

SEX DIFFERENCES IN SPORT-ADAPTATION OF SKELETAL MUSCLE ARTERIOLES AND THE LEFT ANTERIOR DESCENDING CORONARY NETWORK

PhD thesis

Petra Merkely MD

Doctoral School of Basic and Translational Medicine
Semmelweis University



Supervisor: Szabolcs Várbió, MD, DSc

Official reviewers: Gábor Pavlik, MD, DSc
Attila Szendrői, MD, PhD

Head of the Complex Examination Committee:
Prof. György Losonczy MD, DSc

Members of the Complex Examination Committee:
Andrea Székely, MD, DSc
Ákos Jobbágy, MSc, DSc

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LIST OF ABBREVIATIONS

Ade	adenosine
AIx	Augmentation index
CAD	coronary artery disease
CT	computed tomography
d	diastole
DAB	3-3'-diamino-benzidine
E_{inc}	incremental elastic modulus
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
Erk	extracellular signal-regulated kinase
FC	female control
FE	female exercised
FMD	Flow-mediated dilation
FS	fractional shortening
HNE	4-hydroxy-2-nonenal
IST	interval sprint training
LAD	left anterior descendent
LV	left ventricular
LVAWT	left ventricular anterior wall thickness
LVPWT	left ventricular posterior wall thickness
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
MC	male control
ME	male exercised
MHC	Myosin heavy chains
MR	magnetic resonance
mRNA	messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NT	3-nitrotyrosine
PAR	poly(ADP)-ribose
PVW	Pulse Wave Velocity

RF	resorcin-fuchsin
s	systole
SHR	spontaneous hypertensive rat
SMA	smooth muscle actin
SV	stroke volume
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor-1

1. INTRODUCTION

1.1. The effect of regular exercise on the vasculature

As a whole, the body adapts to persistent and regular exercise, and this may be observed on all levels: body fat decreases, muscle mass increases, and the heart muscle reacts via hypertrophy (athlete's heart) (1-6). Due to these positive effects, cardiovascular risk decreases significantly in people who exercise regularly compared to those who do not (7).

It is no longer in debate that the vasculature adapts to sports just as the heart does, therefore "athlete's vasculature" develops just as "athlete's heart" does (8). However, adaptation mechanisms are not uniform along the vasculature, as there are many types of vessels running along structurally different tissue beds serving a variety of purposes. These are thereby affected by sport in a variety of manners; therefore adaptation will differ as well. For example, blood flow decreases in response to physical activity in the splanchnic area (9, 10). When analyzing the sport adaptation of the splanchnic vasculature, we find that during physical activity flow is reduced to the area to a lesser degree than in those who do not exercise regularly (9, 10). In contrast, blood flow to coronaries and vessels of physically active muscles increases during physical activity (as opposed to the splanchnic region). We will discuss the adaptation of coronaries and skeletal muscles to exercise in the chapters following.

1.1.1. The effects of sport on the adaptation of the coronary system

Eighty percent of coronary artery disease (CAD) is caused by unhealthy lifestyle choices, including a lack of regular exercise. CAD morbidity and mortality decrease in response to regular exercise: sports enhance blood flow to the coronaries and the oxygen supply of the heart, therefore the reducing the risk of both angina and myocardial infarction (11-13).

Regarding sport adaptation of coronaries, we must differentiate between different types of coronaries i.e., epicardial (conduit) vessels and resistance arterioles. The role of large epicardial vessels is to forward blood, therefore they effect minimal resistance to flow. In these vessels, sport activities lead to increased tangential stress and consequential flow-

induced, endothelium-mediated dilation (13-15). Regular exercise increases vascular reactivity: nitroglycerin-induced coronary vasodilation is significantly more pronounced in athletes compared to controls, while differences are not observed regarding diameter during resting periods (8). Not only was an increase of the functional reserve capacity found in other studies, but as an effect of endurance training the cross-sectional area of the left and right main and the (LAD) coronary artery increased significantly (16). The diameter of resistance coronary arteries is considerably smaller compared to conduit arteries (<300 μm) and they present a significant resistance to blood flow. These vessel segments are not only responsible for conduction of blood, but they also regulate blood supply to different regions (14). In vitro pressure myography examinations revealed that as a result of regular swim training (following habituation the animals swam 200 minutes a day) resistance arterioles adapt through an increase of wall/lumen ratio, incremental elastic modulus (E_{inc}) and vascular reactivity (17). It is interesting to note that an increase in vascular reactivity and vessel wall thickness (constriction and relaxation reserve) may be observed even following a short period of sport training (i.e., 4 weeks) (18). However, descriptions of the sport adaptation of resistance arterioles are not uniform in the literature: Laughlin et al. found that cross-sectional area and lumen diameter increased in swine following 16 weeks of training (19). Contradictions may be due the fact that experiments were performed on different species, and training type and duration varied as well.

1.1.2. The effects of sport on the adaptation of the arterioles of the skeletal muscle

Literature data regarding sport adaptation of skeletal muscle arterioles is more uniform. However, when examining these vessels it should be noted whether they run within the bed of an active muscle or not. This is important because oxygen demand and blood flow are significantly increased, and these parameters may remain unchanged or decrease in active muscles. This is supported by results from a study of wheelchair-bound athletes where the diameter of the brachial artery increased compared to controls, and also by studies of runners/cyclists where the diameter of the femoral artery increased (8, 20, 21). The explanation for this may lie in the aforementioned fact that training does not have a universal effect on the entirety of the vasculature; during training local responses such as an increase/decrease in flow/tangential stress may affect adaptation. Wall thickness

decreases in active skeletal muscles, and diameter increases in response to persistent training (8).

1.2. Gender differences in the adaptation of the coronary system and skeletal muscle arterioles

Gender differences may be found in almost all human function. One of the most well known is that before menopause cardiovascular morbidity and mortality risk is decreased in women compared to men of the same age. This relative immunity ceases following menopause, marking that the cardioprotective effects of sex hormones may be the underlying cause (22-24). The effects of estrogen are diverse: vasodilation, renal and cardiovascular protection are achieved via the decrease of free radicals and an increase of nitrogen monoxide, anti-apoptotic and anti-inflammatory agents (22, 25, 26).

In consequence, it is not surprising that the sexes adapt to different physiological (i.e., sport) and pathological (i.e., hypertension) stimuli via different mechanisms. However, the role of sex hormones and molecular mechanisms has not yet been established in detail. Physiological left ventricle hypertrophy develops in both sexes as a result of sports activities, however this is more pronounced in females even though the improvement of functional parameters is similar. Stroke volume, contractility and stroke work are increased in both sexes, while active relaxation is more pronounced in males. This may be explained by the following molecular mechanisms: Akt signaling is greater in females, while p44/42 MAPK (Erk), mTor phosphorylation, and myosin heavy chain ratio (α (MHC)/ β -MHC) are increased, while they remain unchanged in males (27). As an effect of chronic left ventricle volume, overload from chronic aorta valve regurgitation leads to left ventricle dilation in both male and female rats (28). However, it may be observed as a feature of gender difference that while male rats develop relative left ventricle wall thickening (the ratio of wall thickness to end-diastolic diameter) compared to controls, this did not occur in females (28). As an effect of chronic angiotensin therapy, relative heart mass and wall thickening was greater in female Wistar rats compared to males (29). At the same time, vascular tone and the vasoconstriction response to thromboxane was greater in male animals, and endothelial dilation was greater in females. As an effect of Angiotensin II therapy, inward eutrophic remodeling was observed in females, while

increased wall stress and elastic modulus were found in males while they maintained vessel morphology (29).

1.2.1. Gender differences in the coronary system

Gender affects the diameter of the left main, anterior descending and circumflex, as well as the right coronary arteries: in males the diameters of all of the major coronaries are greater than those found in females regardless of age, body mass, body mass index, body surface index, left ventricle mass, or body type (30, 31). Earlier, Mátrai et al. analyzed gender differences in resistance coronary arteries in Sprague-Dawley rats (32). Inner and outer diameter do not differ significantly between the sexes in resistance arteries, however the geometry of male and female animals is not uniform: males have greater wall thickness and higher wall/lumen ratio compared to females, while wall stress is reduced in males due to greater wall thickness. Isobaric elastic modulus is smaller at greater pressure values in female animals. Vascular function differs between the sexes as well: vasoconstrictive response is heightened in males compared to females, while endothelial dilation does not differ between the sexes (32). Contraction is significantly decreased in porcine female coronary arteries to thromboxane agonist and endothelin-1, while this is not observed in males. The anticontractile effects of perivascular adipose tissue in females may be responsible (33).

1.2.2. Gender differences in the muscle arterioles

Similar to coronaries, gender differences may be observed in the morphology and function of skeletal arteries. The diameters of the brachial and radial arteries are significantly greater in males compared to females (34). Furthermore, the passive diameter of the gracilis artery of 12-week-old adult rats was significantly smaller in females compared to males (35). Myogenic tone in response to pressure was higher in females than in males, which may be due to the increased presence and activity of NO to estrogen (35). The flow-mediated dilation of the brachial artery (vasodilation mediated by nitrogen monoxide) was significantly greater in females compared to males (36).

1.2.1. Gender differences in the sport-adaptation of coronaries

The research of sport-adaptation of different vessel types has become quite popular, however data concerning gender differences is significantly more scarce.

Our research group examined differences regarding adaptation of resistance coronaries following 12 weeks of persistent swim training (17, 37). As an effect of swim training, the inner diameter of the examined coronaries decreased while outer diameters remained unchanged, leading to an increase in wall-to-lumen ratio, and the incremental elastic modulus increased in both sexes as well (17). However, significant gender differences were found regarding vascular function: spontaneous tone and response to thromboxane was increased in trained females, while endothelial dilation improved in males (17, 37).

1.2.2. Gender differences in the sport-adaptation of arteries in skeletal muscle

Green et al. have previously analyzed the sport-adaptation of skeletal muscles using brachial and popliteal arteries. Vascular diameter proved to be increased in males compared to females following 12 and 14 weeks of training in both vessel segments (38). Vasorelaxation to dynamic leg exercises was greater in females compared to males (39). These results demonstrate that data from the literature are not uniform regarding gender differences in skeletal muscle arteries following sport-adaptation. Some of the contradictions may be due to the fact that vessel segments, animal species, and degree of loading differed, however further analysis is necessary to unveil gender differences.

1.3. Examination methods of the vasculature

There are several methods for examining blood vessels, from non-invasive procedures (such as ultrasound (40, 41), Pulsepen (42), Mogil-O-graph (43), Sphygomocor (44)) to more invasive ones, such as angiography (45, 46), pressure-myograph (47), wire-myograph (48, 49), and network analysis (47)). In the following section we present the methods relevant to our research.

1.3.1. Pressure-myograph

Pressure-myograph examinations allow functional, biomechanical, and morphological examination of the isolated vessel segments. The downside is that a vessel segment of a

few mm in length is needed to perform the examination, so it is limited to small animal experiments, post-mortem analysis, and tissue removed due to pathologies.

The essence of the method is that the isolated vessel segment is cannulated from both sides, and then flow and pressure values may be set. The vessel segment is placed in a tissue bath, where adequate temperature, ion supply and pH are established to ensure viability of the vessel segment (47).

Once the vessel segment is placed in the tissue bath, the image of the vessel may be projected via video microscopy; this allows for exact imaging of the vessel wall and diameter (50). The wall thickness and diameter of the vessel segment alter in response to vasoactive stimuli such as changes in pressure, flow, and vasoactive agents to promote dilation and constriction; these alterations are recorded. Using mathematical formulae, biomechanical, morphological, and functional characteristics may be calculated from measured vessel wall and diameter parameters, making the effects of therapeutic measures comparable (51).

1.3.2. Network analysis

Evolution of micropreparation techniques made network analysis possible in contrast to individual segment analysis of coronaries (47). During micropreparation, the branches of the LAD coronary are dissected down to the 80 μm outer diameter segments while maintaining vessel function. Adequate temperature and ion supply are assured during micropreparation of the vessels. The dissected network is then cannulated and physiological pressure levels are ensured via normal Krebs Ringer solution. Images are obtained of the vessel segments at different levels of magnification while taking care to have the lens of the microscope at a right angle to the photographed vessel. During off-line analysis the pixel data is conformed to μm with the help of a micrometer gauge (52). This technique allows for analysis of network geometry: the examination of different segments and abnormalities, and measurement of diameter and wall thickness. Also, theoretically dividing the network into 50 μm segments allows “ring analysis” to be performed. Here, the ring segments are placed in a frame of reference and we may determine the exact location of each segment from its orifice, and we may categorize them based on diameter and wall thickness (52). This technique allows collection of data

on not only vascular function and morphology, but adaptation mechanisms to physiological (53) or pathological (54) stimuli as well.

1.3.3. Histology and immunohistochemistry

Histological and immunohistochemical methods allow not only for collection of data regarding the morphological characteristics of the vessel wall, but also for examination of the quantity and type of different receptors and proteins. During this process the dissected vessel segments are fixed in 4% formalin, they are placed in paraffin, and they are cut into 4-5 slices. During histological analysis different structural elements of the vessel wall may be marked through staining procedures i.e., smooth muscle cells through SMA (alpha smooth muscle actin) staining, collagen-elastin through resorcin-fuchsin (RF) staining, nitrative and oxidative stress markers like poly(ADP)-ribose (PAR), 3-nitrotyrosine (NT), 4-hydroxy-2-nonenal (HNE), endothelial nitric oxide synthase (eNOS), and a variety of receptors like VEGFR-1 (vascular endothelial growth factor receptor 1), AdeA2A-R (adenosine A2A-R), and ER (estrogen receptor). Immunohistochemical staining may be presented in different ways i.e., 3-3'-diaminobenzidine (DAB) staining. The slices may be analyzed with the help of specialized software (i.e., ImageJ, Pannoramic Viewer) (55-57).

