The effects of testosterone on isolated sheep coronary artery

İzole koyun koroner arterinde testosteronun etkileri

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ABSTRACT

Objective: Although estrogens have been shown to be vasoactive hormones, the vascular effects of testosterone are not well defined. Little is known about the *in vitro* effects of testosterone on isolated arteries. The purpose of this experimental prospective study was to assess the direct effect of testosterone on isolated sheep coronary artery *in vitro.*

Methods: We evaluated whether the vascular effects of testosterone were altered in the presence of endothelium or it changed in accordance to gender or the concentration of testosterone. Coronary arterial rings were precontracted by KCl (30mM). After plateau contraction levels were reached, testosterones (10^{-7}-10^{-4}M) were added in a cumulative manner. The effects of testosterone were also tested in the presence of NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 10^{-4}M), and L-arginine (10^{-4}M). In statistical analysis, Student's t-test was used for comparison between two groups and one-way ANOVA test for comparison among multiple groups.

Results: Testosterone relaxed sheep coronary artery. Testosterone-induced relaxation was dependent of sex [male endothelium (ME(+)): 24.48±4.13; -29.36±6.41; female endothelium (FE(-)): -3.02±1.04: p<0.05); (ME(+): -24.61±4.14; -24.48±4.13; -29.36±6.41; FE(-): -3.64±0.67: p<0.01); (FE(-):-4.91±0.67: p<0.001)] and endothelium (ME(+): -9.23±2.4; FE(+): -6.33±1.5; -8.43±0.49; -8.83±0.9: p<0.05, p<0.01). L-NAME decreased relaxations in the male (ME(+): 0.77±0.72; 0.086±0.012; 0.0768±0.083; 0.51±0.44: p<0.01, p<0.001) and female (FE(+): 0.0318±0.052; 0.52±0.49; 0.029±70.05: p<0.05, p<0.001) with endothelium groups but only female without (0.01±0.011; -1.14±0.59; -1.23±1.21: p<0.01, p<0.001) endothelium group. L-arginine decreased relaxations especially in the male with endothelium (ME(+): -1.7±0.91; -3.02±1.42; -2.51±1.46; -6.68±2.15; p<0.01, p<0.001) group and in part in the female with (FE(+):-1.73±0.83; p<0.05) or without (FE(-): -1.14±0.59; p<0.01) endothelium groups.

Conclusion: Testosterone induces endothelium and sex-dependent relaxation on sheep coronary artery *in vitro*. Acute testosterone-induced coronary vasodilatation is mediated in part via endothelium-derived NO. Non-genomic mechanisms may be considered to play a role in the vasodilatory effect of testosterone especially in the female group. *(Anadolu Kardiyol Derg 2011; 11: 343-50)*

Key words: Testosterone, vasodilatation, endothelium, coronary artery, sheep

ÖZET

Amaç: Her ne kadar östrojenlerin vazoaktif etkileri olduğu gösterilmiş olsa da testosteronun vasküler etkileri iyi tanımlanmamıştır. Testosteronun *in vitro* izole damar preparatları üzerindeki etkileriyle ilgili bilgiler yeterli değildir. Bu deneysel prospektif çalışmanın amacı testosteronun *in vitro* olarak izole koyun koroner arteri üzerindeki doğrudan etkilerini değerlendirmekti.

Yöntemler: Çalışmamızda testosteronun vasküler etkilerinin damar endotelinin var olup olmamasına, cinsiyet farklılığına veya uygulanan testosteron konsantrasyonuna bağlı olarak değişiklik gösterip göstermediğini araştırdık. Koroner arter halkalarının 30 mM KCl ile kasılması sağlandı. Kasılma cevapları plato düzeyine ulaştıktan sonra testosteron (10^{-7}-10^{-4}M) kümülatif olarak uygulandı. Ayrıca testosterona bağlı cevaplar nitrik oksit sentez inhibitörü Nω-nitro-L-arginine methyl ester [L-NAME (10^{-4}M)] ve L-arginin (10^{-4}M) varlığında da değerlendirildi. İstatistiksel analizde ikili grupların karşılaştırılmasında Student's t-testi, çoklu grupların karşılaştırılmasında one way ANOVA testi kullanıldı.

