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COMBINED EFFECTS OF HYPERTENSION AND TESTOSTERONE DEFICIENCY ON CORONARY RESISTANCE ARTERIES

PhD thesis

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LIST OF ABBREVATIONS

1K1C	1-kidney-1-clip
ADMA	asymmetric dimethylarginine
ADP	adenosine diphosphate
AII	angiotensin II infused group
AII+OCT	angiotensin II infused plus orchidectomized group
ANOVA	analysis of variance
AT_2R	angiotensin II type 2 receptor
BW	body weight
Ca^{2+}	calcium ion
CAD	coronary artery disease
CHD	coronary heart disease
Co	control group
CVD	cardiovascular disease
DHT	dihydrotestosterone
DOCA	deoxycorticosterone acetate-salt
DSS	Dahl salt-sensitive
dTGR	double transgenic rat
EGFR	epidermal growth factor receptor
eNOS	endothelial nitric oxide synthase
EPCs	endothelial progenitor cells
GHR	genetically hypertensive rat
HDL	high-density lipoprotein
HW	heart weight
LDL	low-density lipoprotein
L-NAME	N-nitro-L-arginin-methyl-ester
MAP	mean arterial pressure
mRNA	messenger ribonucleic acid
NO	nitric oxide
OCT	orchidectomized group

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$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PKC	protein kinase C
PWV	pulse wave velocity
RAAS	renin-angiotensin-aldosterone system
ROCK	Rho-associated kinase
S1P	sphingosine-1-phosphate
SHR	spontaneously hypertensive rat
SHRSP	stroke-prone spontaneously hypertensive rat
TG	triglyceride
TGF-β	transforming growth factor-β
TxA2	thromboxane A2
WKY	Wistar-Kyoto

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1. INTRODUCTION

1.1. Vascular effects of hypertension

One of the most serious health problems nowadays is the high number of cardiovascular deaths. Plenty of risk factors are known to increase the risk of cardiovascular disease, one of the best known of which is hypertension. A myriad of factors have an effect on blood pressure, including uncontrollable factors such as age, sex hormones, sex chromosomes, and factors which can be influenced such as lifestyle (1). The most important lifestyle factors that increase high blood pressure and thus CVD risk are alcohol (moderate to high intake), smoking and obesity (1). It is important to mention, that there are significant gender differences in the risk of CVD including high blood pressure. Namely, women exhibit lower cardiovascular risk, which disappears after menopause (1-4).

Hypertension results in damage to the arteries, which appears in both the large and small arteries (atherosclerosis, arteriosclerosis, and arteriolosclerosis) (5). However, the reverse is also true, structural and functional changes in the vascular system can precede and even cause hypertension.

1.1.1. Effect of hypertension on large arteries

The two main characteristic manifestations of hypertensive vascular damage are atherosclerosis and increased arterial stiffness (6, 7). Atherosclerosis is a chronic, mostly local inflammatory lesion, characterized by endothelial dysfunction, subintimal lipid accumulation, and the appearance of inflammatory cells (8). Intracoronary thrombosis and acute coronary syndrome may be provoked when a plaque along the atherosclerotic coronary vessel segment is ruptured (9). Another characteristic hypertensive lesion in large vessels is the increase in vessel wall stiffness. Arterial stiffness may increase irreversibly as a result of hypertension, because hypertension causes structural remodeling in the arterial wall, which is characterized by atherosclerosis, smooth muscle cell hyperplasia/ hypertrophy, and collagen deposition (6). In regards to cardiovascular mortality and disease it has also been described to be an independent predictive factor, its value increases with age (10, 11). Pulse Wave Velocity (PWV) is the measurement used

to quantify stiffness of the vessel segment. The augmentation index is also used - it may be measured with a relatively simple technique, the Arteriograph, and its 24-hour version, the mobile O-graph (12-15).

1.1.2. Effect of hypertension on resistance arteries

Arterial segments where the diameter of the lumen is <350 μm and arteriole segment where it is<100 μm have a pivotal role in regards to blood pressure regulation, accounting for almost 50% of total peripheral resistance, for this reason they may be referred to as "resistance" arteries (5). As a result of essential hypertension, the resistance arteries are remodeled, which manifests in altered morphology, biomechanics, and function of the vessels.

1.1.2.1. Morphological adaptation

As a result of high blood pressure, the vessel wall thickens and the lumen narrows, this leads to the increase in the ratio of wall thickness values and lumen measurements. However, the cross-sectional area –quantifying the sum of vessel wall tissue around the lumen–does not change initially, thus inward eutrophic remodeling occurs. This type of remodeling is characterized by the same amount of vessel wall tissue being rearranged centering around a decreased size lumen (16, 17). Such remodeling is found in SHR-, 2-kidney-1-clip Goldblatt-, and Ang II -induced hypertension animal models (17). However, hypertrophic remodeling for hypertension has also been described for a prolonged period of time and for very severe hypertension: DOCA-salt rats, 1-kidney-1-clip (1K1C) Goldblatt rats, in Dahl salt sensitive rats (17-21), and in elderly SHR animals (in young SHR animals eutrophic remodeling occurs) (22).

The morphological adaptation due to hypertension is the result of a very complicated and complex process. In short, hypertension activates vasoactive peptides, such as angiotensin, endothelin (it is important to note that the reverse is also true, vasoactive peptides lead to hypertension). Vasoactive peptides, and in part, increased oxidative stress cause vasoconstriction, smooth muscle cells become hypertrophied, and finally, inflammation and vascular fibrosis lead to remodeling of the vessel wall (17, 23-25).

1.1.2.2. Biomechanical adaptation

The most important parameters characterizing the mechanical function of blood vessels are the tangential (circumferential) wall stress, the distensibility, and the elastic modulus. The value of tension measured in the vessel wall demonstrates a direct and proportional relation to the values measured regarding transmural pressure and regarding inner radius of the vessel wall. Wall tension has also been proved to show an inverse and proportional relationship to wall thickness (26). Under hypertension, intraluminal pressure increases, and as a result, wall tension increases in the vessel wall. However, due to the increased vascular tone detailed above, the wall tension remains at an optimal level (27). Over time, not only does the spontaneous tone increase, but inward eutrophic remodeling occurs, which also prevents an increase in tangential wall stress. The term distensibility can be used to characterize the degree of blood vessel distension, and the term elastic modulus describes the elasticity of the material of the vessel wall. High distensibility and low elastic modulus indicate high expansive – capacity and flexibility. In large elastic arteries, the distensibility decreases with hypertension (28). In case of resistance arteries, however, contradictory results can be found. In resistance saphenous arteries distensibility decreases, elastic module increases due to hypertension (29). In contrast, in coronary resistance arteries increased blood pressure results in increased distensibility and decreased elastic modulus in the high pressure range (27). An explanation for this phenomenon may be the diversity of the vascular network within the body: vessels might have different functions, and the vessels located in different vascular beds are affected by very different effects. In addition, the extent and duration of the hypertensive stimuli also influence vascular adaptation, as, for example a milder and earlier hypertension initiates inward eutrophic remodeling, whereas hypertrophic remodeling develops in a later phase.

1.1.2.3. Functional adaptation

The most characteristic vascular adaptation to hypertension is the increase in myogenic tone and vasoconstrictor response (30). The physiological background of increased myogenic tone is very diverse. TGF-β (transforming growth factor-β) stimulates EGFR (epidermal growth factor receptor) expression in smooth muscle cells (31). Furthermore, increased myogenic tone seems to require Ca^{2+}-influx through L-type voltage-gated

Ca^{2+}channels (32). Another important factor in the increased resistance of coronary vessels is the damage to endothelium-dependent dilatation. Basal NO bioavailability and function decrease in hypertension, however, there is no difference in stimulated NO - release and bioavailability (33). Furthermore, endothelium-independent dilatation (by the nitrovasodilator linsidomine) is also not different between normotensive and hypertensive animals (34). Similar to myogenic tone, hypertension is also characterized by an increased vasoconstrictor response. Response to thromboxane is increased in coronary resistance vessels, serotonin sensitivity increases in mesenteric resistance vessels, and maximal contraction elicited by norepinephrine also increases in saphenous resistance vessels in angiotensin II-induced hypertension (27, 35, 36).

1.1.3. Animal models of hypertension

Models to replicate conditions of hypertension using animals are extremely diverse (37). In various studies, the list of hypertension models extends through different genetic models to models generated by high salt diet that mimics the diseases of civilization. The most frequently used models include:

- Genetic models (SHR, spontaneously hypertensive rat; SHRSP, stroke-prone spontaneously hypertensive rat; GHR);
- Salt-sensitive models (DSS, Dahl salt-sensitive; DOCA-salt, deoxycorticosterone acetate-salt);
- Renovascular models (1-Clip Goldblatt; K2-1C; 2-kidney, 1-clip; Subtotal nephrectomy);
- NO dependent models (L-NAME (N^{ω}-nitro-L-argnine-methyl-ester) induced hypertension: SHR/L-NAME);
- Ang II-dependent models (REN2 transgenic rats; dTGR, double transgenic rat; AngII infused hypertension rat model) (37).

One of the most suitable methods for studying hypertensive lesions is the angiotensin –infused hypertension rat model. This model is often generated by subcutaneous angiotensin administration via an osmotic minipump (implanted under the skin) containing angiotensin II, which causes chronic hypertension in two to three weeks. This animal model has several shoulder species, depending on how much angiotensin II dose the animals receive per kilogram of body weight. Accordingly, this model can be divided

into 3 groups: 100 ng / min / kg, 400 ng / min / kg and more than 1000 ng / min / kg. Low dose angiotensin II infusion of 100 ng / min / kg results in chronic hypertension after 2-3 weeks without an acute increase in blood pressure – this corresponds to an early hypertensive animal model (38). A dose of 400 ng / min / kg may mimic the gradual development of primary hypertension in humans (39). Doses above 1000 ng / min / kg have been used to test the function of angiotensin II type 1 receptor, however, this dose represents a pharmacological level, much more above the levels of angiotensin II observed in human subjects with high blood pressure. It produces a marked increase in blood pressure and renal damage after 4 weeks of treatment by lymphocyte activation (37).

1.2. Vascular effects of testosterone deficiency

The principal androgen, testosterone, is a vasoactive steroid hormone produced primarily by the testes; it provides a vital hormonal basis for the development of genitalia and secondary sexual character in males, and also maintenance of reproductive function. Its anabolic effect is basically manifested in stimulating the production of skeletal muscles and bone.

Testosterone levels may be reduced due to a number of diseases. The prevalence of testosterone deficiency varies widely: 2,1-38,7% in men who have reached middle-age or are elderly and this value may rise to 50% in diabetes or obesity (40). Primary causes include testicular disease, secondary causes include lesion of the hypothalamus-pituitary axis. The term ‘functional causes’ is used for inflammatory conditions, obesity, or chronic diseases (41). Interestingly, testosterone deficiency itself is very often associated with diabetes, obesity, cardiovascular disease, or osteoporosis, which in themselves lead to testosterone-deficiency too. This creates a vicious circle as testosterone deficiency increases abdominal fat deposition, increasing the chances of obesity, type II diabetes mellitus or metabolic syndrome, which may have an adverse effect on hypothalamic-pituitary function,; this in turn has a consequential detrimental effect on testosterone levels (40).

To understand the effects of testosterone-deficiency, we must summarize the pillars of genomic and non-genomic testosterone effects.

1.2.1. Genomic and non-genomic testosterone effects

However, beside the major well-known effects, testosterone also has a significant effect on the function of the arteries and thus, on the cardiovascular system. This is evidenced by numerous literature data on the association between testosterone and cardiovascular risk. It is important to mention, that testosterone exerts its effects as a steroid hormone both genomically and non-genomically. Classical (genomic) effects are slow to develop and the effect is bound to androgen receptors, which modulate gene transcription and protein synthesis as transcription factors (42). In contrast, non-genomic effects develop rapidly, and these effects are independent from the intracellular androgen receptor. Interestingly, although the testosterone membrane receptor has already been identified, it is not yet entirely clear how all non-genomic androgen effects are mediated to the cardiovascular and other non-reproductive systems and organs (42). Its genomic actions include effect on cardiac morphology and function, increase in myocardial volume, and increase in cardiac output, possibly by reducing left ventricular afterload (43, 44). It is important to mention that androgens have a direct effect on angiotensin II type 2 receptors (AT_2R): AT_2R mRNA and expression of proteins in the aorta is lower in males than in females, and dihydrotestosterone (DHT) treatment also reduced AT_2R levels in females. Also, castration significantly increases AT_2R mRNA and protein expression (45, 46).

We emphasize that amongst its non-genomic effects there is an androgen receptor independent acute vasodilatory effect (47). DHT raises the levels of endothelial factor along the vasculature and it also has a beneficial effect on the migration, adhesion and proliferation, and capability of progenitor endothelial cells (45, 48). Its vasodilatory effect has also been described on mesenteric segments, aortic rings, basilar arteries and coronary arteries, in various animal species (49). Testosterone produces endothelium-dependent responses at physiological concentrations and endothelium-independent responses at supraphysiological doses (49). Furthermore, in coronary arteries, after removal of the endothelium, vasodilatation remained unchanged after testosterone administration, i.e., smooth muscle cells also participate in testosterone-induced relaxation (50). The observed vasodilation response occurs in both female and male animals (51, 52).

Physiological levels of testosterone inhibit $PGF_{2\alpha}$-induced Ca^{2+}influx – this was demonstrated in smooth muscle cell cultures – this is also an acute and non-genomic

testosterone effect. This could possibly play a role in vasodilation induced by testosterone (53). Furthermore, testosterone may contribute to the acute vasodilatory effect by activating potassium channels or inhibiting Ca^{2+}channels in smooth muscle cells (54). Beyond these vasodilatory effects, both the activity and the expression of endothelial nitric oxide synthase (eNOS) is also increased by testosterone; this in turn results in the production of nitric oxide (NO) (55, 56).

A further non-genomic effect is a rapid increase in the levels of calcium intracellularly. Besides this rapid increase in intracellular calcium, it should be noted, that in general low testosterone levels, e.g. hypogonadism, predispose to osteoporosis and osteopenia (45). There may be some interactions between receptor-mediated genomic and acute effects. Androgen receptor mRNA content in the aortic wall shows a positive correlation with blood testosterone levels (57), and in aortic smooth muscle cell culture testosterone increases androgen receptor expression, which was partially inhibited by androgen antagonists (58). In contrast, castration reduces alpha-1 adrenergic receptor expression in male rabbits and this decrease could be prevented by testosterone administration (59). In a very interesting study, sudden cardiac death caused by coronary heart disease (CHD) was studied in men and it was found that androgen receptor expression in the coronary wall is reduced in CHD patients, suggesting that androgens could play a beneficial role in cardiovascular function (60).

1.2.2. Testosterone deficiency and endothelial dysfunction

Cardiovascular complications caused by testosterone deficiency may be mediated by abnormal vascular function resulting from the deficiency itself. This pathological change can be detected in the alterations of the morphological, biomechanical and functional properties of blood vessels. Testosterone deficiency increases intimal hyperplasia and vascular wall stiffness (61-63). Interestingly, there is no literature data on the biomechanical and morphological changes of resistance coronary arteries caused by testosterone deficiency.

Testosterone affects the vascular function: both relaxation and contraction (55). Myogenic tone decreases in male castrated animals. Endothelium-independent (adenosine diphosphate, ADP) dilation in cerebral arteries does not change, while constriction caused by N-nitro-L-arginin-methyl-ester (L-NAME) decreases upon castration (55). Furthermore, endothelium-dependent relaxation decreases in the aorta as a result of castration. In the absence of testosterone, the degree of relaxation was similar to the degree of relaxation after endothelial removal (64).

Decreased endothelium-dependent relaxation due to testosterone deficiency may be associated with endothelial dysfunction (65). This may be explained by the previously mentioned fact, that testosterone increases the expression and activity of eNOS, which is responsible for NO production (56). Sphyngolipids may establish the link between testosterone deficiency and eNOS expression and activation (65). A sphingosine-1-phosphate-1-receptor (S1P1) is found on endothelial cells from mammals, while S1P2 and S1P3 receptors are found on smooth muscle cells from mammals (66, 67). S1P1 is responsible for relaxation, while S1P2 and S1P3 are responsible for contraction (65, 68). Castration decreases the expression of both S1P1 mRNA and receptors, while in the corpus cavernosum of the rat the expression of S1P2 and S1P3 mRNA and receptors are increased (69). The correlation of testosterone deficiency and sphyngolipids is shown in **Figure 1**. Testosterone deficiency related endothelial dysfunction may also be brought about by an increase in the levels of an endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA). Upregulation of mRNA expression of the protein arginine N-methyltransferase, which is responsible for the production of ADMA, may also further add to endothelial dysfunction in testosterone deficiency (65, 70-72). Furthermore both clinical and basic research demonstrates that testosterone levels are positively correlated

with endothelial progenitor cell (EPC) levels, these cells are an integral part of the repair system of the endothelium. Their level rises in vascular damage and endothelial dysfunction. Due to this property, EPCs are known markers of vascular damage, such as cardiovascular disease and atherosclerosis (73). Both adhesion and proliferation of EPC cells is increased significantly by dihydrotestosterone. This suggests that in hypogonadism testosterone treatment may have a significant beneficial effect on the dysfunction of the endothelium via modulation of EPCs (65, 74). Therefore, we may hypothesize that a deficiency of testosterone impairs NO production via down-regulation of eNOS and increasing ADMA levels. It also reduces the function of the endothelial repair system, which together lead to endothelial dysfunction and decreased endothelial dilatation (65).

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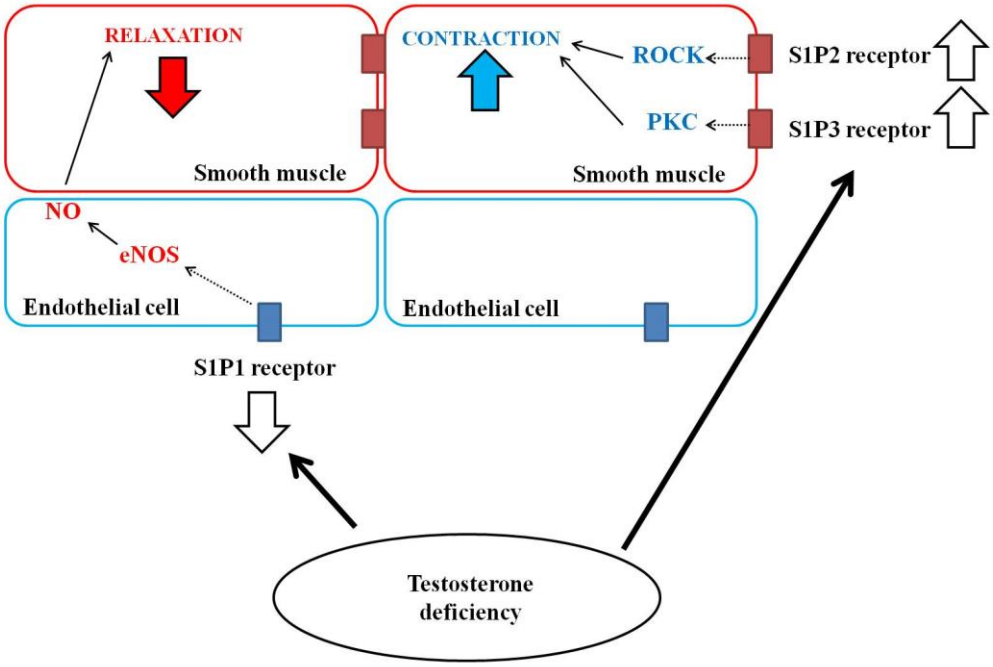

Figure 1. Correlation of relaxation of the smooth muscle cells and testosterone deficiency through sphyngolipids. The degree of Sphingosine-1-phosphate-1-receptor expression, responsible for dilation through eNOS activation, decreases with testosterone deficiency. In contrast, levels of sphingosine-1-phosphate-2 and 3 receptors, which are responsible for contraction in smooth muscle cells, increase with testosterone deficiency. Abbreviations NO, nitric oxide, NO; endothelial nitric oxide synthase, eNOS; Rho-associated protein kinase, ROCK; protein kinase C, PKC; sphingosine-1-phosphate, S1P. Upon Hotta et al (65).