2. OBJECTIVES

“Athlete’s heart” is a well known phenomenon. It means that the heart adapts to chronic physical load both structurally and functionally. However, cardiovascular adaptation mechanisms not only effect the heart but the entirety of the vascular system. As different segments run along different types of tissue beds and they serve varied functions, adaptation mechanisms will differ as well.

The major characteristics of athlete’s heart have been described previously; however we do not have such detailed information regarding gender differences of adaptation mechanisms along different vessel segments. Therefore, our research group aims to analyze the sport-adaptation of the vessel system using a physiological left ventricle small animal model and to concentrate on gender differences in this region. In this Study we focused on network mapping of the coronaries (LAD) and the biomechanical and functional adaptations of the artery feeding the gracillis muscle.

Wistar rats were examined following 12 weeks of swim training (58). The rats were divided into 4 groups following a 7-day acclimatization: male and female control, swim-trained male, swim trained female. The animals that underwent training swam five times a week, and the duration of training was increased every two days to a maximum of 200 minutes. The members of the control group swam five times a week for five minutes under the same conditions.

The first step in our analysis was to perform a transthoracic echocardiograph, where we established/demonstrated the hypertrophic adaptation of the heart. As a next step, the blood vessel supplying the gracilis muscle (a. gracilis) was prepared and a pressure myograph performed to measure biomechanical, functional, and morphological analysis (vessel diameter, vessel wall thickness, vessel reactivity to pressure and vasoactive agents). We also mapped the network of the prepared coronaries via videomicroscopy. Changes regarding fiber composition we examined through histological examination.

Questions:

- 1) Does the coronary vessel network adapt to persistent training, and what differences may be observed regarding the adaptation based on gender?

- 2) Do the vessels located in the muscles – i.e., the gracilis arteriole – adapt to persistent training, and what differences may be observed regarding the adaptation based on gender?

3. RESULTS

3.1. Body weight and cardiac changes

Sex differences in body weight and cardiac changes

The body weight of the male control (MC) animals of similar ages was higher than that of the female control (FC) group. However, the ratio of the heart weight and body weight measured post-mortem was significantly higher in the female animals than in the male control group. (**Table 1**).

Table 1 shows the echocardiographic data. FC rats had a higher LV (left ventricle) mass index than the MC group did; a greater relative heart hypertrophy was associated with the female sex (similarly to the results regarding heart weight/body weight ratio measurements). Greater ejection fraction (EF) and fractional shortening (FS) were found in FC animals than in MC rats. A lower SV (stroke volume) was detected in FC rats than in MC animals. In contrast with SV, a significantly higher SV index was found in FC animals than in MC rats. No significant difference showed in the thickness of the LV wall between the MC and FC groups, except in the wall thickness of the left ventricular posterior in systole (LVPWTs), which was lower in the case of females. There was no difference in the mean arterial pressure between the groups (**Table 1**).

Training adaptation in the body weight and cardiac changes

During the 12 weeks of observation a significant increase in body mass was detected, while a significantly lower elevation in body mass was observed in the male exercised (ME) group compared to the MC group. In the body mass of the FC and FE groups, no difference was detected. A significantly increased ratio of heart weight to body weight was measured post-mortem in the ME and female exercised (FE) groups in comparison with control groups (**Table 1**).

Table 1. Echocardiographic data of the experimental groups

Variable	Male	Male	Female	Female
	Control	Exercised	Control	Exercised
Basic characteristics				
Body weight ¹ (g)	309 ± 8	298 ± 5	210 ± 3 ^c	214 ± 4 ^d
Body weight ² (g)	496 ± 12	431 ± 9 ^a	290 ± 4 ^c	289 ± 4 ^d
Heart weight / body weight (g/kg)	3.36 ± 0.05	3.95 ± 0.11 ^a	3.79 ± 0.05 ^c	4.49 ± 0.13 ^{bd}
Echocardiographic data				
LV mass index (g/kg)	2.36 ± 0.08	3.05 ± 0.08 ^a	3.07 ± 0.08 ^c	3.64 ± 0.10 ^{bd}
EF (%)	73 ± 1.0	81 ± 1.2 ^a	79 ± 0.8 ^c	82 ± 0.9 ^b
FS (%)	44 ± 0.9	52 ± 1.3 ^a	49 ± 0.8 ^c	52 ± 1.1 ^b
SV (μl)	245 ± 9	271 ± 6 ^a	185 ± 6 ^c	213 ± 9 ^{bd}
CO (ml/min)	78 ± 5	90 ± 3 ^a	62 ± 3 ^c	73 ± 4 ^{bd}
SV index (μl/g)	0.49 ± 0.02	0.62 ± 0.02 ^a	0.64 ± 0.01 ^c	0.74 ± 0.03 ^{bd}
LVAWTd (mm)	2.00 ± 0.01	2.17 ± 0.08 ^a	1.93 ± 0.03	2.13 ± 0.04 ^b
LVAWTs (mm)	3.25 ± 0.06	3.77 ± 0.10 ^a	3.17 ± 0.07	3.45 ± 0.10 ^{bd}
LVPWTd (mm)	1.87 ± 0.05	2.03 ± 0.04 ^a	1.86 ± 0.04	1.92 ± 0.03 ^d
LVPWTs (mm)	3.10 ± 0.06	3.30 ± 0.10 ^a	2.80 ± 0.06 ^c	3.01 ± 0.06 ^{bd}
MAP (mmHg)	124 ± 9	123 ± 8	105 ± 7	110 ± 5

Table note: ¹: Body weight at the beginning of the training program, ²: Body weight at the 12th week, CO: cardiac output, LV mass index: LVM (left ventricular mass) standardized for body mass, EF: ejection fraction, FS: fractional shortening, SV: Stroke volume, LVAWT: Left ventricular anterior wall thickness, LVPWT: Left ventricular posterior wall thickness, d: Diastole, s: Systole, MAP: mean arterial pressure. Two-way ANOVA with Tukey's post hoc test by SPSS Sigma Stat software. Data are expressed as the mean (SEM) values; N=8-10-10-10 (for MC, ME, FC and FE groups). The significance levels of two-way analysis of variance (ANOVA) and Tukey's post hoc tests between the four groups are shown. aP<0.05 MC vs. ME; bP<0.05 FC vs. FE; cP<0.05 MC vs. FC, and dP<0.05 ME vs. FE (50, 53).

The echocardiography-determined, calculated index of LV mass indicated cardiac hypertrophy in ME and FE animals in comparison with the control groups (similarly to heart weight/body weight) (**Table 1**). Exercised rats (ME and FE groups) had a significantly increased systolic function of the heart affected by hypertrophy (determined by the fractional shortening, the ejection fraction, and stroke volume) compared to sedentary rats. Significantly higher stroke volume values were observed in the ME and FE animals than in the corresponding sedentary groups. In addition, a significantly increased SV index was found in ME and FE animals. The left ventricular wall thickness values were found to be significantly higher in the ME and FE rats at the end of the exercise period. The data above indicate significant hypertrophy in the left ventricles in male as well as female exercised rats. There is no difference in mean arterial pressure between groups (**Table 1**).

Sex differences in body weight and cardiac changes after training

Similar to the gender differences found in the control groups, males demonstrated greater body weights compared to females. At the same time, the difference in weight gain between males and females in the exercised group was smaller. Furthermore, similarly to sex differences found in the control group, the ratio of heart weight to body weight measured postmortem was significantly higher in female exercised animals than in male exercised rats (**Table 1**).

Regarding the data gained through echocardiography we found that similarly to the control groups a higher LV mass index was detected in FE animals than in the ME group; the stroke volume was lower in FE animals compared to the ME rats; and the SV index was significantly higher in FE animals than in ME rats. However, following training, in contrast to the control group the EF and FS did not differ between the trained groups, but LVPWTd, LVAWTs, and LVPWTs were found to be significantly smaller in exercised female rats than in exercised male rats (**Table 1**).

3.2. Network analysis

3.2.1. Bifurcations

Murray's law applied to the lumen diameters of bifurcation: in the case of daughter branches, the sum of the cubes of the lumen radii approached the cube of that of the mother branch. No differences were found in the deviations from theoretical values between the four groups in any of the comparisons (**Fig. 1**). Similarly, branching angles (defined as the angle between the axis of the daughter branch and that of the mother branch) increased with the lumen increase of the daughter branch. Negative correlations between the ratios of diameters (r_m/r_d) and the angles of axes were equally characteristic of the four groups (**Fig. 2**).

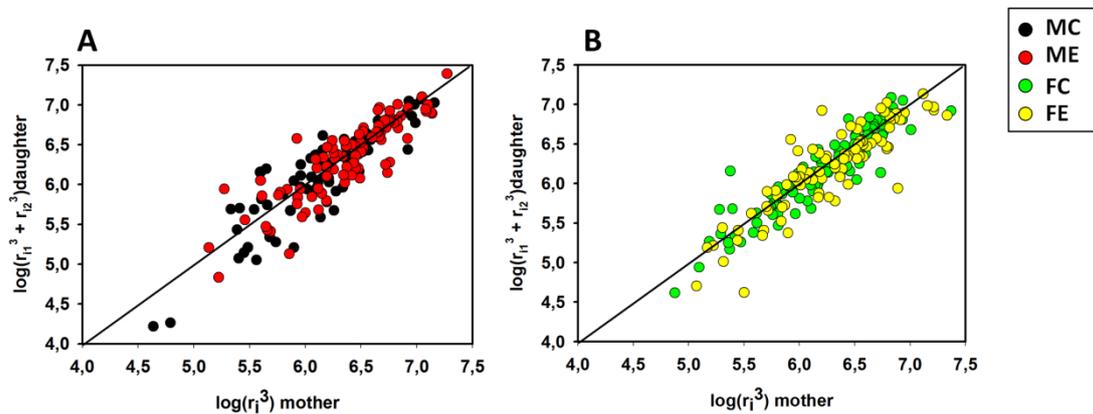


Figure 1. Analysis of bifurcations (branchings)

A) Control and exercised male groups. B) Control and exercised female groups. The validity of Murray's law in the LAD coronary resistance artery network was examined. The sum of the cube of the lumen radii of daughter branches was compared with the cube of the lumen radius of the mother branch in a logarithmic scale. The line connects points where Murray's law is valid. Substantial adherence to the line in all four groups can be noted. Deviations from the theoretical value did not significantly differ in any of the comparisons (53).

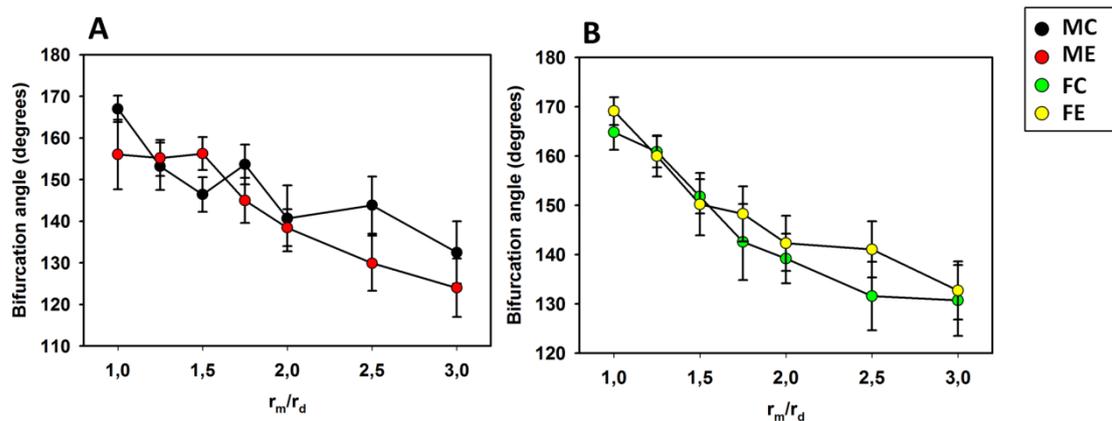


Figure 2. Analysis of bifurcations angle

A) Control and exercised male groups. B) Control and exercised female groups. Angle of the axis of the daughter branch with the axis of the mother branch as a function of the ratio of lumen radii ($r_{\text{mother}}/r_{\text{daughter}}$). Smaller daughter branches deviate more from the course of the mother branch (angles closer to 90°), while larger daughter branches follow the course of that of the mother branch (ca. 180°). In all four groups, Pearson correlations were found to be significant, with no significant differences between the groups (53).

3.2.2. Ring unit analysis

Sex differences in the ring unit analysis

Positive correlations of outer diameter with wall thickness in all four groups were detected (**Fig. 3**). The walls were slightly but significantly thinner in the FC group than in the MC group (not shown explicitly in the Figure) (**Fig. 3**). Histograms of ring frequency (number of ring units of $50 \mu\text{m}$, pooled for 10 animals) were detected as significantly different among sedentary animals ($p < 0.01$ with the χ^2 probe, **Fig. 4**). The numbers of ring units were not different in the four groups (718 ± 68 , 780 ± 47 , 730 ± 45 and 705 ± 30 ring unit elements per heart for the MC, ME, FC and FE groups, respectively, n.s.). Ring frequency spectra differed significantly between MC and FC rats ($p < 0.01$ with the χ^2 probe). MC networks had fewer 200- to 250- and 400- μm vascular units and more 350- and $>500\text{-}\mu\text{m}$ units (**Fig. 4C**).