Bulgular: Testosteron koyun koroner arterlerinde gevşeme cevabı oluşturdu. Testosterona bağlı gevşeme cevapları cinsiyet [erkek endoteliyum (ME(+)): 24.48±4.13; -29.36±6.41; kadın endotelıyum (FE(-)): -3.02±1.04: p<0.05); (ME(+): -24.61±4.14; -24.48±4.13; -29.36±6.41; FE(-): -3.64±0.67: p<0.01); (FE(-):-4.91±0.67: p<0.001)] ve endotelle (ME(+): -9.23±2.4; FE(+): -6.33±1.5; -8.43±0.49; -8.83±0.9: p<0.05, p<0.01) ilişkili olarak değişiklik gösterdi. L-NAME testosterona bağlı gevşeme cevaplarını erkek [ME(+): 0.77±0.72; 0.086±0.012; 0.0768±0.083; 0.51±0.44: p<0.01, p<0.001] ve dişi (FE(+): 0.0318±0.052; 0.52±0.49; 0.029±70.05: p<0.05, p<0.001) endotelli gruplarda azaltırken sadece dişi endotelsiz (0.01±0.011; -1.14±0.59;

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-1.23±1.21: p<0.01, p<0.001) grupta azalma cevabı gözlenmiştir. L-arginin ise gevşeme cevabını özellikle endotelli erkek (ME(+): -1.7±0.91; -3.02±1.42; -2.51±1.46; -6.68±2.15; p<0.01, p<0.001) grupta azaltırken endotelli (FE(+): -1.73±0.83; p<0.05) ve endotelsiz (FE(-): -1.14±0.59; p<0.01) dişi gruplarda kısmi bir azalma meydana getirmiştir.
Sonuç: Testosteron uygulanması *in vitro* koyun koroner arterinde endotele ve cinsiyete bağlı gevşeme cevabı oluşturmuştur. Testosterona bağlı akut gevşeme cevabı kısmen endotel kaynaklı NO aracılığı ile olmaktadır. Özellikle dişi grupta, testosterona bağlı gevşeme cevaplarında non-genomik mekanizmalarında rolü olduğu düşünülebilir. *(Anadolu Kardiyol Derg 2011; 11: 343-50)*
Anahtar kelimeler: Testosteron, vazodilatasyon, endotel, koroner arter, koyun

Introduction

The incidence of coronary heart disease and hypertension is much greater in men than in premenopausal women. Lipid-lowering and antioxidant effects of estrogen have been proposed to protect from the development of coronary artery disease (CAD) by many epidemiological and experimental studies. It is generally accepted that the incidence of morbidity and mortality from CAD depends on both gender and age (1, 2). However, a woman and a man, aged around 65 years are similar in terms of cardiovascular dysfunction. This has been reported to be occurred due to the unfavorable effects of testosterone. Testosterone is often considered to aggravate the development of CAD because the incidence of CAD is higher in men than women and some studies have linked the level of plasma testosterone with the incidence of CAD. However many other clinical and epidemiological studies have also reported that testosterone inhibits CAD development. Testosterone has been reported to have a beneficial effect on blood lipid profile and against atheroma formation. Hypotestosteronemia in men for example, has been associated with CAD risk. Due to testosterone-induced coronary vasodilatation testosterone replacement therapy appears to reverse CAD risk (2). Fibrinogen, plasminogen activator inhibitor-1, known as the risk factors for CAD, have also been found to be negatively correlated with serum testosterone levels (3). Furthermore, hypotestosteronemia is associated with myocardial infarction in men (4, 5) and testosterone was found to relieve exercise induced depression of the ST segment by electrocardiography (6-9). Lower testosterone levels have been found to be associated with carotid-intima media thickness, lower extremity peripheral arterial disease and aortic atherosclerosis (10-13). However, it is unclear whether lower testosterone levels independently predict morbidity and mortality from cardiovascular disease (14). In the light of these clinical and epidemiological studies, the relationship between testosterone and CAD remains controversial and detailed studies are required to better understand the relation between testosterone and CAD.

Both *in vivo* and *in vitro* animal studies have reported that testosterone acts as a direct coronary vasodilator in a variety of species. Recent evidence suggests that the vasodilatory effects of testosterone occur via non-genomic mechanisms, independent of the classical androgen receptor (AR) (2, 15-17).

Thus, the purpose of the present study is to compare the effect of pharmacological concentrations of testosterone (10^{-7}-10^{-4}M) on vascular reactivity in isolated sheep coronary artery and also to explore whether the vascular effect of testosterone was altered in the presence of endothelium or it changed in accordance to gender. We also investigated whether the vascular effect of testosterone was altered via nitric oxide synthase inhibitor (NOS) Nω-nitro-L-arginine methyl ester L-NAME, and L-arginine. Nitric oxide (NO) is endothelium-derived vasoactive mediator. It is formed from the amino acid precursor L-arginine by the enzyme endothelial nitric oxide synthase (eNOS).

Methods

Tissue preparation

Six-month-1 year old sheep hearts were used in this experimental prospective study. All procedures were applied in accordance to the Guide for the Care and Use of Laboratory Animals Published by the US National Institutes of Health (1996).

