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Effects of testosterone on orchiectomy-induced oxidative damage in the rat hippocampus

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ABSTRACT

The aim of this study was to investigate the morphological changes of the hippocampus after orchiectomy and the protective effects of testosterone on these changes.

Animals were divided into 3 groups. The rats in group I were used for sham-orchiectomy. Orchiectomy was performed on the rats in group II. The rats in group III were administrated testosterone propionate 0.5 mg/kg/day for 30 days after the orchiectomy. Some of the hippocampal tissues were used for determination of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) enzyme activities, and malondialdehyde (MDA) levels. The remaining hippocampal tissue specimens were stained with routine histological methods and examined under the light microscope. Additionally, the samples were immunohistochemically stained by using avidin–biotin-peroxidase for determination of bax immunoreactivity.

The SOD and GSH-Px enzyme activities of the hippocampus were decreased, and MDA levels were increased in group II rats compared to the sham-orchiectomy group. In the light microscopic evaluation of the tissue specimens from group II, significant increases were detected in the number of picnotic cells and in bax immunoreactivity compared to the sham-orchiectomy group. However, an increase was observed in activities of SOD and GSH-Px enzymes and a decrease of the MDA levels in animals with orchiectomy, but having externally administered testosterone. It was determined that the increase of bax immunoreactivity and histopathological changes in this group were regressed by testosterone.

The results of our study revealed that orchiectomy-induced oxidative damage and morphological changes in the hippocampal tissue were suppressed by testosterone.

1. Introduction

The hippocampus is divided into four areas, known as CA1, CA2, CA3 and CA4, due to their cellular variances. CA1 is the nearest area to the cubiculum while CA3 is the nearest area to the gyrus dentatus. Short-term memory, which is recognized as the location for the storage of new information, is closely related to the hippocampus (Songur et al., 2001; Andersen et al., 2007; McHugh et al., 2008). Furthermore, the right hippocampus shows a higher degree of activity in functions related to visual memory, whereas the left hippocampus shows a higher degree of activity in functions related to verbal memory. Any lesions that may occur at this region of the brain lead to loss of memory in varying degrees (Kandel et al., 2000; Andersen et al., 2007).

Oxidative stress consists of a continuous level of oxidative damage in a cell, which is caused by free radicals. A shift in the critical balance between the production of free radicals and the cellular antioxidant defense system in favor of free radicals might lead to the development of oxidative stress. The cellular antioxidant defense system consists of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and other related enzymes. Increased activity of these enzymes prevents the harmful effects of oxidative stress (Karla et al., 1991; Elsayed and Bendich, 2001; Ozyurt et al., 2006). The level of malondialdehyde (MDA), a product that forms as a result of lipid peroxidation, is another indicator of oxidative damage (Kamal et al., 1989).

Testosterone is an androgenic hormone with a chemical formula equal to 17β-hydroxy-4-androstene-3-on, and recognized

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as a 19 carbon (19C) steroid (Moore and Persaud, 1998; Flück et al., 2003). It also regulates reproduction, and sexual and aggressive behaviors (Wilson, 2001; Bancroft, 2005). Additionally, testosterone is capable of providing analgesic and anxiolytic activities, leading to variations in personal mood and awareness (Janowsky, 2006; Barreto et al., 2007). Besides having neuroprotective properties, testosterone plays a major role in the functioning of the central nervous system. It has been reported that testosterone was able to prevent neuronal death in experimental models that involved injuries of the nerve system. Any disorders that may interrupt the secretion of this hormone, which provides the development and growth of neurons, may trigger the formation of apoptosis in neural tissues (Hammond et al., 2001; Estrada et al., 2006; Barreto et al., 2007). Testosterone is capable of reducing the death of neurons in the hippocampus that is occurring because of β-amyloidal toxicity and oxidative stress (Rhodes and Frye, 2004). Additionally, testosterone has been found to demonstrate a preventive activity against oxidative stress that was induced by ethanol (Celec et al., 2003). Experimental studies have demonstrated damage in the brain tissue as a result of oxidative stress, and an immediate decrease in antioxidant enzyme levels has also been detected. Testosterone administration also prevented tissue damage and improved antioxidant enzyme levels (Schmidt et al., 2002; Morimoto et al., 2005; Schmidt et al., 2005). Furthermore, there are many studies showing the effects of testosterone on learning and memory associated hippocampal region (Leranth et al., 2003; Janowsky, 2006; Sandstrom et al., 2006). However, there are limited studies showing low testosterone levels are linked orchiectomy caused by oxidative stress in the rat brain. Therefore, we examined oxidative damage that may occur in the hippocampal tissue after an orchiectomy and planned effects of testosterone on the apoptosis of hippocampal cells.

