

The Angiogenic Effect of Testosterone Supplementation on Brain of Aged Mice ^[1]

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Abstract

The aim of this study was to determine, by histological and molecular techniques, the effects of testosterone hormone treatment on angiogenesis in brain of aged mice. A total of 30 mice were used in 3 study groups: Sham operation group (Control), gonadectomy group (G) and gonadectomy and testosterone supplementation group (GTS). The capillary number and the inner diameter of larger capillary vessels in brain were measured by light microscope. The levels of VEGF (vascular endothelial growth factor) mRNA in brain tissues were determined by RT-PCR. After gonadectomy operation, capillary densities of brain decreased in male ($P<0.05$) but did not change in female. Testosterone supplementation to gonadectomized aged mice caused a mild increase on capillary number in female brain but did not affect in male. Gonadectomy caused a decrease in VEGF mRNA levels of brain in male and female mice. Interestingly, testosterone replacement caused an important decrease ($P<0.05$) in the expression of VEGF of brain in male compared to control group whereas resulted with no change in female. The present results showed that testosterone hormone has different angiogenic effect on brain in male and female old mice. The gonadectomy operation in old male mice has a negative effect on angiogenic events. However, testosterone replacement in male was not sufficient to convert this change and did not increase angiogenesis.

Keywords: Angiogenesis, Testosterone, Old mice, Brain

Yaşlı Farelerin Beyninde Testosteron Takviyesinin Anjiyogenik Etkisi

Özet

Bu çalışmanın amacı yaşlı farelerin beyinde testosteron hormon tedavisinin anjiyogenik etkisini moleküler ve histolojik olarak tespit etmektir. Toplam 30 fareden 3 çalışma grubu oluşturulmuştur. Bunlar; yalancı operasyon yapılan (kontrol) grup, gonadektomi yapılan (G) grup ve hem gonadektomi hem de testosteron takviyesi yapılan (GTS) gruptur. Beyin dokusunda kapillar sayısı ve geniş kapillarların iç çapı ışık mikroskopunda ölçülmüştür. Beyin dokusundaki VEGF (vasküler endotelial büyüme faktörü) mRNA seviyesi RT-PCR ile ölçülmüştür. Kısırlaştırmadan sonra erkeklerde beyindeki kapillar yoğunluk azalmış ($P<0.05$) dişilerde ise değişmemiştir. Kısırlaştırılmış hayvanlarda testosteron takviyesiyle dişilerde kapillar sayısı hafif bir artış gösterirken erkeklerde bir değişim görülmemiştir. Kısırlaştırmayla beyindeki VEGF mRNA seviyesinde hem dişi hem de erkek farelerde bir azalma saptanmıştır. Testosteron takviyesiyle erkeklerin beyindeki VEGF mRNA seviyesinde kontrol grubuyla karşılaştırıldığında önemli bir azalma ($P<0.05$) görülürken dişilerde bir değişim görülmemiştir. Bu çalışmayla testosteronun erkek ve dişilerin beyinde anjiyogenik etkisinin farklı olduğu saptanmıştır. Yaşlı erkek farelerde kısırlaştırmanın anjiyogenik olaylarda negatif etkisinin olduğu belirlenmiştir. Ayrıca erkeklerde testosteron takviyesi bu değişiklikleri düzeltmek için yeterli olmamış ve anjiyogenezi artırmadığı saptanmıştır.

Anahtar sözcükler: Anjiyogenezis, Testosteron, Yaşlı fare, Beyin

INTRODUCTION

Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vessels.

Angiogenesis, a normal process in growth and development, is also effective in tumor development. In absence of angiogenesis, cardiovascular and cerebrovascular diseases can occur. Angiogenic factors, found in a lot of organs and



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tissues, are made of proteins and stimulate angiogenesis. In these factors include vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) [1]. There are many factors for increase of synthesis or stimulation of angiogenic agents. It is reported that especially the estrogen into many hormones effected to the factors [2]. It is known; the estrogen increased the vascularization in some organs (uterus, heart, brain etc.) by expression and releasing of angiogenic factors in female [3-5]. There are few and limited studies about the effect of testosterone on angiogenesis [6,7]. In addition to the factors mentioned above angiogenesis are also affected by the age. It is now known that the interruption and changes in angiogenesis by aging play as a major factor [8,9]. There is no literature about how aging and testosterone hormone affect the angiogenesis in mammalian brain. The aim of this study was to determine by histological and molecular techniques the effects of testosterone hormone treatment on angiogenesis in brain of aged mice.