Hearts from sheep of either sex were collected from a local abattoir in cold, modified Krebs-Henseleit solution (composition: NaCl: 118.3mM, KCl: 4.7 mM, $MgSO_4.7H_2O$: 1.2 mM, $CaCl_2$: 2.5 mM, K_2HPO_4: 1.2 mM, D(+) Glucose monohydrate: 11.1 mM), and transferred immediately to the laboratory. We used two left circumflex coronary artery rings from one sheep and six sheep in each group. Left circumflex coronary arteries were dissected free of fat and connective tissue and cut into 0.2-0.3 cm ring segments. Endothelial layers of some rings were removed by gentle rubbing with a wooden probe. Ring samples were then mounted on two stainless steel hooks one of which was attached to the end of a bathing tube and the other to a transducer [TDA 96 Transducer Data Acquisition System (May Com)-Ankara, Turkey] in 10 ml organ baths under a resting tension of 1g (18). Changes in arterial tensions were recorded isometrically by the transducer. Successful removal of the endothelium was confirmed by the lack of relaxant response to Ach (10 mM). Krebs-Henseleit solution was gassed with a mixture of 95%O_2 and 5%CO_2, and adjusted to pH:7.4. The temperature was maintained at 37°C.

Before the start of experiments, the arterial rings were allowed to equilibrate for 60-90 min. and they were washed every 15 min prior to the protocols being undertaken. In the set of experiments, after the equilibration period, tonus of the coronary arterial rings became constant. 1g of resting tension values were considered as baseline values. After that arterial rings were precontracted with KCl (30 mM) (18). Contraction levels were recorded when the contractions reached to the plateau. This period was approximately 15-30 min. The difference between the plateau values and 1 g resting tension values were considered as the contraction values provided by KCl (30 mM). Then cumulative concentrations of testosterone (10^{-7}-10^{-4}) were

added. Relaxations to cumulative concentrations of testosterone were recorded. Each cumulative concentration was applied after the relaxation to previous concentration reached to plateau. This period was 5-15 min. Testosterone was dissolved in dimethyl sulfoxide (DMSO) + distilled water (1/5v/v). The results were calculated by eliminating the effects of DMSO initially recorded in each preparation by excluding the vasodilatory response of DMSO from observed testosterone vasodilatory response. NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 10^{-4}M) solved in distilled water and e-NOS stimulant L-arginine (10^{-4}M) solved in distilled water, were added to the organ bath 5 minutes before being precontracted with KCl (30 mM).

The groups we used in this study were; Group 1: male, with endothelium, (n=6); Group 2: female, with endothelium, (n=6); Group; 3: male, without endothelium, (n=6); Group 4: female, without endothelium, (n=6).

Chemicals

1. Testosterone propionate (Organon, Istanbul, Turkey).
2. Potassium chloride (KCl) (Merck; 64271, Darmstad, Germany).
3. Nw-Nitro-L-arginine Methyl Ester (L-NAME) $C_7H_{15}N_5O_4$. HCL (Sigma; St Louis MO, 63178, USA).
4. L-arginine $C_6H_{14}N_4O_2$ (Sigma; St Louis MO, 63178, USA).
5. Dimethylsulfoxide $C_2H_6O_5$ (Sigma; St Louis MO, 63178, USA).
6. Acethylcholine chloride $C_7H_{16}NO_2Cl$ (Sigma; St Louis MO, 63178, USA).

All drugs were prepared on the day of the experiment.

Statistical analysis

All statistical analyses were applied using a computer statistical package (SPSS software for Windows, version 15.0, Chicago, IL, USA). The property of our data distribution has been investigated using the Kolmogorov-Smirnov and Shapiro-Wilk tests. As a result of the tests, our results showed normal distribution. Therefore, parametric tests were used. All results are expressed as mean±S.E.M. Relaxation is expressed as percentage relaxation of contraction induced by KCl (30 mM). Comparisons between two groups and within groups were evaluated using Student's t-tests for paired and unpaired data. Comparison among multiple groups were made by using a one-way ANOVA test. Differences were considered significant at p<0.05.

Results

The removal of the endothelium abolished testosterone-induced relaxation in the male (10^{-7},$^{-4}$M) group (p<0.05, p<0.01) (Fig.1) and significantly decreased in the female ($10^{-7,-5,-4}$M) group (p<0.05) (Fig. 2). The relaxation to testosterone ($10^{-6,-5,-4}$M) in the male with endothelium group was more than that of female group with endothelium (p<0.05, p<0.01) (Fig. 3). But in the female without endothelium group testosterone ($10^{-6,-5,-4}$M)

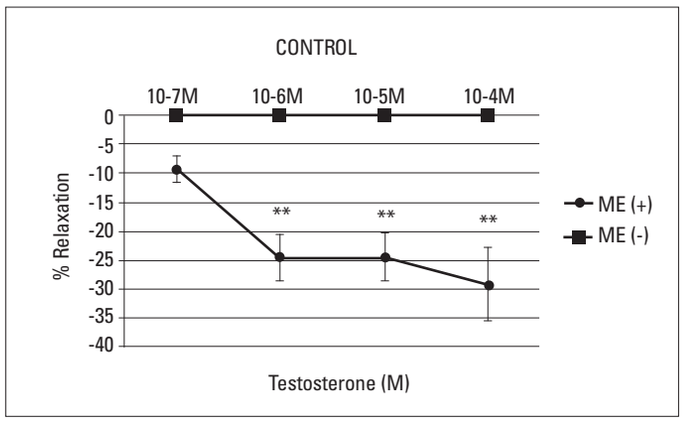

Figure 1. Cumulative relaxation of male endothelium to testosterone propionate (10^{-7}-10^{-4}M) in coronary artery rings precontracted with KCl (30mM)
Data are presented as mean±SEM
*Student's t- test, * p<0.05, ** p<0.01: compared with ME(-)
TesME(+) - testosterone male with endothelium, tesME(-) - testosterone male without endothelium

