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

Ph.D. thesis

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1. Introduction

The different arterial segments adapt to long-term exercise training, however the mechanism of adaptation depends on the type and size of the blood vessels. Vessels run in different vascular beds, and perform varied functions. They are subject to different hemodynamic forces during exercise therefore adaptation cannot be uniform. Vascular morphology and function may be characterized based on analysis performed on isolated vascular section (e.g. pressure-myograph) – this allows for examination of vascular morphology and function, however this does not demonstrate coronary network adaptation, which may be important regarding cardiovascular risk and sudden cardiac death among athletes. In addition to coronary arteries, sport-adaptation of skeletal muscle arteries has long been established. However, sex differences in the sport adaptation of these vessels is a scarcely studied area. Regarding cardiovascular risk, there is a gender difference between women and men before menopause. Sex differences may also be found in the sport adaptation mechanisms of many vessel segments. Thus, it may be assumed that there are gender differences in the sport-adaptation of the left anterior descending coronary (LAD) and gracilis arteries.

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 the serve varied functions so adaptation mechanisms will differ as well.

In this study, we focused on network of the left anterior descending (LAD) coronary arteries and biomechanical and functional properties of gracilis arteries following 12 weeks of intense swim training.

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. Methods

3.1. Animals

The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th edition, 2011) and the EU conform Hungarian Law on Animal Care (XXVIII/1998). All procedures and handling of the animals during the study were approved by the Animal Care Committee of Semmelweis University as well as by state authorities (PEI/001/2374-4/2015). The animals had access to tap water ad libitum. Rats were housed in a room under constant temperature (22 ± 1°C) with a 12-hour light-dark cycle. 50-54-day-old male and female Wistar rats were divided into four groups: male control (MC), female control (FC), male exercised (ME) and female exercised (FE).

3.2. Swim training protocol

Following 7 days of acclimatization, the trained animals participated in swim training: the time spent in the water was increased by 15 minutes every second day until a maximum of 200 minutes was reached. The swim program lasted 12 weeks, during which the swim trained animals swam 5 times a week. Control animals swam 5 minutes a day, 5 times a week, for 12 weeks.

3.3. Echocardiography

After 12 weeks, the rats were anesthetized with 1-2% isoflurane with 100% oxygen and transthoracic echocardiography examination was performed (GE, Healthcare, Horten, Norway). Mid-papillary level short-axis B-mode images were analyzed and LV morphology and function were checked.

3.4. Geometric analysis of LAD

Under pentobarbital anesthesia (45 mg/kg body weight, ip) the heart was removed. The geometry of the left coronary artery system was analyzed on pressure-perfused, microsurgically prepared resistance artery networks using in situ video microscopy (Wild M3Z preparation microscope). All segments over > 80 μm in diameter were studied using 50-μm-long cylindrical ring units removed from the network. We analyzed the branches, segments and the distribution of the ring elements of the LAD. N=8-10-10-10 (for MC, ME, FC and FE groups).

3.5. In vitro pressure-arteriography of gracilis arterioles

At the end of the swim training program, under pentobarbital anesthesia the hearts were removed and the gracilis arterioles were carefully dissected and removed under a microscope magnification. The gracilis arteriole's biomechanical properties and functional reactivity (myogenic tone, norepinephrine- and testosterone induced vasoconstriction) were tested via pressure arteriography.

Biomechanical and functional parameters were calculated as follows:

Wall thickness, $h = r_o - r_i$;

Wall/lumen ratio, $Q = h/d_i$;

Wall stress, $\sigma = P*r_i/h$, according to the Laplace–Frank equation.

Incremental tangential elastic modulus of the cylindrical segments, $E_{inc} = (2r_o r_i^2 * \Delta P)/((r_o^2 - r_i^2) * \Delta r_o)$.

