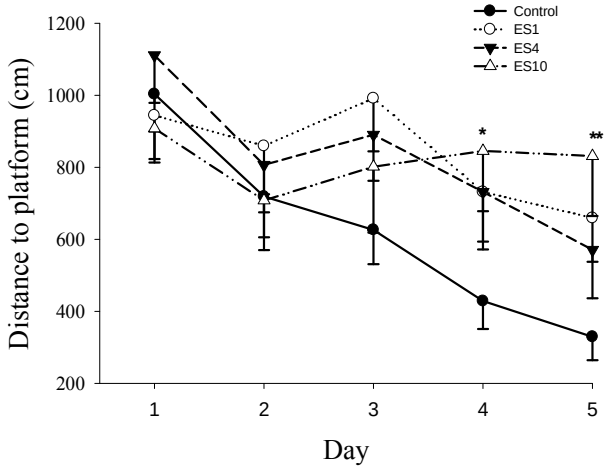

Fig. 3. Mean path length to the platform for male castrated rats (n=8) injected weekly with sesame oil (control), estradiol valerate 1, 4, 10 mg/kg (ES1, ES4, ES10, respectively). During 5 days of acquisition trials ES10 group showed a significant increase in path length on the fourth and fifth days of testing as compared to the control group. Results are shown as mean-SD. *<0.05 and **<0.01 versus control.

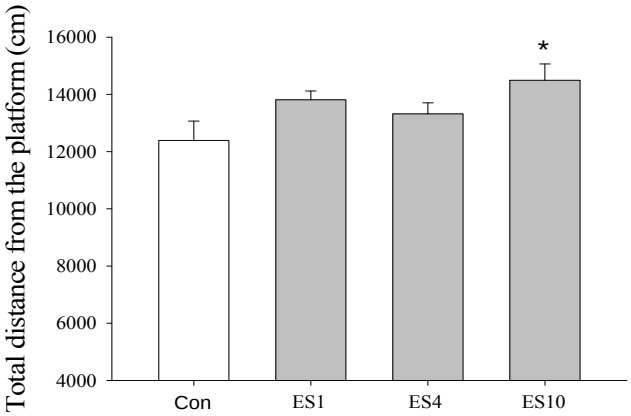

Fig. 4. Effect of estradiol on performance of male castrated rats (n=8) in the Memory Recall Test. Animals are injected weekly with sesame oil (control) and estradiol valerate 1, 4, 10 mg/kg (ES1, ES4, ES10, respectively). Search error (total distance from the platform) is significantly increased in ES10 as compared to the control group. Values are presented as mean+SD. *<0.05 versus control.

One way ANOVA showed that the total distance to the platform does not differ for different doses of TES (F(3,28)=2.15, P=0.23) but as evident in Fig. 2, the effects of TES is not parallel in different doses and an All Pairwise Multiple Comparison showed that TES at 40 mg/kg and TES at 20 mg/kg were significantly different from the control group (P<0.05 for both).

Effects of estradiol on learning performance on the Morris water maze

In ES treated groups ANOVA showed a significant effect for days (F(4,112)=6, P<0.001) on the distances swum to the platform. There was also a significant effect for dose (F(3,28)=3.01, P=0.048) while the interaction of the dose-day was not significant (F(12,112)=0.65, P=0.79). Post hoc comparison showed a significant difference on the fourth (P<0.05) and fifth (P<0.01) days of treatment between control and ES 10 mg/kg groups (Fig. 3).

Fig. 4 shows the results of the probe trial in estrogen treated rats. One way ANOVA showed that the total distance to the platform differs for different doses of ES (F (3,28)=4.3, P=0.01) and post hoc comparisons indicated that estrogen at a dose of 10 mg/kg increased the total distance of the animals to the platform (P<0.05) compared to the control group.