2. Materials and methods

2.1. Animals and treatments

Adult male Wistar rats (230–250 g) were obtained from Experimental Research Centre of Firat University (Elazig, Turkey). The animal studies were carried out according to the guidelines of European Community Council for experimental animal care. Animals were housed individually on a 12-h light:12-h dark cycle (lights on at 08.00 h), at a temperature of 21 ± 1 °C and 50% humidity. Food (standard pellet diet) and tap water were supplied ad libitum. The animals were divided into 3 equal groups. The rats in group I ($n = 7$) were used as the sham-orchiectomy group (control). Surgical orchiectomy was applied to rats in group II ($n = 7$). Group I (control) and group II (orchiectomy) were given vehicle (0.1 ml sesame oil, subcutaneously) daily for 30 days. A dose of 0.5 mg/kg/day of testosterone propionate (in 0.1 ml of sesame oil, administered subcutaneously), was given to rats in group III ($n = 7$), one week after orchiectomy.

The surgical procedures were performed under general anaesthesia using a combination of ketamine (60 mg/kg) and xylazine (5 mg/kg) intraperitoneally. Orchiectomy was performed through a midline scrotal raphe incision. The testicles were then exposed through a scrotal incision. The ductus deferens was isolated and ligated. Then the testicles were removed bilaterally. The incision was closed and sutured. The sham operation involved the exposure of the testicle without isolation. The incision was closed and sutured (Ward and Abdel-Rahman, 2006).

At the end of the 30-day experimental period, all rats were decapitated. The brains of the rats were removed and the hippocampus tissues obtained from all the brain specimens. The right hippocampal specimens were washed twice with cold saline solution, placed into glass bottles, labelled, and stored frozen (−30 °C) for eventual determination of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) production. The left hippocampal specimens were used for immunohistochemical evaluation.

2.2. Biochemical analyses

For biochemical analysis, the tissues were weighed and homogenized in 4 volumes of ice-cold Tris–HCl buffer (50 mM, pH 7.4) containing 0.50 ml/l Triton X-100 with a homogenizer (IKA Ultra-Turrax T 25 Basic) for 2 min at 13,000 rpm. All procedures were performed at +4 °C. Tissue homogenates were centrifuged at 5000 × g for 60 min to remove debris, and the clear supernatant fluids were separated and kept at −40 °C until the enzyme activity measurements were performed (about a week later).

2.2.1. Determination of superoxide dismutase activity

Total (Cu–Zn and Mn) SOD (EC 1.15.1.1) activity was determined based on the method of Sun et al. (1988). The principle of the method is based on the inhibition of nitro blue tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the supernatant after 1 ml of an ethanol–chloroform mixture (5:3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. The SOD activity was expressed as U/g protein.

2.2.2. Determination of glutathione peroxidase activity

Glutathione peroxidase (GSH-Px, EC 1.6.4.2) activity was measured by the method of Paglia and Valentine (1967). The enzyme reaction in the tube containing NADPH, reduced glutathione (GSH), sodium azide, and glutathione reductase was initiated by the addition of H_2O_2, and the change in absorbance at 340 nm was monitored by a spectrophotometer. Activity was expressed as U/g protein.