MATERIAL and METHODS

A total of 15 female and 15 male Swiss albino mice are enrolled. The animals have been obtained from the Animal Experimental Unite of Veterinary Medicine Faculty in Adnan Menderes University, Turkey. Ethic committee approval was taken from Adnan Menderes University (with no: B.30.2.ADÜ.0.00.00.00/050.04/2011/098). Mice were grown up 405 days (12 months + 10 days + 30 days) in cages under normal conditions (20-24°C and 50-60% humidity) and were fed ad libitum with available commercial pelleted feeds. Each 3 group consisted of 10 mice. Out of 10 mice of each group, 5 were male and 5 were female. Among the experimental groups, the first group of animals (control) exposed to the same stress sham operations at 12 months (only skin incision and closure) was performed. The second experimental group (G) had gonadectomy at the age of 12 months but did not have testosterone supplementation. The third experimental group had both gonadectomy at the age of 12 months and testosterone supplementation. Third group of animals (GTS) had testosterone supplementation for one month beginning after ten days of post-operative recovery period. For the animals in GTS group, 0.01 cc testosterone (250 mg/mL Sustanon 250®, Organon) was administered by subcutaneous injection as a single dose. Mice were anesthetized by intraperitoneal administration of (ip) Ketamine (90 mg/kg)/Xylazine (10 mg/kg). All animals were sacrificed and their blood and brain tissue were taken at the age of 405 days. Testosterone levels in bloods were measured using ELISA Kit (for Mouse Testosterone (T), USCN Life Science Inc.® Wuhan) in accordance with the manufacturer's instructions. Blood was taken by exsanguinations from all animals after hormone supplementation period. Brains were dissected from the mice and cut in the middle of transversal plane. Half of the organs were collected for histological examination and

the other half was devoted to molecular investigation. For histological examination, the tissue samples were kept in 10% buffered formalin buffer and were embedded in paraffin after appropriate tissue tracking. For each tissue from paraffin blocks of 5 µm three serial sections were taken with an interval of 30 µm. After applying the process deparaffinization, sections performed triple (Mason trichrome method) staining [10]. The number of capillaries and inner diameter of larger capillary vessels in brain tissues was measured. The slices were analyzed and photographed under a light microscope (Leica DMLB) that is equipped with a calibrated digital camera (Leica DC200 CD camera and Q-win Standard imaging analyses programme). Histo-morphometric analyses were performed at a magnification of x 40-100. Both capillary number and inner diameter were measured and averaged results of 15 microscopic sites.

For molecular investigation, total RNA extraction from tissue samples (brain and hearth) was done using geneJET RNA Purification kit (Fermentas) according to the manufacturer's instructions. Reverse transcription using 2 µg of total RNA, was done with revertAid First Strand cDNA Synthesis kit (Fermentas) containing M-MuLV reverse transcriptase enzyme following manufacturer's instructions. The resulting cDNA was used for real time PCR amplification. For RNA extraction and PCR procedures of standardization and control the housekeeping transcript (GAPDH) was used. Genes were amplified using QuantiTect SYBR PCR Kit (ABM) as defined by Shidaifat et al. [6].

Statistical analysis was used in the SPSS 19.00 software package. Distributions of data were analyzed using Shapiro-Wilk test. Nonparametric distribution the data was checked using the Kruskal-Wallis test. Bonferroni- corrected Mann-Whitney U test was applied as post-hoc test. The presence of a correlation between the angiogenic events in brain tissue and the levels of testosterone was also tested by Spearsman's test. A *P*-value less than 0.05 were considered significant.