Effect of hormonal treatment on AChE activity in hippocampus

The specific activity of AChE was determined in the hippocampus of control and hormone treated rats after behavioral tests were finished. As shown in Fig. 5, the activity levels of AChE compared to the control increased significantly in ES 1 mg/kg (P<0.05), ES 4 mg/kg (P<0.01) and ES 10 mg/kg (P<0.001) groups and it was decreased in TES 40 mg/kg (P<0.01) treated rats.

Effects of testosterone and estradiol on thigmotaxis parameters in MWM

To find the role of TES doses or passing days on thigmotaxis of the animals a two way repeated measure ANOVA was performed on the distance each animal moved beside the wall of the pool. The results showed that there was a significant effect for days (F(4,112)=34.9,P<0.001) but the effects of dose (F(3,28)=2.63, P=0.12) and dose-day interaction (F(12,112)=0.74, P=0.63) were not significant (Fig. 6A).

For ES treated rats in a similar way there is a significant effect for days (F(4,112)=27.93, P<0.001) and a non-significant effect for dose (F(3,28)=1.15, P=0.56). Also there was not an interaction between the dose and the days (F(12, 112)=0.87, P=0.32) (Fig. 6B).

Effects of testosterone and estradiol on speed of the animals

Fig. 7 shows the results of the speed of animals in the 5-day test trials for ES and TES treated rats. ANOVA showed that there was not a significant effect for dose (F(3,28)=0.82, P=0.49) , day (F(4, 112)=0.59, P=0.66) and dose-day interaction (F(12,112)=0.97, P=0.47) in TES treated animals (Fig. 7A). ANOVA results for ES showed that the speed of the animals did not change in days (F(4,112)=0.86, P=0.59) and was not statistically significant among different doses of the drug (F(3,28)=0.75, P=0.38). Also there is not an interaction between days and doses (F(12,112)=0.64, P=0.23) for ES treated rats (Fig. 7B).

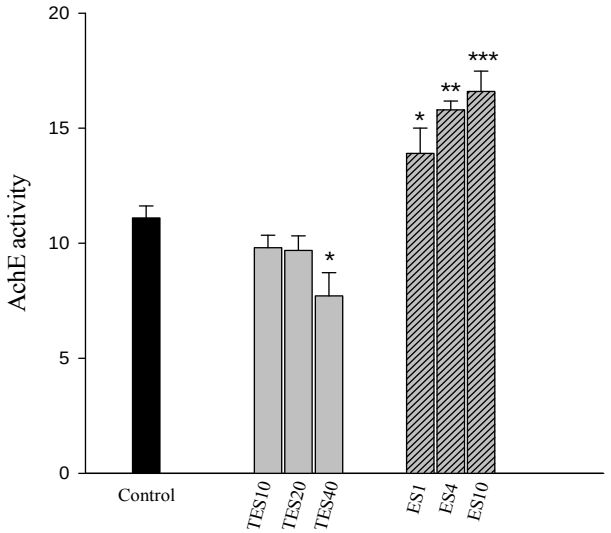

Fig. 5. Effect of sex steroids in the *in vitro* AChE activity in hippocampus. Male castrated rats were injected weekly with sesame oil (control), testosterone enanthate 10, 20, 40 mg/kg (TES10, TES20, TES40, respectively) and estradiol valerate 1, 4, 10 mg/kg (ES1, ES4, ES10, respectively) for 6 weeks. AChE activity was measured separately in the homogenates of hippocampus of control and treated rats (n=8) after maze tests. Values are mean + SD. *<0.05, **<0.01 and ***<0.001 compared to the control.