1.2.3. Testosterone deficiency and coronary artery disease

Levels characteristic of normal total and free testosterone levels in young healthy men are laboratory and test dependent, with some laboratories considering the lower limit of normal to be 280-300 ng/dl for total testosterone and 5-9 pg/ml for free testosterone (75). However, testosterone levels decrease with age (andropause) (76). Based on literature data, it can be seen that this decrease, similar to menopause, has a harmful effect on cardiovascular disease (77, 78). Several studies regarding men have demonstrated the relationship between androgen levels and coronary heart disease. These studies concluded that optimal testosterone levels have a protective effect on the cardiovascular system. It has been demonstrated that when healthy men were compared with those suffering from

coronary artery disease their free androgen index, free and bioavailable testosterone levels were markedly higher (77). CAD severity and testosterone levels from the plasma were correlated inversely (79). It should be noted that beside testosterone levels estradiol levels were also measured to be lower in men with CAD (79). Testosterone may have a protective effect against CAD because due to its in vivo vasodilator effects (demonstrated in vitro). In human angina experiments administration of testosterone administration led to a reduction of symptoms and it also improved the ischemia itself as well (80). Beside the vasodilative effect of exogenous testosterone, we must emphasize that endogenous testosterone has been shown to potentiate the effects of the exogenous testosterone. This was demonstrated in a swine castration model where acute replacement of testosterone resulted in markedly lesser increases regarding coronary flow and a decrease in conductance of the coronaries compared to intact specimens (81). The potentiating effect of endogenous testosterone has been demonstrated to be independent of androgen receptor expression (81). A study conducted on homozygous twin men found that CAD and endogenous sex hormones were not associated phenomena (82). The differences between the studied groups themselves (homozygous twin men vs. men selected into the study at random from the population) and also the timing of blood sample collection (state of health vs. following CAD) may be responsible for the discrepancies. When reviewing results from the literature we may state that in men the risk of CAD is increased with the decrease of testosterone levels.

1.3. Vascular effects of hypertension & testosterone deficiency

Castration has an effect on blood pressure; however, the results are quite contradictory. In evaluating the various studies examining the effect of testosterone deficiency on blood pressure, a distinction should be made between normotensive and hypertensive conditions and between males and females. The differences regarding blood pressure regulation between the genders have been described a long time ago: Higher blood pressure values have been measured in men vs. women of the same age before menopause (83, 84). Testosterone contributes significantly to the above phenomenon: in men, an inverse relationship can be found between plasma testosterone levels and blood pressure, moreover, while lower testosterone levels in men are associated with high blood pressure, high testosterone levels in women increase arterial stiffness (85-87). Loh S. et al.

compared the mean arterial pressures (MAP) of female and male normotensive Wistar-Kyoto (WKY) and Spontaneous Hypertensive (SHR) rats produced by gonadectomy in both sexes, and by gonadectomy and testosterone replacement in females (85). Significantly higher levels of MAP were measured in intact animals in males compared to females. Gonadectomy led to a reduction of blood pressure values in both gender both in the normo- and the hypertensive groups. In female animals, after gonadectomy, 6 weeks of testosterone treatment increased MAP. This demonstrates that testosterone has a pivotal role in raising blood pressure (85). In another study in female and male SHR rats, blood pressure was also higher in males, and gonadectomy reduced blood pressure 7 months later, whereas no such effect was observed in females (88). In contrast, we can read about the high blood pressure caused by testosterone deficiency and the anti-hypertensive effect of endogenous testosterone (89, 90). Endogenous and exogenous androgens in long-term have antihypertensive effect on systolic blood pressure, which is estrogen independent, partly non-genomically, partly genomically regulated by reducing the expression of the renin-angiotensin- aldosterone system (89). According to these studies, this antihypertensive effect is also explained by the above mentioned systemic acute vasodilatory impact of testosterone (90). A further explanation for the blood pressure decrease after castration may be, that in the aorta, AT_2R mRNA expression is lower in intact males than in females. In females AT_2R levels were reduced by androgens. The effects of testosterone and testosterone deficiency on blood pressure requires further research. Both testosterone deficiency and hypertension significantly increase cardiovascular risk. However, the joint presence of these two noxa may not be independent of each other. Regardless of age, hypertension is associated with lower total testosterone levels in men (91).

Based on literature data, it is known that hypertension and testosterone deficiency have harmful effects on vascular function separately. However, the combined effect of the two noxa has not been studied in resistance coronary vessels to date, although an estimated 1.39 billion people worldwide is affected by hypertension in 2010, and the prevalence is expected to increase globally (92), furthermore, the prevalence of testosterone deficiency varies between 2,1-38,7% in elderly men and those who have reached middle age. This number appears to rise to approximately 50% when the men suffer from obesity or diabetes (40). The co-existence of the two noxa is not independent of each other, in

hypertension the odds ratios adjusted for other factors (morbidities and age) are 4.66 (93). Studies in mesenteric vessels, aortic rings, and limb arteries report primarily changes in vascular function, while no literature data can be found on morphological and biomechanical adaptations. Similar to the topic of testosterone deficiency and blood pressure, the available information on the combined effects of these 2 noxa on vascular function is contradictory: spontaneous tone and serotonin-induced vasoconstriction are reduced, and endothelial dilatation is increased (94, 95). Moreover, vascular damage caused by hypertension (worsening of endothelial dilatation) was partially restored by castration, whereas testosterone replacement reduced the rate of dilatation (96).

In answering the above question, both optimal testosterone levels and cardiovascular state may be the clue. Too high androgen levels, or taking anabolic steroids has been shown to have a harmful effect on the cardiovascular system (30, 97, 98), however, the number of cardiovascular complications increases in testosterone deficiency in elderly men as well (91, 99, 100).

Based on clinical experience and a large body of literature data, we know that hypertension and testosterone deficiency separately increase cardiovascular risk by both stimuli damaging vascular function. Hypertension leads to atherosclerosis and arteriolosclerosis, while testosterone deficiency leads to endothelial dysfunction. The vascular damaging effect of the 2 noxa is shown on Figure 2.

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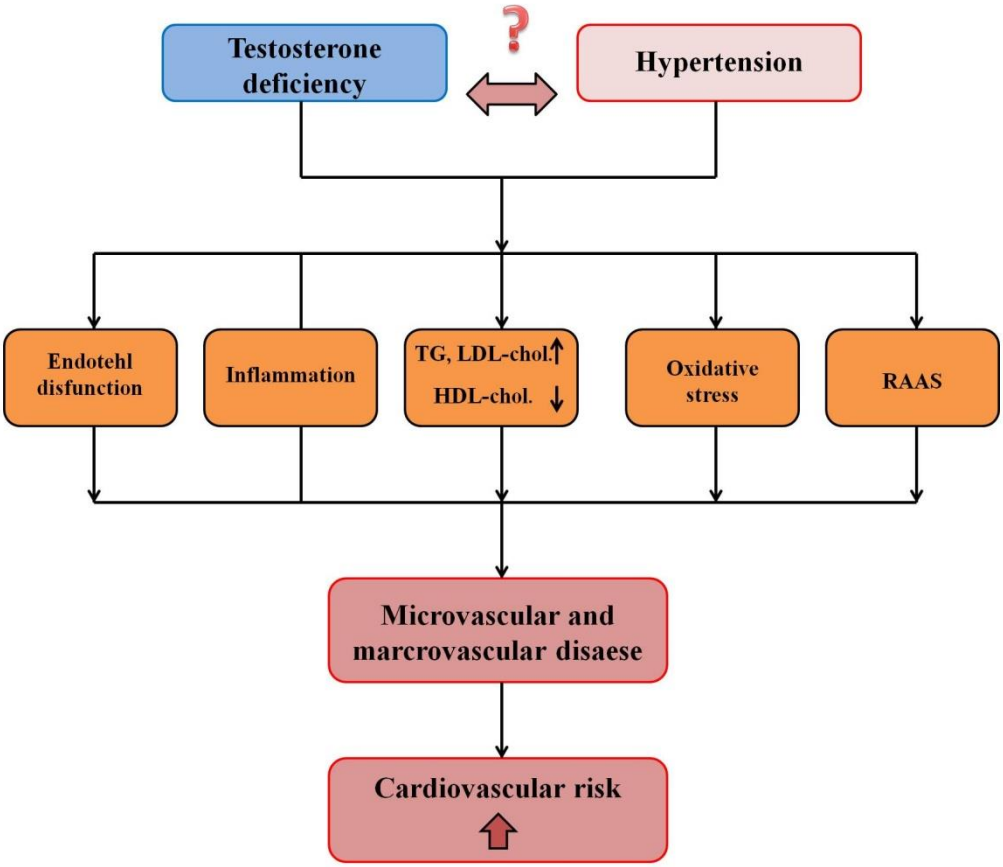

Figure 2. Vascular damaging effects of testosterone deficiency and hypertension. The two noxa lead to separate micro- and macrovascular damage which increases the risk of cardiovascular disease. Abbreviations: triglyceride, TG; low-density lipoprotein, LDL; high-density lipoprotein, HDL and renin-angiotensin-aldosterone system, RAAS.

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2. OBJECTIVES

Both andropause and hypertension have harmful effects on vascular function, each also increases the risk for coronary damage independently. The biomechanical characteristics of coronaries or small resistance arteries have sparsely been studied in this regard until now. However, dual effects of male hormone -depleted state and chronic angiotensin II administration on the function of coronary resistance arteries has not yet been analyzed, nor has the above dual effect been proposed to influence cardiovascular vulnerability. Therefore, in our present study, we focused on the functional, biomechanical and morphological changes in the resistance coronary vessel segments when both noxa are present.

In our andropausal hypertension four groups of Sprague-Dawley rats were analysed: control male (Co, n=10), orchidectomized male (OCT, n=13), angiotensin (AII) hypertensive male (AII, n=10) and AII hypertensive plus orchidectomized male (AII + OCT, n=8). In the OCT and AII + OCT groups surgical orchidectomy was performed. In the AII and AII + OCT groups chronic administration of angiotensin II was achieved via an osmotic minipump with an infusion rate of 100 ng/ min/ kg. Following 4 weeks of treatment by AII and anesthetization, direct blood pressure measurement was performed via right carotid artery cannulation. Following these procedures, the heart of the rats was extracted and vessel segments with diameters of around 200 μm in situ were dissected under magnification and careful microsurgical techniques from the intramural coronaries. The selected vessel segments were secondary branches of the left anterior descending coronary artery. Spontaneous tone, biomechanical properties (wall thickness to lumen diameter ratio, tangential wall stress and outer diameter), and functional characteristics (bradykinin- and thromboxane induced tones) of these segments were evaluated via in vitro pressure microarteriography.

In the above andropausal hypertension model, we aimed to investigate:

1. Both the biomechanical and the morphological characteristics of intramural resistance coronaries when testosterone – deficiency and hypertension are both present together. We also aimed to analyze a possible relationship between the vascular damage

caused by these two joint noxa especially regarding biomechanical properties of the investigated vessel segments.

2. In similar circumstances we aimed to test the contractility of intramural resistance coronaries, and the interactions between hypertension and testosterone deficiency from a functional aspect.

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3. METHODS

3.1. Animals

All animals used in the experiments were housed and catered in accordance with the National Society for Medical Research's Principles of Laboratory Animal Care as well as the Institute of Laboratory Animal Resources' "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23, revised 1996). The Semmelweis University Animal Care Committee and the Hungarian government both confirmed their approval for all of the study's protocols and animal handling (permission number: PEI/001/820-2/2015). 41 male Sprague-Dawley rats that were 2 months old and sexually mature were kept in conventional lab settings with 12-hour light-dark cycles.

The rats were randomized into four groups: control male (Co, n=10); orchidectomized male (OCT, n=13), angiotensin induced male (AII, n=10) and AII induced plus orchidectomized male (AII + OCT, n=8). After general anaesthesia, the rats underwent a sterile surgical procedure: an osmotic minipump was inserted dorsally into the subcutaneous layer. In the angiotensin-treated groups (AII and AII + OCT) the osmotic pump was filled with AII acetate (100 ng/bwkg/min).

The reason We chose this model was to explore early hypertensive vascular alterations since previous research showed that this sub-pressor dose caused chronic blood pressure elevation after 2 to 3 weeks without acute blood pressure elevation (36, 38, 101). Orchidectomy in the OCT and AII + OCT groups was also carried out under the same pentobarbital anaesthesia, using standard surgical methods.

3.2. Pressure arteriography of the coronary arterioles

After four weeks of AII treatment, intraperitoneal pentobarbital anaesthesia (45 mg/kg body weight) and right carotid artery cannulation was used obtain blood pressure data. After removal and weighing of the heart, careful microtechniques were used to isolate the intramural coronary arterioles, secondary branches of the left anterior descending coronary artery (with an in situ outer diameter of around 200 μm) (102). These 2 mm long arteriolar segments were taken out, transferred into a vascular chamber filled with nKR, and both ends of the segments were cannulated with plastic microcannulas. They

were then stretched out to their natural, in vivo, in situ length. The bath was maintained at a constant temperature of 37 °C and bubbled with a gas combination of 5% CO2, 20% O2, and 75% N2, which kept the pH at 7.4. The intraluminal pressure was set using two servo-controlled roller pumps (Living Systems, Burlington, VT, US) attached to the plastic microcannulas. The arterioles were pressurized in a no-flow mode. Microangiometry was used to determine inner and outer radius and wall thickness of the vessels. To visualize the changes in diameter, the inferior part of the tissue bath was transparent, and it was installed on the stage of an inverted microscope (Leica). The vessels were photographed under magnification using a DCM 130 E camera. The obtained microscopic images were analyzed and processed in a later phase using a particular image-analyzing program (ScopePhoto). Using a micrometer etalon (Wild, Heerbrugg, Switzerland), regular length calibration was carried out.

3.3. Vascular protocols

At first, the coronary arterioles were allowed to equilibrate for 30 minutes at 50 mmHg in a normal Krebs solution, and then, the steady-state vascular diameter was determined. Two subsequent conditioning pressure cycles (2-90-2-90-2 mmHg) were performed right at the beginning of the protocol. Thereafter, the pressure-diameter curves were obtained as follows: initially, the pressure was raised to 30 mmHg, and then it was raised to 50, 70, and 90 mmHg. At each stage, the inner and outer diameter- data were recorded. The nKR was next given a TxA2 agonist (U46619, at a concentration of 10-6 M), and after another 10 minutes of incubation at 50 mmHg, the steady-state diameter was once more captured on camera. The pressure diameter curves were again obtained similarly. Without rinsing out U46619, bradykinin (BK) was added into the vascular chamber, and an additional 20 minutes of incubation at 50 mmHg preceded the capturing of the steady-state diameter. The pressure diameter curves were noted once again as detailed above. In the end, all the medications were replaced with calcium-free Krebs-Ringer solution, and the tests were finished by taking the pressure-diameter curves after 30 minutes of incubation at 50 mmHg. At each stage, the wall thickness, inner diameter, and outer diameter were measured (103, 104).

3.4. Calculations related to biomechanics

The following equations were utilized to determine the biomechanical parameters (data were obtained from measurements in calcium-free Krebs solution (103):

- Wall thickness (h) = r_o-r_i
- Wall thickness to lumen diameter (Q): $Q=h/d_i$
- Cross sectional area (A_w): $A_w=(r_o^2-r_i^2)*\pi$
- Tangential wall stress (σ): $\sigma=(P*r_i)/h$), according to the Laplace-Frank equation
- Incremental tangential elastic modulus of the cylindrical segments (E_{inc}): $E_{inc}=(2r_o ri^2*\Delta P)/((r_o^2–r_i^2)*\Delta r_o)$

where h denotes the thickness of the wall, r_o and r_i denote the actual values of the outer and inner radii, d_i denotes the inner diameter, P denotes the transmural (intraluminal) pressure, and Δr_o denotes the change in the outer radius caused by a rise in ΔP, according to Cox(105).

3.5. Calculations of contractility

The following parameters were derived based on the pressure-diameter data (104):

- Spontaneous tone: $T_{nKR}=(r_{iCa\text{-}free}–r_{inKR})/r_{iCa\text{-}free}*100$ (%)
- U46619induced constriction: $C_{U46619}=(r_{inKR}\text{-}r_{iU46619})/r_{iCa\text{-}free}*100$(%)
- Bradykinin-induced relaxation: $R_{BK}=(r_{iBK}\text{-}r_{iU46619})/r_{iCa\text{-}free}*100$(%)

where, at a pressure of 50 mmHg, $r_{iCa\text{-}free}$ and r_{inKR} are the inner radii measured in a calcium-free solution and nKR solution, respectively. The inner radii assessed after TxA2 agonist (U46619) and bradykinin at 50 mmHg are $r_{iU46619}$ and r_{iBK}, respectively.

3.6. Statistics

Data measurements were compared using SPSS Sigma Stat program for statistical analysis. Data are presented as the mean ± SEM. The Shapiro-Wilks method was used to verify the normal distribution. Two-way analysis of variance (ANOVA) with the factors "hypertension" and "castration" was carried out in the case of a normal distribution. We used one-way ANOVA if there were interactions between "hypertension" and "castration" (pint< 0.05) in the two-way ANOVA. Mixed-effect models were applied in

case of spontaneous tone, U46619-induced constriction, and bradykinin-induced relaxation. Tukey's post hoc test was applied in one-way ANOVA, two-way ANOVA, and mixed-effect models as a post hoc analysis. Kruskall-Wallis test with Dunn's post hoc test was utilized in case of ~~a~~ non-normal distribution. A P value of < 0.05 was considered as the criterion for statistical significance.

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4. RESULTS

4.1. Basic physiological properties

The weight of the heart was demonstrated to have increased significantly in the AII groups compared to controls following the four-week AII treatment regime **(Table 2.)**. Measurements regarding blood pressure was performed by cannulating the right carotid directly. Both MAP and systolic blood pressure were found to be significantly increased in the AII rats compared to control animals **(Table 2.)**. Significant decrease was measured regarding diastolic blood pressure in the AII + OCT rats compared to values from the AII groups **(Table 2.)**.

Table 2.Basic characteristic and blood pressure parameters of the study groups.

Variable	Co	OCT	AII	AII + OCT
Basic characteristic				
BW (g)	393 ± 9	396 ± 5	416 ± 9	401 ± 13
HW (g)	1.120 ± 0,03	1.195 ± 0.03	1.29 ± 0.02#	1.22 ± 0.08
HW/BW (g/kg)	2.85 ± 0.06	3.02 ± 0.07	3.08 ± 0.07	3.09 ± 0.13
Blood pressure data				
Systolic blood pressure	122 ± 6	118 ± 7	149 ± 7#	136 ±9
Diastolic blood pressure	109 ± 6	94 ± 7	126 ± 7	102 ± 7§
Mean arterial pressure	114 ± 6	102 ± 7	134 ± 7#	114 ± 8

BW, body weight; HW, heart weight. TWO-WAY ANOVA (factors: hypertension, orchidectomy) with post hoc Tukey's test. Values are the means ± SEM. #P <0.05 Co. vs. AII; §P < 0.05 AII vs. AII + OCT (103).

4.2. Morphological parameters of intramural coronary resistance arteries

Even though all harvested segments were identical in regard to both morphology and anatomy during dissection, significant differences were found regarding relaxed outer diameter between the groups **(Fig. 3)**. The OCT group demonstrated significantly lower outer diameter values than the control rats. Outer diameter decreased significantly in the AII + OCT group also compared to both the AII, and the OCT groups **(Fig. 3)**. Wall thickness was measured to be markedly reduced in the OCT rats compared to the control animals **(Fig. 4)**. Wall thickness to lumen ratio was significantly higher in the AII + OCT group, compared to AII group, and also the OCT animals **(Fig. 5)**. As a further effect of orchidectomy, a decrease regarding wall cross-sectional area was found compared to both the control and AII rats **(Fig. 6)**. This indicates that inward hypotrophic remodeling occurred in both the OCT and AII + OCT groups.

