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Sex differences in sports adaptation of the renal and femoral arteries

PhD thesis

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LIST OF ABBREVATIONS

α-SMA	α-smooth muscle actin
Ach	Acethylcholine
COX	Cyclooxygenase
COX2	Cyclooxygenase-2
CVD	cardiovascular disease
DAB	3,3'-diaminobenzidine
DMSO	Diluted Dimethyl-Sulfoxide
EDCF	Endothelium-Derived Contracting Factor
EDHF	Endothelium-Derived Hyperpolarizing Factor
eNOS	Endothelial nitric oxide synthase
FSed	Female Sedentary
FTr	Female Trained
INDO	Indomethacin
L-Name	Nitro-L-arginine Methyl Ester
LV	Left Ventricular
LVEDD	Left Ventricular end-diastolic diameter
LVESD	Left Ventricular end-systolic diameter
MSed	Male Sedentary
MTr	Male Trained
NIH	National Institutes of Health
NO	Nitrogen Monoxide
OD	Optical Density
NT	Nitrotyrosine
NSAIDs	Nonsteroidal antiinflammantory drugs
PGG2	Prostaglandin G2
PGH	Prostaglandin H

PGH2	Prostaglandin H2
Phe	Phenylephrine
RF	Resorcin-Fuchsin

1. INTRODUCTION

Regular exercise is observed to have beneficial effects on various body functions in most mammalian species, including humans. The impact is evident in the adaptation of the cardiovascular system (1) and the lipid metabolism, where it increases the HDL / LDL ratio and lowers stress (2-4). Exercise also plays a key role in maintaining an appropriate body weight and body fat percentage (5). Furthermore, studies have shown that regular exercise Reduces the risk of developing metabolic syndrome, osteoporosis, and even depression (6).

Adaptation of the cardiovascular system is most visible in the complex structural, functional, and electrical remodeling of the heart, referred to as the athlete's heart (7-9). Ventricular diameters increasealong with myocardial mass and stroke volume, while resting heart rate is lowered (9-12).

An increase in in the cardiac output is also observed as a result of regular exercise, while oxygen consumption increases up to 20-40% (VO2 max) (13).

Significant alterations in the cardiovascular system may be observed while engaging in aerobic activity. The redistribution of blood circulation is the mechanism through which oxygenated blood is sent to organs that are actually working. Elevated cardiac output and increased blood flow to the active skeletal muscles are two common changes that occur during exercise (14). Although cerebral blood flow is untouched, blood flow in inactive muscles and visceral vessels, such as the renal artery and splanchnic region, declines (14-16).

1.1. Fundamentals of the regulation of vascular responses

In respect of their regulation, blood vessels essentially comprise two main types of cells: endothelial and vascular smooth muscle cells. Blood vessel development and the mature blood vessel homeostasis is dependent on the proper functioning and interaction of these endothelial cells and vascular smooth muscle cells (17). Due to the complexity of the vascular system, examination of its several sub-units is necessary, with particular focus on the endothelial and smooth muscle cells, and also the location of the blood vessels (18).

In producing both vasoconstriction and vasodilation factors, the endothelium acts as one of the main regulators of a vessel’s vascular reactivity. Clearly, vasoconstriction

factors play a role in pathological processes associated with cardiovascular diseases, stroke, and ischemic heart attack (19, 20). Meanwhile, it should be noted that endothelium-derived vasoconstriction is also a factor in physiological processes, for instance, brain autoregulatory processes (21).

Endothelium-derived contracting factors (EDCF) include prostaglandins (PG) and thromboxane A2 (TXA2). The most common precursor of PGs is arachidonic acid, a fatty acid released by phospholipase A2 from the phospholipid of the cell membrane. This is then further metabolized by several other enzymes, including prostaglandin H (PGH) synthase, lipoxygenases and cytochrome 450 monooxygenase, or non-enzymatically, in a radical catalyzed non-enzymatic fashion, into isoprostanes. PGH synthase is the primary and rate-limiting enzyme in the pathway of PG synthesis. PGH synthase possesses both cyclooxygenase (COX) catalytic activity resulting in prostaglandin G2 (PGG2) formation and peroxidase activity catalyzing the reduction of PGG2 to prostaglandin H2 (endoperoxide, PGH2) (22).

The two main types of COX are COX-1 and COX-2. The two enzymes differ in respect of how their activity and expression are regulated, and they are able to function independently even within the same cell type. Within blood vessel walls, both endothelial and smooth muscle cells may be observed to contain COXs. In healthy blood vessels, the ratio of these enzymes is higher in endothelial cells than in smooth muscle cells (23).

The substrates of COX-1 are fatty acids (such as arachidonic acid), while the substrates of COX-2, in addition to fatty acids, are a2-arachidonyl glycerol. Accordingly, COX-2 can synthesize products that COX-1 cannot. The activity of both COX-1 and COX-2 is determined by the level of lipid peroxidases, but COX-2is activated by a concentration of hydroperoxide ten times lower than that required for activation of COX-1. This means that COX-2 can act in the presence COX-1 without activating COX-1 (22, 24, 25). COX-1 is constantly expressed in most tissues, but it can be overproduced, for example, as a result of shear stress (26).

Similarly, COX-2 is constantly expressed in many organs, but can be induced by inflammation or shear stress. COX-1 and COX-2 are both expressed in endothelial cells (22, 26, 27), which, if cultured under static conditions, exhibit a predominance of COX-1ower COX-2 (28-30).

Metabolites produced by COX enzymes may result in vasoconstriction or vasodilation effect. Prostaglandin G2, followed by prostaglandin H2 are first produced from arachidonic acid under the action of the COX enzyme. In turn, prostaglandin H2 then forms prostaglandin I2 (prostacyclin), prostaglandin D2, and prostaglandin E2 The last two of these then form prostaglandin F2 alpha, before finally, thromboxane A2 forms from prostaglandin H2. Prostacyclin, prostaglandin D2, prostaglandin E2 have a vasodilator effect and also inhibit platelet aggregation and lymphocyte migration. Meanwhile, vasoconstrictor prostaglandins include: thromboxane A2and prostaglandin F2 (which also have an effect causing platelet aggregation). COX-2, when acetylated, differs from native COX-2 in terms of catalytic activity (acetylated COX-1 has no catalytic activity (31)). Acetylation of COX-2may be triggered by some nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin), via production of various hydroxyeicosatetraenoic acids (HETE) e.g. 15R-hydroxyeicosatetraenoic acid (15R-HETE), 5S,15R-dihydroxyeicosatetraenoic acid (5S,15R-diHETE) (32-34). By reducing the degree of vascular relaxation, these compounds lead to vasoconstriction (35).

COX enzymes may be inhibited either selectively (selective COX-2, e.g. NS398, rofecoxib) or non-selectively (e.g. indomethacin) with NSAIDs. Non-selective COX inhibitors act on both COX-1 and COX-2, while selective COX-2 inhibitors do not affect COX-1. Selective COX-2 inhibitors are preferred for treatment of joint complaints and rheumatic diseases. Of particular interest is the association between selective COX-2 inhibitors and increased cardiovascular risk (36, 37). This is because COX-2 is the dominant producer of prostacyclin (38), which has a vasorelaxant effect. When COX-2 is selectively inhibited, the vasoconstrictor/vasorelaxant factors tend towards vasoconstriction (39).

