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Research Article

The Effect of Testosterone Supplementation on Capillar Morphometry and VEGF Expression Level of the Heart in Aged Mice [1]

İlknur DABANOĞLU [1,a] Bülent BOZDOĞAN [2,b] Şadiye KUM [3,c]

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[1] Adnan Menderes University, Faculty of Veterinary Medicine, Department of Anatomy, TR-09016 Aydin - TURKEY
[2] Adnan Menderes University, Rekombinant DNA ve Protein Araştırma Merkezi (REDPROM), TR-09100 Aydin - TURKEY
[3] Adnan Menderes University, Faculty of Veterinary Medicine, Department of Histology and Embryology, TR-09016 Aydin - TURKEY
[a] ORCID: 0000-0001-7776-1092; [b] ORCID: 0000-0003-2469-9728; [c] ORCID:0000-0001-6586-4596

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Abstract

The aim of this study was to determine the effect of testosterone hormone supplementation on the capillary morphometry and VEGF expression level of the heart of old mice using histological and molecular techniques. A total of 30 old mice were enrolled in 3 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 the heart were measured by light microscope in terms of angiogenic processes. The levels of angiogenic factor, VEGF (vascular endothelial growth factor), mRNA in the heart tissue were determined by RT-PCR. The capillary densities decreased in female mice, in which G and GTS ($P<0.05$) groups compared to the control group, whereas there was no alteration in male mice. Gonadectomy led to a reduction in VEGF expression. Interestingly, testosterone replacement (in GTS group) caused even more reduction in VEGF expression in male and female ($P<0.05$) compared to the control group. The present results showed that there was a variation affected by gender in the regulation of angiogenesis by testosterone in the heart. However, testosterone replacement was not sufficient to restore the effects of gonadectomy on the heart in old mice.

Keywords: *Angiogenesis, Testosterone, Aged mice, Heart*

Testosteron Takviyesinin Yaşlı Farelerin Kalbinde Kapillar Morfometri ve VEGF Expresyon Seviyesine Etkisi

Öz

Bu çalışmanın amacı yaşlı farelerin kalbinde testosteron hormon takviyesinin 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. Kalp dokusunda kapillar sayısı ve geniş kapillar damarların iç çapı ışık mikroskobunda ölçülmüştür. Kalp dokusundaki VEGF (vasküler endoteliyal büyüme faktörü) mRNA seviyesi RT-PCR ile ölçülmüştür. Dişi farelerde; kontrol grubuna göre G ve GTS ($P<0.05$) grup farelerin kalbindeki kapillar yoğunluk azalmış, erkeklerde ise değişmemiştir. Kısırlaştırma farelerde kalpteki VEGF mRNA seviyesinde bir azalmaya yol açmıştır. Testosteron takviyesiyle (GTS grup) kalpteki VEGF mRNA seviyesinde kontrol grubuyla karşılaştırıldığında erkek ve dişilerde ($P<0.05$) daha fazla azalma görülmüştür. Bu çalışmayla testosteronun erkek ve dişilerin kalbinde anjigenik etkisinin farklı olduğu saptanmıştır. Yaşlı erkek farelerde kısırlaştırmanın anjiyogenik olaylarda negatif etkisinin olduğu belirlenmiştir. Bununla birlikte testosteron takviyesi yaşlı farelerin kalbinde gonadekteminin etkilerini düzeltmek için yeterli olmamıştır.

Anahtar sözcükler: *Anjiyogenezis, Testosteron, Yaşlı fare, Kalp*

INTRODUCTION

Angiogenesis, a physiological process for vessel growth and remodeling, is not only a normal process in the regulation of growth and development, but also involved in tumor development. The lack of angiogenesis may cause cardiovascular and cerebrovascular diseases. The angiogenic factors, found in a lot of organs and tissues, are consisted of proteins and they stimulate angiogenesis. Some of the latter factors are vascular endothelial growth

İletişim (Correspondence)
+90 506 6247526
dabanf@yahoo.com

factor (VEGF), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) [1].