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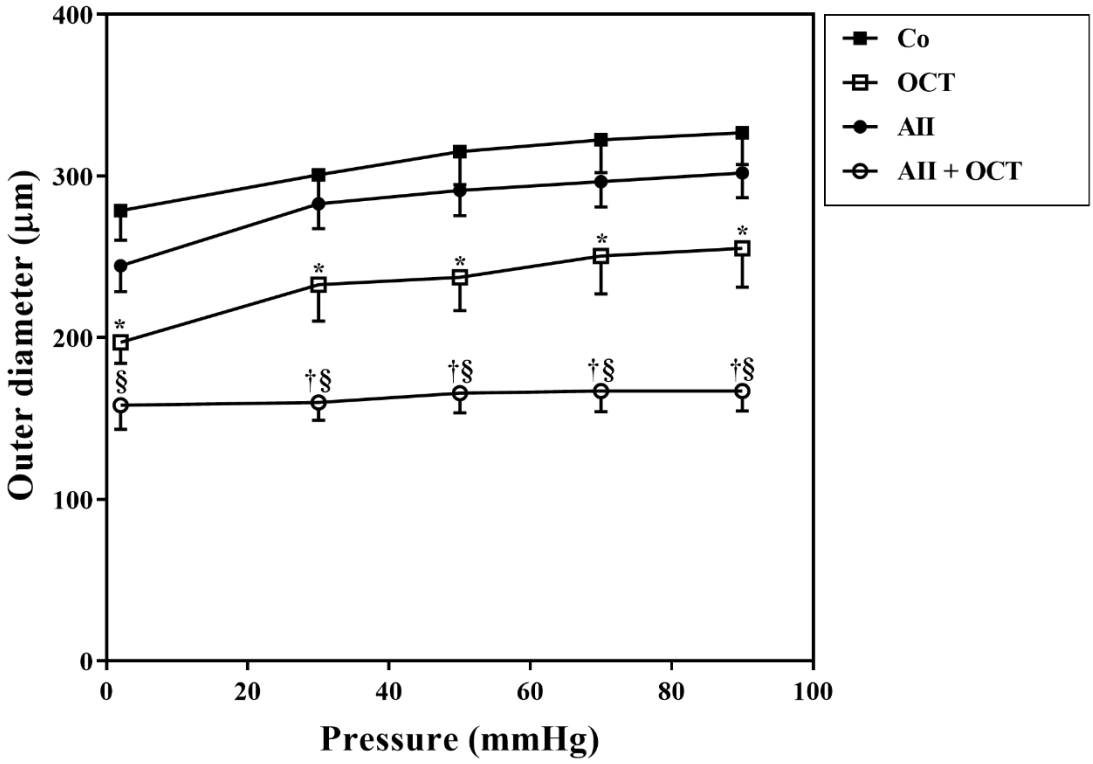

Figure 3. Outer diameter of the intramural coronaries as a function of intraluminal pressure measured in a passive condition (in calcium-free Krebs solution), Co (n=10), OCT (n=13), AII (n=10) and AII + OCT (n=8). Outer diameter values were measured to be decreased in the OCT groups compared to control animals. Outer diameter values were also decreased in the AII + OCT group compared the AII and OCT groups. TWO-WAY ANOVA performed at the same pressure values (factors: hypertension, orchidectomy) with post hoc Tukey's test. All values are expressed in mean ± SEM. *P < 0.05 Co vs. OCT; †P < 0.05 OCT vs. AII + OCT; §P < 0.05 AII vs. AII + OCT (103).

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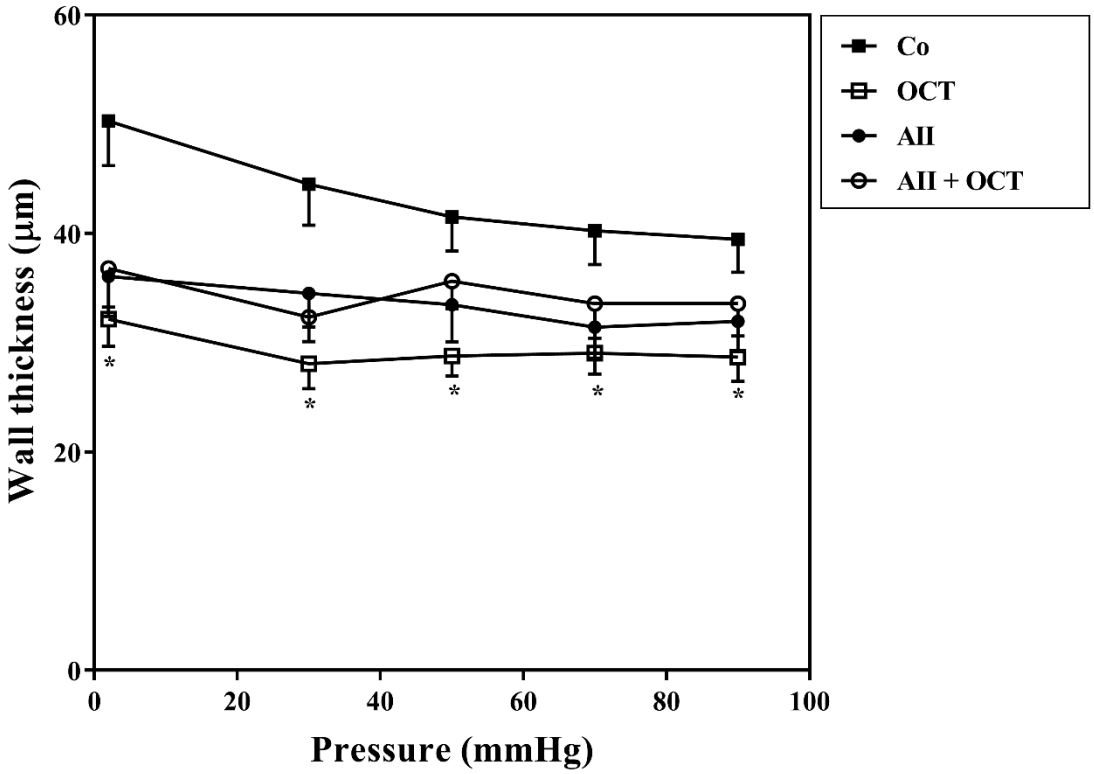

Figure 4. Wall thickness of the intramural coronary arteries as a function of intraluminal pressure measured in a passive condition (in calcium-free Krebs solution), Co (n=10), OCT (n=13), AII (n=10) and AII + OCT (n=8). Values regarding wall thickness was decreased in the OCT group compared the Co group. ONE-WAY ANOVA (because of the P interaction was smaller than 0.05 in TWO-WAY ANOVA) at same pressure with post hoc Tukey's test. All values are expressed in mean ± SEM. *P < 0.05 Co vs. OCT (103).

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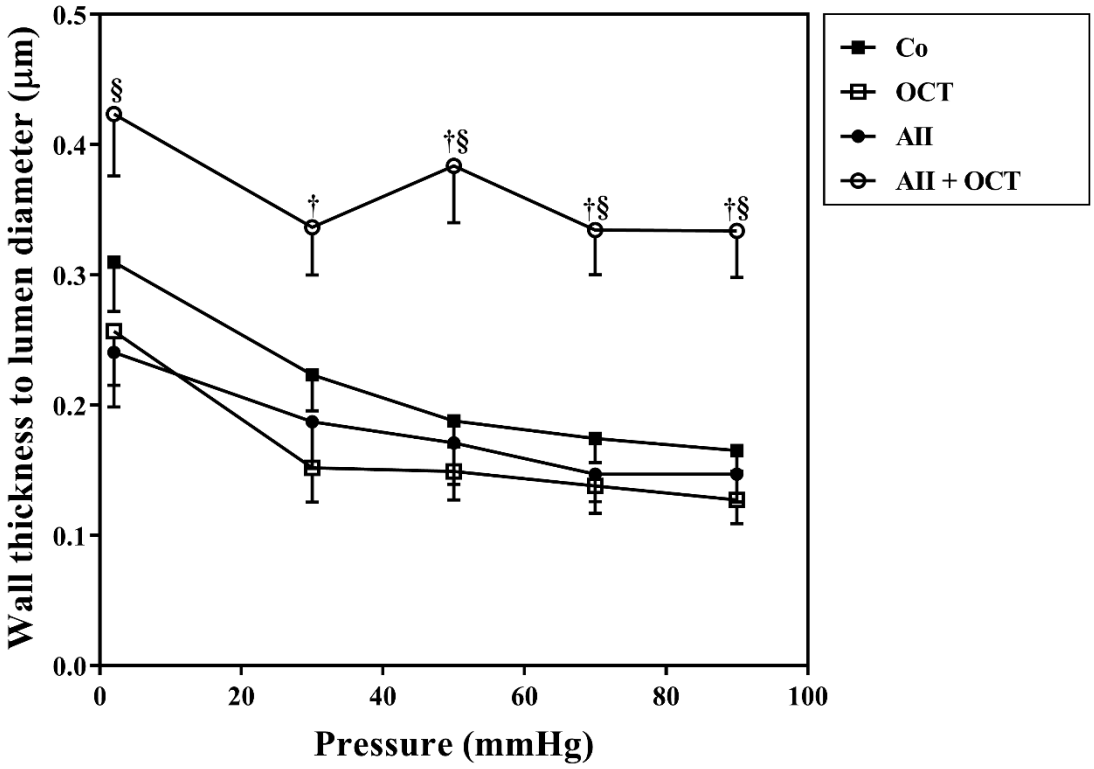

Figure 5. Wall thickness to lumen diameter of the intramural coronary arteries as a function of intraluminal pressure measured in a passive condition (in calcium-free Krebs solution), Co (n=10), OCT (n=13), AII (n=10) and AII + OCT (n=8). Wall thickness to lumen ratio was increased in the AII + OCT rats compared to AII and OCT groups. KRUSKAL-WALLIS test at the same pressure with post hoc Dunn's test. All values are expressed in mean ± SEM. †P < 0.05 OCT vs. AII + OCT; §P < 0.05 AII vs. AII + OCT(103).

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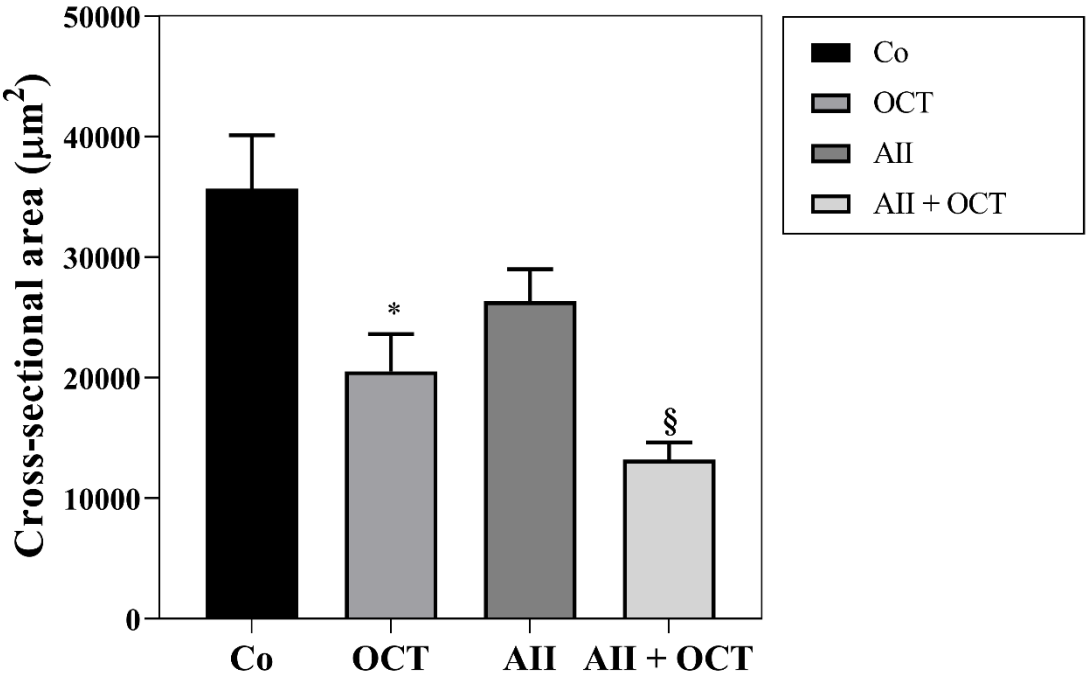

Figure 6. Wall cross-sectional area of the intramural coronary arteries at 50 mmHg measured in the passive condition (in calcium-free Krebs solution), Co (n=10), OCT (n=13), AII (n=10) and AII + OCT (n=8). Values regarding wall cross-sectional area were decreased in the OCT group compared to the controls. Wall cross-sectional area was also decreased in the AII + OCT rats, compared to the AII animals. TWO-WAY ANOVA (factor: hypertension, orchidectomy) with post hoc Tukey's test. All values are expressed in mean ± SEM. *P < 0.05 Co vs. OCT; §P < 0.05 AII vs. AII + OCT(103).

Even though all harvested segments were identical in regards to both morphology and anatomy during dissection, significant differences were found regarding the inner radius of the vessels **(Fig. 7).** Measurements regarding inner radius of the vessels were reduced in the AII + OCT rats compared to all the other animals (control, the AII and OCT).

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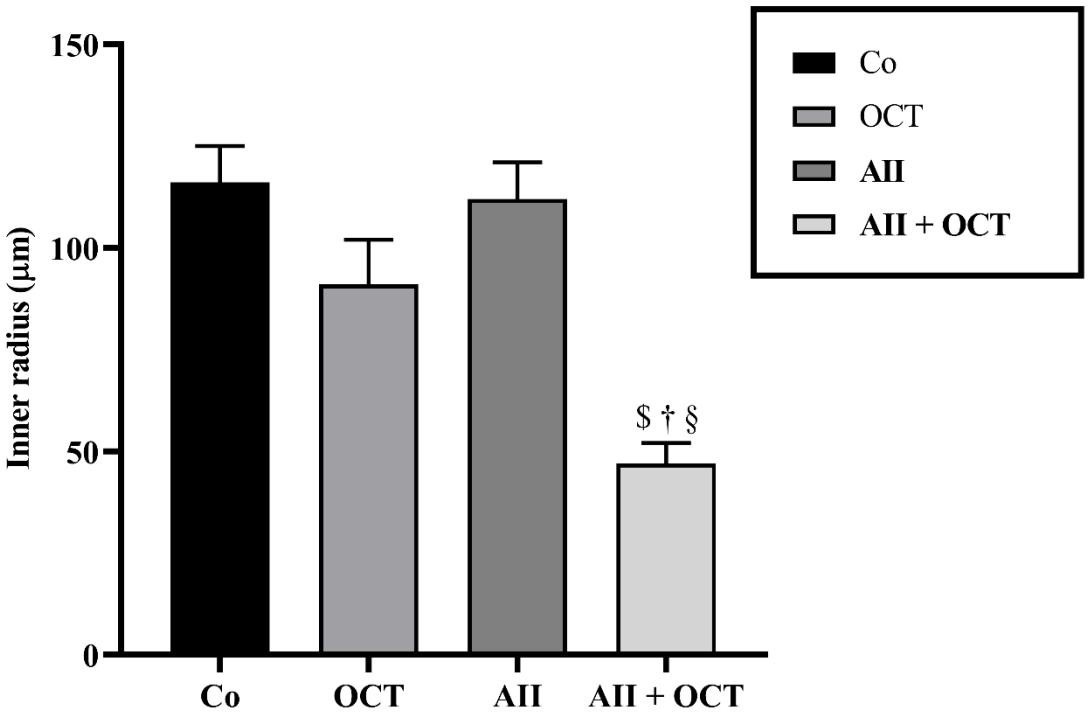

Figure 7. Inner radius of the intramural coronary arteries at 50 mmHg measured in the passive condition (in calcium-free Krebs solution), Co (n=10), OCT (n=13), AII (n=10) and AII + OCT (n=8). Measurements regarding inner radius was decreased in the AII + OCT rats compared to the other animals. ONE-WAY ANOVA with post hoc Tukey's test. All values are expressed in mean ± SEM. $P < 0.05 Co vs. AII + OCT; †P < 0.05 OCT vs. AII + OCT; §P < 0.05 AII vs. AII + OCT(103).

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4.3. Biomechanical characteristics of intramural coronaries

In the AII + OCT rats tangential wall stress (marking mechanical loading of the coronary wall) was significantly decreased compared to the AII rats, and also the OCT animals **(Fig. 8)**. No significant differences were found regarding incremental elastic moduli of the vessels **(Fig. 9)**.

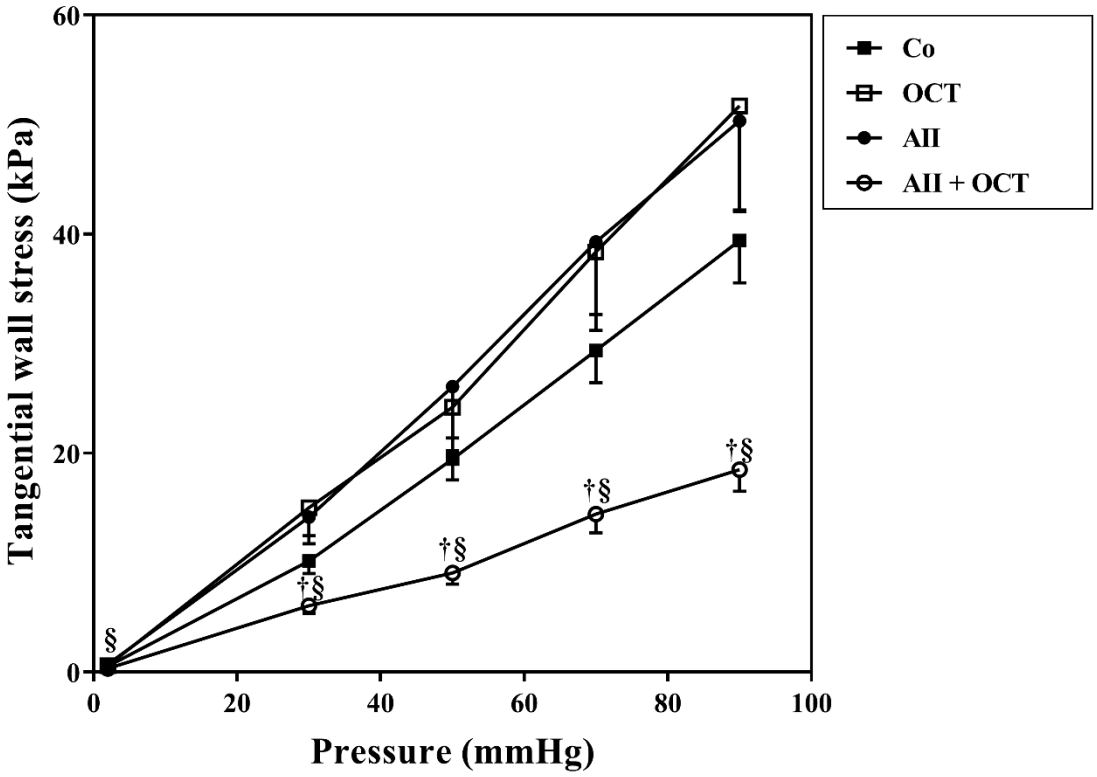

Figure 8. The tangential wall stress of rat intramural coronary resistance arteries as a function of intraluminal pressure measured in the passive condition (in calcium-free Krebs solution), Co (n=10), OCT (n=13), AII (n=10) and AII + OCT (n=8). Tangential wall stress was measured to be decreased in the AII + OCT animals compared to AII and OCT groups. KRUSKAL-WALLIS test at the same pressure with post hoc Dunn's test. All values are expressed in mean ± SEM. †P < 0.05 OCT vs. AII + OCT; §P < 0.05 AII vs. AII + OCT(103).