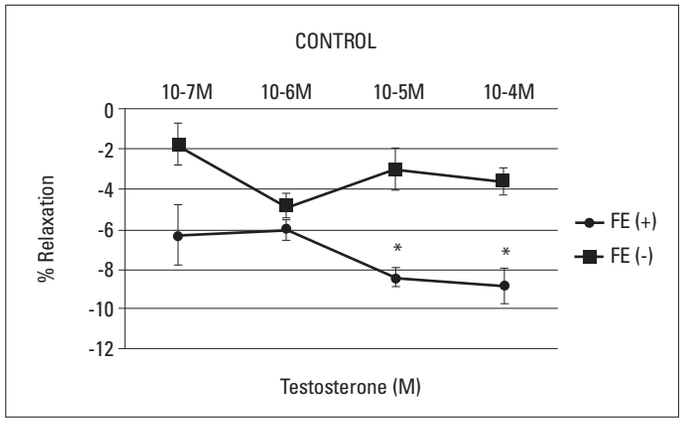

Figure 2. Cumulative relaxation of female endothelium to testosterone propionate (10^{-7}-10^{-4}M) in coronary artery rings precontracted with KCl (30mM)
Data are presented as mean±SEM
*Student's t -test. * p<0.05: compared with FE(-)
TesFE(+)- testosterone female with endothelium, tesFE(-)- testosterone female without endothelium

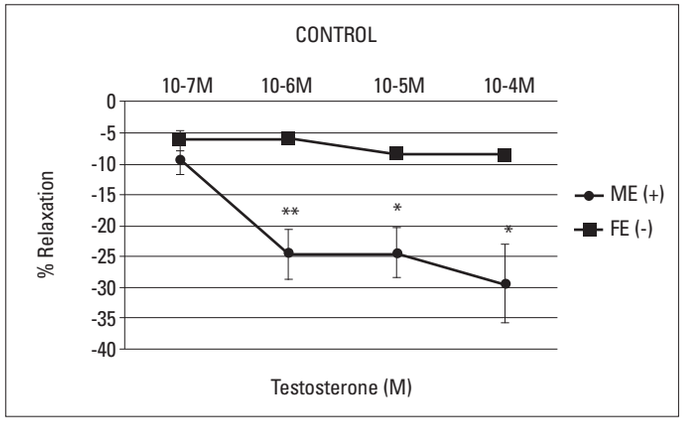

Figure 3. Cumulative relaxation to testosterone propionate (10^{-7}-10^{-4}M) in coronary artery rings precontracted with KCl (30mM)
Data are presented as mean±SEM
* Student's t -test. * p<0.05, ** p<0.01: compared with FE (+)
TesME(+) - testosterone male with endothelium, tesFE(+) - testosterone female with endothelium

showed significant vasorelaxant activity ($p<0.05$, $p<0.01$, $p<0.001$). There was no relaxant effect in the male group without endothelium (Fig. 4).

In the male with endothelium group; relaxations to testosterone ($10^{-7,-4}$M) were significantly prevented by L-NAME (10^{-4}M) ($p<0.01$, $p<0.001$) and L-arginine (10^{-4}M) ($p<0.01$, $p<0.001$) respectively. There were no significant difference between the L-NAME (10^{-4}M) and L-arginine (10^{-4}M) groups (Fig. 5).

In the male without endothelium group; there were no relaxant effect of testosterone in the groups control, L-NAME (10^{-4}M) and L-arginine (10^{-4}M) (Fig. 6).

In the female with endothelium group; relaxations to testosterone ($10^{-7,-6,-5}$M) were more than that of the L-NAME (10^{-4}M) ($p<0.05$, $p<0.001$) and (10^{-7}M) the L-arginine (10^{-4}M) ($p<0.05$) groups respectively. In the L-arginine (10^{-4}M) group relaxation to testosterone ($10^{-6,-5}$M) was more than that of the L-NAME (10^{-4}M) group ($p<0.05$, $p<0.001$) (Fig. 7).

In the female without endothelium group; relaxations to testosterone ($10^{-7,-6,-5}$M) were more than that of the L-NAME (10^{-4}M) ($p<0.01$, $p<0.001$) and (10^{-6}M) the L-arginine (10^{-4}M) ($p<0.01$) groups respectively. In the L-arginine (10^{-4}M) group relaxation to testosterone ($10^{-7,-6}$M) was more than that of the L-NAME (10^{-4}M) ($p<0.05$) group (Fig. 8).