2.2.3. Determination of malondialdehyde level

The tissue malondialdehyde (MDA) level was determined using a method from Esterbauer and Cheeseman (1990), based on its reaction with thiobarbituric acid (TBA) at 90–100 °C. In the TBA test reaction, MDA and TBA react to produce a pink pigment with an absorption maximum at 532 nm. The reaction was performed at pH 2–3 and at 90 °C for 15 min. The sample was mixed with 2 volumes of cold 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was centrifuged and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling-water-bath for 10 min. After cooling, the absorbance was read at 532 nm. Results were expressed as nmol/g of wet tissue, by reference to a standard curve prepared from measurements made with a standard solution (1,1,3,3-tetramethoxypropane).

2.3. Histological examination

The hippocampus tissue specimens were fixed in a neutral formalin solution (10%). After dehydration procedures, the tissue specimens were embedded in paraffin wax and sectioned (thickness, 5 μm). Levels of sections and CAl, CA2 and CA3 areas in the hippocampus were found with the help of the stereotaxic atlas (Paxinos and Watson, 2007), and were stained with Hematoxylin-Eosine and Bax. Measurements were obtained from 4 images per rat for the left hemispheres (coordinates were adjusted to 2.64–3.00 mm posterior from the bregma, according to the Paxinos and Watson (2007)).

In stained with Hematoxylin-Eosine areas, the numbers of picnotic neurons in the pyramidal cell layer were counted using light microscopy (Olympus BX 50) under a 40-fold magnification objective with the help of the eyepieces graticules. Four sections were taken from each animal to obtain an average number of picnotic cells in a fixed field.

2.4. Immunohistochemical procedure

For the immunohistochemical staining of Bax (a marker protein of apoptosis), paraffin sections were cleaned in xylene, hydrated, and then placed in phosphate-buffered saline (PBS; pH 7.6). Antigen retrieval was performed by boiling for 15 min in citrate buffer (0.01 M). Sections were treated with 3% hydrogen peroxide for 5 min to quench endogenous peroxidase activity, rinsed with deionized water, and then washed with PBS. Sections were incubated, first with 1% pre-immune rabbit serum to decrease non-specific staining, and then with a monoclonal antibody against Bax protein (1:200 dilution, Dako, Carpinteria CA, USA) at 23 °C in a moist chamber for 1 h. Detection of the antibody was performed using a biotin–streptavidin detection system (Bio-Genex, San Ramon CA, USA) with 3-amino 9-ethyl carbazole (AEC) as chromogen (Dako, Carpinteria CA, USA). Sections were counterstained with Mayer's hematoxylin, dehydrated, and then cover-slipped with Permount.

Immunoreactivity for the Bax protein was scored semi-quantitatively. Stained sections were evaluated using a light microscope, with the results expressed as a score based on the percentage of the total field stained positively with monoclonal antibody against Bax protein. Scores were based on the following scale: heavy (5+): over 80% of the field showing positive staining; strong (4+): 60–79%; moderate (3+): 40–59%; low (2+): 20–39%; minimal (1+): 1–19%; and no staining (0) (Zararsiz et al., 2007). The brightness setting of the light microscope was optimized to show more clearly all the apoptotic cells in the groups. The background staining was not considered for assessment.

2.5. Statistical analysis

All statistical analyses were carried out using SPSS, version 15.0 (SPSS, Chicago, IL, USA). All biochemical and immunohistochemical data are expressed as means ± SD. Distributions of the groups were analyzed with a one-sample Kolmogorov–Smirnov test. Because the number of rats in each group was seven and the Kolmogorov–Smirnov test indicated non-normal distribution, the data were considered to be non-parametric. Differences in parameters among the 3 groups were

Table 1
SOD, GSH-Px and MDA values in the hippocampus of all groups ($n = 7$ for per group).