Selectively and non-selectively inhibiting COX enzymes allows for more accurate examination of vascular function and endothelium during vascular physiology (e.g. wire-miograph (40) experiments: using inhibitors allows for deduction of the various adaptations based on the overexpression of COX-1, COX-2 or both COX enzymes.

Nitric oxide is an Endothelium-derived relaxant factor, formed from L-arginine by guanylate cyclase activity in smooth muscle cells. (41). Endothelial cells may also secrete endothelium-derived hyperpolarizing factor (EDHF), which is also responsible for endothelium-related relaxation (42).

1.2. The adaptation of blood vessels due to exercise

During exercise, significant changes take place in the circulatory system. As well as elevated cardiac output, blood flow in working muscles and organs is elevated through a process, called redistribution of blood circulation.

While this phenomenon occurs, blood flow in inactive muscles and visceral vessels, like the renal artery and splanchnic area, declines (14-16). However, cerebral blood flow remains unchanged.

1.2.1. Exercise adaptation and sex differences of the arterioles of the visceral arteries

The renal arteries provide blood to the kidneys, which play an important role in homeostasis, blood volume, and blood pressure regulation.

The adaptation of visceral arteries resulting from sport is a poorly understood area, with related studies often controversial (43-46). We are aware that visceral arteries constrict during exercise, albeit athletic individuals experience less of this contraction (44, 46). Possible causes include diminished sympathetic tone, reduced plasma levels of vasopressin and angiotensin II, less reactivity to norepinephrine, and enhanced NO-related vasodilation (43).

Before menopause, there is also a recognized and significant sex-related difference between men and women in terms of cardiovascular disease. This connects to estrogen's protective properties. On the basis of a similar assumption, it may be questioned if a sex-based difference may be found in how the vascular system responds to routine athletic exercise. In past studies, we explored the sex differences in sport adaptation across several artery types, including coronary arteries (47-49) or vessels that supply muscles involved in active work (a. gracilis) (50). In response to the 12-week training, the two types of blood vessels adapt, and the following sex differences have been recognized: In coronary arteries, females' capacity to contract increased while males' ability to relax increased (47, 48); females had more significant tangential stress and noradrenaline-induced contraction than males (50).

Regarding visceral vessels, however, we know considerably less about sport-specific adaptations and gender differences. The extent of muscle reflex-mediated renal vasoconstriction during static exercise (handgrip and quadriceps contraction) did not differ significantly between men and women in previous studies examining the response

of the renal arteries to static exercise (handgrip and quadriceps contraction) (51). Nonetheless, a sex difference in splanchnic blood flow was observed following a 9-12-week program of intense aerobic training. Despite the fact that splanchnic blood flow decreased during exercise in both sexes, this exercise-induced reduction in splachnic circulation became less pronounced in men after the 9-12-week program, whereas no such change was observed in women (52).

1.2.2. Exercise adaptation and sex differences of the arterioles of the skeletal muscle arterioles

The femoral artery provides the main blood supply to the lower limb. Training causes a significant increase in blood flow to the muscles participating in the training (53).

Physical activity causes an increase in arterial pressure, which in turn triggers a cyclical increase in vessel wall tension, resulting in morphological, biomechanical, and functional adaptations of the vessel. Increased tension in the circumferential wall and sustained shear stress causes an increase in endothelial nitrogen-oxide synthase (eNOS), resulting in an increase in NO-related vasodilation (54-56).

In peripheral musculocutaneous artery branches, vessel diameter grows and the vessel wall thins within a single workout session. At repose, the dilated vessel diameter is not always visible (54, 57). Regarding the functional sport-related adaptation of peripheral vasculature, findings conflict. In multiple investigations, increased flow-mediated vasodilation was observed, and this effect has been documented in both acute and chronic exercise contexts (58-60).

A previous study examined how the male rat gracilis muscle arteriole responded to short-term treadmill training. A modest increase in myogenic tone, no change in norepinephrine-induced tone, and a slight decrease in adenosine-induced relaxation were observed. However, a significant increase was reported in acetylcholine and L-arginine (a precursor of NO) dilation degree (61). In addition, short-term training was found to increase the endothelial cells' sensitivity to shear stress, producing a heightened dilation response (62). However, as described by Green et al. there is a so-called "athlete paradox," in which the endothelium function first improves as a result of regular exercise but then reverts to the baseline levels as a result of subsequent structural adaptation. Indeed,

following an extended program of regular exercise, there were no significant variations in the vascular functioning of the trained and control arteries in this study (57).

2. OBJECTIVES

Cardiovascular benefits resulting from exercise are well known. During aerobic exercise, hemodynamic alterations are observed: blood flow in skeletal muscle arteries is observed to increase while it is observed to decrease in visceral vessels due to mesenterial vasoconstriction.

Given that preserving renal blood flow during strenuous exercise is a priority, our objective was to examine sex differences in respect of renal blood flow during exercise, with a view to improving knowledge and clarity in a less-studied area currently characterized by conflicting data.

Pertinent to this objective, we subjected male and female rats to a swimming exercise training program, in order to monitor and compare changes in vascular reactivity and histology specific to the isolated renal and femoral arteries.

The experiment was thus devised to test the hypothesis that sex differences exist in terms of how these blood vessels adapt to regular intensive exercise, over a prolonged course of time.

Vascular reactivity and histology of male and female Wistar rodents were examined before, during and after a swimming program over a duration of 84 days. The rats were organized into 4 groups: male sedentary (MSed), male trained (MTr), female sedentary (FSed) and female trained (FTr). Examination of the isolated renal and femoral artery rings was conducted by wire myography. We also performed histological and immunohistological measurements on the vascular rings.

In the above trained model, we aimed to investigate:

1. Does the renal artery adapt after long-term exercise, and, in this respect, are there observable and significant differences between the sexes?
2. Does the femoral artery adapt after long-term exercise, and, in this respect, are there observable and significant differences between the sexes?

3. METHODS

3.1. Animals

The animals were all subjected to identical living conditions: temperature was maintained at 22ºC-25ºC, with a relative humidity of 40%to 70%, with a 12-hour lightness/darkness cycle; the animals had access to laboratory-standard feed and potable water. In line with protocol, all reasonable steps were taken to reduce animal suffering and inconvenience to a minimum. Animal Care Committee of Semmelweis University approved experimental protocols (Permission Number: PEI/001/2374-4/2015) that complied with European Union (Directive 2010/63/EU) regulations for the care and use of animals in research. Each animal was treated in strict accordance with the National Institutes of Health (NIH Publication No. 86-23, revised 1996.) guidelines for the 'Guide for the Care and Use of Laboratory Animals.

3.2. Chemicals

Anesthesia was administered using pentobarbital (Euthasol, CEVA Santé Animale, Liboure, France).

Chemicals used in our experiment were sourced from Sigma-Aldrich (St. Louis, MO, USA).