Discussion

We have demonstrated that testosterone ($10^{-7,-4}$M) has relaxant activity in isolated sheep coronary arteries precontracted with KCl (30 mM). Endothelium has an important role in testosterone induced vasorelaxant activity and NO play a modulatory activity in the present study.

It was noticed that the vasorelaxant activity of testosterone is higher in the male ($10^{-6,-5,-4}$M) with endothelium group than female with endothelium group ($p<0.05$, $p<0.01$) (Fig. 3). But relaxation to testosterone in the female without endothelium group was more than that of male without endothelium group ($p<0.05$, $p<0.01$, $p<0.001$) (Fig. 4).

Testosterone analog experiments established that relative polarity and lipid solubility of the androgen molecule are important determinants of its vasorelaxation efficacy and potency (16). Testosterone propionate is a polar, least lipid-soluble, and smallest molecular weight (MW: 344.5) analog (2). Polar, non-permeable testosterone analogues have been shown to elicit greater vasodilatation than non-polar, permeable analogues and non-genomic testosterone analogues have also been shown to elicit greater vasodilatation than genomic-acting analogues (19).

To date, studies have employed varied species (human, monkey, pig, rabbit, rat and guinea pig), vascular beds (coronary, femoral and aortal), disease states (normal, experimental atherosclerosis and experimental hypertension) and androgen exposures (chronic, acute, physiological and supraphysiological) (1, 20). Testosterone is recognized to act as a rapid coronary

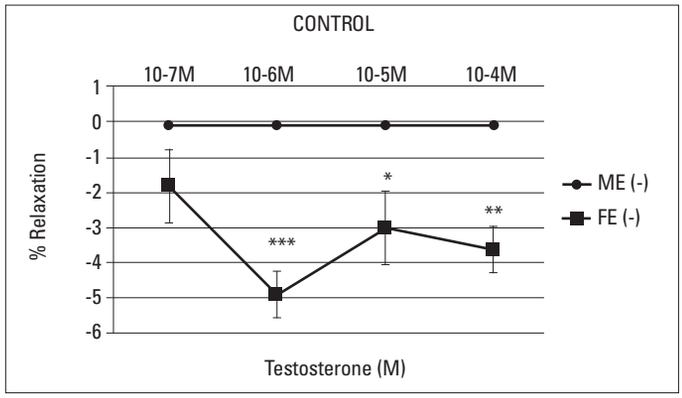

Figure 4. Cumulative relaxation of male and female without endothelium to testosterone propionate (10^{-7}-10^{-4}M) in coronary artery rings precontracted with KCl (30mM)

Data are presented as mean±SEM

* Student's t- test.* $p<0.05$, ** $p<0.01$, *** $p<0.001$: compared with ME(-)

TesME(-)- testosterone male without endothelium, tesFE (-)- testosterone female without endothelium

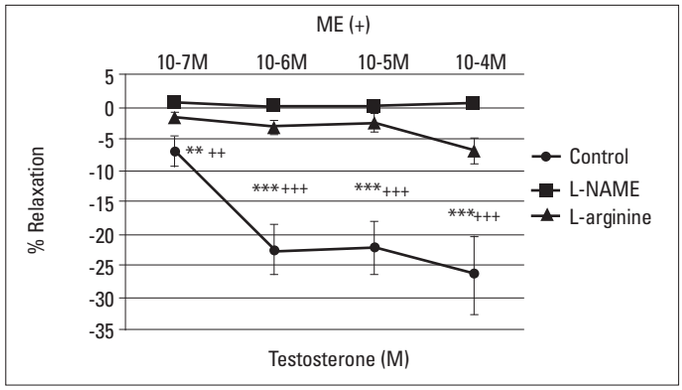

Figure 5. Comparison of the cumulative relaxant responses to testosterone propionate (10^{-7}-10^{-4}M) in coronary artery rings precontracted with KCl (30 mM), after preincubated with L-NAME (10^{-4}M) and L-arginine (10^{-4}M), male with endothelium

Data are presented as mean±SEM

*One-way ANOVA analysis, **$p<0.01$, *** $p<0.001$: compared with L-NAME (10-4M), ++ $p<0.01$, +++ $p<0.001$: compared with L-arginine (10^{-4}M)