Groups	SOD (U/g protein)	GSH-Px (U/g protein)	MDA (nmol/g protein)
Control	46.50 ± 8.06	39.62 ± 1.29	14.45 ± 1.72
Orchiectomy	37.79 ± 5.40*	30.30 ± 0.83*	21.52 ± 0.69*
Orchiectomy + testosterone	65.21 ± 4.71	41.67 ± 2.04	18.62 ± 2.27

Values are expressed as mean ± SD.
* $p < 0.05$ significant differences compared to other groups.

Table 2
The numbers of picnotic cells per unit area in pyramidal cell layer of CA1, CA2 and CA3 regions of the hippocampus of all groups.

Groups	CA1	CA2	CA3
Control	0.66 ± 0.02	2.00 ± 0.11	3.50 ± 0.9
Orchidectomy	2.81 ± 0.12*	6.65 ± 1.46*	8.05 ± 1.55*
Orchidectomy + testosterone	0.85 ± 0.06	3.71 ± 0.17	4.57 ± 1.25

Values are expressed as mean ± SD.
* $p < 0.05$ significant differences compared to other groups.

analyzed by a Kruskal–Wallis test. Dual comparisons between groups that presented significant values were evaluated with the Mann–Whitney *U* test. *P*-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Biochemical results

SOD, GSH-Px enzyme values, and MDA levels were determined in the hippocampus tissue samples of male rats. It was observed that the SOD and GSH-Px activities of the rat hippocampus included in the orchiectomy group were significantly decreased in a statistical pattern when compared with the control group ($p < 0.05$). It was determined that tissue MDA values, which are considered as an indicator of oxidative damage, increased in rats who had undergone orchiectomy when compared to rats in the control group (Table 1). Biochemical data from the group that received testosterone after orchiectomy showed an increase in SOD and GSH-Px values and a decrease in MDA levels ($p < 0.05$) (Table 1).

3.2. Light microscopic and immunohistochemical results

When the hippocampus tissue samples of rats that had undergone an orchiectomy procedure were studied, a statistically significant increase was observed in the number of picnotic cells versus that in the control group ($p < 0.05$) (Fig. 1A and B, Table 2). A decrease in the number of picnotic cells was also observed in the rats that had undergone orchiectomy, but had received testosterone (Fig. 1C, Table 2). The hippocampus sections were immunohistochemically stained with Bax, and apoptosis in the tissue was determined by assessing the intensity of the reaction. Bax-stained cells were not observed (0) in the control group (Fig. 1D). However, a high number of Bax-stained cells (+4) were determined in the hippocampus structure in rats that went under an orchiectomy process (Fig. 1E). It was also observed that the bax-staining of the hippocampus in the testosterone group after orchiectomy was minimal (1+) (Fig. 1F).

4. Discussion

In this study, we investigated both the changes that occurred in the hippocampus structure after an orchiectomy procedure and the effect of externally administered testosterone. We used biochemical methods in the present study and investigated the activity of testosterone on the hippocampus and especially on oxidative damage. Additionally, tissue samples of the hippocampus were assessed by an immunohistochemical procedure.

The antioxidant defense systems play a protective role against oxidative stress caused by pathological processes or physiological conditions. At this effective cellular level, enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) can be present (Kanter et al., 2004). The present study revealed that SOD and GSH-Px enzyme levels in tissue samples of the hippocampus statistically decreased significantly after orchiectomy when compared with the samples of the control group. The decrease demonstrated that the antioxidant defense mechanisms in the hippocampus tissue deteriorated after orchiectomy and that,