Incremental distensibility was $D_{inc} = \Delta V/V\Delta P$

where h is the wall thickness in μm, r_o and r_i are the actual values of the outer and inner radii in μm, d_i is the inner diameter in μm, P is the transmural (intraluminal) pressure, Δr_o is the alteration of the outer radius during a pressure rise of ΔP according to Cox (Cox, 1974), and ΔV is the change in vessel lumen volume relative to the initial volume of V in response to a pressure change of ΔP. Lumen volumes were computed from the inner radii, assuming cylindrical symmetry.

$$\text{Myogenic tone (\%)} = (r_{iCa^{2+}free} - r_{inKR})/r_{iCa^{2+}free}*100$$

$$\text{Constrictions to NE (\%)} = (r_{iCa^{2+}free} - r_{iNE})/r_{iCa^{2+}free}*100$$

$$\text{Testosterone contraction (\%)} = (r_{inKR} - r_{iT})/r_{inKR} *100$$

where $r_{iCa^{2+}free}$ and r_{inKR} are the inner radii measured in a calcium-free solution and in a normal Krebs–Ringer solution at the same pressure, and r_{iNE} and r_{iT} are the inner radii measured after noradrenaline and testosterone at the same pressure, respectively. N=9-10-10-11 for MC, ME, FC and FE groups

3.6. Immunohistochemistry

Coronary arteries

The heart was placed in 4% formaldehyde for fixation. Following dehydration, the tissues were embedded in paraffin and were cut into 5-μm slices. Immunohistochemistry was performed against ribose (PAR), 3-nitrotyrosine (NT), endothelial nitric oxide synthase (eNOS) as well as stained for vascular endothelial growth factor receptor 1 (VEGFR-1), adenosine A2A-R (AdeA2A-R) and estrogen receptor (ER). The non-calibrated optical density of specific staining was measured by ImageJ software (NIH, Bethesda, MA, USA). N=3-4 in each group.

Gracilis arteries

Biomechanical measurements were performed on gracilis arteriole segments. first the segments placed in 4% buffered formalin. The dehydrated and paraffin-embedded tissues were cut into 5-mm sections. The tissues were stained, using MOVAT pentachrome stain (Russell modification), resulting in black (nuclei and elastic fibers), yellow (collagen and reticular fibers), red (muscle and fibrin), and blue (ground substance and mucin) areas. Sections were photographed with a Nikon Eclipse Ni-U microscope with a DSRi2 camera (Nikon Minato, Tokyo, Japan) at 10 x magnification. To evaluate results, pentachromic staining was separated into individual color channels on the images, using ImageJ software. After converting the separated images to black and white, the degree of staining was determined using non-calibrated optical density (OD). N=3-15 in each group.

3.7. Statistical evaluation

SPSS Sigma Stat software, GraphPad Prism 5 software and Excel functions were used for statistical analysis. All data are presented as the mean ± SEM. Normal distribution was checked with the Shapiro-Wilk test. Two-way analysis of variance (ANOVA) was used with post hoc Tukey test for paired comparisons. In case of non-normal distribution, the Kruskal–Wallis test was performed with Dunn's post hoc test. P-values < 0.05 were considered to indicate statistically significant differences. Ring unit diameter distribution histograms of pooled data were compared via $\chi 2$ test.

4. Results

4.1. Cardiac changes

The LV mass index (g/kg, MC: 2.36±0.08; ME: 3.05±0.08; FC: 3.07±0.08; FE: 3.64±0.1), ejection fraction (%, MC: 73±1.0; ME: 81±1.2; FC: 79±0.8; FE: 82±0.9), fractional shortening (%, MC: 44±0.9; ME: 52±1.3; FC: 49±0.8; FE: 52±1.1) and SV index (µl/g, MC: 0.49±0.02; ME: 0.62±0.02; FC: 0.64±0.01; FE: 0.74±0.03) significantly increased in trained animals and the LV mass index and the SV index significantly greater in female animals compared to males (in control and trained groups also) (two-way ANOVA with Tukey's post hoc test, $p<0.05$).

4.2. Branching

No significant differences regarding bifurcation geometry could be identified between either the sedentary and trained groups or the sexes. All four groups adhered fairly well to Murray's law.