TesME(+)- testosterone male with endothelium

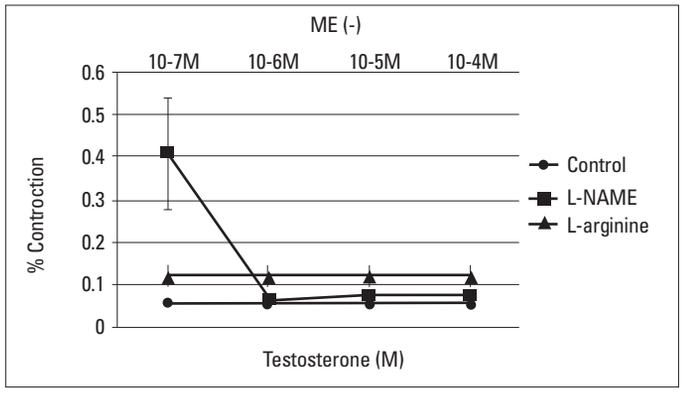

Figure 6. Comparison of the cumulative relaxant responses to testosterone propionate (10^{-7}-10^{-4}M) in coronary artery rings precontracted with KCl (30 mM), after preincubated with L-NAME (10^{-4}M) and L-arginine (10^{-4}M), male without endothelium

Data are presented as mean±SEM

*One-way ANOVA analysis

TesME(-) - testosterone male without endothelium

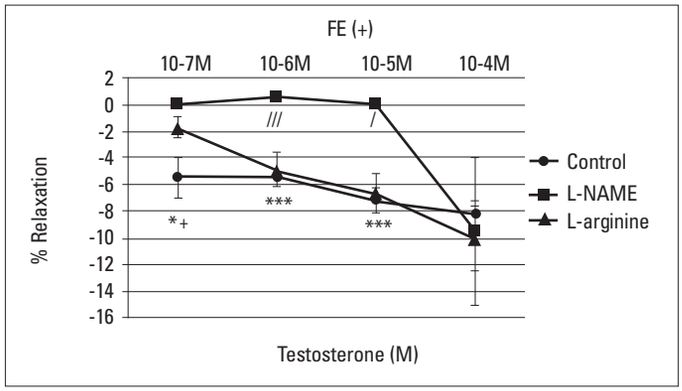

Figure 7. Comparison of the cumulative relaxant responses to testosterone propionate (10^{-7}-10^{-4}M) in coronary artery rings precontracted with KCl (30 mM), after preincubated with L-NAME (10^{-4}M) and L-arginine (10^{-4}M), female with endothelium

Data are presented as mean±SEM

*One-way ANOVA analysis, *p<0.05, ***p<0.001: compared with L-NAME (10^{-4}M), +p<0.05: compared with L-arginine (10^{-4}M), /p<0.05, ///p<0.001: compared with L-arginine (10^{-4}M)

TesFE(+) - testosterone female with endothelium

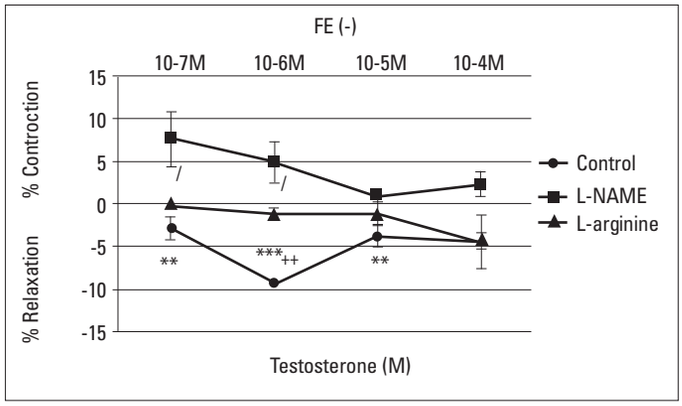

Figure 8. Comparison of the cumulative relaxant responses to testosterone propionate (10^{-7}-10^{-4}M) in coronary artery rings precontracted with KCl (30 mM), after preincubated with L-NAME (10^{-4}M) and L-arginine (10^{-4}M), female without endothelium

Data are presented as mean±SEM

*One-way ANOVA analysis

**p<0.01, ***p<0.001: compared with L-NAME (10^{-4}M), ++p<0.01: compared with L-arginine (10^{-4}M),/p<0.05: compared with L-arginine (10^{-4}M)

TesFE (-) - testosterone female without endothelium

vasodilator both *in vitro* and *in vivo* in animal and human preparations (7-9, 21-24).

Previous studies have investigated various mechanisms [possible role of endothelium, cyclic GMP (cGMP), vasodilator prostanoids, testosterone receptors, potassium conductance, calcium influx] in the response to testosterone (1, 2, 7, 8, 19, 25, 26).

Inhibition of NO synthesis by L-NAME (10^{-4}M) decreased testosterone-induced relaxation both in the male ($10^{-7,-4}$M) (p<0.01, p<0.001) and female ($10^{-7,-5}$M) (p<0.05, p<0.001) with endothelium groups (Fig. 5, 7).

Preincubation with L-arginine (10^{-4}M), decreased testosterone-induced relaxation ($10^{-7,-4}$M) in the male with endothelium group (p<0.01, p<0.001) (Fig. 5). In the male group without endothelium, we did not observe testosterone induced relaxation in the control, L-NAME (10^{-4}M) and L-arginine (10^{-4}M) groups (Fig. 6).