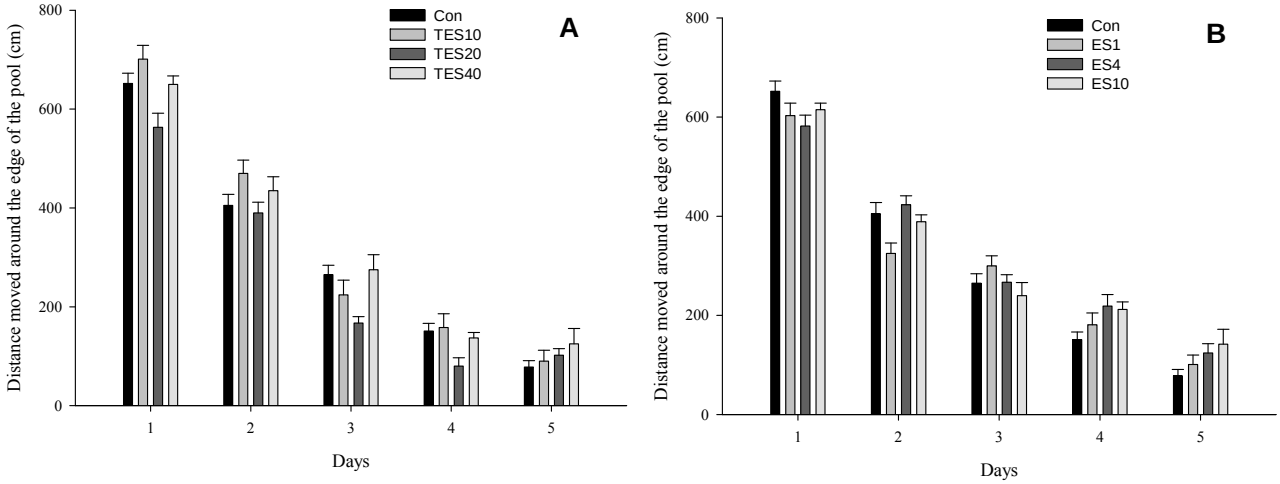

Fig. 6. Mean daily path length within the thigmotaxis region. Male castrated rats were injected weekly with sesame oil (control), testosterone enanthate 10, 20, 40 mg/kg (TES10, TES20, TES40, respectively) and estradiol valerate 1, 4, 10 mg/kg (ES1, ES4, ES10, respectively) for 6 weeks. Thigmotaxis distance decreased across days for both A; testosterone and B; estradiol treated rats but there was not a change for dose or dose-day interaction. Data are shown as mean + SD.