4.3. Wall thickness

Wall thickness correlated positively with diameter in all four groups. Exercise effect was dependent on vessel diameter (interaction, $p<0.001$) **(Figure 1)**. Note increased wall thickness in trained male animals, while in females a slight but significant reduction in wall thickness could be observed. Vessel segments from the females had significantly thinner walls than males in the 100 to 300-µm range in the sedentary groups and in the entire segment-size range of the trained groups (not shown on the figure, ($p<0.05$).

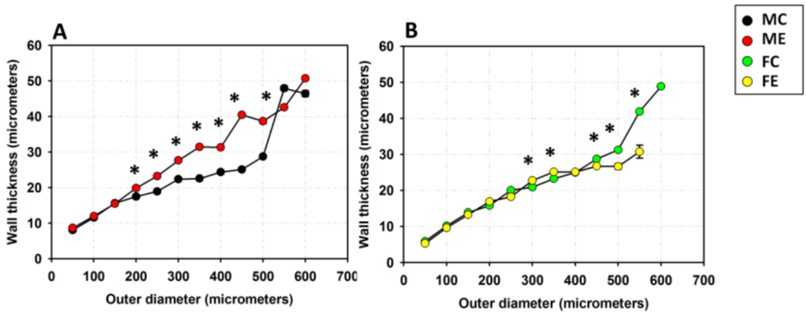

Figure 1. Analysis of ring elements of the whole left anterior descending coronary artery system A) Control and trained male groups. B) Control and trained female groups. Wall thickness as a function of outer diameter. * $p<0.05$ between sedentary and trained. Two-way ANOVA with post hoc Tukey's test test. Data are expressed as the mean (SEM) values; N=8-10-10-10 (for MC, ME, FC and FE groups).

4.4. Frequency of ring units making up the network

Ring frequency spectra (**Figure 2.**) of trained animals were significantly different from the sedentary ones in both males and females. Note doubling of the 400-µm vessel units and substantial elevation of 200-µm vessel units in males in response to training. In females, the number of 150-µm vessel units was elevated, while there was a reduction in the 350-µm range. The ring frequency spectra of male animals were significantly different from the female ones in both the sedentary and trained groups. In the control groups, the number of larger (300–600 µm) vessels was greater, and the number of smaller (200–250 µm) ring units was smaller in the male rats compared to the female rats. In the trained groups, there were more 350- to 600-µm vessels and fewer 200- to 250-µm vessels in male animals compared to female animals.

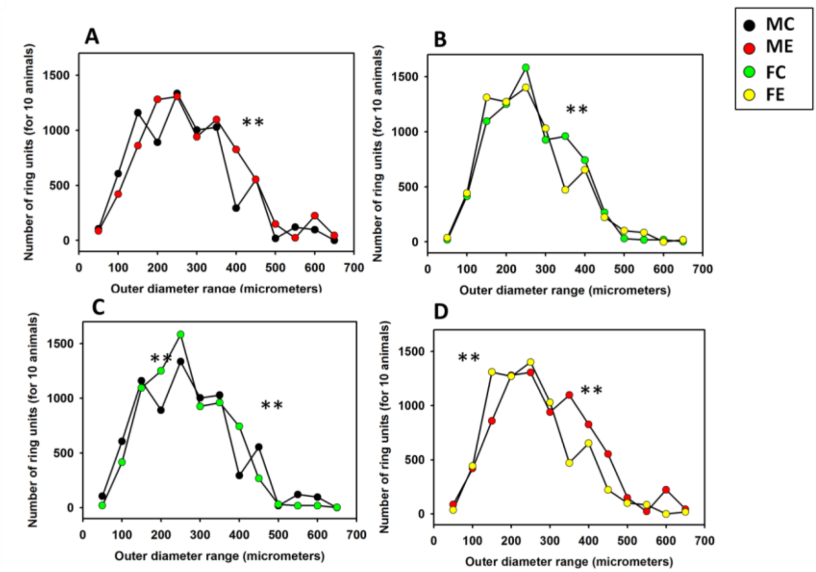

Figure 2. Ring unit analysis. Numbers of 50 µm-length unit sin the networks in different outer diamater ranges. A) Control and exercised males. B) Control and exercised females. C) Control males and females. D Exercised males and females. Pooled data for 10 animals, with a total of 29,390 ring units included. ** $p<0.01$ with the $\chi 2$ probe.