Preincubation with L-arginine (10^{-4}M) decreased testosterone-induced relaxation only (10^{-7}M) (p<0.05) and (10^{-6}M) (p<0.01) in the female with and without endothelium groups respectively. Testosterone-induced relaxations were statistically different between the groups L-NAME (10^{-4}M) and L-arginine (10^{-4}M) in the female with ($10^{-6,-5}$M) (p<0.05, p<0.001) and without ($10^{-7,-6}$M) (p<0.05) endothelium groups respectively (Fig. 7, 8).

Endothelium is a fine layer of cells that covers the luminal surface of all blood vessels and plays a crucial role in the regulation of cardiovascular homeostasis (27). Vascular endothelium regulates the underlying smooth muscle layer and vascular tone by release of endothelium-derived relaxing factors such as nitric oxide (NO), prostaglandins, and endothelium-derived hyperpolarizing factor (EDHF) as well as vasoconstricting factors such as endothelin, superoxide (O2-), and thromboxane (25, 28-32). Nitric oxide (NO) is a relatively stable gas, with ability to easily diffuse through the cell membrane. NO plays an important role in many physiological processes and is important in regulation of vascular system (27, 31, 32). The main receptor for NO is guanylyl cyclase leading to formation of smooth muscle cyclic guanosine monophosphate (c-GMP) and relaxation (25, 30, 33). Nitric oxide is mainly, but not exclusively, responsible for the vasodilatation. Some of the previous studies demonstrated that removal of the endothelium greatly reduced the response, but a dilatation was still observed (34). Most agents that activate release of NO from the endothelium also hyperpolarize both endothelial and smooth muscle cells (35).

The majority of studies have demonstrated that testosterone induced an equal degree of relaxation in vessels with and without endothelium (1, 2, 7, 19-21, 24, 26, 36-38). L-NAME, an inhibitor of EDRF synthesis did not affect the relaxation by testosterone. Methylene blue, an inhibitor of EDRF induced increase of cGMP, also had no effect on relaxation induced by testosterone (7, 22, 24, 26).

Some of these studies also report that indomethacin (an inhibitor COX), has no inhibitory effect on testosterone-induced relaxation (1, 2, 7-9, 19, 39, 40).

Therefore our results suggest that the *in vitro* relaxation of sheep coronary arteries by testosterone, seems to be generally mediated via endothelium-dependent NO release and may involve other endothelial vasodilator factors (EDHF, prostaglandins etc).

Our results do not agree with those from previous studies which have utilized other *in vivo* and *in vitro* animal models to demonstrate that the vasodilatory action of testosterone is not solely reliant upon the presence of intact endothelial cell layer and blockade of endothelial NOS with L-NAME had no impact upon the coronary vasodilatory efficacy of testosterone (7, 22, 26, 36, 37, 39, 41).

Even in the few studies in agreement with our observations, that do demonstrate a reduction in the efficacy of testosterone following such interventions, the observed attenuation is modest, with a sizeable portion of the vasodilatory response remaining (1, 19, 26, 36, 38, 41, 42).

In addition previous studies have also demonstrated that testosterone induced a sex-independent vasodilatation *in vivo* and *in vitro* (7, 8, 43). However, according to our study, we consider that testosterone induces sex-dependent vasorelaxation in isolated sheep coronary arteries with or without endothelium (Fig. 3, 4).

On the other hand we have also demonstrated that relaxation to testosterone ($10^{-7,-5,-4}$M) in endothelial denuded ($p<0.05$) (Fig. 2) group was decreased in the female group. But in the presence of L-NAME (10^{-4}M) with ($p<0.05$, $p<0.001$) and also without ($p<0.01$, $p<0.001$) endothelium groups relaxations to testosterone ($10^{-7,-6,-5}$M) were decreased in accordance to the control groups. Relaxations to testosterone (10^{-7}M), (10^{-5}M) were also decreased in L-arginine (10^{-4}M) with ($p<0.05$) and without ($p<0.01$) endothelium groups respectively (Fig. 7, 8).

In the female groups, it seems to be mediated in part via endothelium-dependent vasodilator factors but our findings suggest that testosterone-induced coronary vasodilatation may also occur with some other mechanisms except the endothelial vasodilator factors.

Especially in the male but partly in the female groups relaxations to testosterone were not statistically different between the groups; control, L-NAME (10^{-4}M), and L-arginine (10^{-4}M) (Fig. 5-8). NO is generated by endothelial NO synthase (NOS) using L-arginine as a substrate. Nitric oxide-derived oxidants [nitrate, nitrite, peroxynitrite ($ONOO^-$)] plays important roles in cardiovascular physiology in numerous animal species and in human (28).

Previous studies have demonstrated different results about the vascular effects of L-NAME and L-arginine. Inhibitors of NOS synthase have been demonstrated not to fully inhibit the production of NO. Thus, in their presence, residual NO can be produced by endothelial cells and contribute to relaxation and/or hyperpolarization of the underlying vascular smooth muscle (28, 44).

