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A Behavioral and Molecular Study of Androgen Effects on Memory Impairments in Mature Male Rats Afflicted by Alzheimer's Disease

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Abstract

The results of a number of studies show that sexual hormones particularly testosterone have protective effects on brain neurons in neurodegenerative diseases such as Alzheimer. The present study shows the important effects of testosterone and androgen receptors on memory impairment caused by intracerebroventricular (ICV) injection of streptozotocin.

The study was carried out on male Wistar rats. An experimental model of Alzheimer's disease was induced by bilateral intraventricular injection of streptozotocin and was assessed two weeks after the first injection by using a passive avoidance test. In the Castration group, surgery was performed to remove the testes and a behavioral study was conducted after 4 weeks. Following the experiments, microscopic slides of different brain regions were prepared to investigate the beta amyloied plaques.

The results showed that ICV injection of STZ could induce marked memory impairment ($p<0.001$). Also, the castrated rats' memory decreased significantly compared to the intact and sham animals. The testosterone replacement therapy restored the castrated rats' learning and memory up to those of the intact animals. On the other hand, drugs, such as flutamide, letrozole and tamoxifen in control rats caused significant changes in learning and memory disorders.

The results showed that androgen could reduce memory impairment and decrease the beta amyloied plaques in experimental rats.

Keywords: Testosterone • Flutamide • Letrozole • Tamoxifen • Alzheimer disease

Introduction

Alzheimer's disease (AD) is a type of neurodegenerative disease afflicting the elderly. Its common symptoms are memory impairment [1], language disorder [2], spatial vision disorders [3], and sensory-motor disturbances [3]. Androgens, in most of the bodily tissues, especially in the brain, perform specialized functions. AD is the result of the deposition of Aβ protein as well as the dystrophy of nervous cells in the terminal areas of neocortical and other cerebral areas [4]. Androgen receptors' action on neurons and glial cells take place at the areas sensitive to androgen in the brain [5].

A number of studies have shown that testosterone can fulfill the functions of the production and regulation of Aβ protein level in both invitro [6,7] and invivo conditions [4]. AD is also characterized by hyperphosphorization of intra-neural tau protein, which causes the development of neural tangles. Testosterone is capable of preventing the hyperphosphorization of tau protein [8]. There have been conflicting results from conducting studies on the issue. Some studies indicate that testosterone replacement can not bring castrated rats' spatial memory back to normal in the 'arm maze' test [9,10]. A study on age-matched controls and AD demonstrates that the reduction of neurosteroids in most areas of the body, especially in cerebral neurons, is the cause of the disease [11].

There are two types of internal androgens in the body: testosterone and its metabolite, dihydrotestosterone [12], each of which performs a variety of functions in the central nervous system [13]. Aromatase is an enzyme that converts androgen into estrogen and regulates the cerebral estrogen level in the brain. The activity of aromatase was first detected in the limbic system of the brain of the human fetus as well as in the rats' hippocampus [8]. A number of subsequent studies show the activity and distribution of the

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aromatase enzyme of the central system in some species of vertebrates. Thus, androgen's actions in the brain's central system are mediated by both indirect mechanism (i.e. aromatization of testosterone and its conversion into estrogen) and direct mechanism (i.e. through androgen receptors) [11].

Interestingly enough, there is a relationship between steroidal hormones and neurodegenerative diseases so that replacement with estrogen causes the decline of AD's progression [14]. The protective effects of estrogen are brought about by the reduction of Aβ protein production [5,15].

Anti-androgens are of two general types: steroidal and non-steroidal [16,17]. The present study uses a non-steroidal anti-androgen (i.e. Flutamide). Recent studies show that androgen receptors play a role of protecting the neurons which operate through testosterone and dihydrotestosterone receptors [18,19]. On the other hand, the results of the studies suggest that the androgen's versatile function of neural protection is dependent on direct and indirect mechanisms. In this study, the effects of neuron protection are investigated by using an antagonist of androgen receptors (i.e. Flutamide), an antagonist of estrogen receptors (i.e. Tamoxifen) and an aromatase inhibitor (i.e. Letrozole).