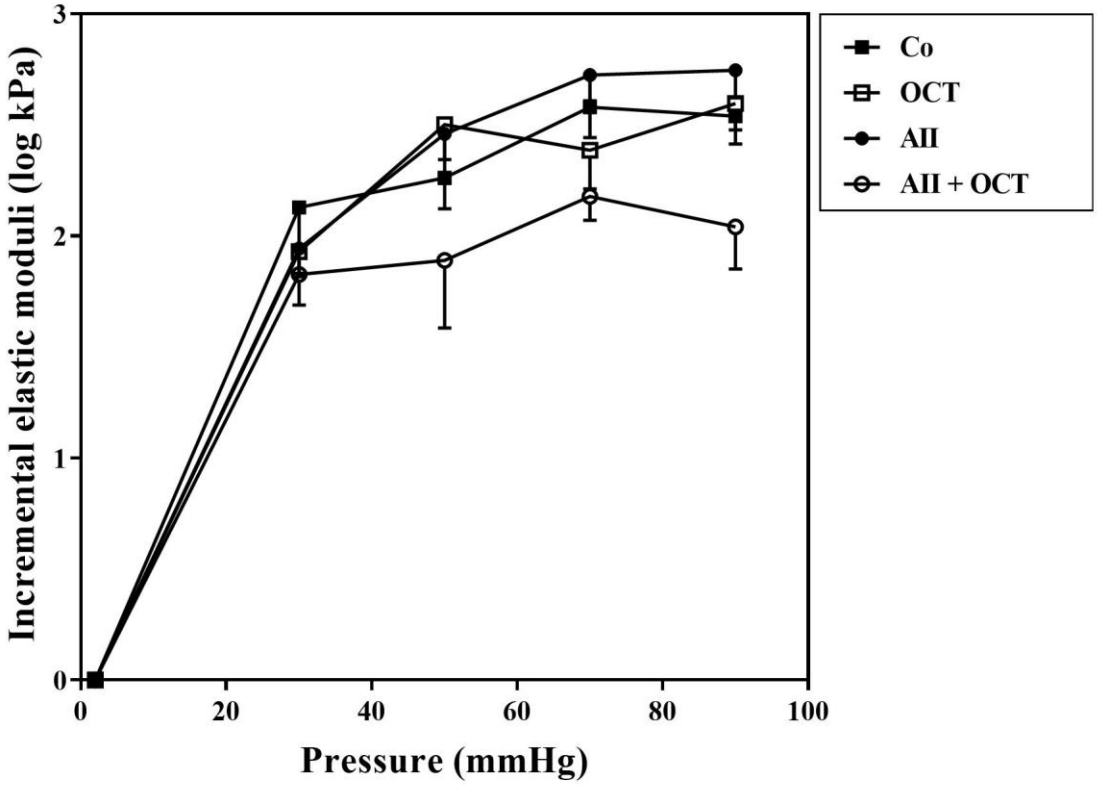

Figure 9. The incremental elastic moduli of rat intramural coronary resistance arteries as a function of intraluminal pressure measured in the passive condition (in calcium-free Krebs solution), Co (n=10), OCT (n=13), AII (n=10) and AII + OCT (n=8). The incremental elastic moduli of the intramural coronary resistance arteries measured in the passive condition (in calcium-free Krebs solution). The logarithm of the incremental tangential elastic modulus is shown as a function of the intraluminal pressure. KRUSKAL-WALLIS test at the same pressure with post hoc Dunn's test. All values are expressed in mean ± SEM (103).

4.4. Vascular reactivity of intramural coronary resistance arteries

The spontaneous tone of the coronaries from the AII groups was significantly increased compared to that of the segments taken from the Co and the OCT animals. The spontaneous tone did not change only as an effect of orchidectomy. Spontaneous tone was however significantly increased in the AII + OCT animals compared to the OCT rats **(Fig. 10).**

Contraction induced by U46619 was significantly decreased in the OCT group vessel segments compared to control animals. Contraction to U46619 was also significantly decreased in the AII + OCT animals compared to f the Co and AII groups. Contraction to U46619 did not differ in the OCT group compared to the AII + OCT group **(Fig. 11)**.

The bradykinin induced relaxation did not differ in AII animals compared to the Co rats. Due to testosterone deficiency, relaxation to bradykinin was significantly decreased in the OCT animals compared to Co and AII rats (Co vs. OCT and AII vs. OCT group). In the AII + OCT group, the bradykinin relaxation was lesser compared to the Co and the AII animals (Co. vs. AII + OCT and AII vs. AII + OCT group). However, relaxation did not differ in OCT rats compared to the AII + OCT animals **(Fig. 12)**.

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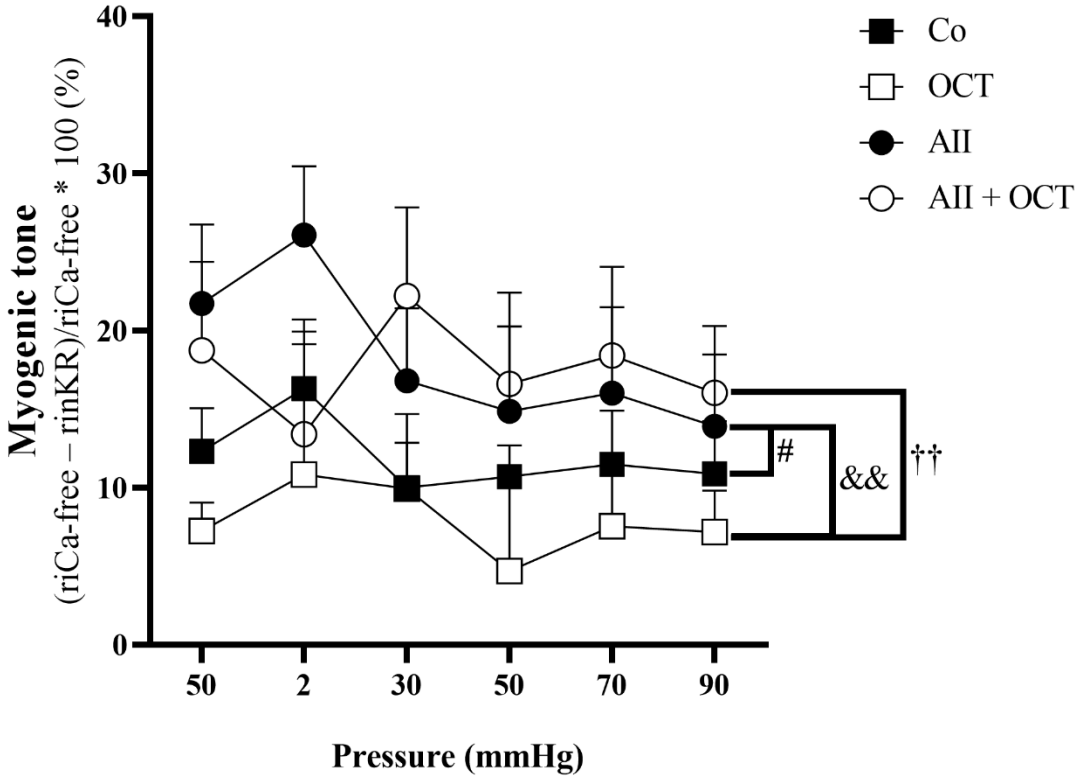

Figure 10. Spontaneous tone of rat intramural coronary resistance arteries in normal Krebs-Ringer solution as a function of intraluminal pressure, Co (n=10), OCT (n=12), AII (n=10) and AII + OCT (n=8). Values regarding spontaneous tone were measured to be significantly higher in AII and AII + OCT animals, compared to rats from the other groups. The MIXED-EFFECTS analysis was applied to the repeated measures data (pressure curves) with post hoc Tukey's test. All values are expressed in mean ± SEM. #P <0.05 Co. vs. AII; &&P < 0.01 OCT vs. AII; ††P < 0.01 OCT vs. AII + OCT(104).

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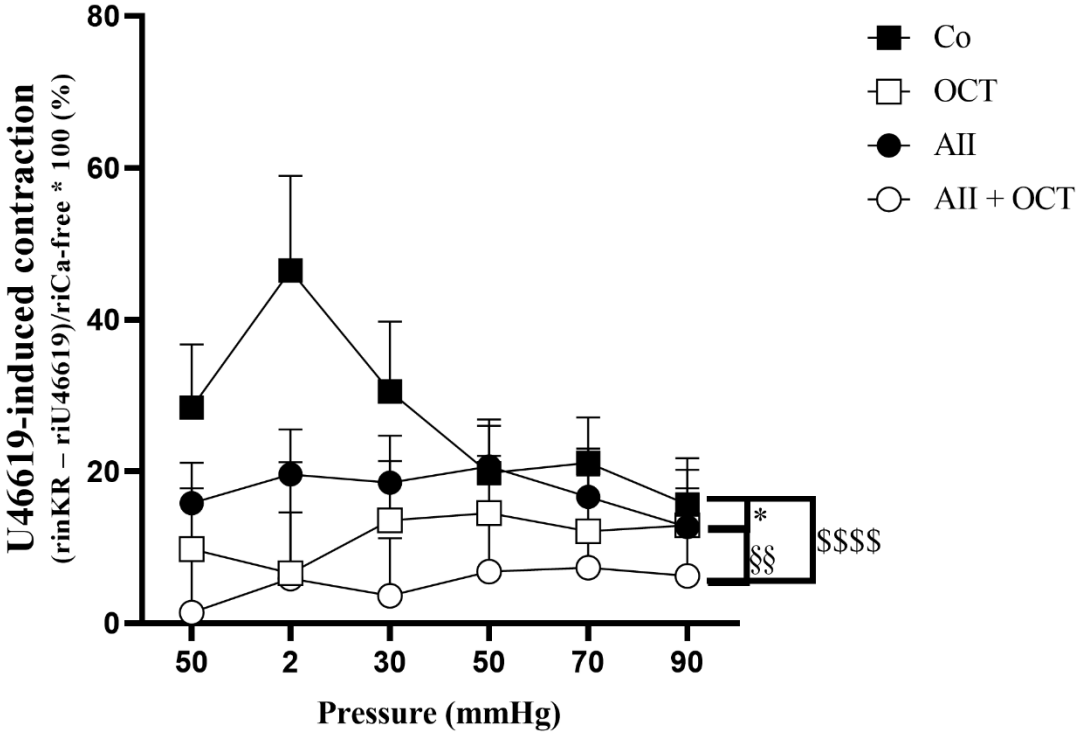

Figure 11. U46619-induced contraction of rat intramural coronary resistance arteries as a function of intraluminal pressure, Co (n=10), OCT (n=12), AII (n=10) and AII + OCT (n=8). The TxA_2 receptor agonist U46619 induced contraction was significantly reduced in the OCT and AII + OCT groups, compared to Co and AII rats. The MIXED-EFFECTS analysis was applied to the repeated measures data (pressure curves) with post hoc Tukey's test. All values are expressed in mean ± SEM. *P < 0.05 Co vs. OCT; $$$$P < 0.0001 Co vs. AII + OCT; §§P < 0.01 AII vs. AII + OCT(104).

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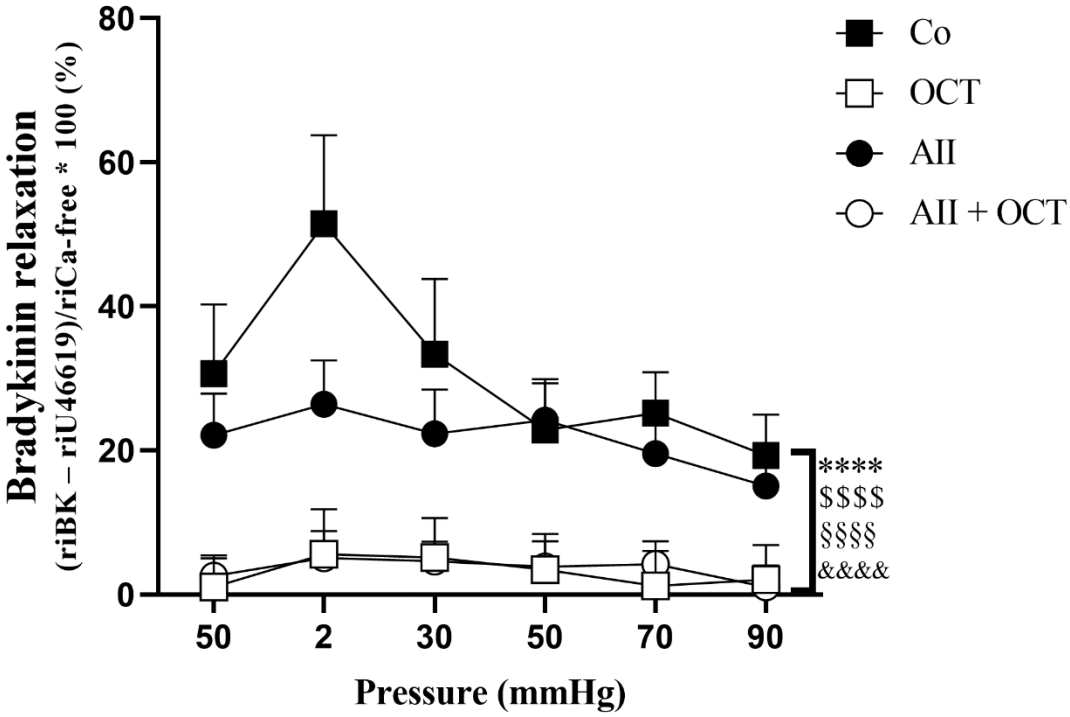

Figure 12. Bradykinin relaxation of rat intramural coronary resistance arteries as a function of intraluminal pressure, Co (n=10), OCT (n=12), AII (n=10) and AII + OCT (n=8). Bradykinin induced relaxation was significantly decreased in the OCT and AII + OCT rats compared to other animals. The MIXED-EFFECTS analysis was applied to the repeated measures data (pressure curves) with post hoc Tukey's test. All values are expressed in mean ± SEM.****P < 0.0001 Co vs. OCT; $$$$P < 0.0001 Co vs. AII + OCT; &&&&P < 0.0001 OCT vs. AII; §§§§P < 0.0001 AII vs. AII + OCT (104).

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5. DISCUSSION

The high number of cardiovascular morbidity and mortality is a major health problem today. One of the main risk factors for cardiovascular disease is hypertension which unfortunately affects over 30% of the adult population (106).

It has long been established that in terms of CAD significant differences exist between women and men, and that this difference disappears after menopause due to estrogen deficiency. However, it is less known and investigated, that testosterone deficiency in men in old age (andropause) also has a harmful effect on the cardiovascular system (91, 99, 100). High blood pressure and testosterone deficiency alone impair coronary function, but what effect can they have together?

Our research team therefore aimed to investigate the effects of testosterone deficiency on morphology, biomechanics and function of coronary resistance arteries in a known and accepted rat hypertension model.

Hypertension was achieved in our animals with a subcutaneous osmotic minipump containing angiotensin II, which caused a stable, prehypertensive state in our animals for 4 weeks. Testosterone deficiency was achieved surgically by removing the testes. Then, the biomechanical characteristics and function of the resistance coronary vessels were assessed using a unique method, microangiometry, which examined the vascular responsiveness, biomechanical and morphological properties of locally intact and responsive vessels independent of the effects of autonomic innervation. Our results demonstrate that not only menopause in women, but also andropause in men has a harmful effect on coronary arteries and the combined presence of testosterone deficiency and hypertension has an additive effect on intramural coronary resistance arteries.

5.1. Effect of hypertension on resistance arteries

Hypertension leads to cardiac hypertrophy of the animals, as evidenced by others and our own research (27, 107, 108). This cardiac hypertrophy is due to a pathological process, and is not a real hypertrophy of cardiomyocytes – but connective tissue proliferation (interstitial fibrosis) (107, 109).

Hypertension damages the vessel wall, which can be traced to changes in both vascular function and vascular biomechanical characteristics. One of the major public health risks

of vascular damage caused by hypertension is calcification of the large and small arteries (atherosclerosis and arteriolosclerosis) (5). The detrimental effects of hypertension are mediated through the endothelium and the vascular wall via humoral and biomechanical factors (110).

With respect to hypertension, significant gender differences can be found in both human and animal studies. There are several animal models for generating hypertension: high salt diet, spontaneously hypertensive rats, chronic inhibition of NOS or angiotensin II-induced models (2, 111, 112). Gender differences have also been described in the angiotensin II-induced model of our choice: low-dose angiotensin II infusion produces hypertension in wild-type male mice, whereas it is absent in female mice. In animal models of hypertension, male animals have higher blood pressure compared to females. Amongst other mechanisms the kidneys also have a substantial role regarding the development of gender differences (2, 3). However, it is important to highlight that 4 weeks of angiotensin treatment results in hypertension in both female (27, 36) and male animals (38, 113-115). In our own study, male rats had significantly higher mean arterial pressure in the AII-treated group (in group AII, but not in group AII+OCT) compared to control animals, indicating, that our 4-week treatment was a successful hypertensive stimulus in our animals. The above difference is consistent with literature data.

On the ground of hypertension, the morphology and function of the coronary arteries change. The vascular network in the body cannot be considered uniform in different vascular beds, so by interpreting certain effects it is important to note the type of vessels used in the experiment. Remodeling of the vessel wall occurs due to high blood pressure. Due to the rise in transmural pressure, the tangential wall tension increases in the vessel wall. Tangential wall tension acts along the circumference of the vessels along the vasculature. Tangential wall tension is also dependent on both the diameter of the segment and the thickness of the vessel wall. Pulse pressure is increased in high blood pressure and therefore wall tension increases. This is compensated for by a remodeling of the vessel wall through thickening. This type of remodeling serves to prevent further increases regarding wall tension. The wall tension decreases with age (116). Under experimental conditions an increase in wall tension leads to consequential increase regarding contractility of the studied segments. Meanwhile, decreased wall tension leads to a reduction of vascular contractility (117). In hypertension, the increased intraluminal

pressure increases the constrictor tone, contractility increases, which leads to increased wall tension (118).

As a part of the defense against increased pressure, a rebuilding of the vessel wall occurs, resulting in inward eutrophic remodeling, during which the lumen narrows, the wall thickens without changing the “wall-volume” (17, 119-121). It is important to note, that hypertrophic remodeling has also been reported in connection with hypertension, the essence of which is that in addition to wall thickening, the cross-sectional area (“wall-volume”) also increases. Hypertrophic remodeling has been found in the coexistence of hypertension and type 2 diabetes, as well as in elderly spontaneously hypertensive animals (in young spontaneously hypertensive animals eutrophic remodeling was observed) (22, 122). This may suggest that either with prolonged persistence of hypertension, or even over shorter period of time under marked stimulus eutrophic remodeling transforms into hypertrophic remodeling. In the present studies, although spontaneous tone increased during 4 weeks of treatment due to hypertension, even eutrophic remodeling was not yet observed.

Increased transluminal pressure due to hypertension would increase wall tension in the vessels. Spontaneous vascular tone increases to maintain optimal wall tension. As a result, the vessel wall thickens and the lumen narrows, causing the tangential wall tension to remain at a normal level. Over time, with the long duration of the hypertensive stimulus, eutrophic remodeling transforms to hypertrophic remodeling and later the process is decompensated and wall tension increases again (27, 29). In the present study, the wall tension did not differ in the angiotensin-treated-only group compared to the control animals, suggesting that wall remodeling was successful (wall thickness increased, lumen narrowed) and optimal wall circumferential tension was efficiently maintained. The increase and decrease of the incremental elastic modulus, a parameter characterizing the elasticity of blood vessels, has also been described in response to hypertension in various vessels. Intramural saphenous arterioles have increased isobaric elastic modulus (29), while intramural coronary arterioles have decreased elastic modulus (27). In the present study, we found that incremental elastic modulus was not altered by angiotensin treatment.