Vascular endothelial cells have a capacity to produce superoxide and H_2O_2 from several intracellular sources, including eNOS. Superoxide anion has been reported to inactivate NO and this may attenuate endothelium-dependent relaxation. On the other hand superoxide has been reported to degrade to H_2O_2 spontaneously which is known to have a direct relaxing effect on the vascular smooth muscle or peroxynitrite, formed from rapid reaction between NO and superoxide, causes relaxations in some coronary arteries. The potency of the observed peroxynitrite effects is approximately 40 to 200-fold less than that of nitric oxide. These results demonstrate that depending on the tissue and the experimental conditions, vasoconstrictor and vasodilator factors possess dilator or constrictor properties, and can hyperpolarize or depolarize vascular smooth muscle cells (VSMCs) (28, 44).

Therefore, it is often difficult to reach a conclusion about the real importance of endothelium-dependent responses because of the use of non specific pharmacological tools and the lack of electrophysiological measurements.

Testosterone-induced vasorelaxation in the female groups may be mediated via more than one mechanism. Increasing lines of evidence indicate that the vasodilatory effect of testosterone might be occurred via non-genomic mechanisms, independent of the classical nuclear androgen receptor (AR). It has been demonstrated previously in a rabbit model that coronary vasodilatory action of testosterone is not affected by AR blockade. The majority of the vasodilatory action of testosterone is affected by a direct action on the vascular smooth muscle. At present, experiments aimed at finding the mechanism underlying this response support two contradictory hypotheses, 1. Potassium (K^+) channels activation or 2. Calcium (Ca^{2+}) channel antagonism (1, 2, 8, 19-21,26, 37, 38, 42, 43, 45-47).

There is a complex-mixture of vessel-type dependent mechanisms reported in previous studies about the type of the K^+ channel involved in the vasodilatory action of testosterone. In rabbit, dog, and pig coronary conduit arteries, testosterone has been shown to act via non-selective large conductance Ca^{2+}-activated potassium channel (BKCa) and voltage-sensitive potassium channel (KV) opening action, but testosterone-induced vasodilatation has been shown to occur via ATP-sensitive potassium channel (KATP) opening action in dog coronary resistance arteries. In human internal mammary artery (IMA) testosterone acts via BKCa opening action (2, 7, 8, 39). Testosterones thus induce rapid effects that appear to represent acute non-genomic effects, possibly transmitted by membrane receptors independent of the classical nuclear androgen receptor (8, 19, 20, 45).

The difference between our study and the previous studies discussed in proposing a vasodilatory mechanism for testosterone is not apparent, but the variance in the species, vascular preparations used, region of the vessel and *in vitro* and *in vivo* models is likely to be contributory.

As stated by Seyrek et al. (2) solvents (e.g. ethanol and dimethysulphoxide) used in different human and animal studies and concentrations have nonselective effects on ion channel activity, within milliseconds, which could interfere with the selective actions of testosterone. So in our experiment we eliminated the effects of DMSO by excluding the vasodilatory response of DMSO from observed testosterone vasodilatory response.

In addition, previous studies have also demonstrated that *in vivo* studies and also *in vitro* organ bath studies demonstrate the effects at only supraphysiological levels (1, 7, 8). Testosterone-mediated coronary vasodilatation *in vivo* is reported at concentrations around dilatation 100 nM and isolated vessel studies commonly require high (10-100μM) micromolar concentrations of testosterone to induce vasodilatation. In order to elicit dilatation in an isolated vessel, testosterone must first diffuse to the vessel surface and then permeate into a large enough number of smooth muscle cells within the vessel to produce a significant response (20).

Study limitations

The most important limitation of our study was the difficulty to supply the sheep from the local abattoir. Our study included a relatively small number of sheep due to the difficulty of supplying the sheep from the same genus and same age range. On the

other hand, in the present study we did not planned to research primarily some other mechanisms (cGMP, vasodilator prostanoids, potassium conductance, calcium influx etc.) in the response to testosterone.

Conclusion

In conclusion, the results of the present study show that the vasorelaxant effects of testosterone in sheep coronary arteries are mediated via the vascular endothelium in the male group. But in the female group testosterone induces both endothelium-dependent and endothelium-independent relaxation. Testosterone -induced relaxation is greater in male with endothelium group than female with endothelium group. Also in the female without endothelium group relaxation to testosterone is more than that of male without endothelium group. Testosterone-induced relaxation in female without endothelium group may be considered to be mediated in part via the vascular endothelium and also via non-genomic mechanisms. Our results are insufficient to show that there are non-genomic effects of testosterone. So this is a major gap in the current study. Further investigations, especially in different species, different vascular preparations and different *in vivo* and *in vitro* models are required to clarify the complete mechanisms of the effects of testosterone.

Conflict of interest: None declared.

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