4.5. Network distribution

Bidimensional histograms of ring unit frequencies based on outer diameter and flow, and the distance from the orifice are shown in **Figure 3.** In males, note the increased number of 200-μm elements following training - this appears to be the result of the morphological dilation of the 100 to 150-μm elements that were a moderate distance from the orifice. Clusters of 400-μm elements appear close to the orifice. Thus a younger network is formed (negative correlation between diameter and flow distance). In case of females, the network in sedentary animals was fairly well organized, while 150 to 200-μm vessel elements appeared close to the orifice in the trained network - not improving but loosening the pattern.

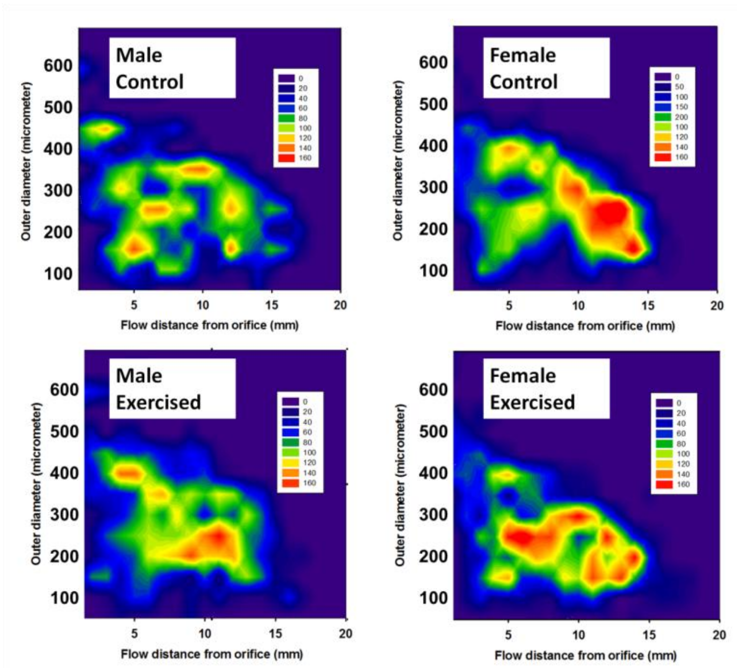

Figure 3. Ring unit analysis. Distribution of different vascular elements in the network. Bidimensional histograms of ring unit frequencies according to outer diameter and flow distance from the orifice. Pooled data for 10 animals, with a total of 29,390 ring units included.

4.6. Immunohistochemical examination of the heart

We found no difference regarding staining of Anti-HNE, Anti-PAR, Anti-NT, Anti-eNOS and VEGFR-1. AdeA$_{2A}$-R optical density reduced in trained males compared with both control males and trained females (arbitrary unit, MC: 0.04±0.002; ME: 0.03±0.002; FC: 0.04±0.001; ME: 0.04±0.002; two-way ANIVA with post hoc Tukey's test, $p<0.05$). Optical density of the estrogen receptor also reduced in trained males compared to the control group (arbitrary unit, MC: 0.28±0.031; ME: 0.15±0.014; FC: 0.31±0.033; FE: 0.24±0.07, two-way ANOVA with post hoc Tukey's test, $p<0.05$).

4.7. Morphology and biomechanical parameters of gracilis arterioles

The inner radius was increased in FE rats compared to FC and ME animals **(Figure 4A)**. Wall thickness was deceased in the ME group compared to the MC group and this value was higher in MC rats compared to FC animals **(Figure 4B)**. The wall thickness to lumen ratio was significantly smaller in FC and FE rats than MC and ME rats **(Figure 4C)**. Tangential wall stress was significantly increased in the FE rats compared to controls. In addition, this value was significantly higher in the FC and FE rats compared to the MC and ME rats **(Figure 4D)**. As a result of training, the elastic modulus was significantly smaller in the ME rats compared to the MC rats **(Figure 4E)**. Distensibility increased in the FE rats compared to the FC rats **(Figure 4F)**.