Materials and Methods

Chemicals

All chemicals were obtained from Sigma Chemical Co. (USA) except for testosterone enanthate, which was purchased from Daroo Pakhsh (Tehran, Iran). Solutions were prepared freshly on the days of experimentation. Tamoxifen and STZ were dissolved in sterile dimethyl sulphoxide (DMSO) 5%, and artificial cerebrospinal fluid (ACSF: 120 mM NaCl; 3 mM KCl; 1.15 mM CaCl2; 0.8 mM MgCl2; 27 mM NaHCO3; and 0.33 mM NaH2PO4 adjusted to pH 7.2) respectively. Also, testosterone, flutamide and letrozole were dissolved in sterile Castor oil respectively.

Animals

The study was conducted with male Wistar rats weighing 220-250 g. The animals were housed in standard polypropylene cages, four per cage, under 12:12-h L/D program and at an ambient temperature of 25.2 °C, with free access to food and water. The experiments were carried out under the ethical guidelines of Tabriz University of Medical Sciences for the care and use of laboratory animals.

Castration

Animals were anesthetized by intraperitoneal (ip) injection of ketamine (60 mg/kg) and xylazine (6 mg/ kg). The ventral scrotum was shaved and scrubbed with betadine (Behvazan Co, Rasht, Iran). Then, 1.5 cm transverse incision was made at midline scrotum; the testes were exteriorized through the incision; the tubules were tied with 0.4 silk sutures; and finally testes and testicular fat were removed. The sham surgery consisted of exposing the gonads without removing them. The behavioral studies were done 4 weeks after castration.

Intracerebroventricular (ICV) injection of STZ

The animals were anesthetized with a combination of ketamine (60 mg/kg, ip) and xylazine (6 mg/kg, ip). Having been anesthetized, they were mounted in a stereotaxic frame in the flat skull position. The scalp was shaved and swabbed with iodine, and a small central incision was made to expose the skull. Then two bilateral burr hole were drilled through the skull with the coordinates according to the stereotaxic atlas (19): anteroposterior from bregma (AP) = -0.8 mm, mediolateral from the midline (ML) = ±1.6 mm and dorsoventral from the skull (DV) = 3.4 mm. STZ (750 µg/10 µl ACSF/Rat, at days 1 and 3) was infused bilaterally into the cerebral ventricles by using a Hamilton syringe and an infusion pump at a flow rate of 0.2 µl/min [19]. Behavioral investigations were carried out 2 weeks after the first injection of STZ.

Passive avoidance test

The apparatus (Azma Co., Tabriz) consisted of an illuminated chamber connected to dark chamber by a guillotine door. Electric shocks were delivered to the grid floor by a stimulator. On the first and second days of testing, each rat was placed on the apparatus and left for 5 min to become habituated to the apparatus. On the third day, an acquisition trial was carried out. The rats were individually placed in the illuminated chamber. After a habituation period (2 min), the guillotine door was opened and after the rat entered the dark chamber, the door was closed and an inescapable scrambled electric shock (1 mA, 50HZ, 3s once) was delivered.

In this trial, the initial latency (IL) of entrance into the dark chamber was recorded and the rats with IL greater than 60 s were excluded form the study. Twenty-four hours later, each rat was placed in the illuminated chamber for retention trial. The interval between the placement in the illuminated chamber and the entry into the dark chamber was measured as step-through latency (STL1, Cut of time 900s) for short memory. This test was conducted after 3 weeks post-surgery and each rat was tested only once for measured STL2 for long memory.

Molecular analysis

At the end of the experiments, the animals' brains were isolated and placed 48 hours in formalin. Then, they were transferred to the autoprosesor device and embedded in liquid paraffin. After making a cut with a microtome, all the slides were stained by especial methods (e.g. Congo red) for indicating plaques. Then they were laid in Nacl saturated alkaline solution for 20 minutes, and then were laid within Congo red alkaline solution for 30 minutes. After drying and fixation with Antaln, beta amyloied plaques were observed in the hippocampus and prefrontal cortex of the brain.