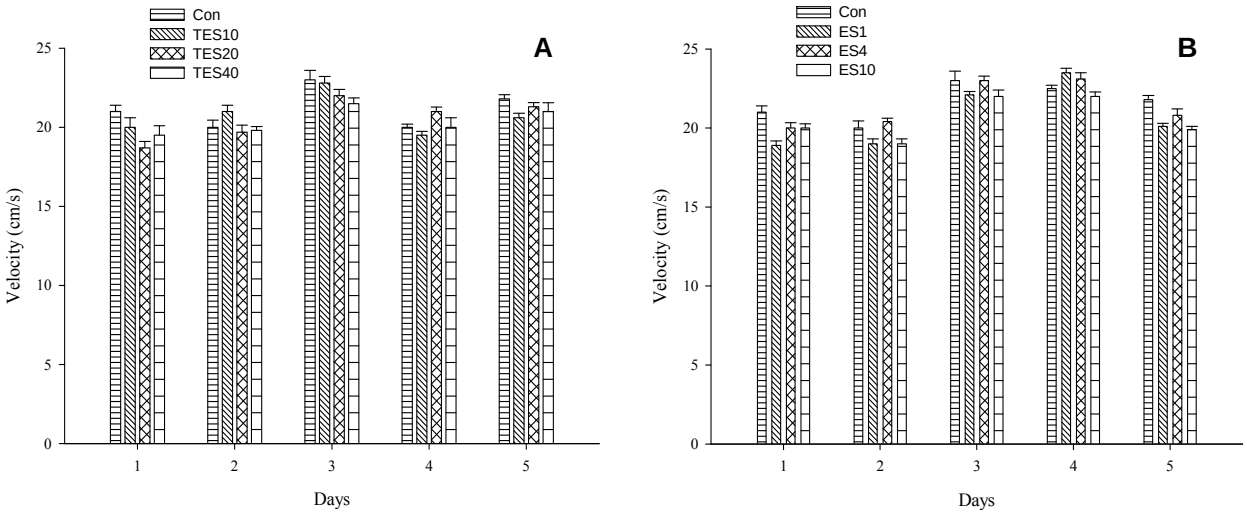

Fig. 7. Mean daily velocity of rats in morris water maze. Male castrated rats were injected weekly with sesame oil (control), testosterone enanthate 10, 20, 40 mg/kg (TES10, TES20, TES40, respectively) and estradiol valerate 1, 4, 10 mg/kg (ES1, ES4, ES10 respectively) for 6 weeks. The velocity did not change across days for both A; testosterone and B; estradiol treated rats and there was not a change for dose or dose × days interaction. Data are shown as mean + SD.

DISCUSSION

The results of the present study indicated that chronic high doses of estrogen results in deficits of learning and memory in castrated male rats and increases cholinesterase activity in the hippocampus. Chronic TES, on the other hand, diminishes cholinesterase activity in hippocampus and has positive effects on learning at the dose of 20 mg/kg.

It has been shown that in many species, including humans and rodents, males are better than females in performing spatial tasks (31-34). In our study we have concentrated on the effects of chronic (6 week) ES and TES treatment in castrated male rats which has not been previously addressed as far as we are aware.

As shown in Figs. 3 and 4, ES (10 mg/kg) caused deficit in both acquisition (learning),

as shown through test trials, and retention (memory), as shown in probe trial. Although lower doses of ES did not result in significant changes in test trials, we observed a trend for the animals to have more distance to the platform with higher levels of ES in acquisition trials on days 3 to 5. The results also showed (Fig. 5) that ES increased the hippocampal AChE activity in a dose dependent manner. It is concluded that ES at 10 mg/kg induced learning deficits possibly through AChE enzyme stimulation, and hence hippocampal ACh depletion. Regarding the role of ES in spatial tasks, researchers have found impairment, enhancement or no effects for this hormone. These discrepancies are attributed to the differences of experimental settings such as the age of animals, dose and route of drug administration, duration of the treatment, and type of the experiment used (49). There are many reports that stipulate an enhancing effect for long term ES on memory (18, 50-52) but none have used either the same task as we used or the male rats. There are basic differences in cellular and cognitive processes underlying different memory tests which may justify our opposing results (51).

ES acts through multiple mechanisms to affect the memory. Its actions are mediated through either genomic or non-genomic signaling pathways (53). Studies show that ES can modulate many aspects of acetylcholine (Ach) neurochemistry. ES can increase neurons that synthesize choline acetyltransferase in the basal forebrain (BF) of female rats. BF is a brain area that sends cholinergic projections to the hippocampus and plays crucial role in mnemonic abilities (54). Estrogen enhances N-Methyl-D-aspartic acid or N-Methyl-D-aspartate receptor binding in CA1 region of the hippocampus in an Ach dependent manner (55). It also potentiates hippocampal ACh levels when this region is activated through learning processes (56).

Previous studies have shown that there is a sexual dimorphism in hippocampal AChE activity with females having greater activity than males (57). This activity is believed to be regulated locally in the hippocampus (58). According to our results, we propose that ES itself may play a role in this regulatory process. High ES concentrations for a long enough time may lead to an increase in AChE activity in the hippocampus and eventually decrease the memory components in MVM test. Lowry and colleagues has reported an enhancement of memory for long term ES treatment in ovariectomized rats in the same MVM test (59). So this discrepancy may point to the possibility of the existence of a sexual dimorphism in estrogen effects on AChE activity. This can be more elucidated by performing same experiments on female rats as we have conducted on male rats in the present study.