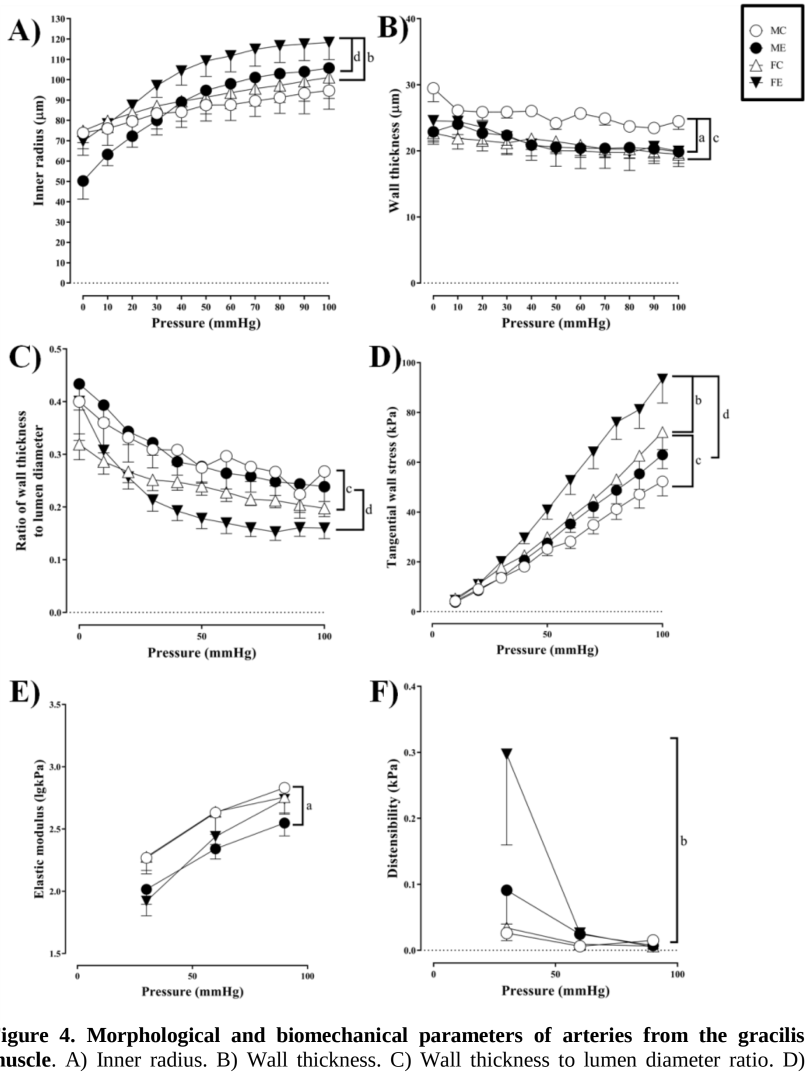

Figure 4. Morphological and biomechanical parameters of arteries from the gracilis muscle. A) Inner radius. B) Wall thickness. C) Wall thickness to lumen diameter ratio. D) Tangential wall stress. E) Tangential elastic modulus. F) Distensibility. The values measured as a function of intraluminal pressure and measured under passive conditions (in calcium-free Krebs solution) of the gracilis muscle arterioles from the MC, ME, FC, and FE animals. 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 shown. aP < 0.05 MC vs. ME; bP < 0.05 FC vs. FE; cP < 0.05 MC vs. FC; dP < 0.05 ME vs. FE. N=9-10-10-11 for MC, ME, FC and FE groups

4.8. Contractility parameters of gracilis arterioles

As a result of training, myogenic tone was significantly smaller in the ME rats compared to the MC rats **(Figure 5.)**. As a result of norepinephrine, vasoconstriction was significantly higher in the FE rats compared to the FC and ME rats **(Figure 6.)**. Contraction to testosterone was significantly lower in females than in males in the control groups. As a result of training contraction to testosterone decreased in the male groups **(Figure 7.)**.