On the day of the experiment, the following reagents were freshly dissolved in physiological saline (0.9% NaCl) or normal Krebs-Ringer (nKR) solution (in mmol/L): NaCl 119, KCl 4.7, NaH2PO4 1.2, MgSO4 1.17, NaHCO3 24, CaCl2 2.5, glucose 5.5, and EDTA 0.034.

3.3. Experiment: grouping, exercise protocols

After an initial seven-day acclimatization period, the animals were grouped accordingly for experimentation: male sedentary (MSed), male trained (MTr, female sedentary (FSed,), and female trained (FTr). The animals were 8 weeks old at the time of commencement of the exercise program.

Male and female trained rats (MTr and FTr) used in an extended 12-week swimming training program as previously described (7). Animals were individually placed in metal basins (45 x 25 x 20 cm) containing potable water (30-32 ºC). It was ensured that the

basins had sufficient depth such that the animals were compelled to swim in order to stay afloat (63-65). It was checked and confirmed that the side walls of the basins offered no purchase, for the same reason. Diving was not permitted.

The duration of the exercise sessions was increased incrementally, from an initial 15 minutes of swimming per day, increasing 15minutes every second day, a up to 200 minutes per day. The entire program lasted 12 weeks, with the animals subjected to swim training 5 days a week. Sedentary rats, meanwhile, were placed in water for 5 minutes each day for 5 days a week to reduce potential differences for swim load.
Throughout the experiment, regular monitoring of body weight, as well as general condition, was conducted.

3.4. Echocardiography

In order to detect myocardial hypertrophy resulting from exercise, echocardiac examinations were performed, as previously (66, 67), in the final week (12 week) of the exercise program. The animals were initially relaxed with 1-2% isoflurane in 100% oxygen. Animal temperature was maintained at 37 ° C through the use of a heating bench. The animals' chests were shaved to allow transthoracic echocardiographic examination (Vivid I Echocardiac Image Analysis System, GE, Healthcare, Unites States) using an ultrasonographic probe. The operator was denied knowledge of the experimental groups, to ensure a blind test. At mid-papillary level, standard two-dimensional short-axis images were acquired using a 13-MHz linear transducer (GE Healthcare, Horten, Norway). Image analysis software (EchoPac v113, GE Healthcare) was utilized. M-mode images measured the left ventricular (LV) end-diastolic (LVEDD) and end-systolic (LVESD) diameters, as well as the left ventricular anterior wall and posterior wall thickness in diastole (LVAWTd and LVPWTd), from which the left ventricular mass (LV mass) was computed using the following formula: LV mass = $[(LVEDD+LVAWTd+PWTd)^3-LVEDD^3]*1.04$

3.5. Myography

Animals were anesthetized with an intraperitoneal administration of sodium pentobarbital (Euthasol, CEVA Santé Animale, Liboume, France, 45 mg/kg) following a 12-week training program. As a preventative measure against intravascular thrombosis,

heparinized nKR solution was perfused through the vasculature. Renal and femoral artery were meticulously prepared under dissection microscope (Wild M3Z) with particular focus directed towards the condition of the vascular rings and endothelium.

Experiments were conducted using a DMT 610 M Wire Myograph system (multi-chamber isometric myograph system, Danish Myo Technology, Aarhus, Denmark) on prepared vascular rings. The myograph system consisted of four organ chambers, each containing 6 ml of nKR and with a temperature of 37 °C that was continuously maintained. Using a gas mixture stabilized at 7.4 pH (O2 95%, CO2 5%), the pH was adjusted by bubbling. Data collection was performed using LabChart software. (ADInstruments, Oxford, UK-Ballagi LTD, Budapest, Hungary)

Having been first isolated, the renal and femoral arteries were then cut into 2 mm equal lengths numbering five pieces, from which 4 vascular rings were prepared before being placed onto the myograph system. Over the course of 1 hour, pretension was gradually increased to the desired value of 10 mN. The fifth piece was fixed using formalin and then embedded in paraffin for the purpose of histological examinations.

3.5.1. Myograph protocol for renal arteries

Animals were grouped for experiment as thus: male sedentary (MSed, n= 16), male trained (MTr, n = 7), female sedentary (FSed, n = 12), and female trained (FTr, n = 12).

Following equilibration, 124 mmol/L K+ was used for a time of 3 minutes (100% contraction) for the purposes of testing contractility of the blood vessel and setting the reference value. To establish the viability of the endothelium, a maximum concentration (10^{-6} mol/L) of phenylephrine and then a maximum concentration (10^{-6} mol/L) of acetylcholine was used.

For the purpose of measuring contraction as a response to alpha-agonist, cumulative concentrations of phenylephrine (10^{-8}–10^{-6} mol/L) were added to the bath. Acetylcholine (Ach) induced vascular relaxation was investigated after phenylephrine (Phe) precontraction (10^{-6} mol/L) (without rinsing out phenylephrine) by applying acetylcholine at progressively increasing concentrations (10^{-8}–10^{-6} mol/L). After 30 minutes of pretreatment with one of the following inhibitors: the cyclooxygenase (COX) inhibitor indomethacin (INDO 10^{-4} mol/L), the cyclooxygenase-2 (COX-2) specific inhibitor NS398 (10^{-5} mol/L), or the NO synthase blocker nitro-L-arginine methyl ester

(L-NAME 10^{-5} mol/L), the procedure was repeated. In contrast, control samples were treated with vehiculum (diluted dimethylsulfoxide, DMSO).

3.5.2. Myograph protocol for femoral arteries

For experimental purposes, animals were grouped into four, as thus: male sedentary (MSed, *n* = 20), male trained (MTr, *n* = 19), female sedentary (FSed, *n* = 21), and female trained (FTr, *n* = 21).

Following equilibration, 124 mmol/L K+ was used for 3 minutes (100% contraction) for the purposes of examining the contractility of the blood vessel and testing the reference value. To establish viability of the endothelium, a maximum concentration (10^{-5} mol/l) of phenylephrine and then a maximum (10^{-5} mol/l) concentration of acetylcholine was used.

For the purpose of measuring contraction ability, the alpha receptor agonist phenylephrine (Phe) was introduced in cumulatively increasing concentrations (10^{-9}-10^{-5} mol/l). It was ensured that organ chambers were triple-washed before and following each change of vasoactive agent. The vasorelaxation prompted by acetylcholine (Ach) was measured following phenylephrine precontraction (10^{-6} mol/L) (with organ chambers unwashed) with increasing concentrations of Ach (10^{-9}-10^{-6} mol/l). After a 30-minute pretreatment with the NO synthase inhibitor nitro-L-arginine methyl ester (L-NAME 10^{-5} mol/L), the cyclooxygenase (COX) inhibitor indomethacin (INDO 10^{-4} mol/L), and the cyclooxygenase-2 (COX-2) specific inhibitor NS398 (10^{-5} mol/L), the protocol was repeated. In parallel, the control vascular rings were treated with only the vehicle (diluted dimethylsulfide, DMSO).

3.6. Hystology and immunohistochemystry

The tissue samples fixed with formalin and embedded with paraffin were divided into 5 sections measuring 5 micrometers each. The density of elastic fibers was examined using resorcin-fuchsin (RF).