Training adaptation in the ring unit analysis

A significant increase in wall thickness occurred in the resistance arteries of ME rats; however, in FE rats the reductions found in the larger arteries were statistically significant (**Fig. 3**). In ME rats, there were almost twice as many 400- μm rings, and the number of

ring units substantially increased within the 200- μ m range in comparison with MC ones (Fig. 4A). In FE animals, there were an increased number of 150- μ m rings, while a reduced number of 350- μ m units was found (Fig. 4B).

Sex differences in the ring unit analysis after training

In trained animals, coronary vessels in FE were found to be significantly thinner than those of the ME group in the total diameter range (Fig. 3., not shown explicitly in the Figure).

The ring frequency spectra were also significantly different between ME and FE rats ($p < 0.01$ with the χ^2 probe). In exercised male rats there were more 350- to 450- μ m units and fewer 150- μ m diameter elements than in the corresponding trained female rats (Fig. 4D).

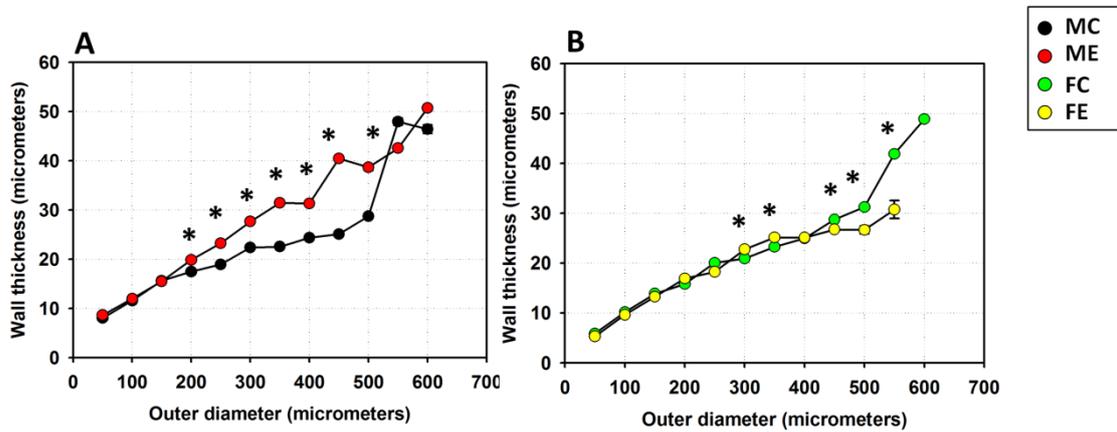


Figure 3. Ring elements of the total LAD coronary artery system

A) Control and exercised male groups. B) Control and exercised female groups. Wall thickness interpreted as a function of outer diameter. In all four groups, positive correlation was detected between wall thickness and diameter. Between sedentary and trained groups, significance levels of paired Tukey’s comparisons are shown, * $p < 0.05$. ‘A’ in the 200–500- μ m range. The effect of exercise is dependent on vessel diameter (interaction, $p < 0.001$). ‘B’ in the 300–350- μ m range. The effect of exercise is dependent on vessel diameter (interaction, $p < 0.001$). Increased wall thickness in exercised male rats, and in female rats a slight but significant reduction in wall thickness was detected. The walls in female vessels were significantly thinner than those of males in the 100- to 300- μ m range in the non-exercised groups and in the total range of the exercised groups (not demonstrated in the figure). Two-way ANOVA with post hoc Tukey’s test by SPSS Sigma Stat software. Data are expressed as the mean (SEM) values; N=8-10-10-10 (for MC, ME, FC and FE groups) (53).

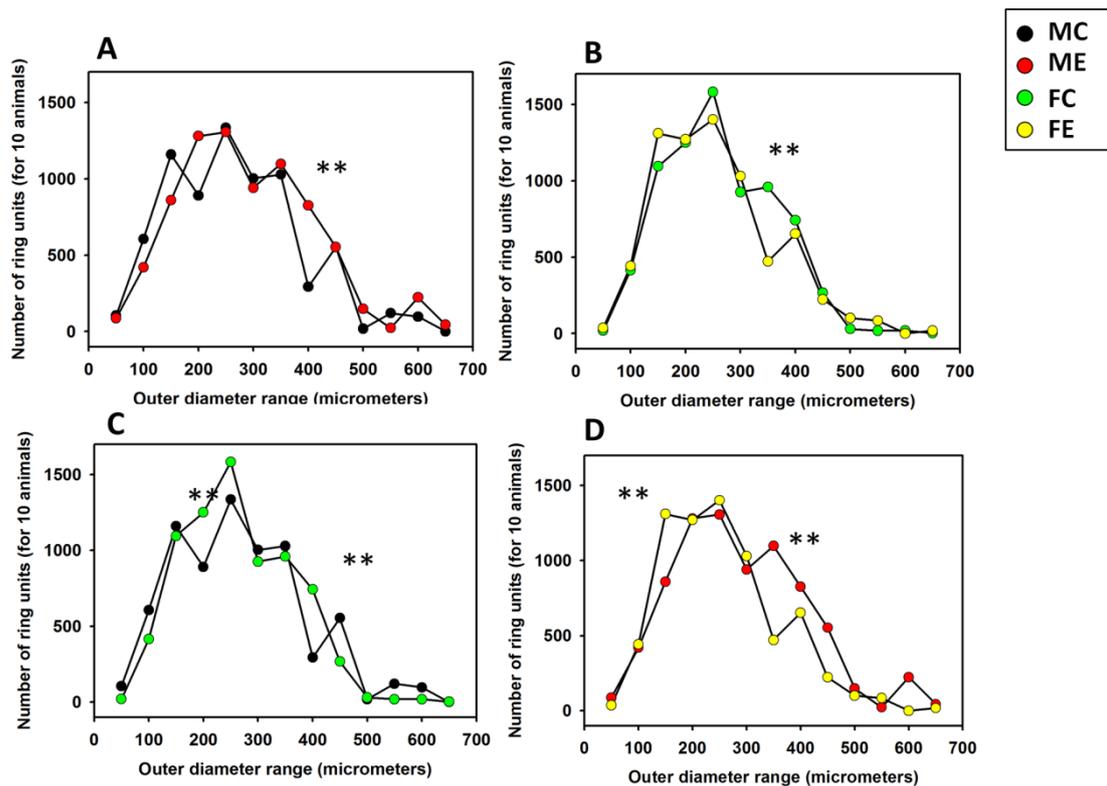


Figure 4. Ring unit analysis. Numbers of 50 μm -length units in the networks in different outer diameter ranges.

A) Control and trained males. B) Control and trained females. C) Control males and females. D) Trained males and females.

Ring frequency spectra of trained animals significantly differed from the sedentary ones in male and female groups (** $p < 0.01$ with the χ^2 probe). Doubling of the 400- μm vessel units and substantial increase in 200- μm vessel units in male groups in response to strenuous exercise. In female groups, the number of 150- μm vessel units was increased, but a reduction in the 350- μm range was found. There was a significant difference in the ring frequency spectra of male rats compared to the female group in the sedentary as well as the trained groups ($p < 0.001$ with the χ^2 probe). There is a higher number of larger (300–600 μm) vessels, and fewer smaller (200–250 μm) ring units are found in male rats than in female ones in the control groups. More 350- to 600- μm vessels and fewer 200- to 250- μm vessels are detected in male animals than in females in the trained groups. SPSS Sigma Stat software. Data pooled for 10 animals; a total of 29,390 ring units included (53).

3.2.3. Network distribution

Sex differences in network distribution

The next point of analysis was the network points where these characteristic differences appeared in the vascular unit frequency. Therefore, histograms demonstrating two-dimensional ring unit frequency were created, where one axis shows the outer diameter

and the other the distance from the orifice following the blood flow route with the number of color-coded units (**Fig. 5**). In sedentary female rats, the network is fairly well organized; (if a characteristic cluster of 150- to 350- μm units at a distance of 10-15 mm can be observed) as opposed to males, where the small-size rings were lacking at a larger distance from the orifice (**Fig. 5**).

Training adaptation in network distribution

In the case of the ME group, there was an elevated number of thicker 400- μm units at the “appropriate” site in the proximity of (3-5 mm) the orifice, while an extension of 200- μm units was observed where it was advantageous, at a distance of 8 to 13 mm from the orifice (**Fig. 5**). In the case of the FE group, 150- to 200- μm vessel elements appear in the proximity of the orifice, loosening instead of improving the pattern (**Fig.5**).

Sex differences in the network distribution after training

Following training, differences between the genders regarding network organization decreased compared to the control groups. The small-size ring population lacking in the control male group located far from the orifice appeared in the trained males (**Fig. 5**).

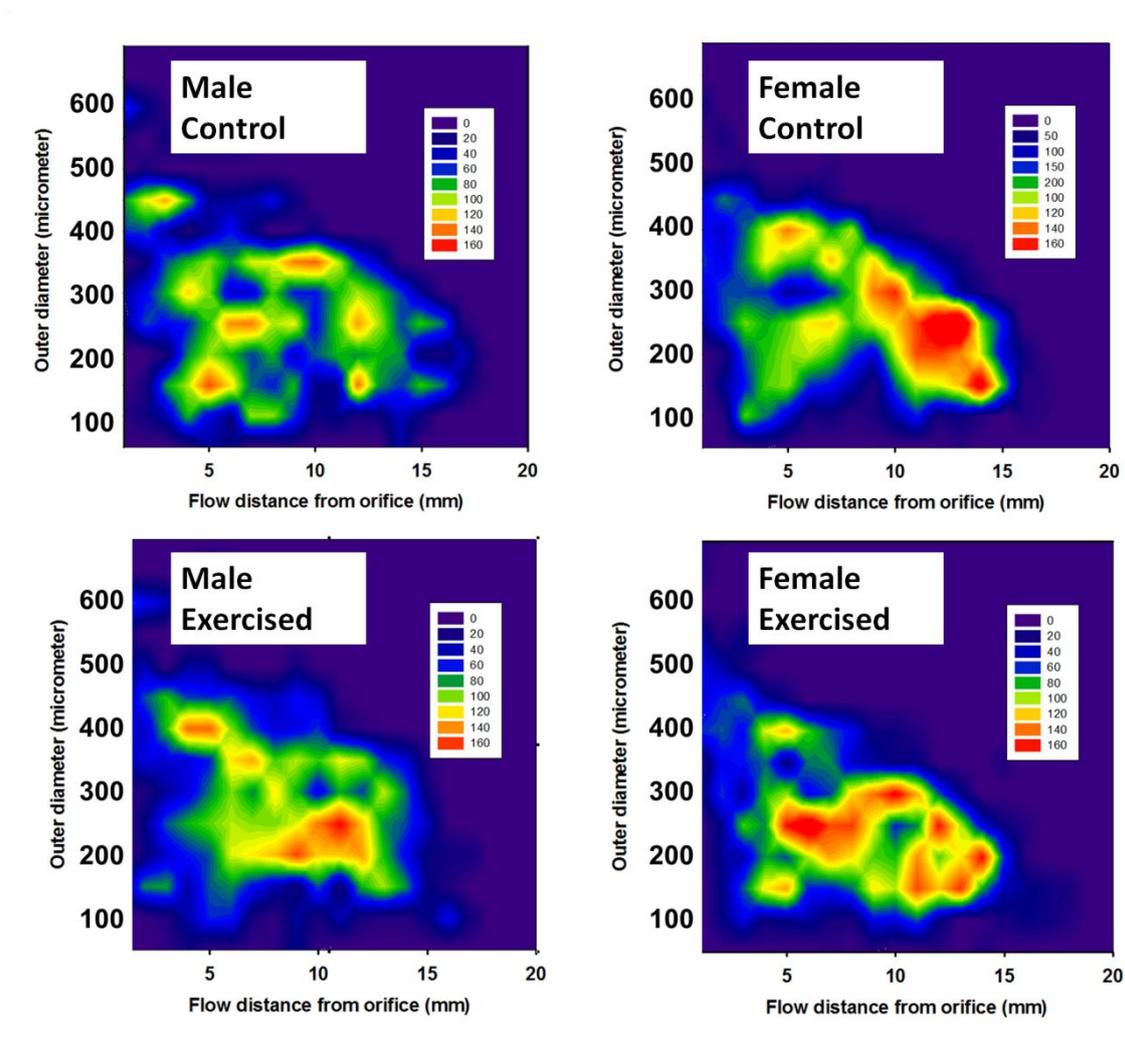


Figure 5. Ring unit analysis. Different vascular elements in the network.

Bidimensional histograms of frequencies in ring unit on the basis of distance from the orifice and outer diameter. In male animals, the elevated number of 200- μm elements after exercise appears to be due to the morphological dilation of 100- to 150- μm elements at a moderate distance from the orifice. Clusters of 400- μm elements appear in the proximity of the orifice. Thus, a younger network appeared (negative correlation between flow distance and diameter). In sedentary female rats, the network is fairly well organized, while in the network of trained ones, 150- to 200- μm vessel elements are observed close to the orifice, not improving but loosening the pattern. SPSS Sigma Stat software. Data pooled for 10 animals, with a total of 29,390 ring units included (53).