Vascular endothelial growth factor, a member of subfamily of growth factors, is an essential catalyst promoting endothelial cell proliferation, initiating angiogenic sprouting and creating vascular structures. VEGF-A is the most important one in mediating endothelial cell proliferation. VEGFs interact with a set of cellular membrane tyrosine-kinase receptors. VEGF-A regulated by androgens is a major mediator of androgen actions on endothelial cell proliferation [2]. In addition, many factors can increase the synthesis of angiogenic agents or directly stimulate them. It has been reported that many hormones, especially estrogen, affect these factors [3]. It is well known that estrogen increases the vascularization in some organs (e.g. the uterus, the heart, and the brain) by releasing angiogenic factors and increasing their expressions in female rats and women [4-6]. There are few and limited studies on the effect of testosterone on angiogenesis [7-11]. Testosterone has protective effects on formation of cells in the arterial vessels [12]. Testosterone has different kinetic effects in vessels; in the first stage testosterone has vasodilator effect, in longer-term exposure, testosterone increases the blood pressure and causes renal function abnormalities [13,14].

In addition to the factors mentioned above, angiogenesis is also affected by the age. It is known that aging plays as a major factor for the interruption and the changes in angiogenic processes [15-17]. Aging is one of the main risk factors for the development of atherosclerosis and, therefore, for coronary artery disease. Age associated remodeling of the vascular wall includes luminal enlargement, intimal and medial thickening, and increased vascular stiffness [18].

There is no literature about how aging and testosterone hormone affect the mammalian heart. Therefore, the aim of this study was to determine, the effects of testosterone hormone on angiogenic process in the heart of aged female and male mice.

MATERIAL and METHODS

A total of 15 female and 15 male Swiss albino mice at 12 months of age were included in the experiments. The mice were obtained from the Animal Experimental Unite of Faculty of Veterinary Medicine, Adnan Menderes University, Turkey. The ethical committee approval was taken from Adnan Menderes University (with no: B.30. 2.ADÜ.0.00.00.00/050.04/2011/098). Mice were housed in standard cages under normal conditions (20-24°C and 50-60% humidity) and were fed *ad libitum* with unlimited access to the commercial pelleted diet and water. Among the experimental groups, the mice in the first group (control) exposed to the same stress (sham operated) with other experimental groups at 12 months of age (only skin incision and closure). The mice in the second experimental group (G) were gonadectomized at the same age with the control group, but they did not receive testosterone replacement. The mice in the third experimental group (GTS) received both gonadectomy surgery and testosterone replacement once a day for one month after post-operative day 10. Each group consisted of 10 mice (5 males and 5 females). For the animals in GTS group, 0.01 mL testosterone (250 mg/mL Sustanon 250®, Organon) was administered by subcutaneous injection as a single dose [19]. Mice were anesthetized by intraperitoneal administration of ketamine (90 mg/kg)/xylazine (10 mg/kg) combination. Mice were considered as old at 12 months of age (365 days). At 365 days of growth, the gonadectomy surgery was done. After 10 days of healing period, testosterone replacement therapy was applied for 30 days (365 + 10 + 30 = 405 days). All mice were then sacrificed at day 405 after they were born. Whole blood was collected by exsanguination from all animals. Testosterone levels in the blood samples were measured using ELISA kit (for Mouse Testosterone (T), USCN Life S**c**ience Inc.® Wuhan) according to the manufacturer's instructions. The hearts were removed from the mice and cut in the middle of transversal plane. Half of the organs were collected for the histological examination and the other half was devoted to the molecular investigations.

For histological examination, the tissue samples were kept in 10% buffered formalin solution 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 were stained with Mason trichrome method [20]. The number of capillaries and inner diameter of larger capillary vessels were measured in the heart tissues. 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). Histomorphometric analyses were performed at a magnification of x 40-100. Both capillary number and inner diameter were measured and averaged results of different 15 microscopic fields.