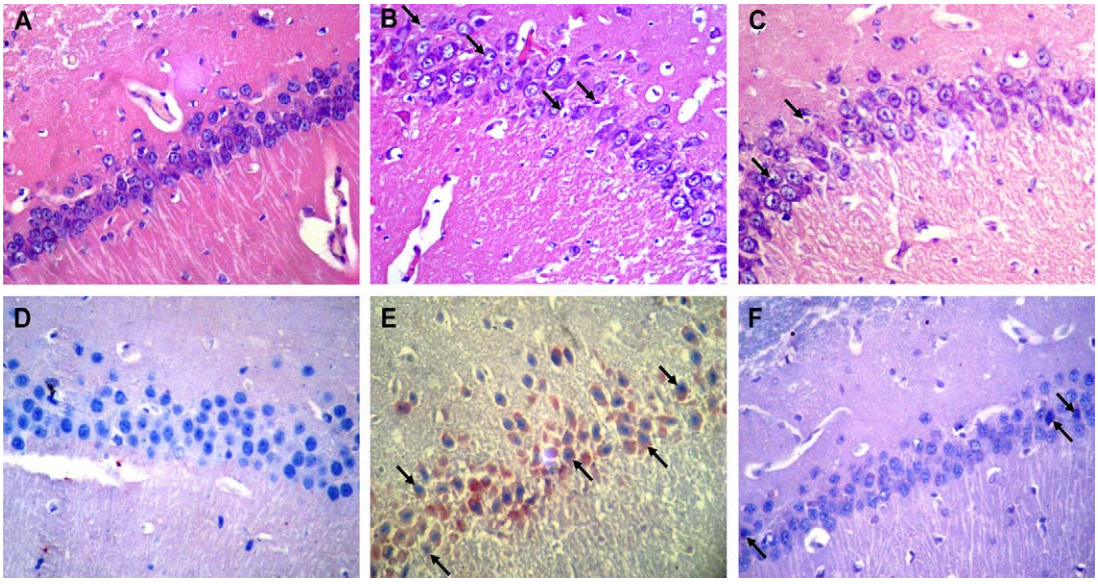

Fig. 1. Histological appearance of the hippocampal CA2 regions from control group showing picnotic cells (arrows) in normal quantity (A), in a great number in the orchiectomy group (B) and in a small number (arrows) in the testosterone administered group (C) (H&E, magnification 40×). No bax-staining in the cells from control group (D), intense bax-staining cells (arrows) from orchiectomy group (E) and minimal bax-staining cells (arrows) from testosterone administered group (F) in the CA3 regions of the hippocampus (magnification 40×).

as a result, oxidative damage had occurred. Another indicator of damage that occurred due to oxidative stress can be seen in the malondialdehyde level (MDA), a product that forms as a result of lipid peroxidation (Kamal et al., 1989). In our study, we determined that hippocampus MDA levels significantly increased in the orchiectomy group as compared to the control group. The increase observed in the MDA level demonstrates that oxidative damage occurred in the tissue as a result of lipid peroxidation after orchiectomy.

Recently, studies performed on rats have showed that gonadectomy caused a decrease in the antioxidant enzyme levels and, consequently, initiated an apoptotic process inside the cells, leading to cellular death. A study conducted by Klapcinska et al. (2008) showed that the antioxidant defense system was negatively affected after gonadectomy that a decrease occurred in the antioxidant enzyme levels (SOD, GSH-Px), and that an increase occurred in the lipid peroxidase levels. Studies performed on neural tissues have demonstrated that the antioxidant defense system collapsed right after gonadectomy, and that the synaptic intensity of neurons decreased and cell death occurred due to apoptosis (Stoltzner et al., 2001; Leranth et al., 2003; Sandstrom et al., 2006). In our study, we obtained information similar to the above-mentioned results. We noticed that apoptosis occurred in the hippocampus after orchiectomy in our immunohistochemical studies. The bax dye process occurred at a severe degree in the cellular cytoplasm of the hippocampus after orchiectomy. Likewise, our light microscopic studies were successful in determining the presence of a large number of picnotic cells in the same group, located inside the pyramidal cellular layer of the hippocampus.