We find gender differences in different animal models not only in terms of hypertension, but also in terms of morphological and biomechanical adaptation to

hypertension, which has already been studied by our group. Relative heart weight and wall thickness were higher in female Sprague-Dawley rats of the same age, while the inner diameter decreased compared to males (123). Male AII animals were found to have significantly higher values regarding elastic modulus and tangential wall tension (123).

Not only vascular morphology and biomechanics are altered by hypertension, but also vascular reactivity. It will also be important to address gender differences in this regard. As a first step, spontaneous tone increases as a result of hypertension, not only in the coronary arteries, but also in the cerebral, musculocutaneous resistance, and mesenteric vessels (29, 31, 118, 124, 125). The increase in the myogenic tone is a protective mechanism against increased intraluminal pressure and thus the flow autoregulation of both coronary arteries and cerebral vessels shifts towards high pressures (124). In parallel with the literature data, we also observed an increase in spontaneous tone in angiotensin-treated animals.

Gender differences in coronary changes due to angiotensin-induced hypertension have been previously studied by our group: both myogenic and thromboxane-induced tone and contraction were higher in intramural coronaries in male rats compared to females (123). Endothelium dependent dilatation deteriorates in the coronary arteries of male rats (126), but does not change in coronary arteries of female rats (27). Spontaneous tone increases in mesenterial, renal vessels, and musculocutaneous resistance arteries of hypertensive male animals (127, 128), in parallel with which literature data we obtained a similar result in our present studies. Normally, contraction and relaxation are regulated at several points, both on calcium-dependent and calcium-independent pathways. Hypertension impairs these processes, leading to a state of hypercontractility (27, 127, 129). In our study - parallel to the data from the literature, spontaneous tone of intramural coronaries was increased in both the angiotensin-treated and castrated plus angiotensin-treated group, and maximal contraction capacity of the resistance arteries was increased by the thromboxane agonist U46619 in the angiotensin-treated-only group.

5.2. Effect of testosterone deficiency on resistance arteries

More and more research reports, that not only too high, but also too low testosterone levels create a highly unfavourable effect on men: increases the risk of atherosclerosis, peripheral vascular- and cardiovascular disease (62, 77, 130, 131). Based on the literature, we hypothesize that orchidectomy may elicit a bipolar blood pressure response: without basic cardiovascular risk it causes a drop in blood pressure, however, if the patient is already affected by cardiovascular complication, it further increases the chances of cardiovascular target organ damage in human conditions (91, 99, 100).

As a result of testosterone deficiency, the extracellular matrix of the vessel wall changes dysfunctionally, which is a milestone in the course of intimal hyperplasia. This process is further facilitated by the unrestricted migration of vascular smooth muscle cells into the intimal region. Testosterone-deficiency has also been demonstrated to have proinflammatory modulatory effects (61, 62). An inverse relationship was found between matrix metalloproteinase activity and dihydrotestosterone-levels. In vitro the proliferation and migration of vascular smooth muscle cells was shown to have the same relationship (132). Testosterone reduced the elastin/collagen ratio in a cell culture of aorta smooth muscle cells. Vascular stiffness was consequentially increased (63, 133, 134). In an animal experiment where exogenous testosterone was supplemented, relaxation of the aorta and the coronaries inhibited the development of atherosclerotic plaques (63). Beside endothelial cells, smooth muscle cells also express testosterone receptors (63). This may be a part of explaining the multifaceted effects of both testosterone and testosterone deficiency also on the cardiovascular system.

The literature on the effects of testosterone deficiency on vascular morphology and biomechanical function is limited. The article published by our research group, which is part of the present PhD dissertation, is the only one that provides morphological and biomechanical data on testosterone deficiency in resistance vessels. In the present experiment, castration induced a decrease regarding both cross-sectional area and outer diameter values, leading to the development of inward hypotrophic remodeling. Previously, inward hypotrophic remodeling was described in hippocampal arterioles from preeclamptic female rats (induced by a high cholesterol diet (135).The clinical significance of inward hypotrophic remodeling may be that the narrower and thinner

vessel wall due to testosterone deficiency becomes stiffer, which may contribute to vascular vulnerability and increased cardiovascular risk.

The literature holds highly contradictory results regarding the effects of both testosterone deficiency and supplementation on vascular function. Non-genomic acute testosterone effects include an increase in the ability of arteries to dilate: on mesenteric segments, aortic rings, and basilar arteries in various animal species (49). At physiological concentrations, testosterone produces endothelium-dependent responses, while at supraphysiological doses, endothelium-independent responses are elicited (49). Acute administration of testosterone to coronary arteries and the aorta induced endothelial independent vasorelaxation in both female and male rabbits (51). Similarly, intra-coronary testosterone administration elicits an acute sex-independent relaxation response in canine conduit and resistance coronary arteries (52). Acute administration of testosterone in castrated male swine also induces coronary relaxation (136). In contrast, it has also been reported that after a short chronic testosterone treatment, 2 weeks of testosterone administration increased vasoconstriction in coronary arteries in female animals (137, 138). However, in the case of castration and castration plus testosterone replacement, the acute vasodilatory effect of testosterone does not prevail, in the case of chronic deficiency plus replacement we expect a different adaptation response. Myogenic tone is reduced by castration (55). Furthermore, it creates relaxation through eNOS (with NO production) (65, 71). Examining the coronary arteries of male rats in a Langendorff heart model it was found that castration impairs bradykinin-induced vasodilation, which is restored by testosterone replacement at both physiological and supra-physiological doses (49). In contrast, human studies have found that physiological testosterone supplementation impairs vascular reactivity in androgen deficient individuals (139). Mesenterial vessels demonstrated a reduced contraction to electrical field stimulation-induction following castration in a study. The study also described increased vasodilator response (140). To the best of our knowledge, TxA2 receptor expression is enhanced by the presence of testosterone in cerebral vessels (141). Furthermore, changes in contraction have been described in other types of vessels as a result of castration, but not in coronary vessels, where individual adaptation is expected due to the special vascular bed. The TxA2 production of mesentery artery smooth muscle cells increased following castration

(142). Release of TxA2 was also increased shortly following castration in both mesenteric arteries and the aorta (143). In the coronaries of male guinea pigs TxA2 induced vasoconstriction is enhanced by testosterone (144). Exogenous testosterone has a vasodilator effect; this is potentiated by endogenous testosterone. It is interesting to note that this process is not dependent on the expression of androgen receptors (81). A deficiency of testosterone has detrimental effects regarding relaxation via a decrease in the production of NO (65) and through the activation of potassium channels and cyclic adenosine monophosphates (96). In the present study, spontaneous tone and U46619-evoked tone were decreased in the castrated group compared to values measured in the control group. Vasodilation to bradykinin was reduced also.

5.3. Combined effects of hypertension and testosterone deficiency on resistance arteries

Our present study aimed to analyze the effects of testosterone deficiency and hypertension in intramural resistance coronaries in males and also focused on investigating their combined effects. Based on our research of the relevant literature to date this is the first research designed to investigate the combined effects of testosterone deficiency – established through an andropause model using removal of the testes - and AII induced hypertension - established via implantation of an osmotic minipump - on the contractility and biomechanics of resistance coronaries.

The effect of castration on blood pressure is contradictory in the literature: both the effect of increasing blood pressure (89, 90) and the effect of lowering blood pressure (88, 145) have been reported. Hypertension in spontaneously hypertensive animals appears to be androgen dependent because in male animals castration decreases hypertension, in females it does not, and testosterone replacement in both sexes resulted in a male pattern of blood pressure development (145). Additional studies are required to resolve current conflicting results. As mentioned earlier, the literature suggests that orchidectomy may elicit a bipolar blood pressure response. In our present study, as a result of castration the mean arterial mean pressure in the OCT group tended to be decreased compared to values from the control group but did not reach the threshold of significance. The mean arterial mean pressure in the angiotensin II plus OCT group did not differ significantly from either the control or the angiotensin-treated-only groups at week 4, which shows, that upon

hypertensive stimulus, blood pressure set in the direction of hypertension, which was reduced by castration.

Regarding the morphology and biomechanical function of the vessels, in the case of the coexistence of the 2 noxa we found that tangential wall stress was decreased compared to the angiotensin-treated-only and the castrated groups. The underlying rationale is that wall thickness/lumen ratio increased and inward hypotrophic remodeling occurred. This inward type of hypotrophic remodeling of the coronaries as a result of testosterone depletion by castration appears to parallel results from the literature. Inward hypotrophic remodeling caused by castration was observed not only in the normotensive but also in the hypertensive group. The AII + OCT animals did not differ significantly from the OCT rats regarding cross sectional area. However cross-sectional area did differ significantly between the AII + OCT animals and the AII rats. Consequently, testosterone depletion (through castration) mainly led to inward hypotrophic remodeling. Both testosterone depletion (castration) and AII hypertension led to similar alteration within the vascular wall, even though underlying mechanisms were different: castration (testosterone deficiency) lead to the development of inward hypotrophic remodeling with consequent vessel wall stress below in vivo pressure, whereas in AII hypertension vessel wall stress was caused by increased intraluminal pressure. This pressure will eventually lead to inward eutrophic remodeling. As a result of hypertension vascular diameter reduced further in the castrated group compared to the hypertensive only group. We may conclude that hypertension aggravated the vascular damage caused by castration (i.e. testosterone deficiency), which - if similar alterations may be demonstrated in humans - may provide an explanation for the increase in cardiac mortality and morbidity (e.g. ischemic events, acute myocardial infarction) (146).

The effects of combined castration and hypertension have previously been studied in aortic rings, mesenteric vessels, and limb arteries (88, 94, 96, 147, 148). However, the information on this has been proven to be highly contradictory. Autonomic venous tone was reduced significantly following castration in male SHRs (94). In male SHRs weakened serotonin-induced vasoconstriction and slightly worse endothelial dilation were observed following castration (88). However, Vasudevanet et al. found that in male hypertensive animals' castration had a beneficial effect on endothelial dilatation (95). In case of high blood pressure caused by high-salt diet, vasorelaxation was impaired in the

aortic rings (vasorelaxation to forskolin and diazoxid), which effects were restored by bilateral orchidectomy, while concomitant testosterone supplementation restored impaired relaxation (96). Similar to this study, high-salt diet was found to increase vascular tone in limb arteries, which could be prevented by castration, which was restored by testosterone supplementation (147). Furthermore, in case of AII hypertension, vasoconstriction in the mesenteric arteries increased; this phenomenon, however was prevented by performing castration and furthermore it was restored by supplementation of testosterone (148). In the present work we found a decrease in both contractile agents elicited vasoconstriction and a endothelial vasodilation in the orchidectomized groups (OCT, AII+OCT). This also means an impaired range of ventricular tissue flow adaptation and narrowing of coronary vascular reactivity as well. In the present study, development of the combination of the above-mentioned lesions was observed in the double noxa group (AII+OCT): both increased spontaneous tone and a narrowed range of vascular adaptation were detected. The observed effects of AII hypertension stimulus and andropause may be considered to be additive, however, the double harmful noxa does not alter that, which was altered by a single factor. These conflicting results in the literature may in part be explained by the difference in the types of vessels, (coronaries vs. aorta/ mesenteric arteries/ musculocutaneous arteries) or types of animals (Sprague Dawley rats vs. SHR). Further studies are needed to assess how castration affects vascular function.

Interestingly, a narrowed vascular adaptation range (decreased vasoconstriction and vasodilation) was also present in the normotensive OCT group, predicting deterioration in vascular adaptation, resulting in impaired reactivity of small coronary arteries directly responsible for cardiac blood supply and the development of cardiovascular vulnerability in andropause.

Previous research has proven that athletes and bodybuilders who use anabolic steroids and andropausal men have higher risk regarding cardiovascular mortality and morbidity than fertile-age men who have physiological androgen levels (98, 149-151). Our present research may offer an explanation regarding a potential underlying mechanism of the cardiovascular vulnerability observed in andropausal men. Results from previous research are conflicting regarding the relationship between testosterone levels and cardiovascular mortality and morbidity (42, 152). The exact range of testosterone level is

key; levels too high or too low may both lead to harmful consequences. Overly high levels of testosterone (i.e. caused by anabolic steroid intake) compromise endothelial function and increase the likelihood of a possible acute coronary event. It also increases vascular calcification (153), atherosclerosis (154), lowers HDL and increases LDL levels (30), increases the risk of thromboembolism (97) and coronary vasospasm (98, 155). Testosterone levels that are too low have negative effects and raise the risk of a possible stroke or the development of coronary artery disease (156, 157). Too low testosterone levels in elderly men suffering from stable coronary artery disease increased coronary calcification compared to men who had testosterone levels within normal range (78). The positive effects of testosterone dominate when testosterone levels are optimal. In these cases, coronary arteries demonstrate an increased vasodilator response (152). Testosterone replacement restores deficiency-induced damage: in animal studies, the endothelium -dependent (bradykinin) relaxation response of coronary vessels in animals undergoing castration improves following the replacement of testosterone (49).

Based on our results, there is a rationale for supplementing testosterone to physiological levels in andropausal men who have vulnerabilities regarding their cardiovascular system. This exogenous supplementation may have protective effects under both normo- and hypertensive conditions. The effect of testosterone on cardiovascular disease describes a U curve. Both too low and too high testosterone levels have detrimental effects on cardiovascular disease, which is also supported by the cited literature. The point is at the optimal testosterone level. That is why, in case of too low testosterone levels, supplementation (to optimal levels!) can have a protective effect on cardiovascular diseases (99, 100). Naturally, this protective effect is assumed to have an effect at the onset of the hormone deficiency: in women, the positive effects of menopausal hormone replacement develop only when hormone replacement is started within 5 years of the onset of menopause. After 10 years vascular damage is definitive and the expected complications outweigh the benefits of treatment (158-162). In an analogue manner, appearance of similar advantages vs. disadvantages may be expected regarding andropausal men. However, this is much more difficult to determine than in women where the absence of menstruation clearly indicates the onset of a hormone-deficient period, whereas in men we cannot mark such a prominent time. Our line of reasoning based on the results of our current studies must be supported by further clinical

and animal research. However, contrary to the paradigm, our results support that restoring physiological hormone levels in andropause may reduce cardiovascular risk. Testosterone supplementation may also be the subject of future research to determine whether there are similar limitations regarding time elapsed following andropause to the effectivity of androgen replacement in men as there is in hormone replacement in women following menopause.

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6. CONCLUSIONS

In the present study, we examined the effect of testosterone deficiency, and the coexistence of testosterone deficiency and hypertensive stimulus on the morphological, biomechanical, and functional adaptation of intramural coronary resistance arteries.

In our model, we confirmed that

- as a result of hypertension spontaneous tone increases first, even without remodeling of wall structure.

In our model, the following novelties were found:

- testosterone deficiency decreases wall thickness
- testosterone deficiency alone results in inward hypotrophic remodeling, which persists in double noxa
- in case of testosterone deficiency, the hypertensive stimulus further reduces the vessel diameter, the wall thickness to lumen ratio increases and the tangential wall tension decreases
- testosterone deficiency also reduces endothelial vasodilatation and thromboxane agonist constriction
- in the case of the coexistence of the two noxa the spontaneous tone increases, the degree of constriction decreases, the degree of endothelial dilatation decreases, so the harmful effects caused by the two noxa add up. However, the association of two harmful noxa does not alter what was altered by the single factor.
- testosterone deficiency further aggravates vascular damage caused by hypertension.

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7. SUMMARY

The biomechanical and functional adaptation of coronary resistance vessels in the coexistence of testosterone deficiency and hypertension has not been studied in detail. Therefore, we aimed to study the structural and functional adaptation of these resistance arteries under the influence of the two noxa (castration and hypertension). Sprague-Dawley rats were divided into 4 groups: control males, orchidectomized males, angiotensin II infused males, and orchidectomized plus angiotensin II infused males. Testosterone deficiency was achieved by surgical castration, and hypertension was achieved by angiotensin II administration via osmotic minipump (100 ng/min/kg). After 4 weeks of treatment, the biomechanical and functional properties of the coronary vessels were studied with a pressure angiometer. Blood pressure was measured using a cannula inserted into the right carotid artery on the day of the experiment. As a result of the combined effect of the two noxa, the inner diameter of the vessels decreased. The increased wall thickness to lumen ratio resulted in lower tangential wall tension in the orchidectomized plus angiotensin II infused group. Castration resulted in inward hypotrophic remodeling that was independent of the hypertensive stimulus. Spontaneous tone was significantly higher in the angiotensin II infused -, and in the angiotensin II infused + castrated groups than in the control and castrated groups. U46619-induced vasoconstriction was significantly reduced in the orchidectomized groups. Endothelium dependent dilatation was also decreased in testosterone-deficient groups. Vascular impairment caused by testosterone deficiency and by hypertensive stimulus add up during mechanical and functional adaptation. Together, the two noxa produced inward hypotrophic remodeling. As a result of castration, vascular reactivity decreased in the direction of both vasoconstriction and vasodilation. Based on our present study, it can be stated that coronary reactivity deteriorates upon andropause and the effects of these two noxa add up, and it might be one of the mechanisms of increasing cardiovascular risk in elderly men.

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ORIGINAL RESEARCH & REVIEWS

BASIC SCIENCE

Effects of Testosterone Deficiency and Angiotensin II–Induced Hypertension on the Biomechanics of Intramural Coronary Arteries

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ABSTRACT

Background: Andropause and hypertension also increase the risk of coronary artery damage.

Aim: To investigate the effect of testosterone deficiency and hypertension on intramural coronary vessels.

Methods: 4 groups of 8-week-old Sprague-Dawley rats were studied: control male (Co, n=10), orchidectomized male (OCT, n=13), angiotensin (AII) hypertensive male (AII, n=10), and AII hypertensive and OCT (AII + OCT, n=8). Surgical orchidectomy was performed, and an osmotic minipump was inserted for chronic angiotensin II infusion (100 ng/min/kg). After 4 weeks, spontaneous tone and biomechanical properties of the intramural coronary resistance artery were investigated in vitro, by pressure microarteriography.

Outcomes: Morphology and biomechanics of the intramural coronaries were evaluated: the outer diameter, wall thickness–to–lumen diameter ratio, and tangential wall stress in the contracted and relaxed states.

Results: The outer diameter was reduced in OCT and AII + OCT groups (on 50 mmHg 315 ± 20 Co; 237 ± 21 OCT; 291 ± 16 AII, and 166 ± 12 μm AII + OCT). The increased wall thickness–to–lumen diameter ratio resulted in lower tangential wall stress in AII + OCT rats (on 50 mmHg 19 ± 2 Co; 24 ± OCT; 26 ± 5 AII, and 9 ± 1 kPa AII + OCT). Spontaneous tone was increased in the hypertensive rats (AII and AII + OCT groups) (on 50 mmHg 7.7 ± 1.8 Co; 6.1 ± 1.4 OCT; 14.5 ± 3.0 AII, and 17.4 ± 4.1 % AII + OCT).

Clinical Implications: Andropause alone can be considered as a cardiovascular risk factor that will further exacerbate vascular damage in hypertension.

Strengths & Limitations: A limitation of our study is that it was performed on relatively young rats, and the conclusions might not apply to coronary remodelling in older animals with slower adaptation processes.

Conclusions: Testosterone deficiency and hypertension damage the mechanical adaptation of the vessel wall additively: double noxa caused inward eutrophic remodeling and increased tone. **Jósvai A, Török M, Mátrai M, et al. Effects of Testosterone Deficiency and Angiotensin II–Induced Hypertension on the Biomechanics of Intramural Coronary Arteries. J Sex Med 2020;17:2322–2330.**

Key Words: Resistance Coronary Artery; Biomechanics; Castration; Testosterone; Hypertension; Rat Andropause Model

INTRODUCTION

It has long been established that hypertension and testosterone levels too high or too low have harmful effects on the cardiovascular system.[1] However, much less is known about the combined effect of testosterone deficiency and hypertension in men.