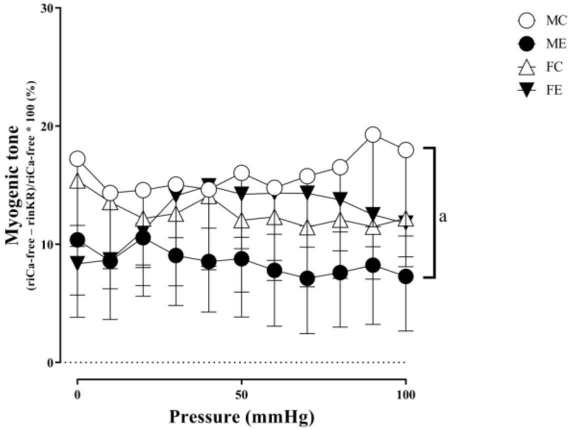

Figure 5. Myogenic tone as a function of intraluminal pressure measured under passive conditions of the gracilis arterioles from the MC, ME, FC and FE animals. The data are expressed as the mean (SEM) values. The significance levels of the two-way ANOVA and Tukey's post hoc tests between the four groups are shown. aP < 0.05 MC vs. ME. N=9-10-10-11 for MC, ME, FC and FE groups.

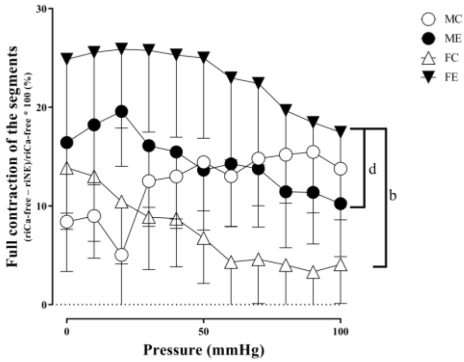

Figure 6. Constrictions to NE (relative difference of the maximally relaxed and maximally contracted radius) as a function of intraluminal pressure measured under passive conditions of the gracilis arterioles from the MC, ME, FC and FE animals. The data are expressed as the mean (SEM) values. The significance levels of the two-way ANOVA and Tukey's post hoc tests between the four groups are shown. bP < 0.05 FC vs. FE and dP < 0.05 ME vs. FE. N=9-10-10-11 for MC, ME, FC and FE groups.

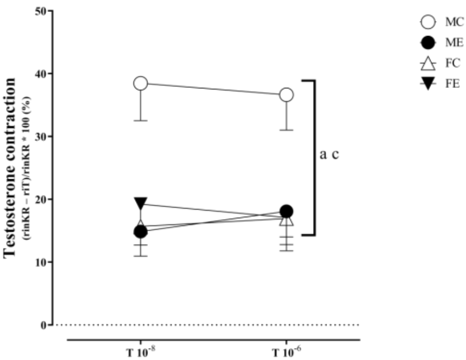

Figure 7. Testosterone contraction of the segments in 10-8 and 10-6 M at 50 mmHg from the MC, ME, FC and FE animals. The data are expressed as the mean (SEM) values. The significance levels of the two-way ANOVA and Tukey's post hoc tests between the four groups are shown.aP < 0.05 MC vs. ME and cP < 0.05 MC vs. FC. N=9-10-10-11 for MC, ME, FC and FE groups.

4.9. Histological changes in the gracilis arterioles

Smooth muscle density was significantly higher in the FE rats than in the MC rats. The density of collagen was significantly higher in the ME group compared to the MC rats. The density of connective tissue was significantly higher in the female control group than in the male control group **(Figure 7.)**.