Deparaffinization of the five fixed sections was performed prior to immunohistochemical staining. Subsequently, immunohistochemistry and colorimetry were used to examine the density of the following: nitro-tyrosine (NT), α-smooth muscle

actin (α-SMA), cyclooxygenase 2 enzyme protein (COX2), and endothelial nitric oxide synthase protein (eNOS).

Antigen retrieval was conducted using heated citrate puffer (pH=6). Neutralization of Endogenous peroxidase activity was achieved using 3% H_2O_2. Standard horse serum in 2,5% dilution (Vector Biolabs, Burlingame, CA, U.S.A.) was used to prevent non-specific binding of the primary antibody.

The use of primary antibodies is recorded thus: (α-SMA: mouse monoclonal antibody 1:10 000 (Abcam 7817, Cambridge, UK); eNOS: mouse monoclonal antibody 1:50, (Abcam 76198, Cambridge, UK), COX2 rabbit polyclonal antibody: 1:200 (Abcam 15191), NT rabbit polyclonal antibody: 1:500 (Merck Millipore AB5411). Monoclonal anti-mouse (in case of eNOS & SMA) IgG antibodies (BA-2001, Vector Biolabs, Burlingame, CA, U.S.A.), anti-rabbit polyclonal antibodies (in the case of NT, COX-2). For secondary labeling, IgG antibodies were utilized (BP-1100-50, Vector Biolabs, Burlingame, CA, U.S.A.) 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA, USA) was utilized for visualization. Background staining was performed with hematoxylin QS (Vector Biolabs, Birmingham, California, United States).

For histological photography, a Nikon Eclipse Ni-U microscope with DS-Ri2 camera (Nikon Minato - Tokyo Japan) was used at 10x magnification for eNOS & α-SMA, and at 20x magnification for NT & COX2 stains. Evaluation of the results was facilitated using ImageJ software (National Institutes of Health (NIH), Bethesda, MA, U.S.A.) to separate immunohistochemistry from background staining (DAB + Hematoxylin). These images having been thus separated, were then converted to black-and-white format. Finally, the degree of staining was evaluated using non-calibrated optical density (OD) in the intima (for evaluation of eNOS and COX2) and for the media for NT and α-SMA evaluation).

3.7. Statistical analysis

GraphPad Prism software (version 8. GraphPad Software, Inc., San Diego, CA, USA) was utilized for data analysis and graphical representation. The data are expressed as a mean ± SEM. The Shapiro-Wilk test was used to determine whether the distribution was normal. For statistical objectives, two-way repeated measures for analysis of variance

(ANOVA) were conducted when a normal distribution was observed. Dunnett's or Tukey's post hoc test was administered for post hoc testing purposes.

Histological and immunohistochemical evaluations were compared using two-way ANOVA with Tukey's post hoc test and Kruskal-Wallis test with Dunn's multiple comparison test. P 0.05 was generally accepted as the level of significance.

4. RESULTS

4.1. Cardiac changes

Measured using echocardiography at week 12, left ventricular heart mass was found to be significantly lower in the FSed group than in the MSed group ($p<0.001$). Following12 weeks of swim training, a significant increase in left ventricular myocardial mass was observed in the FTr and MTr groups ($p<0.001$), indicating that cardiac adaptation was effectively induced in our experimental model. Furthermore, the sex difference observed among the sedentary groups, that is, the higher absolute left ventricular heart mass observed in males, was maintained beyond training termination ($p<0.001$) (LV mass (g): MSed, 1.18±0.029; MTr, 1.31±0.031; FSed, 0.89±0.008 and FEx, 1.05±0.023).

4.2. Renal arteries

4.2.1 Contraction of renal arteries

The contractions of the isolated renal artery segments in progressively higher concentrations of phenylephrine were maintained in the FSed and MSed groups. Significantly higher phenylephrine-induced contraction was observed in MSed animals compared to those in the FSed group **(Figure 1)**. Swim-training in male rats, was observed alongside a decrease in reactivity to phenylephrine, as shown by the decreased Phe-induced contraction **(Figure 1)**. This decrease was not observed in female rats.

In both the FSed and MSed groups, general COX inhibition (INDO) was observed accompanying a decrease in Phe-evoked contraction. In contrast, selective COX inhibition (NS398) decreased contractions in the MSed group but not in the FSed group. Introduction of L-NAME (nitric oxide synthase inhibitor) did not produce any significant difference in animals in either the MSed or FSed groups **(Figure 2A and 2B)**. Following the training period, it was observed that general COX inhibition (INDO) substantially reduced Phe-induced contraction in the MTr group, but had no effect on the FTr group. L-NAME was found to increase Phe-induced contraction significantly in both trained groups **(Figure 2C and 2D)**.

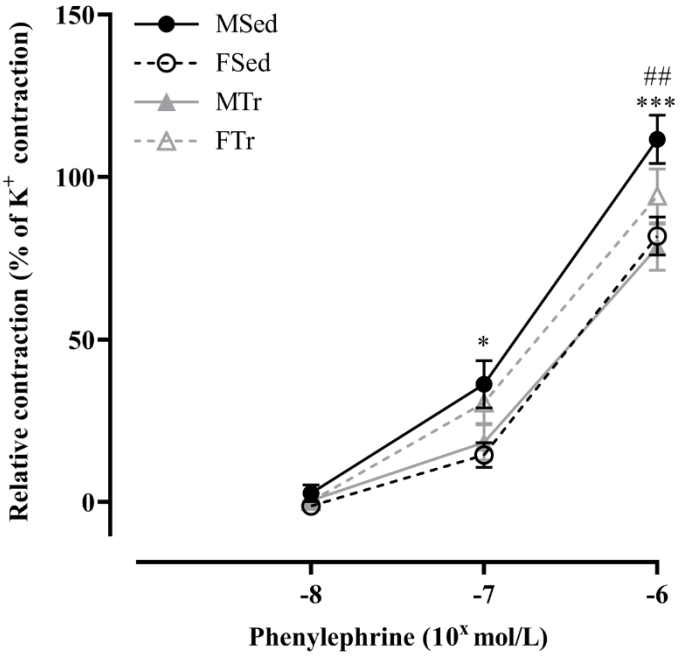


Figure 1. Phenylephrine (Phe) induced contraction of renal arteries. Male sedentary rats showed significantly higher contraction at Phe concentration of 10-7 mol/L and 10-6 mol/L as compared to female sedentary animals. Effect of exercise training, the Phe-induced contraction decreased at Phe concentration of 10-6 mol/L in trained male animals. Data are shown as mean ± SEM. Two-way RM ANOVA, with Tukey post hoc test. N = 5-13 in each group; Pint=0.0285; Pconcentration<0.0001; Pgroup=0.0114; Panimal<0.0001. *, p<0.05 MSed vs. FSed; ***, p<0.001 MSed vs. FSed; ##, p<0.01 MSed vs. MTr, Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary and FTr—female trained (68).