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Hypertension damages the heart not only directly (through cardiomegaly, dilated cardiomyopathy, compensated and decompensated heart failure[2]) but through its effects on the large and small arteries also.[3–6]

It has been demonstrated that optimal testosterone levels are protective, whereas testosterone deficiency increases the risk of atherosclerosis, cardiovascular and peripheral diseases, obesity, hypertension, and dyslipidaemia.[7–10]

As female menopause seems to increase the cardiovascular risk,[11] similar observations can be made in men—andropause also has an adverse effect.[12] Testosterone deficiency may be associated with risk factors for cardiovascular diseases, such as a high level of low-density lipoprotein, high blood sugar level, or high levels of proinflammatory cytokines.[13,14] Hypertension alone greatly increases the cardiovascular risk by damaging small and large blood vessels[6] and by overloading the heart.[15]

Hypertension leads to damage in the blood vessel wall. The damage appears both in function and biomechanical characteristics. The most characteristic biomechanical changes are vascular wall hypertrophy, decrease in tangential wall tension, or decrease in distensibility.[16,17] Tangential wall tension is the force acting on the vessels along the circumference. Its value is dependent on the transmural pressure, inner diameter of the vessel, and vessel wall thickness. In case of a high blood pressure, wall tension increases temporarily because of an increased pulse pressure. This is compensated by a thickening of the vessel wall, which prevents further increase in wall tension. It should also be mentioned that wall tension decreases with age. Increased wall tension leads to increased contractility in blood vessels, whereas decreased wall tension reduces vascular contractility.[18] It is known that hypertension significantly increases the risk of arteriosclerosis in both large and small arteries.[19] It has also been previously demonstrated with numerous studies by our research team that high blood pressure damages the morphology and function of intramural coronary vessels.[17,20–23] The detrimental effects of hypertension on the endothelium and vascular wall are mediated through biomechanical and humoral factors.[24]

The relationship between coronary heart disease and androgen levels in men is well established: optimal testosterone levels are protective, whereas testosterone deficiency increases the risk of atherosclerosis and cardiovascular and peripheral diseases.[7–10] Testosterone deficiency results in intimal hyperplasia (in the vessel wall), which is caused by atypical proliferation of vascular smooth muscle cells. Furthermore, testosterone deficiency also has a proinflammatory (modulator) effect.[25] An inverse relationship was found between dihydrotestosterone levels and matrix metalloproteinase activity, migration, and proliferation of vascular smooth muscle cells in vitro.[26] In aortic smooth muscle cell culture, testosterone reduces the elastin—collagen ratio, thereby increasing vascular wall stiffness.[27–29] Testosterone supplementation in animal experiments relaxed both coronary arteries and the aorta and inhibited atherosclerotic plaque formation.[27] Testosterone receptors are expressed not only in the endothelium of the vessel wall but also in smooth muscle cells,[27] which explains the multifaceted effect of testosterone (and its absence) in the cardiovascular system.

Several studies have shown that optimal testosterone levels have a protective effect. Men with coronary artery disease (CAD) have significantly lower levels of free testosterone, bioavailable testosterone, and free androgen index compared with healthy controls.[9] Furthermore, an inverse relationship between CAD severity and testosterone plasma levels has been described.[30] Interestingly, not only testosterone but also estradiol levels are lower in men with CAD.[30] This protective effect of testosterone against CAD may be due to its vasodilatory effect both in vitro and in vivo. In human angina studies, the administration of testosterone reduced symptoms and improved ischemia.[31] In addition to the vasodilatory effect of exogenous testosterone, endogenous testosterone potentiates the effect of exogenous testosterone.[32] Interestingly, this potentiating effect of endogenous testosterone was independent of androgen receptor expression.[32] In contrast, however, in a study of homozygous twin men, endogenous sex hormones have not been reported to be associated with cardiovascular disease.[33] The discrepancy may be due to the difference between the groups studied (homozygous twin men vs men selected from a random population) and the time of blood sample collection (healthy condition vs after CAD). Based on the literature, it can be stated that low testosterone levels increase the risk of CAD in men.

An adverse synergistic effect may be seen when testosterone deficiency and hypertension coexist: testosterone deficiency increases the risk of arteriosclerosis in older men;[7] testosterone also plays a permissive role in the development and maintenance of angiotensin II (Ang-II)—induced vascular dysfunction, hypertension, and cardiac hypertrophy.[34] There may be several possible explanations for this: on one hand, testosterone regulates the level of angiotensin 1 and 2 receptor proteins in the vessel wall, and, on the other hand, it affects the androgen receptor—mediated ERK 1/2 MAP kinase pathway. In addition, testosterone upregulates the levels of secondary messenger molecules (RhoA, Rho kinase, PKC), which are responsible for the sensitivity of vascular smooth muscle cell contractility.[34–38]

In the present study, we focused on coronary arterioles because separately both testosterone deficiency and hypertension have detrimental effects on coronary arteries, as detailed previously.

We have seen that hypertension has a detrimental effect on vascular function. Furthermore, based on literature data, testosterone deficiency also has a harmful effect on vascular function, but biomechanical properties have only been studied more narrowly in this regard so far. In the present study, we focused on coronary arterioles because separately, both testosterone deficiency and hypertension have adverse effects on coronary arteries.[32,39] Therefore, the question arises as to how the biomechanical function of coronary arterioles changes when they both present together. The present study investigated whether testosterone deficiency has a similar harmful effect on the resistance coronary arteries as estrogen deficiency in menopause. Furthermore, our

intention was to determine whether there *is* a connection between the damage caused by testosterone deficiency and that caused by hypertension with regard to the biomechanics of resistance coronary vessels—vessels that are directly responsible for myocardial blood supply. Therefore, an established andropausal animal model of orchidectomized rats was applied.[40,41]

MATERIALS AND METHODS

Materials

Intraperitoneal administration of Pentobarbital (Euthasol, CEVA Santé Animale, Liboume, France) was used for anesthesia. Long-term 100,000 IU of penicillin (TEVA-Biogal, Retardillin Debrecen, Hungary) was administered intramuscularly to reduce the risk of perioperative surgical site infection. Induction of angiotensin (AII)-dependent hypertension was performed as described elsewhere.[17,20,42] In brief, hypertension was induced using a subcutaneous osmotic minipump (ALZET, 2ML4, Durect Co, Cupertino, CA) containing AII acetate (AII, Sigma-Aldrich Co, St. Louis, MO, and Budapest, Hungary). The composition of the normal Krebs-Ringer solution (nKR) used for in vitro studies was as follows (in mmol/l): 119 NaCl, 4.7 KCl, 1.2 NaH_2PO_4, 1.17 $MgSO_4$, 24 $NaHCO_3$, 2.5 $CaCl_2$, 5.5 glucose, and 0.0345 EDTA. The calcium-free (Ca-free) Krebs solution contained (in mmol/l): 92 NaCl, 4.7 KCl, 1.18 NaH_2PO_4, 20 $MgCl_2$, 1.17 $MgSO_4$, 24 $NaHCO_3$, 5.5 glucose, 2 EGTA and 0.025 EDTA. Salts were purchased from Reanal (Budapest, Hungary).

Animals and Ethical Approval

Throughout the experiments, all animals received care according to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). All procedures and handling of the animals during the study were approved by the Animal Care Committee of Semmelweis University and by the Hungarian state authorities (permission number: PEI/001/820-2/2015).

A total of 41 sexually mature, 2-month-old male Sprague-Dawley rats (Innovo Kft., Gödöllő, Hungary) were housed at a constant temperature (22 ± 2°C) with 12-hour light-dark cycles. They were supplied ad libitum with tap water and standard laboratory rat chow.

After 7 days of acclimatization, the rats were distributed into 4 groups, control male (Co, n = 10); orchidectomized male (OCT, n = 13), AII-induced male (n = 10), and AII-induced and orchidectomized male (AII + OCT, n = 8). The weight of the rats was 280−320 g at the beginning of the study. The AII-treated groups (AII and AII + OCT) were subjected to subcutaneous implantation of osmotic minipump performed under anesthesia (pentobarbital, 45 mg/kg body weight (bwkg)) and sterile conditions. Subcutaneous AII infusion rate of the osmotic pump was 100 ng/bwkg/min. Previous studies reported that this subcutaneous dose led to chronic blood pressure elevation after 2−3 weeks without acute pressure effects, which is why we chose this model to study early hypertensive vessel alterations.[20,21] Orchidectomized animals (OCT and AII + OCT) were anesthetized with pentobarbital (45 mg/bwkg) and then a small incision was made at the posterior tip of each scrotum, through which the testis was exposed via slight compression. The spermatic cord was tied at the height of the vas deferens, and the testes were removed. The incision was closed in a regular fashion. The osmotic pumps were implanted through a small skin incision above the lumbar spine, also followed by regular wound closure. In the AII + OCT group, orchidectomy and osmotic pump implantation were performed in a single session. No notable medical or surgical complications were observed.

Pressure Arteriography of Coronary Arterioles

After 4 weeks of treatment, the rats were reanesthetized (45 mg/kg body weight intraperitoneal pentobarbital). Blood pressure was measured directly by cannulating the right carotid artery. This was followed by opening of the chest and removal and measurement of the heart. After that, intramural coronary arteries (secondary branches of the left anterior descending coronary artery with an in situ outer diameter of around 200 μm) were isolated under high magnification using careful microsurgical techniques and then removed and placed in cold Krebs-Ringer solution.[43] The intramural coronary arterial segment measuring approximately 2 mm in length was microcannulated and mounted in a vessel chamber filled with normal Krebs solution. The constant temperature of the bath was 37°C, and it was bubbled with a gas mixture of 5% CO_2, 20% O_2, and 75% N_2, which stabilized its pH at 7.4. The plastic microcannulas were connected to servocontrolled roller pumps (Living Systems, Burlington, VT) to set the intraluminal pressure. The arterioles were extended to their normal, in situ length and were pressurized under no-flow conditions.

The outer diameter and wall thickness of the arteries were measured by microangiometry. In this setup, the glass-bottom tissue bath was positioned in the light path of an inverted microscope (Leica, Wetzlar, Germany) to enable visualizing the inner and outer diameter changes of the arteriole. Magnified pictures of the vessels were acquired by a DCM 130E (US) camera. Analysis of the vessel pictures was performed off-line with the aid of a specific image-analyzing software (ScopePhoto). Length calibration was made with a micrometer etalon (Wild, Heerbrugg, Switzerland).

The coronary arteries were allowed to equilibrate for 30 minutes at 50 mmHg, in a normal Krebs solution, and the steady-state vessel diameter was measured. Finally, after 30-minute incubation in Ca-free Krebs-Ringer solution at 50 mmHg, the experiments were completed by taking the pressure-diameter curves: the pressure was decreased to 2 mmHg and then

Table 1. Basic characteristic and blood pressure parameters of the study groups

Variable	Co	OCT	AII	AII + OCT
Basic characteristic				
BW (g)	393 ± 9	396 ± 5	416 ± 9	401 ± 13
HW (g)	1.120 ± 0.03	1.195 ± 0.03	1.29 ± 0.02*	1.22 ± 0.08
HW/BW (g/kg)	2.85 ± 0.06	3.02 ± 0.07	3.08 ± 0.07	3.09 ± 0.13
Blood pressure data				
Systolic blood pressure	122 ± 6	118 ± 7	149 ± 7*	136 ± 9
Diastolic blood pressure	109 ± 6	94 ± 7	126 ± 7	102 ± 7**
Mean arterial pressure	114 ± 6	102 ± 7	134 ± 7*	114 ± 8

AII = angiotensin; ANOVA = analysis of variance; BW = body weight; Co = control male; HW = heart weight; OCT = orchidectomized male.
2-way ANOVA with post hoc Tukey's test. Values are the means ± SEM. '*' indicates statistically significant difference compared with the Co group ($P < .05$), and '**' indicates statistically significant difference compared with the AII group ($P < .05$).

increased first to 30 mmHg and then up to 50, 70, and 90 mmHg. The outer diameter and wall thickness were measured at each step.

Biomechanical Calculations

The biomechanical parameters from the Ca-free Krebs solution data were calculated as follows:

- Wall thickness (h): $h = r_o - r_i$;
- Wall thickness—to—lumen diameter (Q): $Q = h/d_i$;
- Cross-sectional area (A_w): $A_w = (r_o^2 - r_i^2)*\pi$;
- Tangential wall stress (σ): $\sigma=((P*r_i)/h)$, according to the Laplace-Frank equation;
- Incremental tangential elastic modulus of the cylindrical segments (E_{inc}): $E_{inc} = (2r_o r_i^2*\Delta P)/((r_o^2 - r_i^2)*\Delta r_o)$;

where h is the wall thickness, r_o and r_i are the actual values of the outer and inner radii, d_i is the inner diameter, P is the transmural (intraluminal) pressure, and Δr_o is the alteration of the outer radius during a pressure rise of ΔP, according to Cox.[44]

The spontaneous tone was calculated as follows: $T_{nKR} = (r_{oCa\text{-}free} - r_{onKR})/r_{oCa\text{-}free} * 100$ (%); where $r_{oCa\text{-}free}$ and r_{onKR} are the outer radii measured in a Ca-free solution and a nKR solution at 50 mmHg.

Statistical Evaluation

For statistical analysis, data measurements were compared by SPSS Sigma Stat software. All data are presented as the mean ± SEM. Normal distribution of data sets was tested with the Shapiro-Wilkes method. In case of normal distribution, 2-way analysis of variance (ANOVA) with the factors 'hypertension' and 'castration' was performed. If there were interactions between 'hypertension' and 'castration' ($p_{int} < 0.05$) in the 2-way ANOVA (wall thickness), we used one-way ANOVA. As a post hoc test, Tukey's post hoc test was used in both one-way and 2-way ANOVA. In case of non-normal distribution (wall thickness to lumen diameter, tangential wall stress and incremental elastic moduli), the Kruskal-Wallis test with Dunn's post hoc test was performed. A P value of $<.05$ was considered as the criterion for statistical significance. GraphPad Prism 6.0 software was used (GraphPad Software, La Jolla, Canada) for creating figures.

RESULTS

Physiological Parameters

After 4 weeks of AII treatment, the heart weight increased significantly in the AII group compared with the control rats (Table 1). The systolic blood pressure and arterial mean pressure were significantly higher in AII rats than in control animals (Table 1). The diastolic blood pressure was significantly lower in the AII + OCT groups than in the AII animals (Table 1).

Morphological and Biomechanical Parameters of Intramural Coronary Resistance Arteries

Despite the fact that all harvested arterial segments were anatomically and morphologically identical at preparation, there was a significant difference between the groups in the relaxed outer diameter of the vessels (Figure 1A). The outer diameter was significantly lower in OCT rats than in control animals. This value was also significantly decreased in the AII + OCT group compared with that in the AII and OCT groups (Figure 1A). The wall thickness was reduced in the OCT group compared with that in the control group (Figure 1B). In AII + OCT rats, we observed significantly greater wall thickness—to—lumen diameter than in AII and OCT groups (Figure 1C). In addition, as an effect of orchidectomy, the wall cross-sectional area was decreased compared with that in the control and AII groups (Figure 1D), indicating the development of inward hypotrophic remodeling in the OCT and AII + OCT groups.

The mechanical loading of the coronary artery wall and tangential wall stress were significantly lower in AII + OCT animals than in AII animals and OCT rats (Figure 2A). There were no significant differences between the incremental elastic moduli of the vessels (Figure 2B).

The spontaneous tone of the coronary arteries harvested from Ang-II acetate—treated groups (AII and AII + OCT) was significantly higher than that of the vessels taken from the Co and the OCT groups (Figure 3).

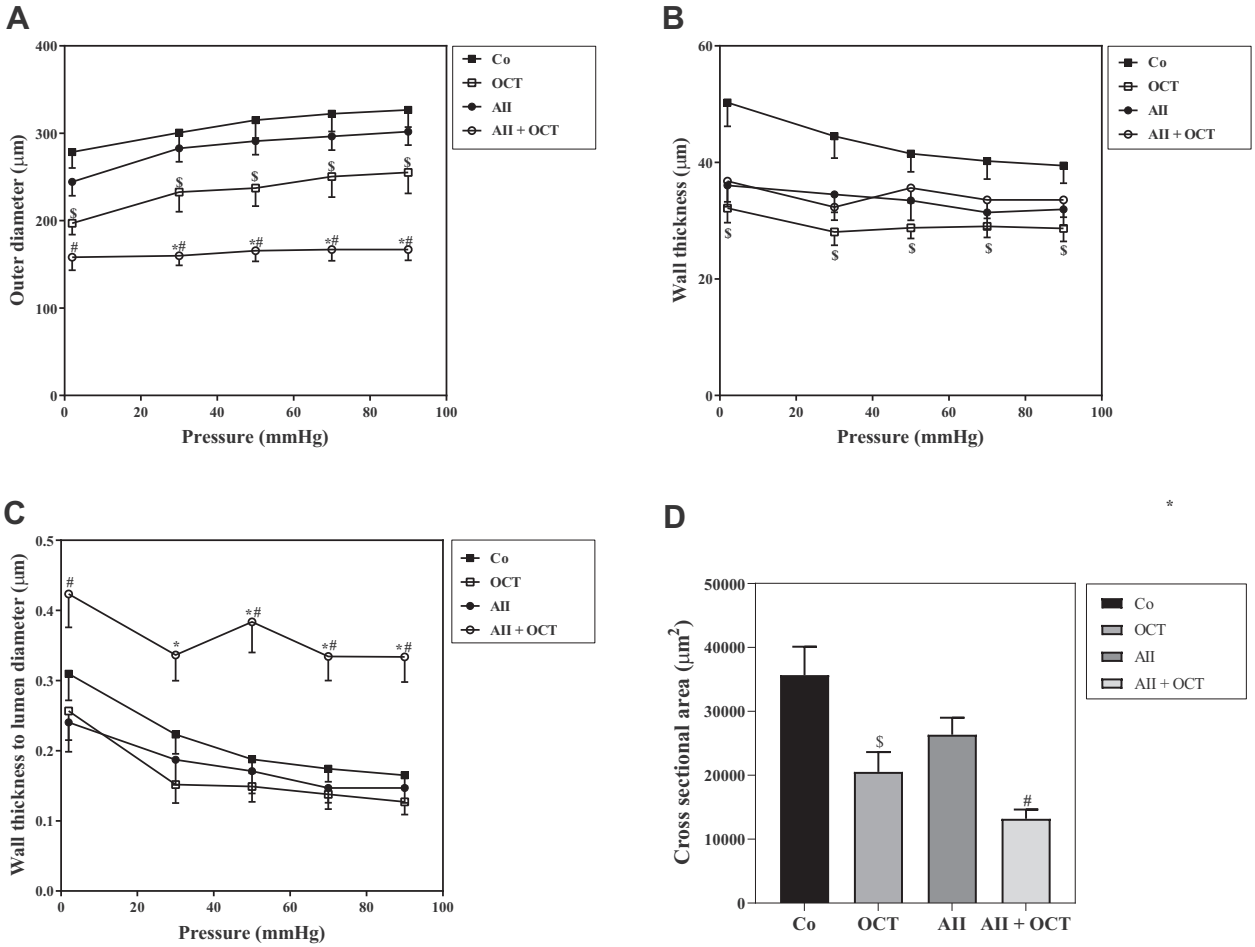

Figure 1. Geometric properties of the intramural coronary resistance arteries from Co (n = 10), OCT (n = 13), AII (n = 10) and AII + OCT (n = 8). (A) The values of the outer diameter as a function of intraluminal pressure measured in a passive condition (in calcium-free Krebs solution). The outer diameter was lower in OCT rats than in control animals. This value was also decreased in the AII + OCT group compared the AII and OCT groups. '$' indicates statistically significant difference compared to Co group, '#' indicates statistically significant difference compared to AII group, and '*' indicates statistically significant difference compared to OCT group. (B) The values of the wall thickness as a function of intraluminal pressure measured in the passive condition (in calcium-free Krebs solution). Wall thickness was reduced in the OCT group compared the Co group. '$' indicates statistically significant difference compared to Co group. (C) The values of the wall thickness to lumen diameter as a function of intraluminal pressure measured in the passive condition (in calcium-free Krebs solution). In AII + OCT rats, we observed greater wall thickness to lumen diameter than in AII and OCT groups. '#' indicates statistically significant difference compared to AII group, and '*' indicates statistically significant difference compared to OCT group. (D) The values of the wall cross-sectional area at 50 mmHg measured in the passive condition (in calcium-free Krebs solution). Wall cross-sectional area was decreased in the OCT group compared to the control group, and was also decreased in the AII + OCT group, compared to the AII group. '$' indicates statistically significant difference compared to Co group. '#' indicates statistically significant difference compared to AII group. For outer diameter and cross-sectional area we used two-way analysis of variance (ANOVA) at the same pressure (factors: hypertension, castration). For wall thickness we used one-way ANOVA at the same pressure, because of the pinteraction was smaller than 0.05 in two-way ANOVA. For wall thickness to lumen diameter we used Kruskal-Wallis test at the same pressure because of non-normal distribution. As a post hoc test, Tukey's test was used for one and two-way ANOVA, and Dunn's post hoc test for Kruskal-Wallis test. All values are expressed in mean ± SEM, a P-value <.05 was deemed significant.