Based on AChE activity results it can be expected that TES at 40 mg/kg caused better performance on tests of learning and memory. The highest dose of TES induced the lowest AChE activity (Fig. 5) and unexpectedly impaired the memory. TES enhanced memory at 20 mg/kg as evidenced by a reduced distance to the platform on probe test (Fig. 2). This suggest that AChE activity may not be an essential determining factor in TES effects on memory. The pattern of TES effect on memory (Fig. 2) seems to fit into a hyperbolic shape. This finding can support human studies in which a curvilinear relation between spatial memory and TES concentrations (60-62) has been observed. McConnell and coworkers compared the effects of serum TES levels on working memory components in different human studies and concluded that there might be a ceiling effect for androgens such that physiologic concentration increases and supraphysiologic levels have no effect on working memory (63). In a series of animal experiments, Spritzer and colleagues has also found an inverted U shape trend for TES in a working memory version of the MWM test in castrated male rats (42). There is not much evidence for the mechanism of TES effects on memory as available on ES. Dihydrotestosterone, a major metabolite of TES, can increase synaptic density and neurogenesis in the hippocampus of male rats (64). It is shown that TES, like ES, can increase choline acetyltransferase synthesizing neurons in BF of gonadectomized male rats who have gone under TES treatment for 28 days (65). Interpretation of TES effects on

memory becomes more complicated when it comes to the fact that TES can be converted to ES by the enzyme aromatase which presents in the rat hippocampal neurons (66,67). It can also be converted to estrogenic compounds such as 5-α androstane 3α, 17 β diol and 5-α androstane 3 β, 17 β diol and act through estrogen receptors (68). Most results with TES indicate that it can enhance spatial working memory in rats but evidences on the TES effects on spatial reference memory are scarce. Spritzer and coworkers showed that administrations of TES on the days of testing in MWM task has no effects on reference memory and TES can enhance memory only if it is administered for a long period of time (7 days) before initiation of testing (42). Other experiments have shown that intrahippocampal TES can decrease reference memory when it is administered on testing days and before each experiment (13). These studies show that the duration of treatment and site of administration are important factors of TES effects on memory.

Although the results of the repeated measures ANOVA indicated no significant effect for the parameter of dose in TES treated rats, it seems that there was a trend for the group received TES 20 mg/kg to perform better than other groups (Fig. 1). It may well be that certain (close to 20 mg/kg) doses of TES do facilitate this type of learning, but the conditions under which our experiments have been conducted has masked the effect.

Our results also shows that in TES- and ES-treated animals the respective blood concentrations of TES or ES are increased in a dose dependent manner (Table 1). So it seems reasonable to attribute behavioral or AChE activity to the hormonal alterations.

The results of the MWM test may be influenced by some interfering factors such as changes in locomotor activity, thigmotaxis, and instinctive tendency of the animals to swim near the wall of the pool (69). As seen in Fig. 7, there is not a statistical difference in locomotor activity, evident from speed of animals, among different doses of ES or TES. For the thigmotaxis behavior the results showed (Fig. 6) that the percentage of time spent in the outer edges of the pool did not change statistically in different treatments and doses. So our analysis of the animals' behavior in MWM test has not been influenced by changes in locomotor acticity or thigmotaxis tendency of the rats. Performance of rats on the visible platform trial on the seventh day was not significantly different ($P<0.05$) among different groups (results not shown). This indicated that the results were not biased because of gross sensorimotor abnormalities in the animals.

CONCLUSION

In summary our results showed that chronic high sc dose of ES decreased the performance of male castrated rats in a reference memory version of MWM test. ES also increased the hippocampal AChE activity in a dose-dependent manner and this may explain its subsiding effects on memory. Chronic high dose of TES (40 mg/kg) decreased hippocampal AChE activity. The effect of TES on memory occurred in an inverted U shape manner which was improved at 20 mg/kg. Similar studies on female ovariectomised rats can reveal if sex hormones can affect reference memory in a sex dependent way.

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