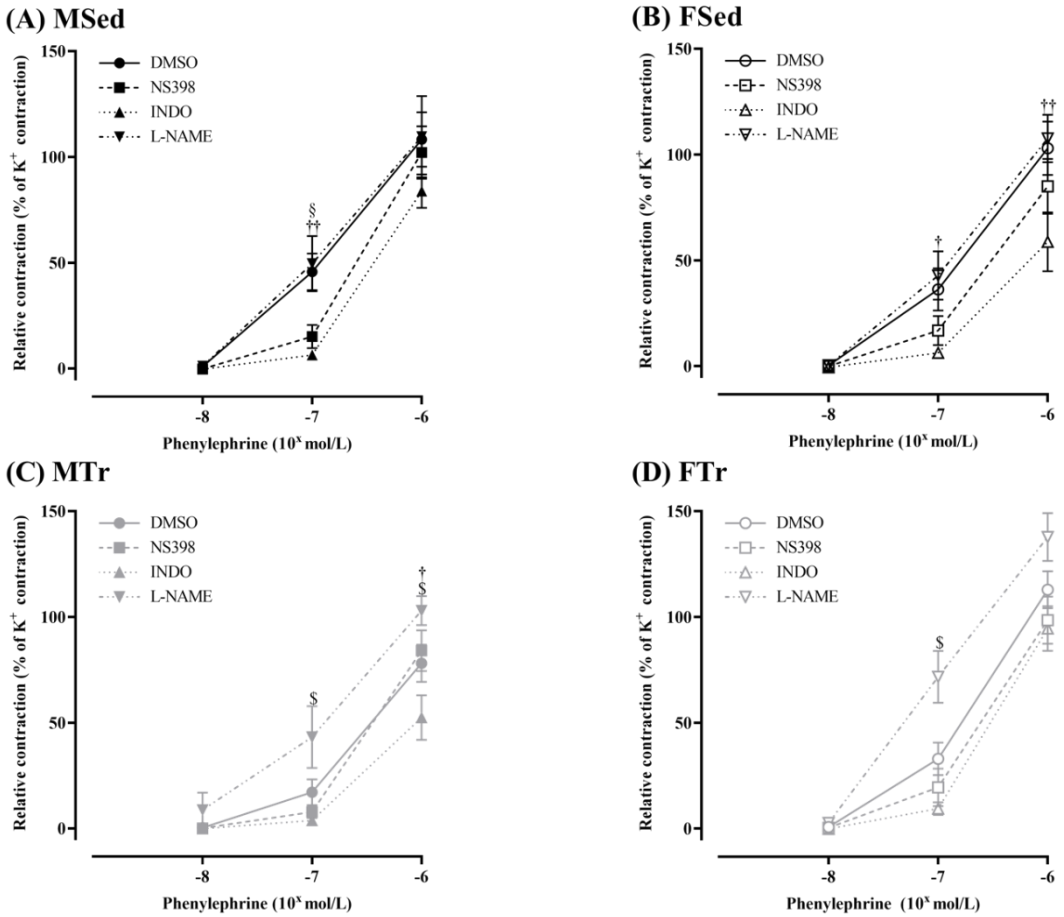


Figure 2. Phe-induced contraction of renal arteries in the presence of the selective COX-2 inhibitor (NS398) or non-selective COX inhibition (indomethacin; INDO), or nitric oxide synthase inhibitor (L-NAME), or their vehicle DMSO (a) in male sedentary rats, (b) in female sedentary rats, (c) in male trained rats and (d) in female trained rats. Non-selective and selective COX inhibition led to decreased contraction in male sedentary animals at 10^{-7} mol/L and non-selective COX inhibition led to decreased contraction at 10^{-7} mol/L and 10^{-6} mol/L in female sedentary ones. The L-NAME caused increased contraction at 10^{-7} mol/L and 10^{-6} mol/L in male and at 10^{-7} mol/L in female trained rats. General COX inhibition led to decreased contraction at 10^{-6} mol/L in male trained animals as well. Data are shown as mean ± SEM. Two-way RM ANOVA, with Dunnett's post hoc test. N = 5-13 in each group; (a) P_{int}=0.0462; $P_{concentration}$<0.0001; $P_{inhibitor}$=0.0265; P_{animal}=0.0045, (b) P_{int}=0.0574; $P_{concentration}$<0.0001; $P_{inhibitor}$=0.0102; P_{animal}=0.0275, (c) P_{int}=0.0186; $P_{concentration}$<0.0001; $P_{inhibitor}$=0.0205; P_{animal}=0.0158 and (d) P_{int}<0.0038; $P_{concentration}$<0.0001; $P_{inhibitor}$=0.0008; P_{animal}=0.0069. †, p<0.05 DMSO vs. INDO ††, p<0.01 DMSO vs. INDO; §, p<0.05 DMSO vs. NS398; $,p<0.05 DMSO vs. L-NAME, Abbreviations: MSed—male sedentary; MTr— male trained; FSed—female sedentary; FTr—female trained; DMSO—diluted dimethyl-sulfoxide; NS398—the cyclooxygenase-2 specific inhibitor; L-NAME—nitro-L-arginine methyl ester; INDO—indomethacin (68).

4.2.2. Relaxation of renal arteries

No significant differences between the FSed and MSed groups were demonstrated bythe Ach induced relaxation **(Figure 3)**. Furthermore, no changes as a result of training were observed in either the sexes in respect of the Ach-induced relaxation **(Figure 3)**.

L-NAME was observed to significantly reduce relaxation in both the FSed and MSed groups but INDO and NS398 did not produce any significant effect **(Figure 4A and 4B)**. Pre-incubation with L-NAME was observed to reduce relaxation in both trained groups. INDO and NS398, meanwhile, were not observed to produce any significant change **(Figure 4C and 4D)**.

In line with expectations, the Ach induced relaxation is for the most part realized by the NO pathway, which is influenced by neither sex, nor training.

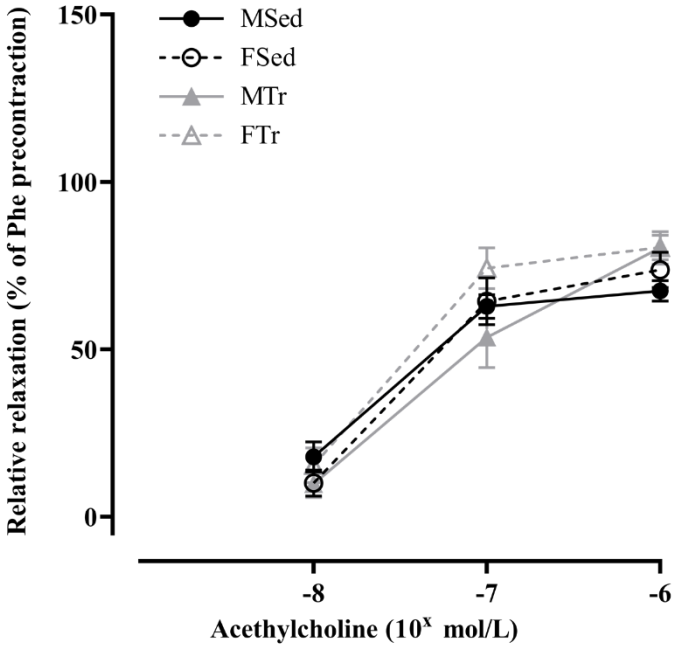


Figure 3. Acetylcholine (Ach) induced relaxation of renal arteries. Data are shown as mean ± SEM. Two-way RM ANOVA, with Tukey post hoc test. N = 7-15 in each group; P_{int}=0.0442; $P_{concentration}$<0.0001; P_{group}=0.3914; P_{animal}<0.0001, Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary and FTr—female trained (68).