3.2.4. Immunohistochemical examinations

Sex differences in the immunohistochemical examinations

When analyzing the results of the immunohistochemical studies measuring nitrative and oxidative stress (HNE, eNOS PAR, and NT markers), no significant difference was found between FC and MC groups (**Fig. 6**). In addition, there were no differences in the staining

applied to VEGFR-1 receptors (**Fig. 6**). AdenosineA_{2A} receptor and estrogen receptor intensity did not differ between the MC and FC rats (**Fig. 7 and Fig. 8**).

Training adaptation in the network distribution

Regarding nitrate and oxidative stress markers (HNE, PAR, eNOS and NT) no significant difference was found between the exercised and control groups (**Fig. 6**). In addition, there were no differences in the staining applied to VEGFR-1 receptors (**Fig. 6**). AdenosineA_{2A} receptor intensity decreased significantly in the ME compared to the MC group. In female animals, no change to exercise was detected in AdeA_{2A} receptor expression (**Fig. 7**). Further, the intensity of estrogen receptors was significantly lower in ME rats in comparison with MC animals (**Fig. 8**).

Sex differences regarding network distribution following training

There were no gender differences regarding staining intensity between the exercised male and female groups regarding oxidative and nitrate stress markers, VEGFR-1 receptors and estrogen receptors (**Fig. 6 and Fig. 8**). The intensity of AdeA_{2A} receptors of ME rats significantly decreased even in comparison with the FE group (**Fig. 7**).

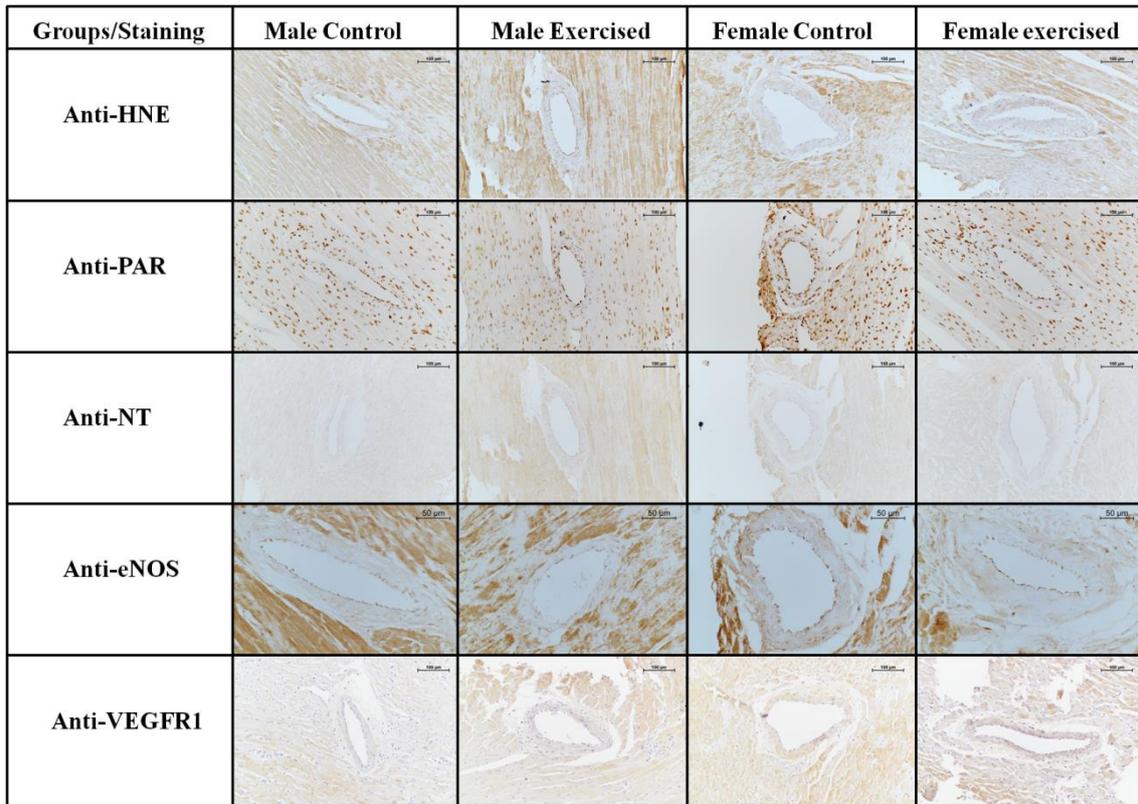


Figure 6. Immunohistochemistry.

Poly(ADP)-ribose (PAR), 4-hydroxy-2-nonenal (HNE), endothelial nitric oxide synthase (eNOS), 3-nitrotyrosine (NT), and vascular growth factor receptor 1 (VEGFR-1) immunohistochemistry sections of MC, ME, FC, and FE rats. Scale bar 50 or 100 μm (53).

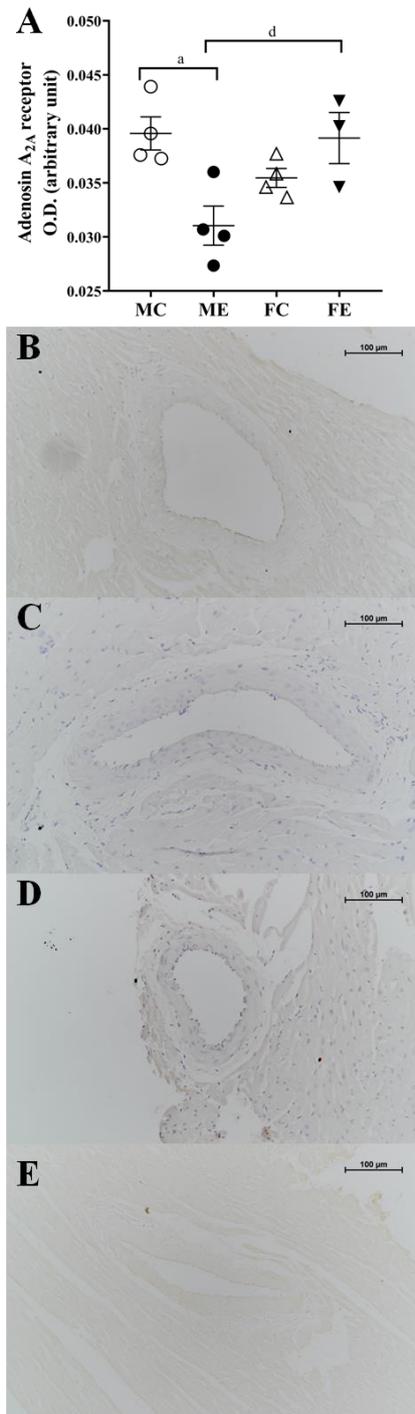


Figure 7. Immunohistochemical staining of AdenosineA_{2A} receptor (AdeA_{2A}-R).

A) Optical density of AdeA_{2A}-R. B-E) Images of arteriole segments stained with AdeA_{2A}-R from MC, ME, FC, and FE groups.

Scale bar of 100 µm. OD decreased significantly in the ME group compared to MC rats. The OD of ME animals was significantly lower compared to the FE animals. Two-way ANOVA with post hoc Tukey's test by GraphPad Prsim software. N=4-4-4-3 for MC, ME, FC, and FE groups (53).

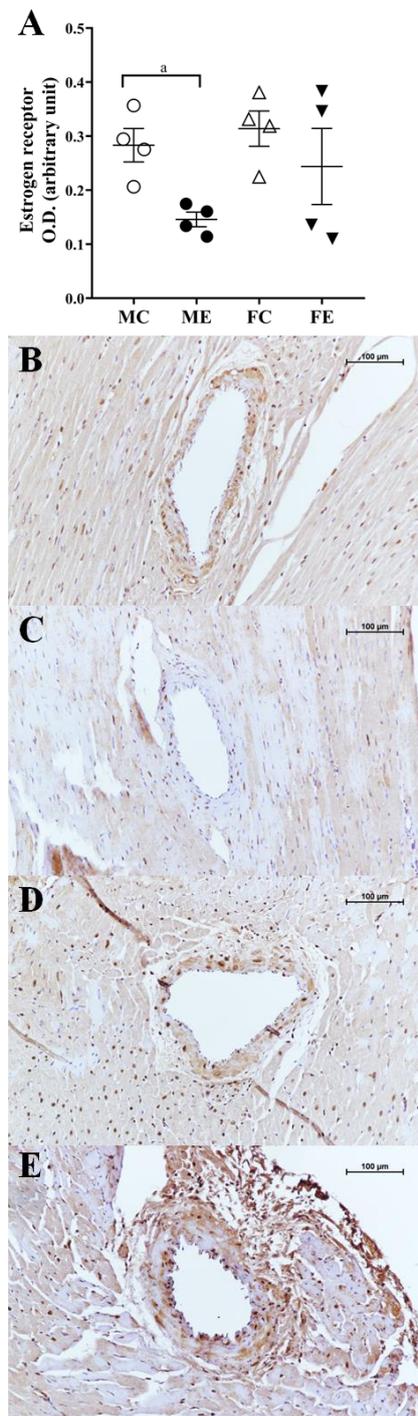


Figure 8. Immunohistochemical staining of estrogen receptor (ER).

A) Optical density of ER. B-E) Images of ER-stained arteriole segments from MC, ME, FC, and FE groups.

Scale bar of 100 µm. OD decreased significantly in the ME group in comparison with the MC rats. The OD of ME rats was significantly lower compared to the FE animals. Two-way ANOVA with post hoc Tukey's test by GraphPad Prism software. N=4-4-4-4 for MC, ME, FC, and FE groups (53).

3.3. Skeletal muscle arterioles

3.3.1. Biomechanical and morphological parameters of gracilis arterioles

Sex differences in the biomechanical and morphological parameters of gracilis arterioles

All arteriolar segments harvested were morphologically and anatomically identical at preparation; no significant difference existed in the internal vessel radii between the groups of MC and FC in the relaxed state (**Fig. 9**). However, the thickness was significantly lower in the FC group in comparison with the MC group (**Fig. 10**). In FC rats, a significantly smaller wall thickness/lumen diameter ratio was observed than those in the MC rats (**Fig. 11**). The tangential stress was significantly higher in FC than in MC rats (**Fig. 12**). The incremental distensibility and elastic modulus (E_{inc}) are an indicator of the stiffness of the vessel wall. The definition of distensibility is the ability of arteries to expand in response to pulse pressure, lower distensibility means greater vessel stiffness. E_{inc} is the slope of the relationship between strain and stress of arteries, which can be calculated based on the COX formula (59)). The incremental distensibility and the elastic modulus did not differ between the MC and FC animals (**Fig. 13 and 14**).

Training adaptation in the biomechanical and morphological parameters of gracilis arterioles

A significant difference was found in the internal vessel radii between the groups in the relaxed state. The internal radius was significantly greater in FE animals than in FC ones (**Fig. 9**). A significantly smaller wall thickness was detected in the ME rats in comparison with that in the MC rats (**Fig. 10**). In addition, due to training, no significant difference was found in the wall thickness/lumen diameter ratio between exercised and control animals in either the female or male groups (**Fig. 11**). The FE group had a significantly higher tangential wall stress than the FC rats. (**Fig. 12**). A significantly reduced elastic modulus was observed in ME rats in comparison with that in MC rats (**Fig. 13**). Following the training regime, a significantly increased distensibility was detected in female animals (**Fig. 14**).

Sex differences in biomechanical and morphological parameters of gracilis arterioles following training

It must be noted that an internal radius increase could only be detected in FE animals. Although no significant difference was observed regarding internal radius between MC and FC, wider gracilis arterioles were found in FE animals than in ME ones (**Fig. 9**). The difference of wall thickness observed in the control group diminished among the exercised rats (**Fig. 10**). We observed a significantly reduced wall thickness/lumen diameter ratio in FE groups than in ME ones, similarly to the results found in the control groups (**Fig. 11**). In female rats, tangential stress increased due to training, while no change of this kind was seen in male specimens, and a significantly higher tangential stress could be detected in FE rats than in ME ones, similarly to the gender differences found in the control groups (**Fig. 12**). Also, exercise-induced reduction in the elastic moduli occurred in male specimens only (**Fig. 13**), although incremental distensibility (also a marker of elastic parameter) increased exclusively in female ones (**Fig. 14**). The elastic parameters did not differ further between males and females in the exercised groups (**Fig. 13 and 14**).

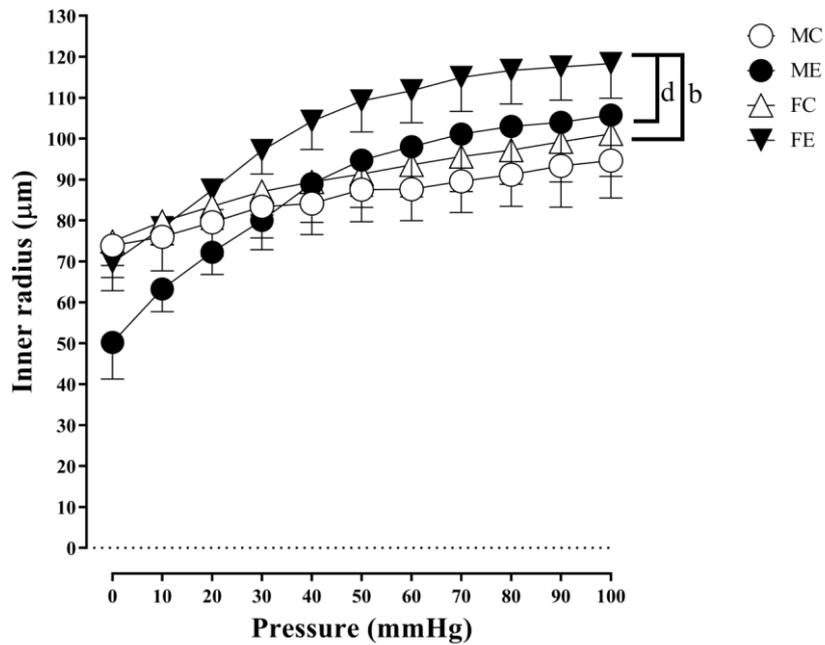


Figure 9. Internal radius of the gracilis arterioles from the MC, ME, FC and FE rats.