For molecular investigation, total RNA extraction from the heart tissue samples was performed using geneJET RNA Purification kit (Fermentas) according to the manufacturer's instructions. Reverse transcription using 2 µg of total RNA, was done with the 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. Primers were designed to be specific for mouse sequence using web-based QuantiProb design software (www.quiagen.com). The forward primer for VEGF was 5'-GGAGATCCTTCG AGGAGCACTT-3' and reverse primer was 5'-GGCGATT TAGCAGCAGATATAAGAA-3'. For RNA extraction and PCR procedures of standardization and control, the house-

keeping transcript (GAPDH) were used. The forward primer for GAPDH was 5′-GAGGGGCCATCCACAGTCTTCT-3′ and reverse primer was 5′-GGAGCCAAACGGGTCATCATCTC-3′. Genes were amplified using QuantiTect SYBR PCR Kit (ABM) as previously described by Shidaifat et al.[7].

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 the 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 are shown in *Table 1*, *Fig. 1*. Among male mice after castration, testosterone levels 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 group G. After hormone supplementation, testosterone level increased ~77.2-fold compared to group G (P<0.001). In female mice after testosterone replacement, the testosterone levels were increased in group GTS when compared to control (P<0.05) and group G (P<0.001) *(Table 1)*.

Capillary Number

No significant change was found in capillary densities after gonadectomy for the male mice *(Table 2, Fig. 2)*. Hormone replacement did not affect the capillary numbers in the heart of castrated mice. In female heart, the maximum capillaries were found in control group animals, and the least in group G followed by group GTS mice *(Table 2, Fig. 2, Fig. 5)*. After ovariectomy, a reduction (~1.15-fold) was found in capillary densities of heart in group G compared to the control group. Testosterone supplementation caused ~1.18-fold decrease (P<0.05) on capillary number in group GTS compared to the control group *(Fig. 5)*. Testosterone supplementation did not increase the capillary density in the heart of the gonadectomized female mice.

Vessel Diameter

Inner diameters of the larger capillary of group G and group

Table 1. *Testosterone levels in male and female groups of mice*

Animal Groups (N)	Testosterone Level nmol/L (Mean±S.D.)	
	Male	Female
Control (10)	1.517±1.173 [a]	0.140±0.101 [c]
G (10)	0.089±0.075 [b]	0.051±0.064 [c]
GTS (10)	1.051±0.478 [a]	3.862±2.838 [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); ***Control:*** *sham operated group,* ***G:*** *gonadectomized group,* ***GTS:*** *gonadectomized and testosterone supplemented group*

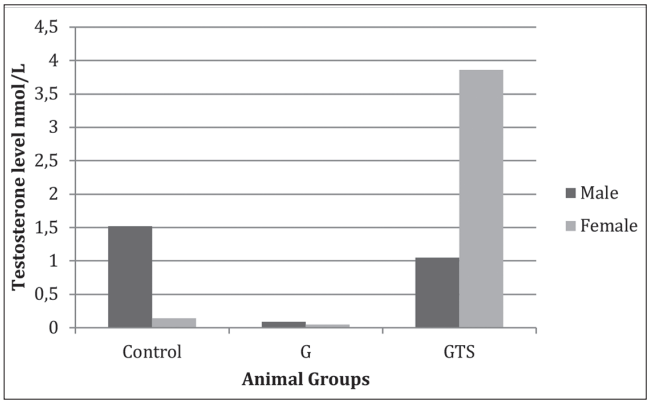

Fig 1. Testosterone levels in male and female groups of mice. Control: sham operated group, G: gonadectomized group, GTS: gonadectomized and testosterone supplemented group

Table 2. *The number of capillaries, inner diameters of larger capillary and VEGF mRNA (ΔCT) values of heart tissues in male and female groups of mice*

Animal Groups (N)		Capillary Number (Mean ± S.D.)	Inner Diameter of Larger Capillary (μm) (Mean ± S.D.)	VEGF mRNA (ΔCT) (Mean ± S.D.)
Male	Control (4)	4.133±0.211	22.228±3.739	4.189±0.929
	G (5)	4.173±0.784	19.023±2.030	2.395±2.154
	GTS (5)	4.306±0.269	18.530±1.868	-0.021±2.492
Female	Control (5)	4.360±0.539 [a]	19.727±9.296	4.357±0.944 [c]
	G (5)	3.773±0.332 [a,b]	17.693±2.829	3.564±0.661 [c,d]
	GTS (5)	3.692 0.102 [b]	15.768±1.712	0.545±2.465 [d]