Statistical analysis

Instate software was used to prepare the descriptive statistics and to compare the differences between the means of the data sets. All the results were expressed as the mean ± SEM and were analyzed through running a one-way ANOVA. Statistical significance was accepted at the level of $P<0.05$. There was a statistical significance at $p<0.05$, and in order to spot where the differences lie, post-hoc Tukey was employed.

Results

Effects of STZ, castration and testosterone replacement therapy on memory impairments

The Step through latency (STL) of ten groups was assessed in intact, STZ (750 µg/10 µl ACSF/Rat, on days 1 and 3), Castrated, Intact+testosterone, STZ+testosterone, Castrated+testosterone, Sham+testosterone, Vehicle of STZ and Vehicle of testosterone. As shown in Figure 1, the STL1 and STL2 related to castration and STZ treated rats decreased significantly (P< 0.001) in comparison with the intact animals.

The figure shows that STL1 and STL2 of the testosterone-treated intact group, Sham group, vehicle of STZ and vehicle of testosterone groups of testosterone-treated rats were the same as those of the normal rats. In the STZ-treated and castrated rats which were treated with testosterone, the STL1 and STL2 were significantly (P< 0.001) higher than STZ-treated and castrated animals (Figure 1).

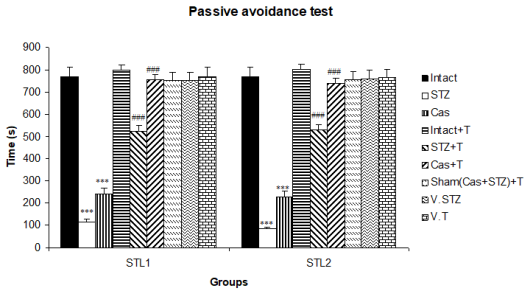

Figure 1. The passive avoidance test STL1 (Short memory) and STL2 (long memory) results of intact, STZ (750 µg/Rat/10µl ACSF at days 1 and 3), castration, sham operated of cast+STZ and vehicle of STZ-lesion rats. ***p<0.001 when compared with intact and sham operated. Also STL1 (Short memory) and STL2 (long memory) results of Intact+T (1 mg/kg, sc, for 6 days of first STZ injection), STZ +T and Cas+T p<0.001 when compared with some groups without testosterone treated. N=8 rats for each group; each bar represent the mean ±SEM of STL time (s). (STZ= Stroptozotocin, Cas=Castration, V=Vehicle, T=Testosterone).

Effect of letrozole, flutamide and tamoxifen and testosterone replacement therapy on memory impairment

As shown in Figure 2A, the STL1 and STL2 in Intact+Flu, Intact +Let, Intact+Tmx groups decreased significantly (P< 0.001) in comparison with the groups which had not been treated with Let, Flu, and Tmx. As Figure 2B demonstrates, the STL1 and STL2 in Intact +flu+T, Intact+Let+T, Intact+Tmx+T groups did not change significantly in comparison with the groups without testosterone treatment. However, the STL1 and STL2 in Castration+Flu+T, castration+Let+T, castration+Tmx+T groups increased significantly (P<0.001) compared to the groups without testosterone treatment (Figure 2).