The values of the internal radii as a function of intraluminal pressure measured under passive conditions (in calcium-free Krebs solution). The internal radius was found to be increased in FE specimens in comparison with FC and ME ones. Data are expressed as the mean (SEM) values. The significance levels of two-way ANOVA and Tukey's post hoc tests between the four groups are demonstrated. bP < 0.05 FC vs. FE; dP < 0.05 ME vs. FE. N=9-10-10-11 for MC, ME, FC and FE groups (50).

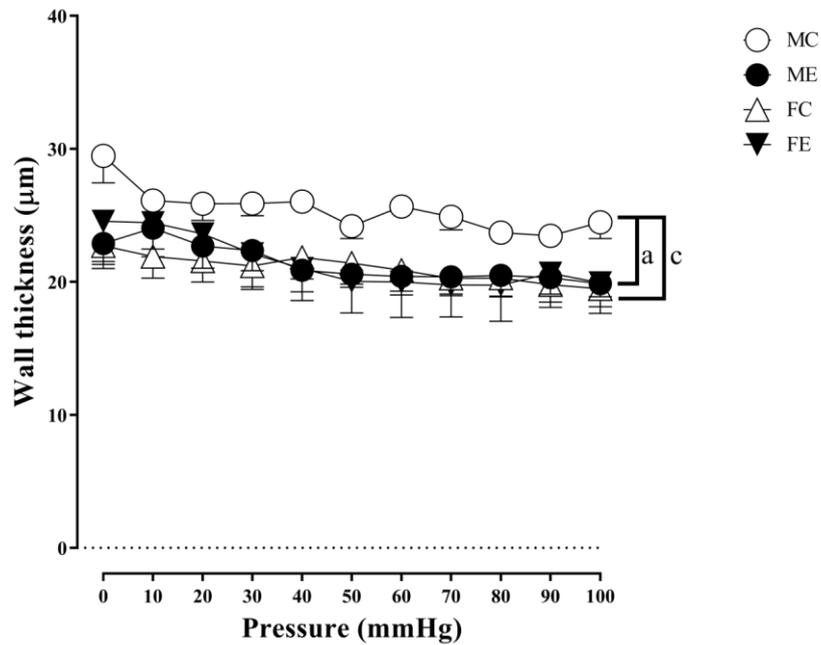


Figure 10. Gracilis arteriole wall thicknesses from the MC, ME, FC, and FE rats.

Wall thickness values as a function of intraluminal pressure measured under passive conditions (in calcium-free Krebs solution). Wall thickness was found to be decreased in the ME group in comparison with the MC group, and this value was higher in MC rats than in FC ones. Data are expressed as the mean (SEM) values. Significance levels of two-way ANOVA and Tukey's post hoc tests between the four groups are demonstrated. aP < 0.05 MC vs. ME; cP < 0.05 MC vs. FC. N=9-10-10-11 for MC, ME, FC, and FE groups (50).

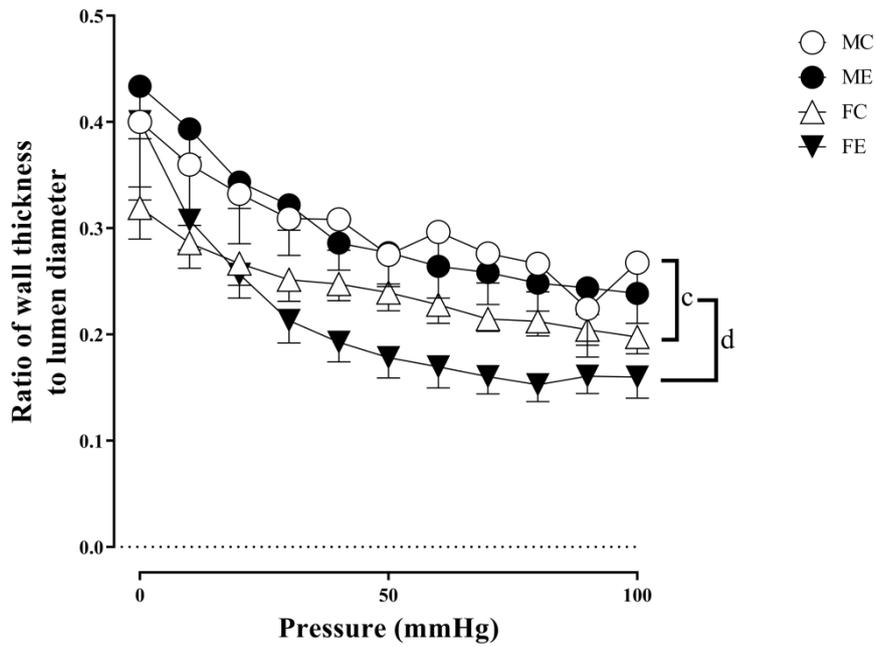


Figure 11. Wall thickness/lumen diameter ratio of the gracilis arterioles from the MC, ME, FC, and FE rats.

There was a significantly smaller wall thickness / lumen ratio in FC and FE animals in comparison with MC and ME ones. Data are expressed as mean (SEM) values. The significance levels of two-way ANOVA and Tukey's post hoc tests between the four groups are demonstrated. $cP < 0.05$ MC vs. FC; $dP < 0.05$ ME vs. FE. $N=9-10-10-11$ for MC, ME, FC and FE groups (50).

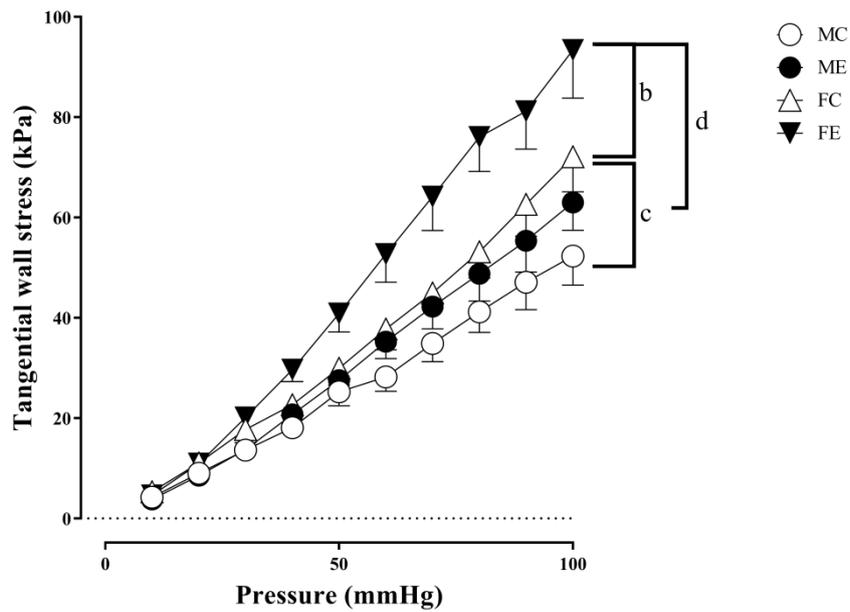


Figure 12. Tangential wall stress as a function of intramural pressure measured in passive conditions of the gracilis arterioles from the MC, ME, FC, and FE animals.

The tangential wall stress was found to be significantly increased in the FE rats compared with the control groups. Also, this value was detected to be significantly higher in the FC and FE specimens than in the MC and ME ones. The data are expressed as the mean (SEM) values. The significance levels of two-way ANOVA and Tukey's post hoc tests between the four groups are demonstrated.

bP < 0.05 FC vs. FE; cP < 0.05 MC vs. FC and dP < 0.05 ME vs. FE. N=9-10-10-11 for MC, ME, FC, and FE groups (50).

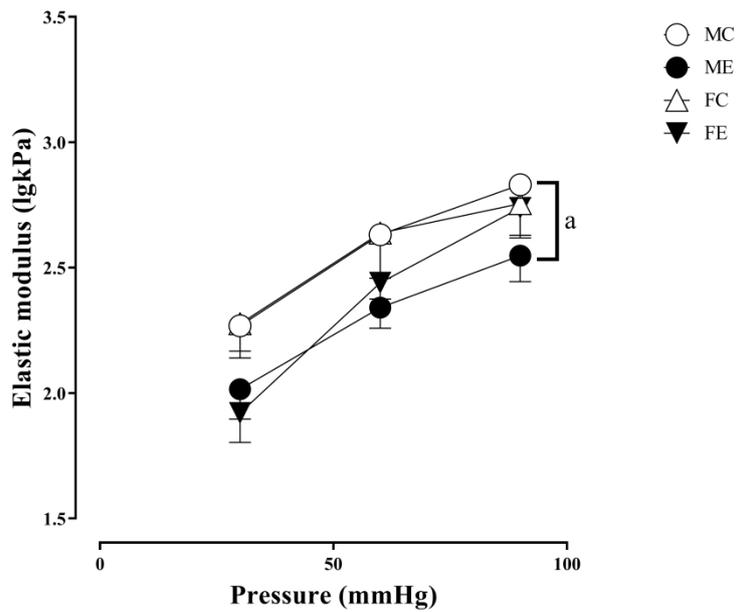


Figure 13. Tangential elastic modulus as a function of intramural pressure measured in passive conditions of the gracilis arterioles from the MC, ME, FC, and FE rats.

Due to exercise, the elastic modulus was found to be significantly smaller in the ME rats than in the MC ones. The data are expressed as mean (SEM) values. The significance levels of two-way ANOVA and Tukey's post hoc tests between the four groups are demonstrated. $aP < 0.05$ MC vs. ME. N=9-10-10-11 for MC, ME, FC and FE groups (50).

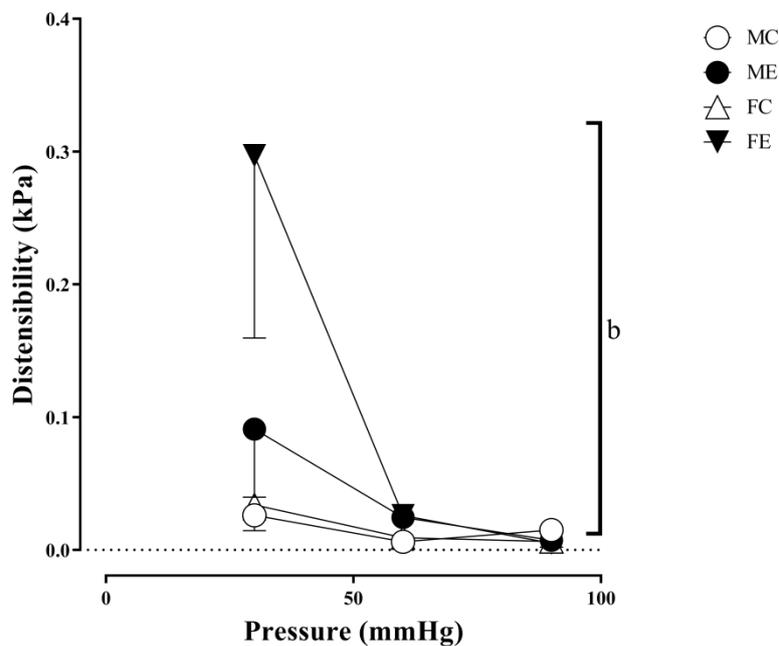


Figure 14. Distensibility as a function of intramural pressure measured under passive conditions of the gracilis arterioles from the MC, ME, FC, and FE rats.

Distensibility was observed to be increased in the FE specimens in comparison with the FC ones. The data are expressed as the mean (SEM) values. The significance levels of the two-way ANOVA and Tukey's post hoc tests between the four groups are demonstrated. $bP < 0.05$ FC vs. FE. $N=9-10-10-11$ for MC, ME, FC, and FE groups (50).

3.3.2. Contractility parameters of gracilis arterioles

Sex-related differences in the contractility parameters of gracilis arterioles

Myogenic tone and contracture to norepinephrine did not differ significantly between MC and FC animals. (**Fig. 15 and 16**). Contraction induced by testosterone was significantly lower in FC specimens than in MC ones (**Fig. 17**).

Training adaptation in the contractility parameters of gracilis arterioles

The myogenic tone observed in the arterioles in the gracilis muscle in the case of the ME group was significantly lower than the myogenic tone found in the vessels taken from the male control rats (**Fig. 15**). Regarding alterations in contractility (NE constriction), the extent of contraction (relative difference of the radius in the maximally relaxed versus maximally contracted state) of the segments increased in FE rats (**Fig. 16**). The levels of testosterone-induced contraction were significantly lower in the male exercised group, showing similar values to those detected in female animals (**Fig. 17**).