[a,b] The number of capillary in female heart significantly decreased (P<0.05) in group GTS compared to the control group; [c,d] The expression level of VEGF mRNA in female heart decreased (P<0.05) in group GTS compared to the control group; ***Control:*** *sham operated group,* ***G:*** *gonadectomized group,* ***GTS:*** *gonadectomized and testosterone supplemented group*

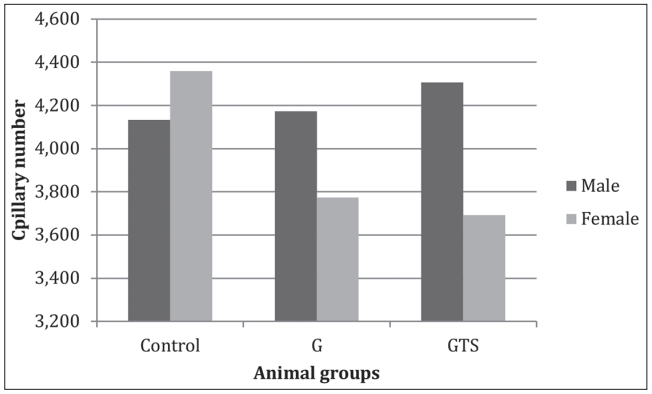

Fig 2. The number of capillaries of heart tissues in male and female groups of mice. Control: sham operated group, G: gonadectomized group, GTS: gonadectomized and testosterone supplemented group

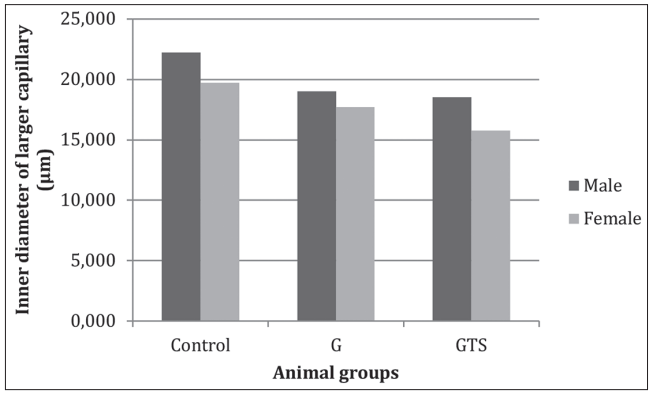

Fig 3. The inner diameters of larger capillary of heart tissues in male and female groups of mice. Control: sham operated group, G: gonadectomized group, GTS: gonadectomized and testosterone supplemented group

GTS were decreased in male heart ~1.17- and ~1.20-fold, respectively, compare to the control group *(Table 2, Fig. 3)*. In female mice, the inner diameter also decreased in group G and group GTS by ~1.11- and ~1.25-fold, respectively, compare to the control group. Testosterone replacement did not affect on the inner diameters of larger capillary of the heart in male and female mice *(Table 2, Fig. 3)*.

Data of RT PCR

It was found that the level of VEGF mRNA (ΔCT) of the male heart was highest in the control group, whereas lowest in group GTS *(Table 2, Fig. 4)*. After castration, VEGF mRNA levels of the heart decreased by ~1.75 fold in group G and testosterone replacement led to a reduction by ~5.21-fold as evidenced by VEGF mRNA levels in group GTS compared to the male controls. VEGF expression decreased by ~3.42-fold in group GTS compared to group G. Similar to that of the male group, the VEGF mRNA levels of the female heart were highest in the control group while were lowest in group GTS *(Table 2, Fig. 4)*. The reduction (~1.22-fold) in VEGF mRNA level of group G was not significant compared to the control group while the reduction (~7.93-fold) in VEGF mRNA levels of group GTS was significant ($P<0.05$). A