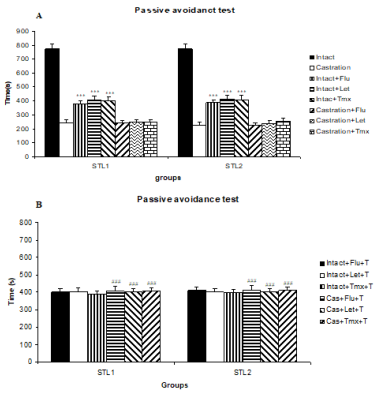

Figure 2. The passive avoidance test STL1 (Short memory) and STL2 (long memory) results of flutamide (10 mg/kg, ip, for 6 days), letrozole (4 mg/kg, ip, for 6 days) and tamoxife (1 mg/kg, ip, for 6 days) with Intact and castration groups. ***p<0.001 when compared with some groups without Flu, Let and Tmx tereated groups (A), also STLs results related of Intact+Flu+T, Intact+Let+T, Intact+Tmx+T, Cas +Flu+T, Cas+Let+T and Cas+Tmx+T groups. p<0.001 when compared with some groups without Testosterone treated groups. N= 8 rats for each group; Each bar represent the mean ± SEM of STL time (s). (STZ=Stroptozotocin, Cas=Castration, V=Vehicle, T=Testosterone, Let=Letrozole, Tmx=Tamoxifen, Flu=Flutamide).

Effects of STZ, castration and testosterone replacement therapy on beta amyloid plaque formation in hippocampus

The effects of STZ, castration and testosterone replacement therapy on beta amyloid plaque formation in hippocampus that induced Alzheimer's disease were also investigated. The results were indicative of significant increase of the beta amyloid plaque formation in hippocampus in the STZ groups. On the other hand, an increase of beta amyloid plaque formation in hippocampus was seen in the castrated and testosterone-depleted groups. The Figure shows that there were no beta-amyloid plaques in the hippocampus of the control group. Similarly, no beta-amyloid plaques were observed in the hippocampus of the castrated groups receiving testosterone. However, the formation of beta-amyloid plaques in the hippocampus of the STZ group receiving testosterone decreased significantly (Figure 3).

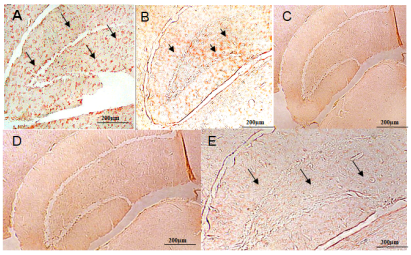

Figure 3. Beta amyloid plaque in hippocampus with Congo red staining, after 4 week, scale size is 200 µm. (A) Streptozotocin group; (B) Castration and testosterone-depleted group; (C) Intact group; (D) Castration group with receiving testosterone (1 mg/kg, sc, for 6 days); (E) Streptozotocin group with receiving testosterone (1 mg/kg, sc, for 6 days).

Effect of letrozole, flutamid, and tamoxifen and testosterone replacement on beta amyloid plaque formation in hippocampus

The effects of letrozole, flutamide and tamoxifen and testosterone replacement therapy on beta amyloid plaque formation in hippocampus that induced Alzheimer disease were also investigated. It was found that the beta amyloid plaque formation in hippocampus increased significantly in the letrozole, flutamide and tamoxifen-treated groups. Also, no significant changes were found in the hippocampus of the groups treated with testosterone replacement (Figure 4).

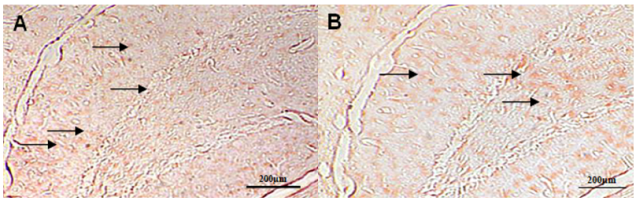

Figure 4. Beta amyloid plaque in hippocampus with Congo red staining, after 4 week, scale size is 200 µm. (A) Letrozole, flutamide and tamoxifen-therated groups; (B) Letrozole, flutamide and tamoxifen group treated with testosterone replacement.

Discussion

AD is a neurodegenerative disease which is caused by the destruction of the neurons of different areas of cerebral cortex, in particular the cholinergic neurons of hippocampus and frontal cortex. Mayer used stroptozotocin for the first time in 1990 to develop a test model of AD in rodents [20]. The results showed that acute administration of stroptozotocin with a dose of 750µg/Rat/ 10 µl ACSF on the first and third days could induce a model of AD [21].