RESULTS

Testosterone Level

Blood testosterone levels were shown in *Table 1*. The highest level of testosterone was detected in testosterone supplemented female mice, followed by control male group. Among male mice after castration, testosterone levels were decreased 18.8 fold ($P < 0.01$) and testosterone replacement increased hormone levels 13.1 fold among castrated mice ($P < 0.01$). In female mice testosterone level was found to be decreased 2.8 fold in ovariectomized female. After hormone supplementation testosterone level increased 77.2 fold compared to ovariectomized female ($P < 0.001$). In female mice after testosterone replacement the testosterone levels were increased in GTS group, in contrast to control ($P < 0.05$) and G group ($P < 0.001$) (*Table 1*).

Capillary Number

The number of capillaries in brain was the highest in control group male animals. The least number of capillary was found in gonadectomized males group followed by GTS male group mice. After castration only 1.20 fold decrease was observed in the capillary densities of brain in both G ($P<0.05$) and GTS ($P<0.05$) group compared to control group. Hormone supplementation did not affect the capillary numbers in brain of castrated animals (Table 2, Fig. 1).

In female brain, the maximum capillaries were found in GTS group animals, and least in control group animals. In female brain, ovariectomy did not affect capillary density but operation and hormone supplementation together caused 1.14 fold increases on capillary number in brain tissue (Table 2, Fig. 1).

Vessel Diameter

Inner diameters of the larger capillary vessel of G groups were decreased in male and increased in female brain 1.26 and 1.03 folds, respectively, compared to control group. Testosterone supplementation did not affect brain vessel diameters in male and female mice (Table 2).

Data of RT PCR

It was determined that the level of VEGF mRNA (Δ CT)

in male brain was highest in the control group and lowest in GTS group animals. Amount of VEGF mRNA in brain tissue were 2.10 fold low in castrated animals compared to control group. In testosterone supplementation group expression level were decreased 8.62 fold and 4.75 fold compared to control ($P<0.05$) and G group, respectively. In female brain, VEGF mRNA levels decreased compared to control group 1.42 and 1.48 folds for G and GTS groups, respectively (Table 2).

The presence of a correlation between the angiogenic events in brain tissue and the levels of testosterone was also tested by Spearmen's test. While there was a positive correlation ($r = 0.528$, $P<0.05$) between the capillary number in brain tissue and the testosterone levels of females, a significant negative correlation ($r = -0.536$, $P<0.05$) was found between inner diameter of large capillary in brain and the testosterone levels of females. This result showed that increased testosterone level in female blood caused elevated capillary number in brain tissue whereas a decrease the inner diameter of large capillary in female brain tissue.

DISCUSSION

Few studies have been made regarding the effects of androgens on angiogenesis [6,7]. It was determined that androgens stimulate erythropoietin production via VEGF in cell culture and endothelial stem cells are regulated hormonally. In the reduced level of these hormones due to castration angiogenesis is also downward as it was shown previously [7]. The changes due to castration includes the decrease in endothelial function of vessel (*Arteria femoralis*) and deterioration in calcium channel activity as a result of decrease in testosterone level and the absence of androgen receptor in testicular feminized mice [11]. Castration in young male mice caused decrease in androgen receptors in the brain up to ten times [12]. Also using ischemic injury in male the effect of endogenous androgens in maintaining neovascularization was shown [7]. The results that castration decreased angiogenesis in male animals by lack of testosterone were led to investigations

Table 1. Testosterone levels in male and female groups of mice. Control; sham operated group, G; gonadectomized group, GTS; gonadectomized and testosterone supplemented group

Animal Groups (N)	Testosterone Level nmol/L (Mean \pm SD)	
	Male	Female
Control (10)	1.517 \pm 1.173 ^a	0.140 \pm 0.101 ^c
G (10)	0.089 \pm 0.075 ^{a,b}	0.051 \pm 0.064 ^d
GTS (10)	1.051 \pm 0.478 ^b	3.862 \pm 2.838 ^{c,d}

^{a,b} In male mice after castration testosterone levels were decreased ($P<0.01$) in G group of mice. Testosterone replacement increased hormone levels in GTS group of male mice ($P<0.01$), ^{c,d} In female mice after testosterone replacement the testosterone levels were increased in GTS group, to compared to control ($P<0.05$) and G group ($P<0.001$)

Table 2. The number of capillaries, inner diameters of larger capillary and VEGF mRNA (Δ CT) values of brain tissues in male and female groups of mice. Control; Sham operated group, G; Gonadectomized group, GTS; Gonadectomized and testosterone supplemented group.