Studies performed on neural tissues have demonstrated an antioxidant property of testosterone. It was reported that testosterone administered by an external route increased enzyme activities in tissue SOD and GSH-Px and led to a decrease in MDA levels (Huh et al., 1994; Guzman et al., 2005; Shao et al., 2006). Oxidative damage that occurred in the cerebellum due to ethanol exposure in rats and to an increase in tissue MDA retrograded right after testosterone administration. In addition, this hormone was the principal substance that prevented the neurofilament loss that occurred because of chronic ethanol intake in the same tissue (Celec et al., 2003). Another experimental study found, that testosterone was capable of increasing the GSH-Px activity in the hippocampus (Schmidt et al., 2002). In another study performed on rats, testosterone administration was found to prevent the apoptosis that occurred due to streptozicin in pancreas cells (Morimoto et al., 2005). The findings of the present study are similar to those obtained from previous studies. Our study indicated that testosterone increased SOD and GSH-Px enzyme activities in the hippocampus, which were reduced after orchiectomy, decreased the MDA level, and showed antioxidant activity.

Many studies have emphasized that testosterone plays a vital role in neural functioning, provides protection of the neurons at normal concentrations (nanomolar level), and simplifies growth and development (Matsumoto et al., 1994; Rubinow and Schmidt, 1996; Hammond et al., 2001). Nevertheless, in a study carried out by Estrada et al., it was reported that testosterone displayed harmful activity at high concentrations (1–10 micro molar level) and initiated the apoptosis process (Estrada et al., 2006). It was shown that testosterone at molecular levels caused an increase in nerve growth factor receptors in the cortical neurons of the rats and reduced the levels of amyloidal β-peptide, which is detected in Alzheimer Disease (Gouras et al., 2000). Therefore, it was believed that a low testosterone level may be responsible for the tendency of the development of Alzheimer disease in elderly individuals (Molsa et al., 1982; Hammond et al., 2001). Low testosterone levels have also been related to various conditions like depression, anxiety, and loss of memory. Apoptosis, also related to low testosterone levels, was prevented by testosterone replacement when testosterone was administered in a normal dose; and symptoms related to the mentioned conditions began to fade (Hammond et al., 2001). Our findings are supported by previous data and make such a contribution that the hippocampal pramidal cell survival after orchiectomy was impaired, and apoptosis occurred. This situation may be associated with low testosterone levels. Indeed, testosterone treatment prevented apoptosis in hippocampal neurons.

Recently several studies have been performed on the activity of testosterone on memory and learning abilities. These studies reported that the neuronal structure and functions of the hippocampus altered after testosterone was administered. A 40–50% decrease was detected in the synaptic intensity of neurons located at the CA1 region of the hippocampus after gonadectomy or after the amount of androgen was reduced. These levels switched back to normal when testosterone and DHT were administered. As reported, this activity occurred by the means of androgenic receptors located in the hippocampus (Leranth et al., 2003; Janowsky, 2006; Sandstrom et al., 2006). A study conducted by Barreto et al. (2007) reported that gliosis control is related to the protective activity of testosterone on neurons. It was also reported that, during brain development, testosterone affects the differentiation of the glial fibrillary acidic protein (GFAP), immunoreactive atrocytes, and astroglias, and regulates the suppression of GFAP in the adult hippocampus tissue. Furthermore, testosterone and its metabolite, DHT, stimulate neurogenesis in the hippocampus (Galea et al., 2006) and in the gyrus dentatus, and increase spinogenesis by enhancing the receptor connections of N-methyl-D-aspartate (NMDA) (Romeo et al., 2005).

At end of this study, which we carried out with biochemical, light microscope, and immunohistochemical levels, we detected oxidative tissue damage inside the hippocampus structure in orchidectomized rats, accompanied by apoptosis. Additionally, dependant on the orchiectomy procedure, we determined that changes detected in the tissue were suppressed and degraded when testosterone was administered externally.

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