Sex-related differences in the contractility parameters of gracilis arterioles following exercise

An exercise-induced myogenic tone reduction could be detected in male specimens exclusively. Sex-related differences in myogenic tone were not statistically significant in the control or the exercised groups (**Fig. 15**). Exercise increased norepinephrine vasoconstriction in female specimens, but not in male ones; consequently, exercised female rats showed more efficient vasoconstriction than the exercised male ones (**Fig. 16**). The testosterone-induced contraction gender difference found in the control groups diminished after the training (**Fig. 17**).

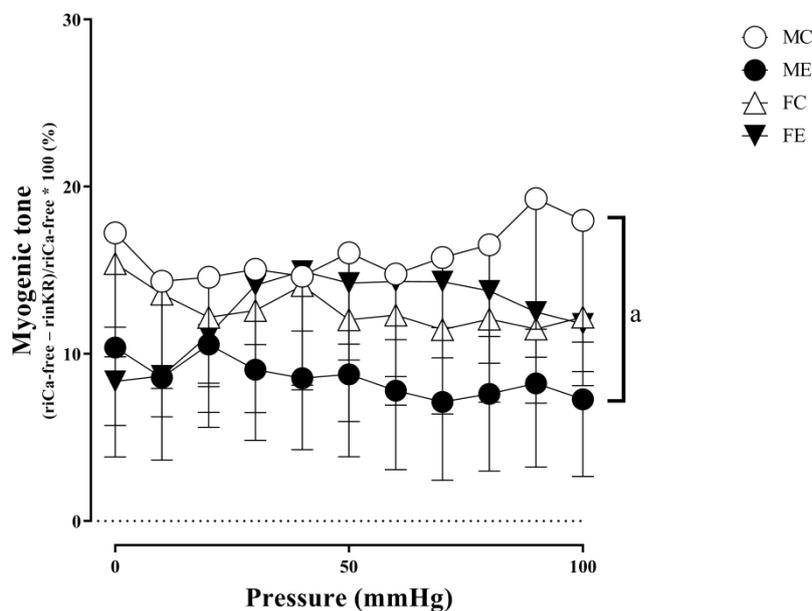


Figure 15. Myogenic tone as a function of intraluminal pressure measured under passive conditions of the gracilis arterioles from the MC, ME, FC, and FE rats.

Due to training, the myogenic tone was significantly reduced in the ME compared to the MC group. The data are expressed as mean (SEM) values. The significance levels of the two-way ANOVA and Tukey's post hoc tests between the four groups are demonstrated. $aP < 0.05$ MC vs. ME. $N=9-10-10-11$ for MC, ME, FC, and FE groups (50).

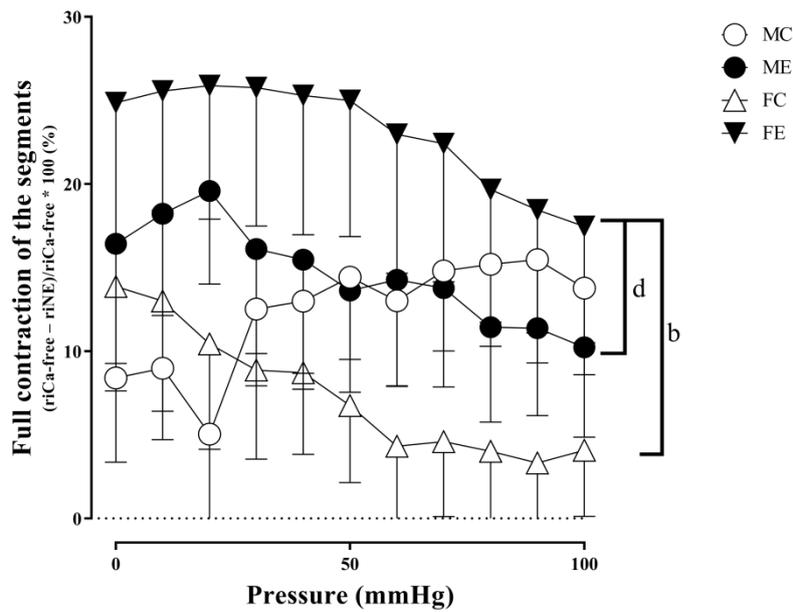


Figure 16. Constriction to NE (relative difference of the radius in the fully relaxed versus fully contracted state) as a function of intraluminal pressure measured under passive conditions of the gracilis arterioles from the ME, MC, FE, and FC rats.

As a result of norepinephrine, constriction was significantly stronger in FE rats in comparison with ME and FC rats. The data are expressed as mean (SEM) values. The significance levels of the two-way ANOVA and Tukey's post hoc tests between the four groups are demonstrated. bP < 0.05 FC vs. FE and dP < 0.05 FE vs. ME. N=9-10-10-11 for ME, MC, FE, and FC groups (50).

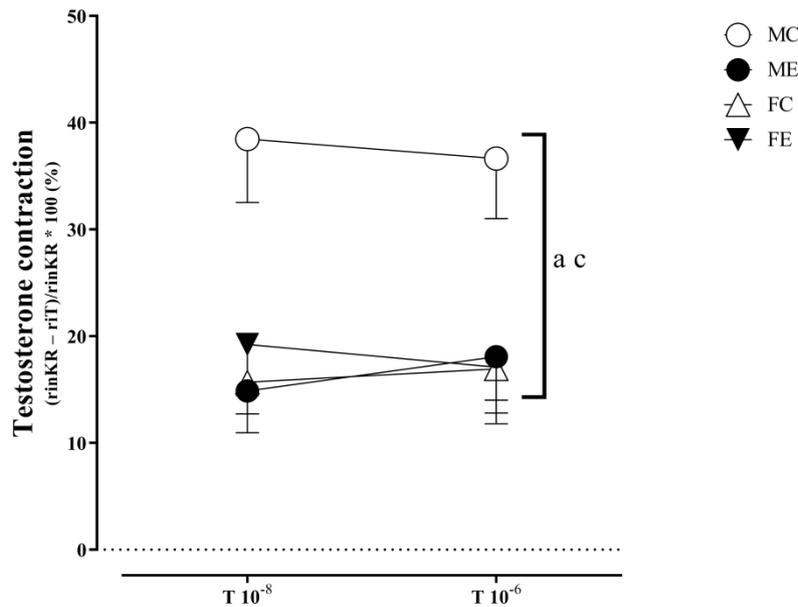


Figure 17. Testosterone contraction of the segments in 10⁻⁶ and 10⁻⁸ M at 50 mmHg from the ME, MC, FE and FC rats.

The testosterone-induced contraction was significantly reduced in the females compared to the males in the control groups. Due to exercise, the testosterone-induced contraction was lower in the male groups. The data are expressed as mean (SEM) values. The significance levels of the two-way ANOVA and Tukey's post hoc tests between the four groups are demonstrated. aP < 0.05 ME vs. MC and cP < 0.05 FC vs. MC. N=9-10-10-11 for, ME, MC, FE, and FC groups (50).

3.3.3. Histological changes found in gracilis arterioles

Sex differences concerning the histological changes of the gracilis arterioles

MOVAT staining did not produce gender differences between the control groups regarding smooth muscle cell and collagen ratio (**Fig. 18A and B**). Staining of the connective tissue was significantly stronger in the FC group compared with the MC group (**Fig. 18C**). **Figs 18D–G** demonstrate representative sections of the gracilis arterioles stained with MOVAT, which have been dissected from the four groups.

Training adaptation regarding histological changes of the gracilis arterioles

Exercise did not significantly change the medial smooth muscle content (**Fig. 18A**). Collagen density was lower in the MC group compared with the ME group (**Fig. 18B**). There was no training-induced change in the overall medial connective tissue content (**Fig. 18C**).

Sex differences concerning the histological changes of gracilis arterioles following training

A significant difference was observed between female trained and male control animals; the MC specimens showed less intense muscle staining of the muscle in their tunica media than the FE ones (**Fig. 18A**).

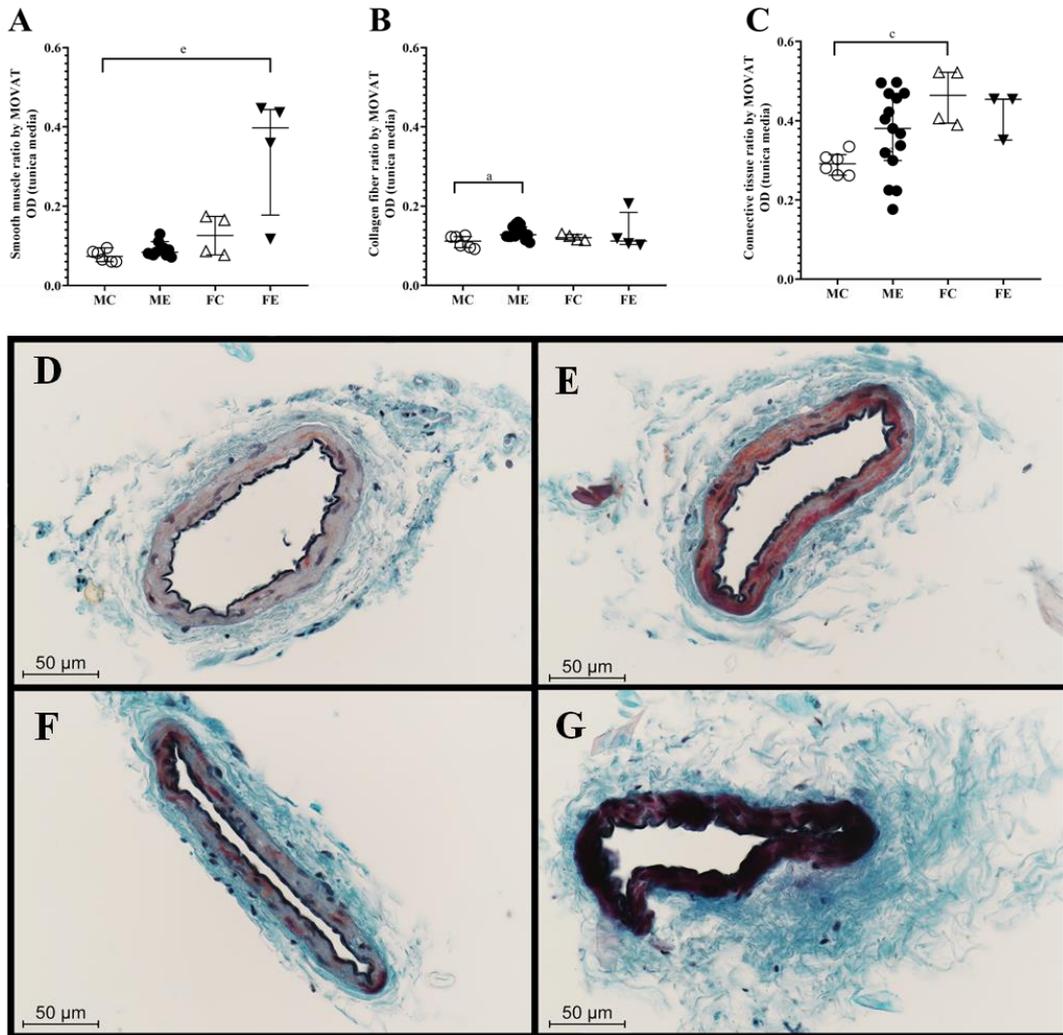


Figure 18. MOVAT staining from the ME, MC, FE, and FC rats.

A) Smooth muscle staining intensity of segments. B) Intensity of collagen staining in the segments. C) Connective tissue staining in the segments. D) Representative staining in the MC animals. E) Representative staining in the ME animals. F) Representative staining in the FC animals. G) Representative staining in the FE animals.

Scale bar, 50 µm. Smooth muscle density was significantly lower in the MC rats than in the FE rats. A significantly higher collagen density was detected in the ME group compared with the MC rats. A significantly higher connective tissue density was observed in the female control group than in the male one. The data are expressed as the median (interquartile ranges) values. The significance levels of Kruskal–Wallis test and Dunn’s post hoc tests between the four groups are demonstrated. $eP < 0.05$ FE vs. MC; $aP < 0.05$ ME vs. MC and $cP < 0.05$ FC vs. MC. $N=3-15$ (50).

4. DISCUSSION

The body adapts to persistent training, and this may be monitored using markers of physical fitness: physiological left ventricle hypertrophy, increased skeletal muscle mass, and a decrease of fat tissue (60). These signs of physical fitness may also be found along different types of vessels – both in terms of morphology and function. However, because the vessels run in different types of tissue beds, they serve different functions and are subjected to a wide variety of hemodynamic forces, so their adaptation mechanisms cannot be uniform. Morphological and functional adaptation may be demonstrated via isolated vessel segment analysis; however they do not offer information regarding network adaptation – which is key in the adaptation mechanisms of coronaries. The unique method applied by our research team – microdissection and videomicroscopy – allows for examination of the coronary network (47, 52). The network adapts to physiological and pathological stimuli, such as aging or hypertension (52, 54). As a consequence we may assume that as a result of persistent training (a physiological stimulus) branches of the LAD will also adapt in the hypertrophic heart muscle. In our current research we examined the effects of persistent training on the adaptation of the LAD network and the gracilis artery (the feeding vessel of the gracilis muscle involved in active muscle work during swimming). We also aimed to analyze if there were gender differences in these adaptation mechanisms similar to those found when examining isolated vessel segments (17, 37).