DISCUSSION

In our current research, we were interested not only in the effects of hypertension or testosterone deficiency on intramural coronary arteries in males but also in their combined effects. To our knowledge, this is the first study investigating the combined effects of testosterone deficiency—andropause model via removal of the testicles—and Ang-II–induced hypertension on coronary artery biomechanics.

We verified our model by measuring the blood pressure, body weight, and heart weight in all animals. We found stabilizing hypertension in the AII groups and concomitant cardiac hypertrophy as a trophic effect of AII.[17]

When analyzing our results, one of our main goals was to identify effects caused by castration and the effects caused by hypertension. We also analyzed how these effects interact and theorized through what mechanism they may be achieved.

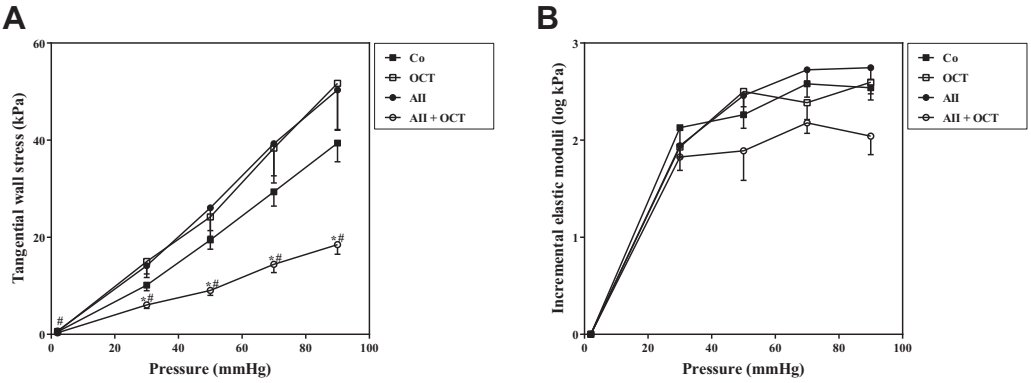

Figure 2. Biomechanical alterations of the intramural coronary resistance arteries from Co (n = 10), OCT (n = 13), AII (n = 10) and AII + OCT (n = 8). (A) The tangential wall stress of rat intramural coronary resistance arteries as a function of intraluminal pressure measured in the passive condition (in calcium-free Krebs solution). The tangential wall stress was lower in AII + OCT animals than in AII and OCT rats. '#' indicates statistically significant difference compared to AII group, and '*' indicates statistically significant difference compared to OCT group. (B) The incremental elastic moduli of the intramural coronary resistance arteries measured in the passive condition (in calcium-free Krebs solution). The logarithm of the incremental tangential elastic modulus is shown as a function of the intraluminal pressure. Kruskal-Wallis test at the same pressure with post hoc Dunn's test. All values are expressed in mean ± SEM, a P-value <.05 was deemed significant.

In our experiments, we found that castration resulted in a decrease in the external diameter and vascular cross-sectional area of coronary arterioles in both the normotensive and hypertensive groups, causing inward hypotrophic remodeling in our testosterone-deficient animals, which was further enhanced by hypertension. The cross-sectional area in the AII + OCT group was not significantly different from the OCT group, but there was a significant difference in this regard between the AII + OCT and the AII groups. Consequently, the inward hypotrophic remodeling was primarily produced by castration, that is, due to the lack of testosterone. There are conflicting research results regarding the relationship between testosterone and cardiovascular morbidity and mortality.[45,46] Overly high levels of testosterone (such as anabolic steroid intake) compromises endothelial function and increases the likelihood of acute coronary events. Furthermore, it also increases vascular calcification and[47] atherosclerosis,[48] lowers high-density lipoprotein and increases low-density lipoprotein levels,[49] and increases the risk of thromboembolism[50] and coronary vasospasm.[51,52] In parallel, too low testosterone levels again predispose to negative effects, and the risk of stroke and CAD is increased[53,54] through mechanisms such as augmented coronary calcifications.[55] In contrast, positive effects dominate when testosterone levels are optimal: coronary arteries have an increased vasodilator response.[45] In testosterone deficiency, testosterone replacement may restore deficiency-induced damage through mechanisms such as the endothelium-dependent (bradykinin) relaxation response of coronary vessels.[56]

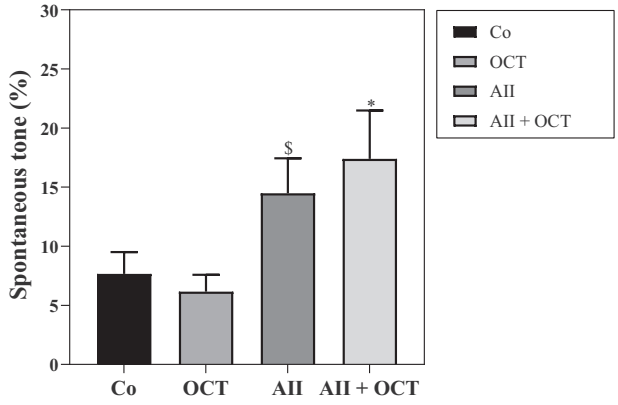

Figure 3. Spontanoues tone of the intramural coronary resistance arteries from Co (n = 10), OCT (n = 13), AII (n = 10) and AII + OCT (n = 8) in vitro at 50 mmHg. The spontaneous tone of the AII and AII + OCT groups was significantly higher than that of the vessels taken from the Co and OCT groups. '$' indicates a statistically significant difference compared to control group, and '*' indicates statistically significant difference compared to OCT group. Two-way ANOVA with post hoc Tukey's test. All values are expressed in mean ± SEM, a P-value <.05 was deemed significant.

Increased wall tension leads to increased contractility in blood vessels, whereas decreased wall tension reduces vascular contractility.[18] In case of wall stress, we have found that in the AII + OCT group, as a part of the adaptation, the tangential wall stress was reduced compared with the AII and OCT groups because of increased wall thickness to lumen ratio and inward hypotrophic remodeling. Our finding of inward hypotrophic remodeling of the coronary arteries caused by castration (ie, testosterone deficiency) seems to parallel the previously described results in the literature. Several mechanisms may play a role in the adaptation mechanisms described previously: testosterone regulates the level of angiotensin 1 receptor and angiotensin 2 receptor proteins in the vessel wall; it affects the androgen receptor—mediated ERK 1/2 MAP kinase—pathway and upregulates the levels of secondary messenger molecules (RhoA, Rho kinase, PKC) that play a role in the setting of vascular smooth muscle cell contractility.[34—38]

As a result of Ang-II—induced hypertension, we have not yet seen a change in the wall structure of the intramural coronary arteries, but an increase in vascular tone has already been observed. Down the line, the chronic morphological adaptation of the wall causes inward eutrophic remodeling in hypertension. In our animal model of Ang-II—induced early hypertension, we were able to detect the first step of classical hypertonic adaptation, an increase in vascular tone. This increase in the spontaneous tone was independent of castration. It has long been described in the literature that AII enhances the expression of adrenergic receptors.[57,58] Contractility was also increased in the coronary arteries through alternative mechanisms here and in our earlier studies.[17,20]

In summary, both AII-induced hypertension and castration result in similar vascular wall changes, but the underlying mechanisms are different: testosterone deficiency leads to inward hypotrophic remodeling with consequent vessel wall tension under in vivo pressure, whereas during high blood pressure, tension in the vessel wall is caused by increased intraluminal pressure, which will subsequently lead to inward eutrophic remodeling. In the castrated group, hypertension further reduced the vascular diameter compared with the hypertensive group, so a high blood pressure further aggravated the damage caused by testosterone deficiency, which may explain the increase in cardiac morbidity and mortality (eg, ischemia, acute myocardial infarction).[59] Consequently, it seems that restoring physiological male hormone levels at the beginning of andropause might have beneficial cardiovascular effects both at a normal blood pressure and in hypertension.

CONCLUSIONS

To our knowledge, we have been the first to investigate the combined effects of hypertension and testosterone deficiency on intramural coronary vessels. Our main observation is that castration results in inward hypotrophic remodeling, whereas hypertension increases the spontaneous tone. Through different mechanisms, both testosterone deficiency and hypertension damage the intramural small coronary arteries directly responsible for the blood supply to the heart, and these effects may add up. In conclusion, andropause alone can be considered as a cardiovascular risk factor that will further exacerbate vascular damage in hypertension.

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SUPPLEMENTARY DATA

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Research article

Additive damage in the thromboxane related vasoconstriction and bradykinin relaxation of intramural coronary resistance arterioles in a rodent model of andropausal hypertension

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ABSTRACT

Hypertension and andropause both accelerate age–related vascular deterioration. We aimed to evaluate the effects of angiotensin-II induced hypertension and deficiency of testosterone combined regarding the resistance coronaries found intramurally.

Four male groups were formed from the animals: control group (Co, n = 10); the group that underwenr orchidectomy (ORC, n = 13), those that received an infusion of angiotensin-II (AII, n = 10) and a grous that received AII infusion and were also surgically orchidectomized (AII + ORC, n = 8). AII and AII + ORC animals were infused with infusing angiotensin-II (100 ng/min/kg) using osmotic minipumps. Orchidectomy was perfomed in the ORC and the AII + ORC groupsto establish deficiency regarding testosterone. Following four weeks of treatment, pressure-arteriography was performed in vitro, and the tone induced by administration of thromboxane-agonist (U46619) and bradykinin during analysis of the intramural coronaries (well-known to be resistance arterioles) was studied.

U46619-induced vasoconstriction poved to be significantly decreased in the ORC and AII + ORC groups when compared with Co and AII animals. In ORC and AII + ORC groups, the bradykinin-induced relaxation was also significantly reduced to a greater extent compared to Co and AII rats. Following orchidectomy, the vasocontraction and vasodilatation capacity of blood vessels is reduced. The effect of testosterone deficiency on constrictor tone and relaxation remains pronounced even in AII hypertension: testosterone deficiency further narrows adaptation range in the double noxa (AII + ORC) group. Our studies suggest that vascular changes caused by high blood pressure and testosterone deficiency together may significantly increase age-related cardiovascular risk.

1. Introduction

Vascular aging is the process during which the structure and function of blood vessels is damaged over time. These changes together may lead to vascular vulnerability and hypertension [1, 2, 3, 4]. Hypertension related damage seems to be a type of accelerated vascular aging, further increasing cardiovascular risk [5]. Characteristic lesions of vascular damage are endothelial dysfunction, vascular wall remodeling,

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inflammation, athero- and arteriosclerosis and increased vascular wall stiffness [4, 5].

It has long been established that in women menopausal estrogen deficiency significantly increases cardiovascular risk [6, 7]. It is also well known that testosterone deficiency (men with hypogonadism/andropause) also increases cardiovascular risk [8, 9]. Similar to accelerated vascular aging caused by hypertension, andropause may correspond to a specific effect on men that also accelerates vascular aging. The risk of cardiovascular disease (CVD) increases not only directly due to testosterone deficiency [10], but also indirectly, since testosterone deficiency increases the risk of metabolic syndrome, type 2 diabetes, obesity, atherosclerosis, dyslipidemia and hypertension [10]. Contradictory results can be found on the effects of testosterone supplementation in the elderly. On the one hand, testosterone supplementation reduced ischemic symptoms in human angina studies [11]. On the other hand, testosterone supplementation in the elderly (in men over 60 years of age) may have a detrimental effect on the cardiovascular system [12]. Frinke et al. found that in case of men at ages 65 years and older the risk for developing either a stroke or a non-mortal infarction within the myocardium increased during the first 90 days of testosterone treatment, however this risk decreased over time [12, 13]. In another publication, testosterone supplementation increased mortality, and the risk of myocardial infarction and stroke in elderly (over 60 years) men who had a preexisting previous heart condition [12, 14].

Testosterone has a relaxing effect on the coronary arteries: testosterone opens the large-conductance calcium-activated potassium channel. Removal of the endothelium on porcine coronary arteries did not significantly affect testosterone-induced coronary artery relaxation; therefore, it is likely that the vascular smooth muscle cell is the primary site of the vasodilatory effect of testosterone in porcine coronary arteries [15]. Testosterone also relaxes thoracic aorta in rat [16], coronary artery and thoracic aorta in rabbit [17]. Based on previous studies, it is known that testosterone-induced vasorelaxation is independent of the classical androgen receptor [18].

Low testosterone levels alone damage small vessel function: both relaxation and contraction. Testosterone deficiency has detrimental effects on relaxation on one hand through decreased NO production [19] and on the other hand through altered cyclic adenosine monophosphate and potassium channel activation [20]. In contrast, testosterone enhanced thromboxane-induced vasoconstriction in the coronary arteries in male guinea pigs [21]. Testosterone leads to relaxation via nitrogen monoxide (NO) through the production of eNOS in human and in male rats [19, 22]. Myogenic tone also decreases with castration in male rats [23]. The internal pudendal arteries from orchidectomized rats exhibited decreased phenylephrine- and electrical field stimulation induced contraction and decreased acetylcholine- and relaxation values when stimulated by an electric field [24].

Animal studies have found, that as a result of castration, not only increases but also decreases in blood pressure may be observed [20, 25, 26]. To resolve conflicting results, further studies are needed.

However, testosterone deficiency does not only directly damage the function of small blood vessels, but also indirectly. Testosterone deficiency increases the risk of obesity, dyslipidemia, reduction of muscle mass and insulin resistance, which can strongly affect vascular aging including vascular function. Connection of these states with elevated cardiovascular risk is obvious. During a 5-year follow-up examination of adolescents, Ryder et al. found that having type 2 diabetes, being obese and demonstrating elevated values for baseline systolic blood pressure early in life leads to an acceleration regarding the risk of premature vascular aging. Being obese, suffering from type 2 diabetes and having elevated systolic blood pressure values lear to an increase in the thickness of the intima and media of the carotid arteries; it also increased the velocity of the pulse wave from the carotid arteries to the-femoral arteries [27]. In adult Caucasian population without cardiovascular disease, insulin resistance showed a positive association with carotid-to-femoral pulse wave velocity and brachial-to-ankle pulse wave velocity and with early vascular ageing (the individuals with values of carotid-to-femoral pulse wave velocity or brachial-to-ankle pulse wave velocity over 90th percenteliles) [28, 29]. The investigated values characterizing lipid profile (low-density lipprotein levels, triglyceride non-high-density, lipoprotein and also total cholesterol levels) strongly correlated with increased pulse wave velocity values and early vascular aging. Furthermore, triglyceride/high-density lipoprotein ratio might be used for prediction to early vascular aging [30]. There is a reciprocal relationship between sarcopenia and vascular aging: advanced vascular aging leads to a decrease in skeletal muscle mass, while sarcopenia (loss of muscle mass and strength) increases cardiovascular burden [31].

Like testosterone, high blood pressure also affects the function of small blood vessels. Hypertension also increases myogenic tone and thromboxane—induced contraction [32, 33], while endothelium -dependent dilatation deteriorates [34]. As an initial step in hypertension in coronary resistance vessels, wall tension and elastic modulus increase in male rats, while inward eutrophic remodeling may be observed in female animals. Vessel tone and contractile responses to thromboxane agonist increase in both sexes, but this is significantly more pronounced in males, while decrease in vasorelaxation is bigger in females [35].

There is significantly less data available regarding effects of a deficiency of testosterone combined with hypertension on resistence arteries [26, 36]. The effects of testosterone-deficiency and hypertension together on the morphology and also on the biomechanical characteristics of resistance coronaries were first examined by our team [37]. In brief: as a result of hypertension, spontaneous tone increases first, even without remodeling of wall structure. Testosterone deficiency alone results in inward hypotrophic remodeling, which persists in double noxa. Hypertensive stimulus with testosterone deficiency further reduces vessel diameter [37]. However, little is known about the functional adaptation of blood vessels affected by the double noxa [26].

In the present study, the effects of a surgically established deficiency of testosterone together with hypertension was investigated on the coronary resistance arterioles.

2. Materials and methods

2.1. Chemicals

Rats were anesthetized with Euthasol (by CEVA Santé Animale, Liboume, France). Hypertension was established via an Angiotensin (AII) modelas described previously [32, 37, 38, 39]. An osmotic minipump (by Alzet, 2ML4, Durect Co, Cupertino, US) containing AII (Sigma-Aldrich Co, St. Louis, Missouri, US and Budapest, Hungary) was implanted subcutaneously. The experiments were conducted under in vitro conditions; physiological Krebs-Ringer (nKR) solution was used. The nKR solution was composed as follows (in mmol/l) 119 NaCl, 1.2 NaH2PO4, 4.7 KCl, 1.17 MgSO4, 2.5 CaCl2, 24 NaHCO3,5.5 glucose and 0.0345 EDTA (Reneal, Budapest, Hungary). The calcium-free Krebs solution - used to achieve total relaxation within the vascular smooth muscle tissue – was composed of the following (in mmol/l): 4.7 KCl, 1.18 NaH2PO4, 92 NaCl, 20 1.17 MgSO4, MgCl2, 24 NaHCO3, 2 EGTA 5.5 glucose, and 0.025 EDTA (Reneal, Budapest, Hungary). U46619 (a TxA2 receptor agonist) and bradykinin-acetate (BK). All chemicals had a purity greater than 98% (Sigma-Aldrich, St Louis, Missouri, US and Budapest, Hungary) and they were all prepared in the nKR solution on the day of the experiment.

2.2. Animals and animal care

Throughout the experiments, the relevant regulations and guideline of the Institute for Laboratory Animal Researches and the National Society for Medical Research (published in the National Institutes of Health Publication, No. 86–23, revised 1996) were adhered to when using and caring for the animals during the experiment series. The study was accredited at Semmelweis University (by the dedicated Animal Care

Committee) and also by the relevant Hungarian authorities (PEI/001/820–2/2015 and PE/EA/1427–7//2018).

Young adult Sprague-Dawley male rats (n = 41, 2-month-old, 280–320 g) were purchased from Innovo Kft (Gödöllő, Hungary); they were housed at room temperature (22 ± 2 °C), and were provided a light-dark cycles of 12 h. Standard rat chow and tap water were available to the animals ad libitum.