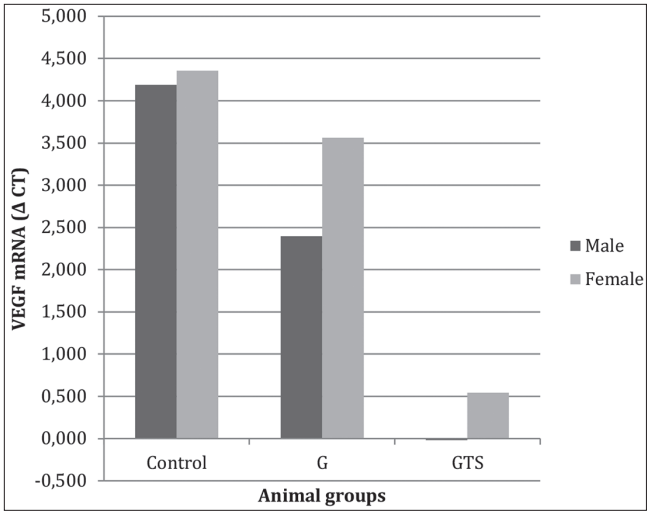

Fig 4. The VEGF mRNA (ΔCT) values of heart tissues in male and female groups of mice. Control: sham operated group, G: gonadectomized group, GTS: gonadectomized and testosterone supplemented group

reduction by ~6.47-fold in VEGF mRNA levels was determined in group GTS compared to group G.

The presence of a correlation between the angiogenic events in the heart tissue and the levels of testosterone was also tested by Spearsman's test. A significant negative correlation (r_s = -0.550, $P<0.05$) was found between inner diameter of larger capillary in the heart tissue and the testosterone levels of female mice, suggesting that increasing testosterone level in female mice leads to a reduction in the inner diameter of larger capillary of the female heart.

DISCUSSION

Limited studies on the effects of androgens on angiogenesis have been found [7,8]. Sieveking et al.[8] reported that androgens stimulate erythropoietin production via VEGF in cell culture and endothelial stem cells. Angiogenesis is also down regulated when the levels of these hormones reduce after castration as stated previously [8]. In addition, an *in vitro* study showed that testosterone affects the development and function of early endothelial progenitor cells, but has no affect on late endothelial progenitor cells [21]. Chen et al.[22] stated that, in the case of experimental myocardial infarcts, estrogen treatment increases in the capillary density by leading bone marrow stem cell mobilization in the heart of ovariectomized rats.

Recent studies showed angiogenic effects of castration and testosterone treatment. Their results showed that there was a reduction in VEGF expression level and capillary density of the heart among castrated rats and testosterone treatment restored the adverse effects. In case of experimental myocardial infarcts among adult rats, capillary density is decreased in castrated rats [23,24]. It was reported that testosterone hormone plays an important

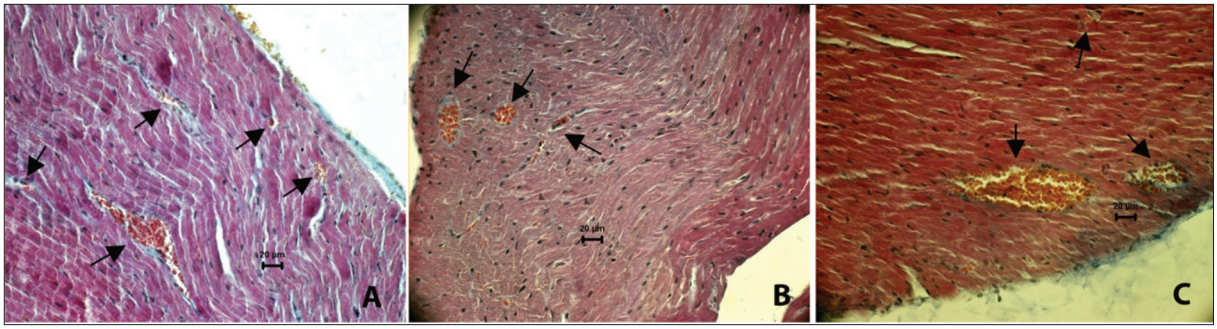

Fig 5. The capillary densities in the heart tissues from female (A, B, C) aged mice. Sections performed triple. A: sham operated group (Control), B: gonadectomized group (G), C: gonadectomized and testosterone supplemented group (GTS). Arrows show capillaries. After gonadectomy operation, capillary densities of the heart decreased in female mice. Testosterone supplementation to gonadectomized aged mice caused a slight decrease in capillary number of the female heart. A significant decrease ($P<0.05$) in group GTS was observed in female heart capillary density compared to the control group