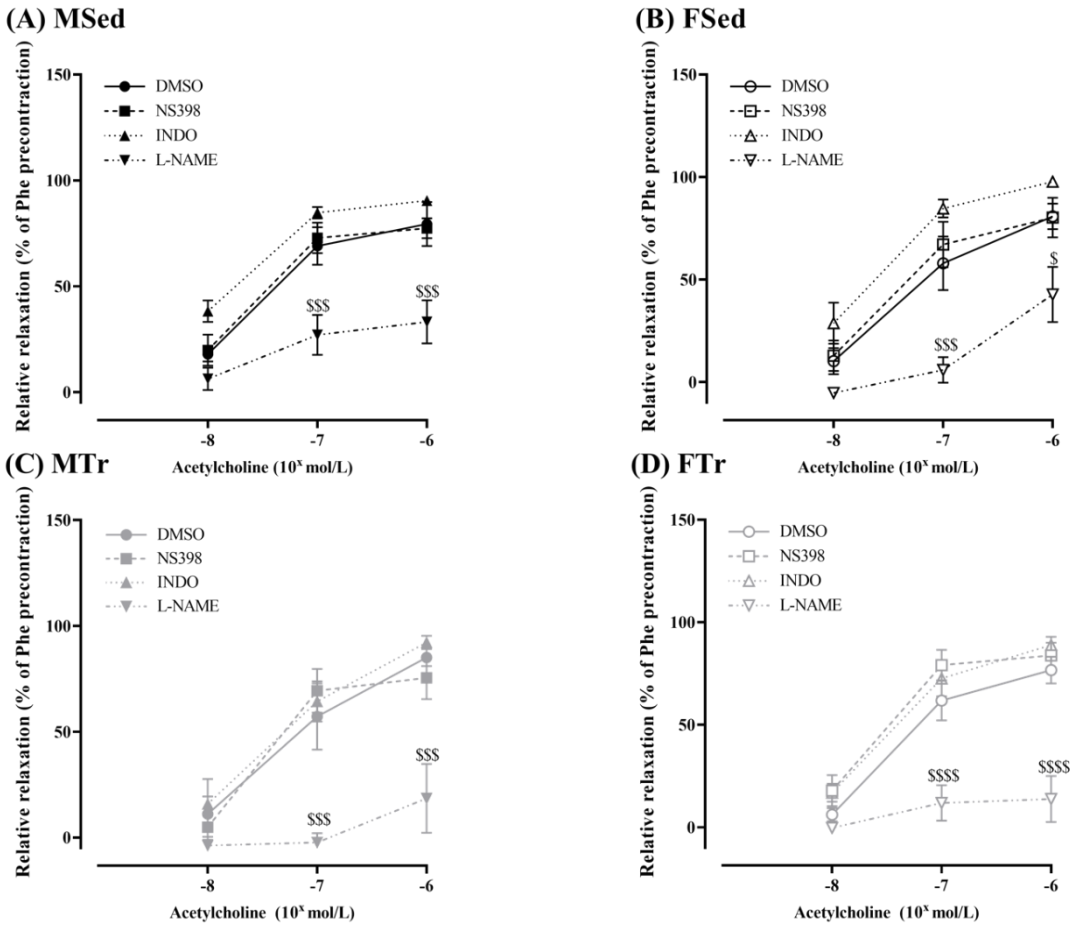


Figure 4. Acetylcholine (Ach) induced relaxation of renal arteries in the presence of selective COX-2 inhibitor (NS398) or non-selective COX inhibition (indomethacin; INDO), or nitric oxide synthase inhibitor (L-NAME), or their vehicle DMSO (a) in male sedentary rats, (b) in female sedentary rats, (c) in male trained rats and (d) in female trained rats. The L-NAME caused decreased relaxation in all experiment groups. Data are shown as mean ± SEM. Two-way RM ANOVA, with Dunnett's post hoc test. N = 4-13 (a) P_{int}=0.02; $P_{concentration}$<0.0001; $P_{inhibitor}$=0.0001; P_{animal}<0.0001, (b) P_{int}=0.0214; $P_{concentration}$<0.0001; $P_{inhibitor}$=0.0001; P_{animal}<0.0001, (c) P_{int}=0067; $P_{concentration}$<0.0001; $P_{inhibitor}$=0.001; P_{animal}=0.0018 and (d) P_{int}<0.0001; $P_{concentration}$<0.0001; $P_{inhibitor}$<0.0001; P_{animal}<0.0001. $, p<0.05 DMSO vs. L-NAME; $$$, p<0.001 DMSO vs. L-NAME; $$$$, p<0.0001 DMSO vs. L-NAME, Abbreviations: MSed—male sedentary; MTr— male trained; FSed—female sedentary; FTr—female trained; DMSO—diluted dimethyl-sulfoxide; NS398—the cyclooxygenase-2 specific inhibitor; L-NAME—nitro-L-arginine methyl ester; INDO—indomethacin (68).

4.2.3. Histological alterations of renal arteries

The optical density (OD) of elastic fiber in sections stained with resorcin-fuchsin was observed to be significantly lower in MSed rats compared with FSed rats **(Figure 5)**. For female rats, swim training resulted in decreased OD of elastic fibers but OD was not observed to decrease in males. OD was observed as significantly lower in FTr rats compared with FSed rats **(Figure 5)**.