After seven days acclimatization, they were divided into the following four groups: control (Co, n = 10); those undergoing surgical orchidectomy (ORC, n = 13), those receiving an infusion of AII (AII, n = 10) and the AII infused and surgically orchidectomized group (AII + ORC, n = 8). The animals that received a treatment of AII (AII and AII + ORC) had osmotic minipumps surgically implanted under anesthesia (45 mg/kg intraperitoneal pentobarbital), subcutaneously into the region above the lumbar spine. The infusion rate of the AII infusion minipump was 100 ng/kg/min, which leads to chronic blood pressure elevation without any acute pressure effects in 2–3 weeks [37, 38, 39]. We choose this AII hypertension model to study coronary arteries alterations in the early hypertensive state. 100 ng/mg/kg/min is a sub-pressor dose; therefore, it does not have acute hypertensive effects. Administered chronically, it leads to the development of hypertension. We choose week 4 in our series to perform experiments as this corresponds with the early stages of hypertension. In our previous publication [37] this AII infusion increased both systolic and mean blood pressure values in compared to the Co rats. Hormone deficiency lead to decreases blood pressure in the group of hypertensive rats (AII + ORC) compared with the rats that did not undergo orchidectomy AII-infused animals. Mean arterial pressure values from the study groups were found to be the following: Co: 114 ± 6 mmHg; ORC: 102 ± 7 mmHg; AII: 134 ± 7 mmHg and AII + ORC: 114 ± 8 mmHg [37].

Orchidectomy is as an established andropause model [37, 40, 41]. In this study it resulted in the following androgen levels: 2.2 ng/ml in intact and 0.1 ng/ml in orchidectomized rats [40, 41]. To perform orchidectomy, animals (ORC and AII + ORC) under anesthesia (45 mg/kg, intraperitoneal), the testis was removed surgically at the posterior tip of each scrotum as described previously [37]. In the AII + ORC group, the removal of testis and osmotic minipump implantation were performed at the same time. Neither medical nor surgical complications occurred during the course of treatment.

2.3. Pressure arteriography of coronary arterioles

AT the end of the experimental series body weight was measured, values were as follows: Co, 393 ± 9 g; ORC, 396 ± 5 g; AII, 416 ± 9 g and AII + ORC, 401 ± 13 g (non significant with two-way ANOVA) [37]. The animals were then anaesthetized as before, the heart removed through the chest and intramural resistance coronary branches with similar in situ outer diameters (ca. 200 μm) were carefully dissected and isolated under a stereomicroscope from secondary branches of the left anterior descending coronary [42]. These arteriolar segments with a length of approximately 2 mm were removed, placed in a vessel chamber filled with nKR, and cannulated at both ends using plastic microcannulas. Finally, they were extended to their normal, in situ, in vivo length. The temperature of the nKR was set at 37 °C and it was also bubbled with predetermined ratio of gasese (20% O_2, 75% N_2 and 5 % CO_2, – this stabilized pH at 7.4). Theisolated cannulated vessel segments were mounted and pressurized on the pressure-servo-systems (Living Systems, Burlington, VT, US) under no-flow conditions.

The arterioles' inner diameter was measured after acquiring microscopic images of the vessels (aka "microangiometry"). This experimental setup contained a glass-bottom tissue bath positioned under an inverted microscope (Leica), centered right into a path of light to visualize alterations of the inner diameter of the arteriole segment. A DCM 130 E camera captured digital images of the isolated segments. Processing and analysis of the acquired microscopic images was performed offline by dedicated image-analyzing software (Scope Photo). A micrometer etalon was used to calibrate length (Wild, Heerbrugg, Switzerland).

Contractile characteristics of the studied isolated vessel segments was performed as follows: equilibration for 30 min at 50 mmHg to allow for establishment of myogenic tone [43]. This step was necessary to check the viability of the vessels. Following equilibration, the steady-state vessel diameter was photographed. Pressure-diameter curves were recorded following two consequent conditioning pressure cycles (2-90-2-90-2 mmHg). Then pressure was increased to 30 mmHg and then to 50, 70 and 90 mmHg. Inner diameter values were measured at pressure value following equilibration. TxA2 agonist (U46619-concentration of 10^{-6} M) was added to the tissue bath and incubation was allowed for 10 min at 50 mmHg; at this point an image of the steady-state diameter was captured. Pressure-diameter curves were then recorded sequentially and repeatedly. Inner diameter values were always measured at each pressure value. Without washing out the U46619, bradykinin (BK) was added in 10^{-6} M concentration, and a further 20 min of incubation at 50 mmHg was allowed before capturing the image of the steady–state diameter. Thereafter, the pressure diameter curves were recorded repeatedly. The inner diameter was measured at each step. Finally, all drugs were washed out with calcium-free Krebs-Ringer solution, and after a final 30 min of incubation at 50 mmHg, an image of the relaxed vessel diameter was taken and the experiments were finished by taking the pressure diameter curves with the fully relaxed muscle (passive state). Inner diameter was measured at each step.

2.4. Contractility calculations

The following characteristic parameters were calculated based on the data from the pressure-diameter curves:

- Mogenic tone (%)

$$T_{nKR} = (r_{iCa\text{-}free} - r_{inKR})/r_{iCa\text{-}free} * 100$$

- U46619-induced constriction (%):

$$C_{U46619} = (r_{inKR} - r_{iU46619})/r_{iCa\text{-}free} * 100$$

- Bradykinin-induced relaxation (%)

$$R_{BK} = (r_{iBK} - r_{iU46619})/r_{iCa\text{-}free} * 100$$

where $r_{iCa\text{-}free}$ and r_{inKR} are values representing inner radii measurements taken in calcium-free and in a nKRr at the same pressure. $R_{iU46619}$ and r_{iBK} are measurement values of inner radii following application of TxA2 agonist (U46619) and bradykinin - at the same pressure points, respectively. Inner radius data can be found in the supplementary data.

2.5. Statistical evaluation

Statistical comparison of the measured data was performed by SPSS Sigma Stat and GraphPad Prism 6.0 softwares. We have presented all of our data mean ± SEM. Shapiro-Wilk method was used to assess normal distribution. In case of repeated measures data (pressure curves) mixed-effects models was performed. We applied Tukey's post hoc in the mixed-effect models. Statistical significance was considered at $P < 0.05$. GraphPad Prism 6.0 software was used to plot Figures. P values and Tukey's post hoc test numbers are found in the supplementary material.

3. Results

3.1. Contractility parameters of intramural coronary resistance arterioles

Vascular contractility was checked by myogenic tone derived from the inner radius of the coronary vessels. In the AII group, myogenic tone was significantly higher compared to both the Co and ORC groups.

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Orchidectomy alone did not alter myogenic tone. However, due to double noxa (in the AII + ORC group), the myogenic tone was significantly higher compared to the ORC group (Figure 1).

Thromboxane induced (U46619) vasoconstriction did not differ between Co and AII groups, but was significantly reduced due to castration in both the ORC and AII + ORC groups compared to Co animals. The AII + ORC group differed significantly different not only from the group of controls (Co) but also from the AII group. Compared to the ORC group, no further reduction in thromboxane-induced vasoconstriction was observed with the combined effect of AII treatment and testosterone deficiency (in the AII + ORC group) (Figure 2).

Endothelial dilatation was tested with bradykinin, which did not change to AII treatment alone compared to the control group (Co vs. AII group). Due to testosterone – deficiency, a decrease in relaxation was observed compared to the Co group and AII groups (Co vs. ORC and AII vs. ORC). Combined noxa of hypertension and testosterone–deficiency decreased bradykinin induced relaxation compared to the Co group and the AII only group (Co vs. AII + ORC group and AII vs. AII + ORC group), but there was no difference compared to orchidectomy alone group (ORC vs. AII + ORC) (Figure 3).

4. Discussion

In our current study, we investigated the effects of a surgically established deficiency of testosterone together with hypertension on the characteristics of intramural coronaries of male rats. The major findings of our investigation may be summarized as follows [1]: Testosterone–deficiency alone impaired vascular reactivity; decreased both thromboxane induced contraction and bradykinin dependent relaxation [2]; the effect of testosterone deficiency on constrictor tone and relaxation can be detected both in AII hypertension and in normotensive animals [3]; double noxa, that is AII hypertension and testosterone deficiency together, resulted in a combination of abnormalities observed in both the AII group and ORC group: myogenic tone increased, capacity for both contraction and relaxation decreased, further narrowing the range of vascular adaptation relative to ORC and AII groups, however, the association of noxa does not alter what was altered by the one factor. The results from our study may well contribute to the better understanding of the initial steps of age-related impairment found regarding

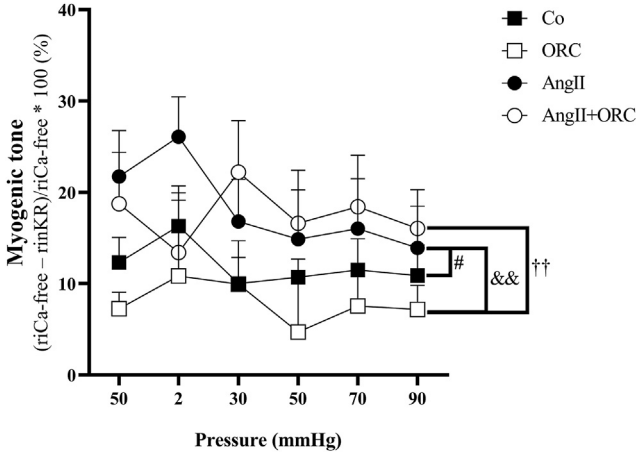

Figure 1. Myogenic tone is plotted on this figure as a function of intraluminal pressure. The inner radii values of the intramural coronaries from the Co, ORC, AII and AII + ORC groups were was measured under passive conditions (in Calcium-free solution) of. Myogenic tone found regarding coronaries in both the AII and in the AII + ORC groups was significantly higher compared to Co and ORC groups. Data in this figure are expressed ase mean (+/-SEM) values. We used mixed-effects analysis at the same pressure. The significant values from Tukey's post hoc tests regarding the 4 investigated groups are shown. #P < 0.05 Co vs. AII; && P < 0.01 ORC vs. AII; ††P < 0.01 ORC vs. AII + ORC.

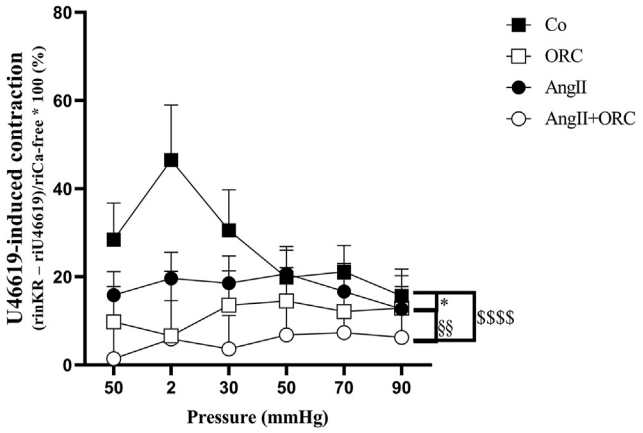

Figure 2. U46619-induced contraction is plotted in this figure as a function of the intraluminal pressure values measured in the intramural coronary arteries from the Co, ORC, AII and AII + ORC animals. Vasoconstriction induced by U46619 was significantly less in the coronaries of the orchidectomized animals (ORC and AII + ORC groups) compared to that Co and AII rats. We expressed data as mean (SEM) values. We used mixed-effects analysis at the same pressure. . The significant values from Tukey's post hoc tests regarding the 4 investigated groups are shown. *P < 0.05 Co vs. ORC; $$$$ P < 0.0001 Co vs. AII + ORC; §§P < 0.01 AII vs. AII + ORC.

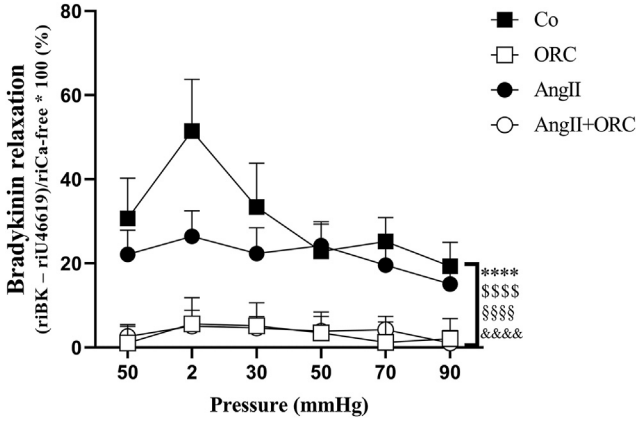

Figure 3. Bradykinin relaxation is plotted in this figure as a function of intraluminal pressure measured of the intramural coronary arteries from the Co, ORC, AII and AII + ORC animals. Relaxation induced by Bradykinin was significantly decreased in the group that underwent orchidectomy (ORC and AII + ORC groups) compared to values from the Co and AII animals. We expressed data as mean (SEM) values. We used mixed-effects analysis at the same pressure. The significant values from Tukey's post hoc tests regarding the 4 investigated groups are shown. ****P < 0.0001 Co vs. ORC; $$$$ P < 0.0001 Co vs. AII + ORC; §§§§P < 0.0001 AII vs. AII + ORC and &&&& P < 0.0001 ORC vs. AII.

vascular function in hypertension and in andropause in males, these result in an increase of cardiovascular morbidity and mortality.

Our animal model provides an opportunity to study early vascular damage caused by hypertension and testosterone–deficiency together. It should be emphasized from our previous results in female animals, that our early hypertension model is suitable for the detection of initial lesions. This is the first study where the effects of both hypertension and testosterone hormone depletion were studied on the functional characteristics of intramural coronaries (contractile and vasodilatative capacity) via a sub-pressor dose angiotensin II-induced hypertension model. The etiopathogenesis of vascular aging is heterogeneous, it is influenced by many factors. Angiotensin II stimulates the formation of superoxide anion $O2^-$ through the activation of membrane-bound NAD(P)H-oxidase with the type 1 angiotensin receptor, which contributes to the reduction

of NO bioavailability, vascular damage and atherogenesis [44, 45]. Furthermore, in diabetic men, decreased levels of testosterone were linked to both elevated total and mitochondrial reactive oxygen species as well as to the decreased function of superoxide dismutase and glutathione S-transferase (expression of both was decreased): testosterone levels and ROS production were found to correlate negatively [46]. Inflammatory cytokines, specifically interleukins (IL-2, IL-6, IL-10, IL-12 and IL-13) were increased when testosterone levels decresed, further, testosterone supplementation decreased interleukin levels [47]. In our model the vascular damaging effects could be studied both independently for hypertension and for lowered testosterone levels in males as well as for their combined action.

In our present study, testosterone-deficiency decreased both contractility and endothelial relaxation in the ORC group. Data in the literature are contradictory regarding the effects of testosterone deficiency and supplementation on the cardiovascular system. One of the acute extra-nuclear effects of testosterone is that it increases the capacity for arterial dilation in the mesenteric arteries, aortic rings and in the basilar arteries of different species [48]. In physiological concentrations these effects are endothelium-dependent, however, supra-physiological doses lead to endothelium-independent dilatory action [48]. Acute doses of testosterone resulted in endothelium-independent vasorelaxation in coronaries and the aorta both in males and females [17]. However, it is not the acute vasodilator effect of testosterone that we observe following castration or castration + testosterone supplementation. Using the Langendorff model it was observed in the coronaries of male rats, that BK induced vasodilation is impaired following castration, and this was restored by supplementation of physiological and supra-physiological doses of testosterone [48]. In contrast, in human studies it was observed, that physiological supplementation of testosterone weakened vascular reactivity in those with androgen deficiency [49]. In an other study castration decreased electrical field stimulation (EFS) induced contraction in mesentery vessels and increased vasodilator response [50]. Changes in contraction on different types of vessels (mesenteric arteries and aorta) following castration have also been described, but site-specific action in coronary arterioles could not be excluded. As a result of castration the quantity of TxA2 produced by smooth muscle cells increases in mesenteric arteries [51]. TxA2 release also increases in the aorta and in mesentery arteries shortly following castration [52]. Furthermore, as a result of orchidectomy, the phenylephrine- and EFS induced contraction and acetylcholine- and EFS induced relaxation decreases in the internal pudendal arteries from rats [24]. In our current study, castration alone did not decrease myogenic tone, however U46619 induced tone were decreased following castration compared to values found in the control group. Bradykinin-induced vasodilation was decreased as well.

An early feature in hypertensive lesions is an increase in myogenic tone [53]. In our current study, similar to our previous publications [37], myogenic tone was significantly elevated in the AII group.

Our studies demonstrated that andropause-induced reduced contraction and relaxation ability appear not only in normotension, however under the conditions of drug-infusion hypertension. Reduced vascular adaptation ability of testosterone plus hypertensive animals significantly differed from those subjected to hypertension alone. Furthermore, in the double noxa group, we found not only the adverse effects caused by testosterone deficiency but also myogenic tone increase caused by AII treatment, so all three differences were observed in the double noxa group (myogenic tone increase, decreased contractility and relaxation ability). The combined presence of the two damaging factors did not alter what was altered by the single factor. The effects of hypertension and castration have been studied previously on mesenteric arteries and on extremity arteries as well [26, 36, 54, 55]. However, data are contradictory. In spontaneously hypertensive male rats, autonomic venous tone was found to be significantly reduced by castration [36]. In mesenteric arteries of spontaneously hypertensive male rats, double noxa, in parallel with our results, weakened the serotonin-induced vasoconstriction, but slightly worsened endothelial dilatation [26]. In Ang II-induced hypertension, contractility increased in mesenteric arteries. However, this was prevented by castration and restored by supplementation of testosterone [55]. These conflicting results may be due to different types of vessels (coronary vs. mesenteric artery, aorta, musculocutaneous arteries), or different types of animals (Sprague Dawley rats vs. spontaneously hypertensive rats).

We know from previous studies, that both bodybuilders and athletes, using anabolic steroids and men with andropause are more at risk for cardiovascular morbidity and mortality than fertile-age men with physiological hormone levels [9, 56]. Based on this, our present study might describe one of the potential mechanisms underlying the cardiovascular vulnerability of andropausal men.

In addition, based on our results, there is rationale for hormone replacement to physiological levels in andropausal men to reduce the vulnerability of the vascular system. Such protective effect can be expected both in normotensive and hypertensive conditions. We suggest to expect the cardiovascular effects of testosterone in form of an “U” shaped curve: both too high and too low testosterone levels can be expected to deviate from the optimum, emphasis should be put to ensure optimal testosterone levels. Therefore, in case of androgen deficiency supplementation of testosterone to an optimal level may have a protective effect in terms of cardiovascular disease [57, 58, 59]. Of course, this protective effect is assumed to start at the onset of hormone deficiency. In an analogue situation of women the positive effects of menopausal hormone replacement are noticeable only if hormone replacement is started within 5 years of the onset of menopause [6]. Beyond 10 years, definitive vascular damage develops, and complications outweigh the benefits of treatment [60]. Similar advantages and disadvantage may also be assumed regarding males in the andropause. Our line of reasoning should be supported by further animal and clinical studies, but contrary to the paradigm, our results support that restoring physiological hormone levels in andropause may reduce cardiovascular risk.

The limitation of our study is the lack of histology or immunoblotting examinations. Direct study of these vessels cannot be studied ethically in humans; therefore animal testing is of paramount importance. Although human conditions cannot be inferred directly from our animal experimental result, they may provide perspective for the design of clinical trials.

5. Conclusion

Based on our present results, both noxa resulted in different and unfavourable changes in vessel function. That is, multiplicative, noxa-specific and consistently developing changes –independent from each other - resembling accelerated vascular aging have been detected in this early model with slight differences. After all, there was an increase in myogenic tone in both AII groups and a decrease in TXA and BK tones in both ORC groups, and all of these were observed together in the double noxa group, however, the association of two harmful noxa does not alter what was altered by the single factor. These are the initial changes that later can be expected to induce definitive target organ damage. Our results call attention to the fact, that similar to female menopause, andropause also increases cardiovascular vulnerability. More importantly, our results were obtained from resistance coronaries - responsible for the blood supply of the heart in a direct manner. The condition of these vessels may also play a key role in heart function and may determine cardiovascular ischemic events.

Declarations

Author contribution statement

Attila Jósvai; Judit Hetthéssy: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Marianna Török: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Máté Mátrai; Anna Monori-Kiss; Jennifer Makk; Márton Vezér; Levente Sára; István Szabó: Performed the experiments; Analyzed and interpreted the data.

Béla Szakács; György L. Nádasy; Szabolcs Várbíró: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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