4.1. Body weight and cardiac adaptation

4.1.1. Gender differences between control groups

Female sedentary animals had lower body weights, consistent with results from previous studies in humans and rodents (61, 62). This is not surprising, because in most mammals and in humans the females are usually smaller than males (63). Even though females were smaller in size, their LV mass index and heart weight/body weight ratios were significantly higher than those measured in males. Similar to LV-mass index results, the SV, FS, and EF indices were also significantly increased in the female control group. Absolute heart size parameters did not differ from males, except for LVPWTs, which

were significantly decreased in female specimens compared to males. Even though the relative heart mass increased, arterial blood pressure was not different between the groups. Data from the literature are contradictory regarding the gender differences between age-matched male and female rats in terms of increased relative heart mass: most studies focusing on hypertrophy to physiological or pathological stimuli did not find gender differences between control groups (27). However, there are studies where, similar to our results, relatively greater heart muscle hypertrophy was found in females (37, 64). Sex hormones may play a role in these differences, but conducting further studies is necessary to pinpoint underlying mechanisms.

4.1.2. Sport-adaptation

Several human and animal studies looked into physiological, exercise-induced left ventricular hypertrophy (27, 65, 66). In order to test the levels of physical fitness of the animals, echocardiographic examinations were carried out. In accordance with the results from most rodent studies (27, 58, 61), increased systolic function (EF and FS) and relative LV mass (LV mass index) were detected in male as well as female specimens following twelve weeks of our swim training program. These changes were the result of ventricular myocyte hypertrophy (58). Despite hypertrophy of the left ventricle, there was no difference in the mean arterial pressure between the groups; this also reflects the physiological nature of athletes' heart.

4.1.3. Gender differences following training

Similarly to our results, Oláh et al. observed decreased body weight in the male exercise groups only, while it was unchanged in the females (27). Lower body weights were measured in the exercise group of females, similar to those found in the control group; this is consistent with results from previous studies of humans and rodents (61, 62). A more significantly increased LV mass index was found in female rats than in males, also in accordance with previous research (27, 61, 67, 68). This observation was also found in other animal experiments (69), but contradicts the human data. The works of Pavlik et al. (70-72) and others (73-80) also indicate that the left cardiac mass relative to body size is slightly smaller in untrained and trained women than in men. Significantly lower absolute cardiac parameters were measured by echocardiography in female specimens, but they

were increased when corrected for the lower weight of female rats. According to the literature, the Akt-protein may increase relative cardiac hypertrophy of female specimens. In our previous research, phosphorylation of Akt showed an increase in both females and males due to training, but was more distinctive in female animals (27). The type beta estrogen receptor may play a part in this difference (61). In addition, Oláh et al. found a difference in the ERK1/2, S6, and mTOR activation levels in the hearts of the exercised female and male specimens (27). In the present study, both sexes in the sedentary state showed a difference in the LV mass index, with females having increased values. In spite of the gender differences in LV index, there were no gender differences identified in connection with systolic function (EF and FS) in the trained groups. Similar results were found in both animal models and also in female and male athletes. In accordance with our findings in rats, women tend to have smaller absolute cardiac sizes than men do (81).

4.2. Network analysis

4.2.1. Gender differences between the control groups

There were no significant differences found between the non-exercised groups in terms of bifurcation geometry. Murray's law applied fairly well in the case of all four groups (82). Murray's law holds that the cube of the lumen diameter of the mother branch should be equal to the sum of the cubes of lumen radii in daughter branches, which ensures that endothelial shear stabilization is efficient in coronary networks, having formed during the trained and sedentary states in both males and females. Murray's law was proved to be valid in the coronary networks of older rats (52) and in female specimens which were made hypertensive by long-term intravenous administration of angiotensin II (54). In the non-exercised groups, a rather negative correlation was found between daughter and mother lumen ratios and daughter branch angles plotted against mother branch axes: larger branches followed the course of the mother branch, whereas more deviation was detected in smaller branches.

As it was described above, entire networks were digitally dissected into 50- μ m ring units, with 29,390 such "ring" units in total for the 38 specimens. Female controls had significantly thinner walls compared to male controls, while MC networks had fewer 200- to 250-, and 400- μ m vascular units as well as more 350- and >500- μ m units. In

female rats, the network was fairly well organized in sedentary animals (typically a cluster of 150- to 350- μm units was observed at a distance of 10-15 mm) compared to male controls, where there was a lack of small diameter rings far from the orifice. The female network is more beneficial compared to the male network (thinner vessels present further away from the orifice). There are substantial differences regarding epidemiology of coronary morbidity and mortality between women and men, especially prior to female menopause (83, 84). Following menopause, the decline in estrogen levels disadvantageously affects the cardiovascular system (85, 86); the underlying adaptation mechanisms need further clarification. Estrogen beta receptor (ER β) stimulation enhanced coronary angiogenesis even in male mice with heart failure (87), and the risk of coronary disease increased due to a loss of ER β activity (87-92). Estrogen acts as a stimulator of angiogenesis in several types of tissues by boosting the expression of VEGF, eNOS, and bFGF (87, 93, 94), thereby enhancing endothelial dilation (95-97).

4.2.2. Sport adaptation

Similar to results found in control groups, we did not find differences in bifurcation geometry following training. Similarly to the control groups, Murray's law applied to the trained groups fairly well (82), ensuring the effectiveness of the stabilization of endothelial shear stress in the coronary network formed due to training in both sexes. Also similar to the control groups, a rather negative correlation was found between daughter and mother lumen ratios and daughter branch angles with the mother branch axis: larger branches followed the course of the mother branch, whereas more deviation was observed in smaller branches. In spite of a substantial elevation in the mass of the left ventricle, no branching deformities evolved, as seen in aged (52) and in hypertensive (54) networks.

Due to exercise, there was a significant increase in wall thickness in the 200- to 500- μm outer diameter range in the case of male animals. It suggests that the increased muscularity triggered an increased range of vasomotion (between fully contracted and fully relaxed states), as observed in some earlier research done by our laboratory (17) using pressure arteriography to examine coronary arteriole segments of rats in the 200- μm range.

Training-induced remodeling of the coronary resistance artery system may be observed on the diagrams showing the frequency of the ring units which form the entire network. In male animals, there were almost twice as many 400- μm units, and a substantial increase in the 200- μm range was also detected. Female animals showed a similar alteration, but in the 150- μm diameter range. In females however, there was a substantial reduction in the number of the 350- μm units. Since no alteration was found in the ring elements themselves, it can be deduced that morphological dilation of previously existing thinner vessels took place. This result is unsurprising because the higher myocardial mass needs increased perfusion, which remodelled coronary circulation may provide (98). Due to endurance training, cardiac performance increases in healthy individuals, and the tolerance for ischemia and reoxygenation injury improves even in patients suffering from LV dysfunction (99-101). The lower ischemic heart disease risk may be associated with the increased density of capillaries and neo-angiogenesis in the cardiac mass due to exercise (102, 103). This may have happened in our case as well, but the small resistance vessels which perpendicularly penetrate the ventricular wall were outside the scope of our study using video microscopy.

Despite the limitations mentioned above, our technique makes it possible to pinpoint the locations in the network where these remodeling processes occur. Research on the two-dimensional flow distance-diameter ring unit frequency plots revealed that the 400- and 200- μm units increased at just the right place in males: in a close proximity – at a distance of 2 mm from the orifice for the thicker vessels of 400- μm , and at a further (8-12 mm) distance from the orifice for the 200- μm vessels. As a consequence, the trained network became healthier and younger (52) (54). A clear, hemodynamically advantageous tendency could be observed: thinner vessels were further away from the orifice, and thicker ones were closer to it. In the female trained group, the network was more organized beforehand and as a result of persistent training, the newly arising population of 200- to 250- μm vessels formed not at the ‘right’ place i.e., far away from the orifice, but 5-8 mm away, thus disrupting the hemodynamically advantageous oblique pattern.

4.2.3. Gender differences following training

Similar to the control group, no gender differences were found in the branching geometry correlating with Murray's law. In summary, we may state that there were no differences regarding branching between the four groups in terms of either gender or training.

There were some significant differences regarding vessel wall thickness: in female (300-350 μm) vessels, exercise-induced wall thickening was observed to be moderate and the thinning at the largest branches proved to be characteristic. There was no agreement in the literature concerning the effect of exercise on the wall thickness of the coronary arteries: alternate regions of the vasculature of the heart may be affected in different manners (8, 18, 104, 105).

The effect of persistent exercise was also found to be different between the two sexes. The training load was similar for both females and males. However, in females, the increase in body mass virtually stopped, and ventricular function improvement was more moderate than in the case of males. Higher wall thickness (muscularity) of the coronary vessels was absent; in effect, a tendency of thinning in the walls of the larger vessels was observed. The remodeling of the network appeared to be less beneficial than in exercised male animals. A possible rationale for this may be the sex differences before menopause (83-97). Furthermore, our research group found similar alterations on isolated vessel segments when examining them via pressure-myograph, namely that trained males developed a more advantageous adaptation compared to trained females: while contractility increased in females, endothelial dilation increased in males (17). This increased endothelial reserve represents a kind of protection regarding cardiovascular disease for males compared to females. Similar results were published from human postmenopausal studies: age increases vascular rigidity, oxidative stress is increased, and NO availability is decreased. This process is called "vascular aging" and it increases the risk of CVD. This process may be slowed by training even if the subject did not exercise regularly in their youth. Simultaneously, there are significant gender differences in this process; in middle aged and elderly men, training reduces oxidative stress and NO bioavailability, while in women in the estrogen-deficient state of menopause this does not occur unequivocally. Estradiol therapy improves vascular aging in women (106). Based on our results, advantageous adaptation may be observed not only on isolated vessel segments, but on a network level as well.

4.2.4. Immunohistochemical analysis

The lack of balance between oxidative/nitrative stress and antioxidants causes damage to cells and tissues at many points. In many diseases and conditions, this balance is significantly upset and shifted toward oxidative stress. All of the above are the aggravating factors or the causes of different diseases, like cardiovascular diseases, inflammatory conditions, or cancerous lesions (107). In general, regular training generally lowers chronic oxidative stress (108). In this current study – regarding the degree of oxidative and nitrative stress – there was no difference found between the two sexes, or between the exercised and the control groups. One explanation for this could be the fact that the mechanisms which are responsible for antioxidant processes may become more noticeable due to long-term exercise (108, 109).

Interestingly, there was no difference found in the staining of VEGFR-1. Previous studies have revealed that VEGF mRNA levels become higher in striated muscles and the myocardium due to training (103). Data in the literature on VEGF, however, is not consistent; Kraus et al. demonstrated that though acute exercise enhances VEGF in trained men, during resting periods long-term exercise does not affect circulating VEGF levels (110). Concerning circulating VEGF levels, no difference was found between trained and untrained individuals, or between the sexes (110, 111). The conflicting results may be explained by the fact that VEGF may affect angiogenesis at the beginning of the exercise, but it does not play a role in the maintenance of the capillary network in long-term exercise (112).

Based on the results gained from the staining of adenosine A_{2A} receptors, we came to the conclusion that in males, exercise decreased staining intensity in comparison with both trained females and control males. The expression of the adenosine A_{2A} receptor becomes higher under ischemic and hypoxic conditions (113, 114). This, together with the fact that in our exercised rats a decreased receptor intensity was observed, gives rise to the suggestion that long-term exercise increased oxygen supply in the myocardium of males. In our study, we examined estrogen receptor intensity by applying immunohistochemical methods. As a consequence of exercise, there was a decrease in estrogen receptor staining intensity in male individuals.

The potential clinical significance of the adaptation of the network pattern to sport is that the resistance artery network geometry affects the blood supply of the ventricular tissue.

If this system keeps to a homogenous pattern, higher metabolic need provokes the proper supply of oxygenated blood in all the areas of ventricular tissue.

4.3. Gracilis muscle artery

4.3.1. Gender differences between control groups

Significant differences between the sexes were seen in the control group concerning wall thickness. Vascular walls were thicker in the male control animals, and the wall thickness to lumen ratio was higher in the male than in the female control group. The higher wall thickness to lumen ratio in the males caused decreased tension in the walls of the gracilis vessels in the control groups.

Regarding biomechanical parameters, only tangential wall stress differed between the control groups: it was greater in female controls compared to males.

Comparing these results to data from the literature, our study of animal coronaries did not find differences between the control groups regarding outer/inner radius, or wall thickness/lumen ratio. However, tangential wall stress was significantly lower compared to gracilis arteries in the control females compared to control male rats (17).

Vascular reactivity like myogenic tone and norepinephrine-induced contraction did not differ between the control groups.

Considerable differences between the sexes were observed regarding the acute vasoactive effects of testosterone; male control animals showed the most intensive contraction. Testosterone influences vascular tone through prostanoid-dependent vasoconstriction and relaxation, NO-dependent vasorelaxation (115), and the relative proportion of these effects may be gender-dependent.

4.3.2. Training adaptation

Though several previous studies have examined the training adaptation of peripheral vessels, some of this research focuses on conduit arteries (116). Changes in small arteries due to exercise have been examined in some earlier publications (117-119).