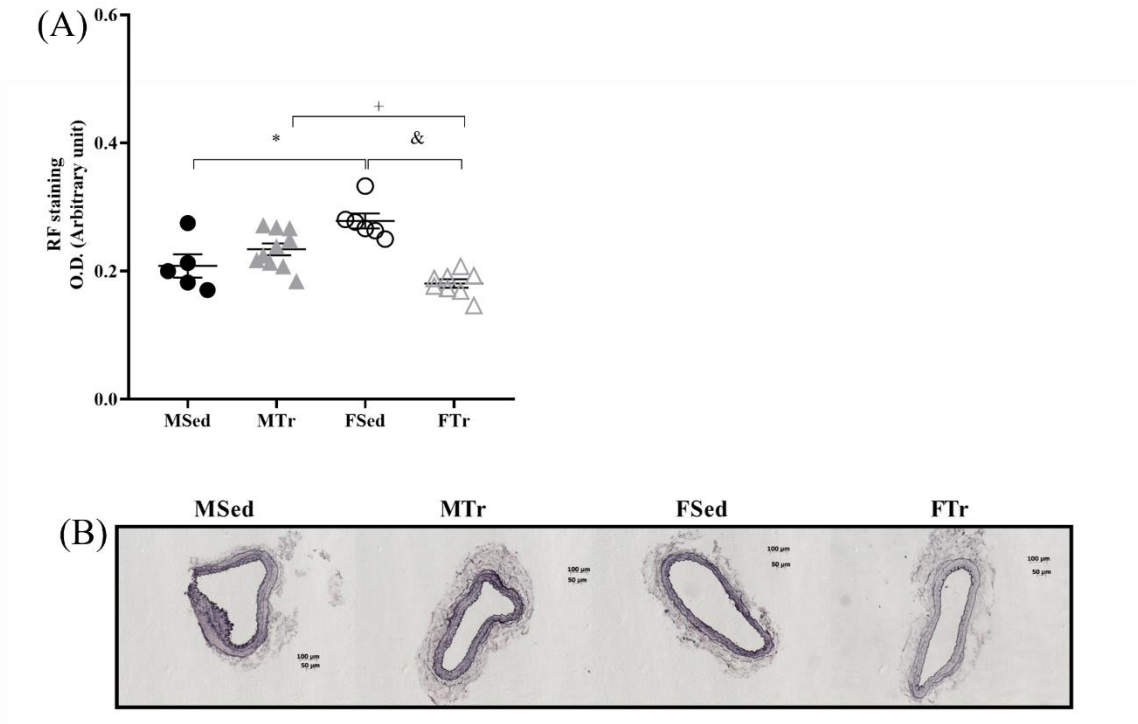


Figure 5. Results of resorcin-fuchsin staining of renal arteries. (A) Optical density of elastica on resorcin-fuchsin-stained segments. (B) Representative images of RF-stained segments from MSed, MTr, FSed and FTr groups. Scale bar, 100 µm. The optical density was significantly lower in male sedentary animals than in female sedentary rats. The OD was significantly reduced in trained female animals compared to sedentary female animals. The optical density was significantly lower in FTr animals than in MTr rats. Data are presented as individual data points, lines represent mean ± SEM. Two-way ANOVA with Tukey post hoc test. N = 5-10 in each group; P_{int}<0.0001, P_{sex}=0.4567, $P_{training}$=0.0036. *, p<0.05 MSed vs. FSed; &, p<0.05 FSed vs. FTr; +, p<0.05 MTr vs. FT, Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary; FTr—female trained (68).

As a consequence of exercise, the OD of smooth muscle actin (SMA staining) decreased in the female group **(Figure 6)**.

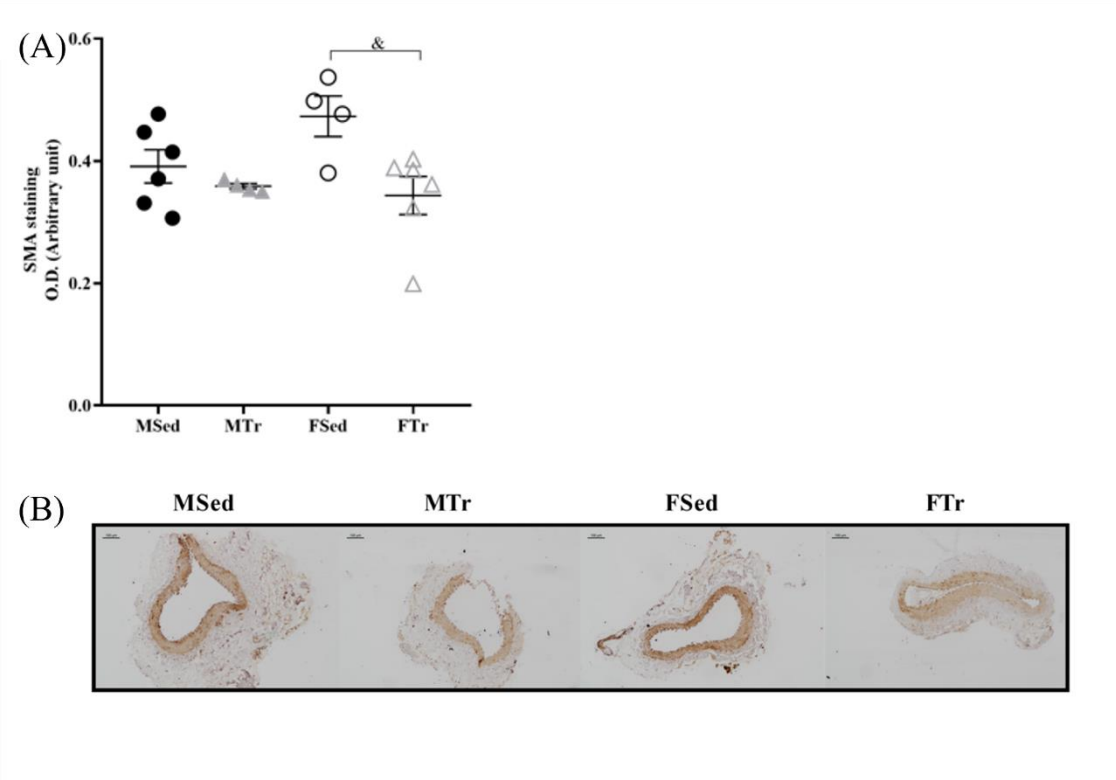


Figure 6. Results of smooth muscle actin (SMA) immunohistochemical staining of renal arteries. (A) Optical density on smooth muscle actin-stained segments. (B) Representative images of SMA-stained segments from MSed, MTr, FSed and FTr groups. Scale bar, 100 μm. The optical density was significantly reduced in trained female animals compared to sedentary female animals. Data are presented as individual data points, lines represent mean ± SEM. Two-way ANOVA with Tukey post hoc test. N = 4-6 in each group; P_{int}=0.1132, P_{sex}=0.2672, $P_{training}$=0.0131. &, $p<0.05$ FSed vs. FTr, Abbreviations MSed—male sedentary; MTr—male trained; FSed—female sedentary; FTr—female trained (68).

No difference between groups was observed in terms of optical density of nitrotyrosine **(Figure 7)**.