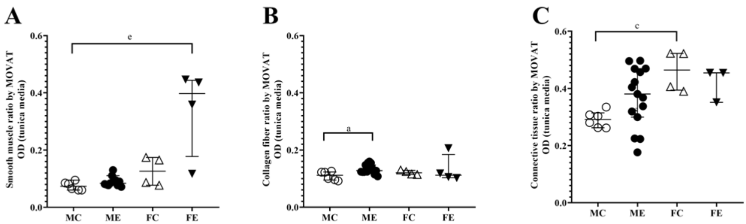

Figure 7. A) Smooth muscle staining intensity of segments. B) Collagen staining intensity of the segments. C) Connective tissue staining of the segments. The data are expressed as the median (interquartile ranges) values. The significance levels of Kruskal–Wallis test and Dunn's post hoc tests between the four groups are shown. eP < 0.05 MC vs.FE; aP < 0.05 MC vs. ME and cP < 0.05 MC vs. FC. N=3-15.

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?

Substantial remodeling of the coronary resistance artery network geometry occurs in the hearts of rats subjected to a strenuous, persistent swim training program. There are a substantial sex differences in this process: in males, wall thickness increases, with a morphological dilation of vessels, increasing the number of 400 and 200-µm units, while in females, a narrowing of larger (350 µm) vessels and the appearance of a new, rich, 200 to 250-µm population close to the orifice are characteristic.

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?

The gracilis arteriole adapts to physical activity and adaptation mechanisms demonstrate sex differences. Wall thickness remained unchanged in females while diameter increased, while in males, diameter remained the same, and thinning was observed in the vessel wall. In males, the elastic modulus decreased due to exercise, while in females, increased distensibility was observed. Myogenic tone decreased in males, whereas maximum contraction was enhanced in females.
The observed processes ensure increased flow during physical activity, defense against increased wall stress during work-dilation, and represent a convenient way to reduce blood flow during inactivity.

6. Bibliography of the candidate's publications

Publications related to the thesis:

Merkely, Petra; Bakos, Marcell; Bányai, Bálint; Monori-Kiss, Anna; Horváth, Eszter Mária; Bognár, Judit; Benkő, Rita; Oláh, Attila; Radovits, Tamás; Merkely, Béla; Ács, Nándor; Nádasy, György László; Török, Marianna and Várbíró, Szabolcs. Sex differences in exercise-training-related functional and morphological adaptation of rat gracilis muscle arterioles. FRONTIERS IN PHYSIOLOGY. 2021.
Impact factor: 4,566

Török, Marianna*; Merkely, Petra*; Monori-Kiss, Anna; Horváth, Eszter Mária; Sziva, Réka Eszter; Péterffy Borbála; Jósvai, Attila; Sayour, Alex Ali; Oláh, Attila; Radovits, Tamás; Béla Merkely; Nándor Ács; Nádasy, György L. and Várbíró, Szabolcs. * these authors contributed equally to this work and are considered first authors. Network analysis of the left anterior descending coronary arteries in swim-trained rats by an in situ video-microscopic technique. BIOLOGY OF SEX DIFFERENCES. 2021
Impact factor: 5,027

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Török, Marianna; Monori-Kiss, Anna; Pál, Éva; Horváth, Eszter; Jósvai, Attila; Merkely, Petra; Barta, Bálint András; Mátyás, Csaba; Oláh, Attila; Radovits, Tamás; Béla Merkely; Nándor Ács; György L. Nádasy and Szabolcs Várbíró. Long-term exercise results in morphological and biomechanical changes in coronary resistance arterioles in male and female rats.BIOLOGY OF SEX DIFFERENCES. 2020.
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Török, Marianna; Horváth, Eszter M; Monori-Kiss, Anna; Pál, Éva; Gerszi, Dóra; Merkely, Petra; Sayour, Alex A; Mátyás, Csaba; Oláh, Attila; Radovits, Tamás; Béla Merkely; Nándor Ács; György L. Nádasy and Szabolcs Várbíró. Chronic swimming training resulted in more relaxed coronary arterioles in male and enhanced vasoconstrictor ability in female rats. JOURNAL OF SPORTS MEDICINE AND PHYSICAL FITNESS. 2020.
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∑IF: 15.766