The results also revealed a significant reduction of STL1, 2 in the group receiving stroptozotocin compared to the control group ($p< 0.001$). Thus, stroptozotocin could contribute to memory and learning disorders in wistar male rats. For this reason, the present study used stroptozotocin with a given dose to create memory and learning disorders. Using stroptozotocin with the mentioned dose could promote resistance to the entry of glucose into the animal's brain without showing any diabetic effects [21].

Research studies show that the level of Aβ protein in blood serum increases in castrated rats [14]. Some recent experiments suggest that low level of testosterone increases Aβ protein 40 in elderly men, causing dementia or memory impairment [22]. On the other hand, according to the findings of this study, the formation of Aβ plaques in different parts of the brain like the prefrontal cortex and hippocampus increases in the castrated groups or those treated with strotozotocin, causing the development of AD. The findings are also indicative of an increase in the formation of Aβ plaques in the groups of animals treated with flutamide, letrozole, and tamoxifen.

In previous studies, the effects of testosterone on the stroptozotocin-receiving groups in relation to androgen or estrogen receptors have not been thoroughly addressed [23]. Therefore, in the current study, the antagonist of estrogen receptors (i.e. Flutamide with the dose of 10 mg/kg. ip, on days 1-6), antagonist of estrogen receptors (i.e. Tamoxifen with the dose of 1mg/kg, ip, on days 1-6), and aromatase inhibitor (i.e. Letrozole with the dose of 4 mg/kg, ip, days 1-6) were employed. The results revealed obvious memory disorders in the four-week castrated rats on the passive avoidance test. These results are in line with those of the previous research conducted on the effects of testosterone on rats' spatial memory [24].

The results of the present study showed that STL1 (short memory) and STL2 (long memory) decreased significantly in the control group and stroptozotocin-receiving group treated with flutamide, tamoxifen, and letrozole, implying the impairment of memory and subsequent development of AD in these groups of animals.

Other studies show that in a number of mammal species, androgens are turned into estrogen through the function of aromatase enzyme, which is among important central functions of steroids in males [25]. Also, research suggests that using estradiol improves the spatial memory in rats [3]. Similarly, intra-hippocampus injection of estradiol improves rats' performance on Moiré's water maze test [26]. Thus, the metabolic role of androgens in improving memory should not be underestimated.

This study showed that testosterone did not produce any significant effect on the time of STL1 and STL2 in the control group; consequently, it could be concluded that testosterone, beyond the physiological limit of the body, does not have any effect on improving the memory and learning of the non-castrated or control groups. Testosterone can improve the memory and learning of the stroptozotocin-receiving rats, but not as much as the control group. Another finding of this study was that olive oil solvents, carboxy methyl cellulous (CMC) and di methyl solphoxide (DMSO), did not show any significant changes in STL1 and STL2 of both control and castrated groups. Therefore, all the significant changes are associated with the replacement of letrozole, flutamide, and tamoxifen in rats.

The antagonist of androgen receptors like flutamide affects the Androgen receptors and blocks them. Applying both types of drugs (i.e. Tamoxifen and Letrozole) to the rats in the control group was the main reason for the significant changes in STLs ($p < 0.001$), which induced memory and learning disorders. The drugs influences testosterone's effect-producing pathways in CNS, causing learning and memory disorders in rats. Also, testosterone, through direct and indirect mechanisms, could help decrease memory impairment in rats. According to the obtained results, castration impairs memory in the rats treated with STZ, while testosterone replacement could serve as a major agent of reducing learning and memory disorders. It seems that androgen and estrogen receptors have synergistic effects; thus, the use of testosterone, as a supplementary drug, during medication with the routine anti-AD drugs could prove highly useful in prevention and treatment of the disease.

Conclusion

This study demonstrates that androgen could reduce memory impairment and decrease the beta amyloied plaques in experimental rats.

Acknowledgments

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