Gender	Animal Groups (N)	Capillary Number (Mean \pm SD)	Inner Diameter of Larger Capillary (μ m) (Mean \pm SD)	VEGF mRNA (Δ CT) (Mean \pm SD)
Male	Control (5)	3.865 \pm 0.226 ^{a,b}	8.527 \pm 1.508	7.375 \pm 0.564 ^c
	G (5)	3.226 \pm 0.193 ^a	6.778 \pm 0.448	3.510 \pm 2.268
	GTS (5)	3.222 \pm 0.234 ^b	6.686 \pm 0.774	-0.241 \pm 2.424 ^c
Female	Control (5)	3.316 \pm 0.400	7.160 \pm 0.410	6.205 \pm 0.624
	G (5)	3.384 \pm 0.237	7.418 \pm 0.758	4.372 \pm 2.500
	GTS (5)	3.770 \pm 0.167	6.916 \pm 0.675	4.186 \pm 1.940

^{a,b} The number of capillary in male brain was decreased in both G ($P<0.05$) and GTS ($P<0.05$) group compared to control group; ^c The expression level of VEGF mRNA was decreased in the testosterone supplementation group ($P<0.05$) compared to control group

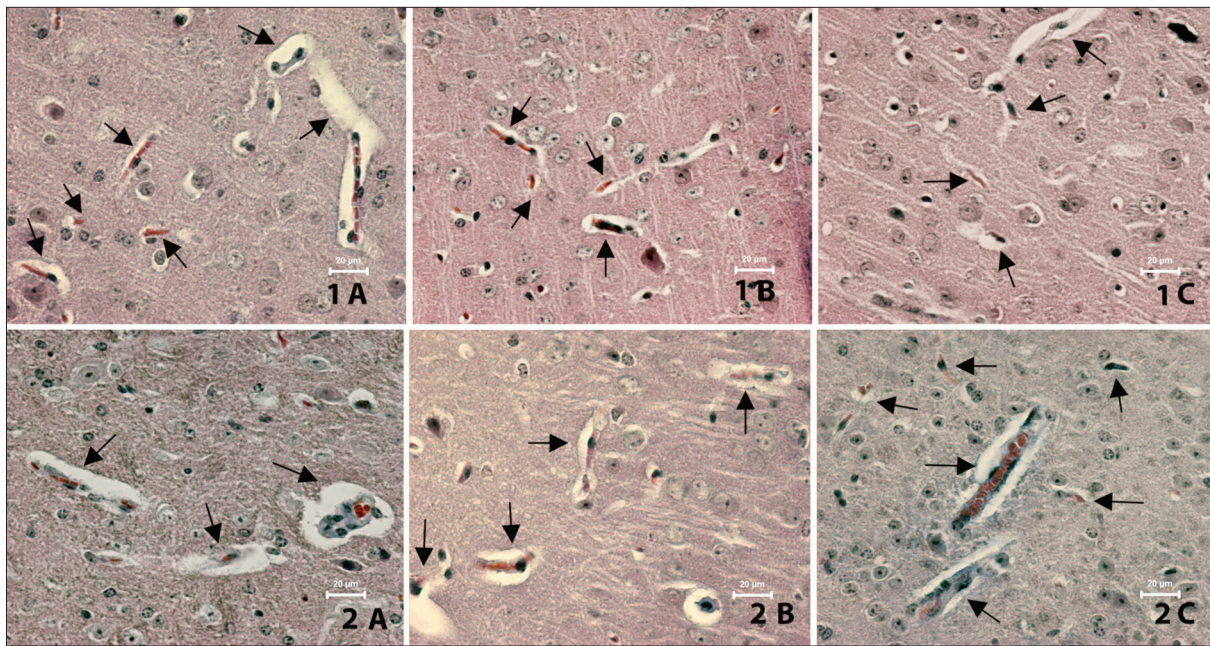


Fig 1. Capillary density in brain tissues from male (1A, 1B, 1C) and female (2A, 2B, 2C) aged mice. Sections performed triple (Mason trichrome method) staining (Crossman, 1937). A; sham operated group (Control), B; gonadectomized group (G), C; gonadectomized and testosterone supplemented group (GTS). Arrows show capillary. After gonadectomy operation, capillary densities of brain decreased in male ($P < 0.05$) but did not change in female. Testosterone supplementation to gonadectomized aged mice caused a slightly increase on capillary number in female brain but did not affect in male

on the effects of cancer tissue. Most of the studies have been made in prostate cancer. According to Hammarsten et al.^[13], castration caused decreased vascular density in the normal tissue surrounding the tumor and consequently increased tumor hypoxia and apoptosis, and moderately decreased tumor growth in prostate. Also an *in vitro* study showed that testosterone affected development and function of early endothelial progenitor cells but there was no effect on late endothelial progenitor cells^[14]. However in the present study, there was a reduction in capillary density and vessel diameter in brain tissue after castration. Hormone supplementation to castrated animals caused no effect on capillary number and vessel diameter in brain. Also in female brain tissue, with ovariectomy there was no change in capillary density and vessel diameters. With testosterone supplementation to ovariectomized animals an increase on capillary number in brain was observed. The discrepancies among previous studies and our study results may be due to the age of the animals. That is why the aged animals were studied in present study to see the effects of the hormone replacement in elderly mice.

Previous studies showed that androgens stimulate erythropoietin production via VEGF in cell culture and endothelial stem cells. In the absence of these hormones by means of castration, angiogenesis is downward^[7]. According to Sordello and colleagues testosterone caused an increase in transcription and biological activity of VEGF in human prostate cell culture^[15]. *In vivo*, a transient increase in the weight of ventral lobes of the prostate