role in the pathophysiology of heart attack in rat and the hormone replacement therapy has positive effects in this regard [25-27].The comparative effects of estrogen and testosterone treatment on castrated rats have been studied and found that the androgen treatment causes an improvement in angiogenesis of the heart muscle more than estrogen treatment does [28]. In our study, castration and hormone supplementation to the ovariectomized mice did not cause significant changes in the heart capillary density and vessel diameter. A slight reduction was found in the inner diameter of larger capillary after castration in male mice. Among female mice, it was found a reduction in the heart capillary density and vessel diameter following ovariectomy. However, a significant difference ($P<0.05$) was observed between capillary number of group GTS and the control group in female mice. Testosterone supplementation did not increase the capillary density and vessel diameter in female heart. The discrepancy among previous studies and our study may be due to the age of the animals. In present study, the aim was to determine the effects of the hormone replacement in elderly mice in contrast to the previous studies, which had been conducted with adult animals.

Previous studies showed that the absence of androgens such as under castration conditions, angiogenesis is down-regulated [8]. In addition, it was reported that the transcription of VEGF increases in cancer cells culture after estrogen and androgen treatments [29] but Sieveking et al.[8] determined an increase in the angiogenesis depending on VEGF in male mice, but not in female mice. Recent studies showed that VEGF can improve recovery from experimental ischemic myocardial injury in adult laboratory animals. VEGF administration was associated with smaller infarct sizes and greater wall thicknesses and greater vascular density in border region of the infarct [30-32]. Chen et al.[22] studied the angiogenic effects of castration and testosterone treatment in the experimental myocardial infarcts. They found that there was a reduction in the expression levels of VEGF and capillary density of the heart tissue in castrated rats. They also found that testosterone treatment restored the adverse effects due to castration [23]. In our study, the amount of VEGF mRNA in old mice (for both sexes) decreased due to gonadectomy. However and unexpectedly, testosterone supplementation caused even more reduction in VEGF expression. The reduction of VEGF mRNA levels of female mice in group GTS was significant ($P<0.05$) compared to the control group.

The effects of androgens on angiogenesis may vary depending on gender. *In vitro* studies showed that the androgens stimulate angiogenic phenomena in males, but not in females. In addition, *in vivo* studies have shown that the endogenous androgens regulate angiogenesis in males, but not in females [8]. Jesmin et al.[5] found a reduction in the vessel density, VEGF levels and VEGF receptors in frontal cortex of the brain in ovariectomized, 44 weeks of age, female rats. They observed a complete normalization in these changes following estrogen treatments [5]. In our study, a reduction was observed in the heart capillary number after gonadectomy in female mice, but not in male mice. Gonadectomy led to a reduction in vessel diameter of the heart tissue in both sexes. Although testosterone replacement of gonadectomized mice caused a mild decrease in vessel diameter of the heart in female mice, male mice were not affected by testosterone replacement. The level of VEGF mRNA decreased after gonadectomy and hormone replacement intensified this effect as the VEGF mRNA levels decreased more after the hormone replacement.

Our results showed that the reduction in testosterone levels in old mice had an important negative effect on VEGF mRNA levels. However, testosterone replacement in male mice was not sufficient to restore this change and to increase angiogenesis. Interestingly, testosterone replacement caused an important reduction in the expression of VEGF in the heart of male mice although previous studies reported increased VEGF expression caused by testosterone supplementation. Further studies are required to show whether the discrepancy between these results is due to the age of the animals because the

response to the androgen treatment may vary depending on the age of the subject.

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