It is well established that physical exercise thickens the walls of peripheral arteries and as a consequence, their diameter increases (8, 120). Maximal diameter does not increase always. While as an effect of training there was a significant increase in the diameter of

the arterioles of the gastrocnemius muscle both in young and older rats, no similar changes could be observed in the arterioles of the soleus muscle (117). A study conducted with the participation of elite squash players revealed that in the non-dominant arm, the brachial artery wall became thinner, but the diameter of the brachial artery remained unchanged; at the same time in the dominant arm, alongside with the thinning of the arterial wall vascular dilation was also observed (121). The explanation for this phenomenon may be that the arteriole in the dominant arm is exposed to higher shear stress for a longer period of time than the arteries of the non-dominant arm (121). An alternative explanation can be that the vascular tone becomes lower in response to exercise, which leads to the thinning of the wall (121, 122). Recently we examined the biomechanical and morphological parameters of gracilis arterioles. The inner diameter of the arterioles did not show any changes in trained males, whereas there was a decrease in wall thickness in females. In spite of this, there was no significant change in the the wall thickness/lumen ratio in either of the sexes.

As a consequence of exercise, the wall stress became higher in females, whereas there was no change detected in males. This could be due to the increase in diameter, and the fact that wall tension is directly proportional to the lumen radius of the vessel, and is inversely proportional to wall thickness. Previous studies found both an increase and decrease in vascular wall tension, which were considered as effects of exercise in different blood vessels (123-125). These conflicting results may be due to the difference in study subjects, artery types, exercise protocols, and research methods. In the present study, an in vitro pressure myograph was used to keep track of the morphological parameters of the arteries in their passive state, and biomechanical parameters were obtained. Elastic parameters are linked to geometry and will be discussed in a later subsection. The training-induced functional adaptation was not uniform throughout the vascular system. Each vessel segment has a different function depending on its size and location within the network, and the type of vascular bed. Smaller vessels and arterioles contract spontaneously; – under common physiological conditions, a spontaneous or myogenic tone may be detected. Previous studies have come to contradictory conclusions about the effects of persistent training on myogenic tone, showing, decreased, increased, and unchanged basal arterial tone (8, 122, 126-128). In control or trained male rats aged 28–30 weeks, no significant differences were found in the gracilis arteriole myogenic tone

(127). However, in healthy young men there was a decrease in the total peripheral resistance index after a month's exercise (128). Sun et al. studied how moderate exercise affects male rats. The study examined gracilis arterioles with pressure arteriography. A slight increase in myogenic tone was detected but the norepinephrine tone did not differ significantly (129). It should be noted that the vessel type examined may have an effect on the extent of myogenic tone. In rats that underwent interval sprint training (IST), arterioles dissected from the gastrocnemius muscle had increased myogenic tone levels compared with feeding arteries of the gastrocnemius muscle (119). The spontaneous tone of arterioles is increased by IST, whereas myogenic reactivity remains the same as that of the control – the diameter is kept almost constant, although there are changes in the intraluminal pressure (119). These observations contradict the results of our study, which proved that after a training program of 12 weeks, the myogenic vessel tone was lower in young adult male specimens, and did not change in females. Additionally, our present research showed that the norepinephrine tone was increased. We also found that age affects the increase of alfa-adrenergic and endothelin-1-mediated contractions of arterioles in the skeletal muscle of rats (130, 131). In older rats, exercise can decrease alpha-adrenergic vasoconstriction (130), meanwhile it has no effect on endothelin-1 vasoconstriction in the arterioles of the soleus gastrocnemius muscle (131).

A decreasing effect of exercise on vasoconstriction was found in young male rats. Exercised old specimens may show a difference in vascular adaptation in metabolically dissimilar muscle types. There is low perfusion in high oxidative profile regions (e.g., red gastrocnemius), although relatively high perfusion occurs in low-oxidative areas (e.g., white gastrocnemius) in comparison with young specimens (132). The matching of oxygen supply to oxidative capacity of the muscle is improved by endurance training in older specimens. Thus, vascular conductance and blood flow are increased in red gastrocnemius; at the same time these parameters are moderate in the white gastrocnemius. (132). Exercise in young specimens was also found to have an increasing effect on the total number of arteries in the gastrocnemius, but the cross sectional region of the feed artery remains unchanged. The reverse happens in older specimens; there was an increase in the diameter of the feed artery, while the number of perforating arteries did not change (132). In the framework of the present research there was no possibility to include the analysis of potential differences in arterial adaptation due to aging.

The differences in exercise strength and duration may influence the outcome of these studies. In our research a 12-week ongoing exercise was enough to reach histological and geometrical remodeling. Additionally, if sufficient time is allowed, an alteration in the sympathetic innervation of these vessels may commence.

An acute vasoactive effect of testosterone can be observed. The extent of this effect is different depending on the types of species and vessels, (57, 133). Contraction of the gracilis vessels was induced by testosterone added to the organ bath in our recent study. Testosterone contraction decreased by exercise, however there was no significant change in female specimens (see next subsection).

4.3.3. Gender differences following training

There was an increase in the inner vascular diameter in exercised female specimens, whereas the wall thickness did not change. The exercised males showed a more prominent increase. The vascular diameters were narrower in exercised males and the males had a higher wall thickness to lumen ratio than females in the control as well as exercised groups. The effect of differences between the sexes of vascular wall thickness to lumen ratio in gracilis and other arterioles has been examined in previous research on high blood pressure (122, 134). The results of these studies were similar to ours, as chronic exercise did not exert a significant influence on the wall to lumen ratio in male Wistar–Kyoto rats. In spite of this, normal values were restored in spontaneous hypertensive (SHR) rats, but not in female SHR rats (122, 134). Green et al. found no differences between the sexes concerning the wall thickness of the brachial and popliteal arteries in young or old athletes, while men had a larger vessel diameter (i.e., the wall thickness to lumen ratio was lower). In addition, after a 12- and 24-week exercise program, the wall to lumen ratio decreased in both males and females (38). The type of exercise influenced the wall thickness to lumen ratio; the result of resistance training is a wall thickness to lumen ratio decrease in brachial arteries, whereas in aerobic exercise it does not change in patients suffering from chronic heart failure (135).

The higher wall thickness to lumen ratio in the male rats caused decreased wall tension of the gracilis vessels in the control as well as exercised groups.

The elasticity of the vessels is characterized by distensibility and elastic modulus parameters. Lower elastic modulus and higher distensibility cause increased vascular

elasticity. Increased elasticity of the gracilis vessels was observed as a result of exercise, but it demonstrated differences between the sexes. Males had a decreased elastic modulus, while females showed increased distensibility at low pressures. In accordance with our findings, elastic modulus was decreased and the distensibility of resistance coronary arteries was increased in rats that exercised on a treadmill (18). The increase in elasticity-related vascular parameters is advantageous regarding hemodynamic adaptation. During exercise, there is a periodical increase in the flow in the moving skeletal muscles; as a consequence, the dilation of the gracilis vessels may be more effective due to their adaptation, leading to increased tissue perfusion.

A previous study examining the coronary arterioles of swimming rats came to similar conclusions, stating that exercised male rats had a significantly lower myogenic tone than females did (17, 37). In comparison, the noradrenaline-caused maximum contraction was found to be significantly higher in our exercised female rats. Previous studies dealt with certain gender differences regarding muscle artery contractions following exercise, but the results show inconsistency (136, 137). Just et al. found that vasoconstriction capacity in females was higher in a steady state than in males, however, during muscle contraction the difference disappeared (136). It has to be noted that Laughlin et al. found that endothelin-1 contraction increased subsequent to chronic training, whereas norepinephrine and KCl contractions did not change in the gracilis arterioles of male miniature swine (138). In male rats, soleus-feeding arteries reacted to norepinephrine with dose-dependent contractions; there was no difference between exercised and sedentary animals (118). Gastrocnemius feed arteries were found to be more sensitive in the control group than in exercised animals (119).

Following a 10-week training period, reduced norepinephrine sensitivity was observed in the aortic rings of rats. This could only be seen after 4 to 10 weeks into training. Endothelium removal diminished, which decreased sensitivity, thus proving its endothelial origin (139).

Based on our observations and the ones described above, it may be concluded that exercise results in increased vascular reactivity in the two sexes; in exercised males, relaxation is more prominent, whereas exercised females show a higher contraction. The regulatory effects of testosterone and estrogen may contribute to these reactions, and further research is needed to explain the sex differences observed in the experiment.

Considerable differences between the sexes could be seen in the acute vasoactive effects of testosterone, with male control animals having the most intensive contraction, which became as low as the level of contraction found in females as a consequence of training. This phenomenon may be explained by the assumption that exercise enhances the metabolism of testosterone to estrogen, which acts as a compensation for the vasoconstrictor effect of testosterone (140, 141). Furthermore, the dominance of vascular effects induced by testosterone may shift toward relaxation. Testosterone influences vascular tone through prostanoid-dependent vasoconstriction, NO-dependent vasorelaxation, and relaxation (115), and the relative proportion of these effects may be different as a consequence of exercise (and not only according to gender).

4.3.4. Immunohistochemical examinations

Our histological analyses also detected differences between the sexes in the exercise-induced histological remodeling of the arterioles in skeletal muscles. While the smooth muscle ratio is improved in females, male rats have an increase in the collagen level in the tunica media. The histological composition differences may be linked to the differences observed in wall contractility and elasticity.

4.4. Limitations and future plans

The main limitation is a general one: the application of the results of the animal model to humans. Biometric differences and life cycles are very different, however there is basis in the literature for this method, and certain procedures may not be appropriate for human research (due to cost, invasivity, lack of comparable specimens, etc.). The type and duration of training, differences regarding musculature, age, sex, vascular bed and vascular function (visceral vessel, muscle vessel, etc.), the nature of the animal model, and many other factors may influence the resulting adaptation. A further limitation is that the exact processes behind the seen adaptations and gender differences have not yet been elucidated: repeated molecular biology studies and experiments performed under hormone-deficient and hormone-replacement conditions are needed to elucidate these underlying mechanisms.

Future plans: our research team is already researching additional vascular sections i.e., visceral vessels (a. renalis) and larger muscle vessels (a. femoralis). Our primary goal is to gather information regarding sport-adaptation of vessels of different functions to learn about adaptation approaches.

Positive vascular effects in terms of function and morphology were induced by exercise. As a preventative measure sport activities are some of the most important. Given the pros and cons of different types of sports, our research may contribute to the evaluation and planning of cardiovascular primary/secondary prevention. Based on our results, it may be beneficial to study the effects of a guideline containing more than one sport, so in addition to cardio, strength training and stretching and regenerative exercises could be included.

5. CONCLUSIONS

Our experiments focused on the following questions:

1. Does the coronary vessel network adapt to persistent training, and what differences may be observed regarding the adaptation based on gender?

Considerable remodelling of the coronary resistance artery network geometry can be found in the hearts of rats exposed to a strenuous, long-term swim exercise programme. A considerable difference between the sexes can be found in this process: wall thickness increases in males, along with a morphological dilation of vessels, thus increasing the number of 400- and 200- μm units, whereas in females, larger (350 μm) vessels get narrower and a new, rich, 200- to 250- μm population close to the orifice appears.

2. Do the vessels located in the muscles – i.e., the gracilis arteriole – adapt to persistent training, and what differences may be observed regarding the adaptation based on gender?

Adaptation of the gracilis arteriole to physical activity was observed, and this adaptation shows differences between the sexes. In females, the wall thickness did not change, while the diameter increased; in males, the diameter remained unchanged, and the vessel wall became thinner. In males, the decrease of the elastic modulus occurred due to exercise, whereas females showed increased distensibility. In males, a decrease of myogenic tone was detected, while maximum contraction enhanced in females.

The processes described above ensure enhanced flow during physical activity, defense against increased wall stress during work-dilation, and represent a convenient way to decrease blood flow during inactivity.

6. SUMMARY

Our research aimed to delineate gender differences in the sport-adaptation of the coronary network and the gracilis artery skeletal artery.

Methods: Wistar rats were divided into four groups: male and female control, and male and female swim-trained. In the swim trained group, the exercise period was increased by daily increments of 15 minutes, and following the habituation period, the animals swam for 200 minutes a day for 12 weeks. The control group animals only swam for 5 minutes a day. Following the training period, the LAD network was dissected down to the 80 μm caliber region, and the gracilis muscle artery was prepared and isolated for pressure-angiometry analysis. Histological analysis and immunochemistry staining were performed to quantify collagen and smooth muscle actin content, oxidative-nitrative stress, and the density of adenosine A1 and estrogen receptors.

The LV mass index was significantly increased in trained animals. In the coronary network, ring frequency spectra were found to be significantly different between male and female rats in the sedentary as well as the exercised groups. Wall thickness was higher in males as a consequence of exercise. Elevations in the populations of 200- and 400- μm vessel units were observed in males; the thinner units formed farther away from – and the thicker ones formed closer to – the orifice. In females, we observed a new population of 200- to 250- μm vessels that appeared uncommonly close to the orifice.

Female animals showed a higher wall tension and inner radius in the gracilis arterioles, whereas wall thickness and elastic modulus decreased in males. Myogenic tone was lower in the ME group, while vasoconstriction induced by norepinephrine was increased in the female exercised group. More prominent collagen staining was shown in the male exercised group than in the male control group. Tangential stress of the gracilis arterioles and relative hypertrophy were increased in females compared to males. Testosterone-induced vasoconstriction was lower in females and decreased as a result of exercise in males.

Conclusion: Sport-induced LV hypertrophy was accompanied by a remodeling of LAD network geometry and structure; reactivity of the gracilis arteriole was different in both sexes. Therefore, sport adaptation of the cardiovascular system is gender dependent.

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8. BIBLIOGRAPHY OF PUBLICATIONS

Publications related to the thesis:

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