gland and 7-fold increase in specific activity of VEGF belong to prostate in testosterone injected rats were seen. After castration a decrease is observed in both translation and transcription levels of VEGF of the prostate gland of the dog^[6]. Also in cancer cells culture, an increase of transcription of VEGF is found after estrogens and androgens treatment^[16]. However, Sieveking et al.^[14] observed difference in the effect of testosterone hormone in male and female, an increase was observed in males in the angiogenesis depend on VEGF but no effect in females. In the present study, amount of VEGF mRNA in brain of both sex decreased due to gonadectomy. However, in contrary of the previous findings our results showed that testosterone supplementation caused an important ($P < 0.05$) drawbacks in these values in male mice groups. However, no change was found in female groups.

Androgens have different effects of angiogenesis by gender. *In vitro* studies showed that the androgens stimulate angiogenic phenomena in male, but not in females. In addition, *in vivo* studies shown that the endogenous androgens regulated angiogenesis in males, but not in females^[7]. Jesmin et al.^[4], applied ovariectomy operation after 44 weeks to female rats and as a result observed that the vessel density, VEGF and receptors of VEGF levels decreased in brain (frontal cortex). Estrogen treatments caused a complete remission in these changes^[4]. In the present study, the number of capillary vessels and diameter of vessels in the brain were decreased in gonadectomized male animals, but there was no change in females. In the brain, VEGF mRNA after gonadectomy is

reduced even more by hormone replacement in males, but there was no change in females.

In the old male mice, androgen deficiency caused a decrease in capillary number of brain tissue. Testosterone replacement possessed no increasing effect on angiogenesis in the male brain. In addition, the latter caused a decrease in the level of VEGF mRNA in male brain after gonadectomy and decrease was even more after hormone replacement. There was no change in the capillary density and vessel diameter in female brain.

Our results showed that the decreased testosterone levels in old mice had an important negative effect on VEGF mRNA levels. However, testosterone replacement in male was not sufficient to convert this change and did not increase angiogenesis. Interestingly, testosterone replacement caused an important decrease in the expression of VEGF in male brain although previous studies reported increased VEGF expression by testosterone supplementation. Further studies are necessary to show if this is a result of a feedback mechanism.

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