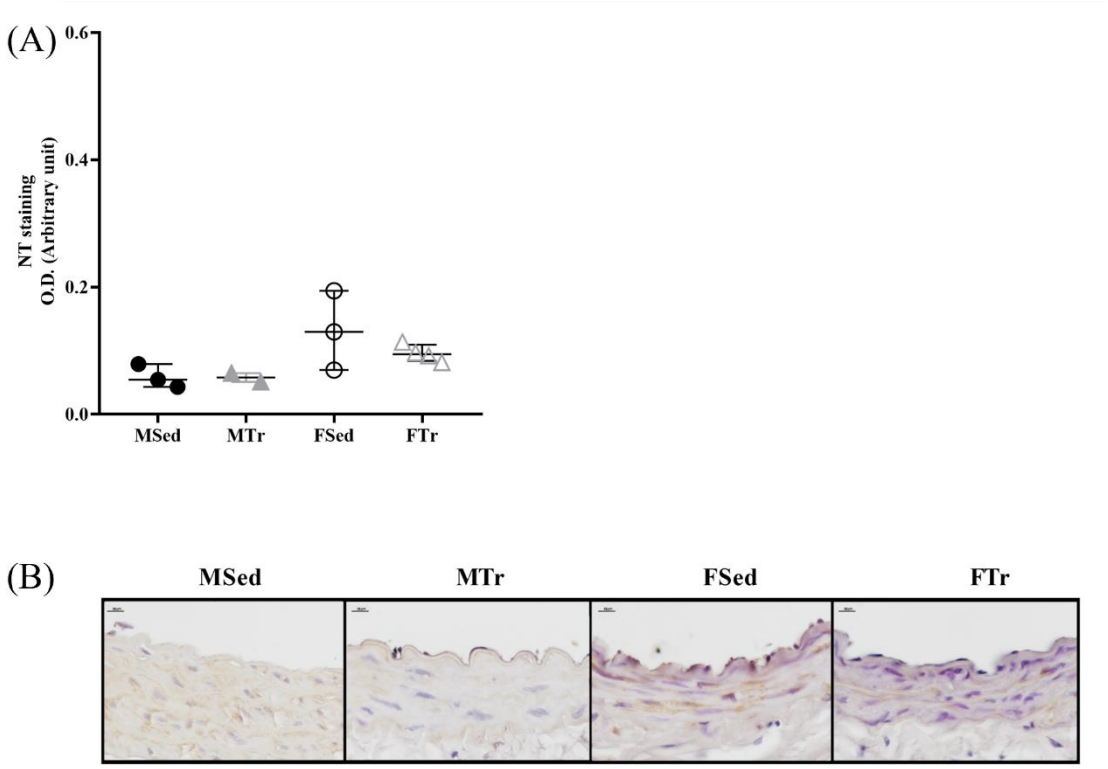


Figure 7. Results of nitrotyrosine (NT) immunohistochemical staining of renal arteries. (A) Optical density on nitrotyrosine-stained segments. (B) Representative images of NT-stained segments from MSed, MTr, FSed and FTr groups. Scale bar, 50 µm. The optical density did not differ between groups. Data are presented as individual data points, lines represent median [IQR]; Kruskal-Wallis test with Dunn post hoc test. N = 2-4 in each group, Abbreviations: MSed—sedentary male; MTr—trained male; FSed—sedentary female; FTr—trained female (68).

After training, COX-2 immunostaining revealed no significant differences between the groups **(Figure 8**).

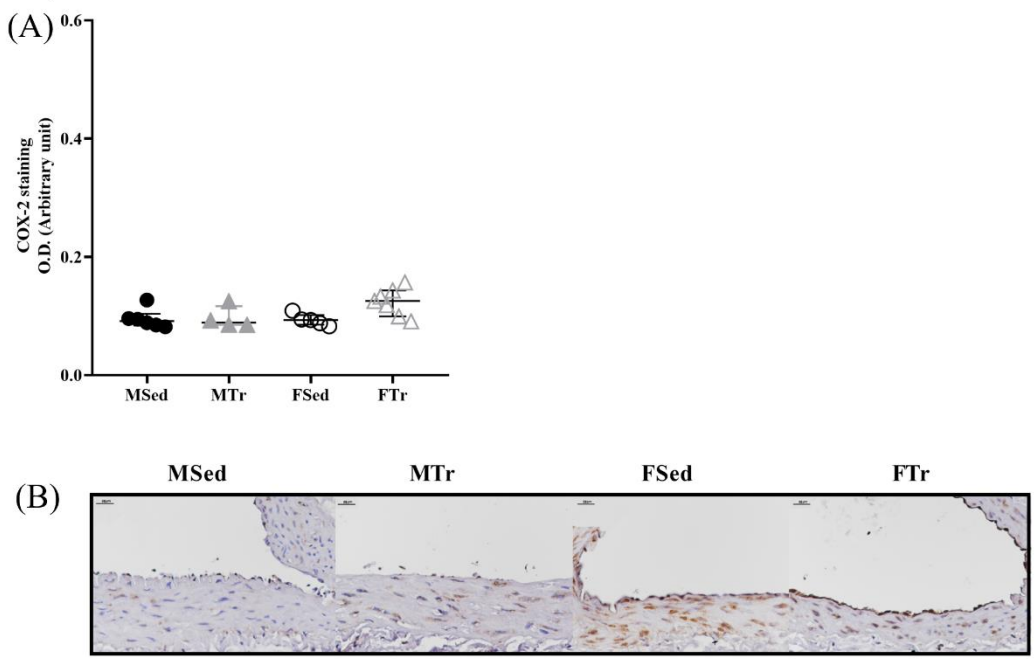


Figure 8. Results of cyclooxygenase-2 (COX-2) immunohistochemical staining of renal arteries. (A) Optical density on COX-2-stained segments. (B) Representative images of COX-2-stained segments from MSed, MTr, FSed and FTr groups. Scale bar, 50 μm. The optical density did not differ between groups. Data are presented as individual data points, lines represent median [IQR]; Kruskal-Wallis test with Dunn post hoc test. N = 4-7 in each group, Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary; FTr—female trained (68).

4.3. Femoral arteries

4.3.1. Contraction ability of femoral arteries

With increasing concentrations of phenylephrine, the ability of femoral arteries to contract was tested. No significant difference was observed between the four groups **(Figure 9)**.

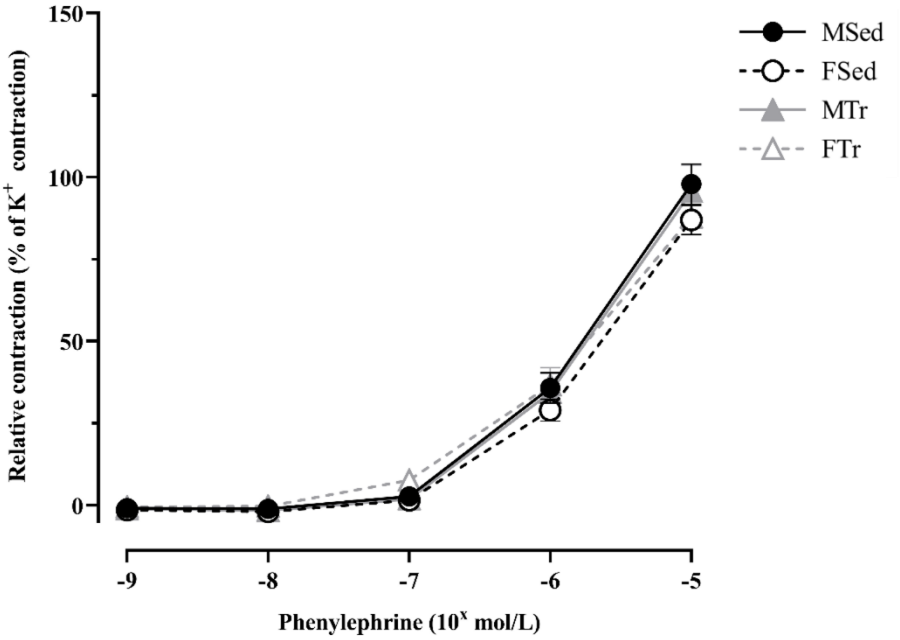


Figure 9. Phenylephrine induced contraction of femoral arteries. Data are shown as means ± SEM; *n* = 18–20 in each group; analysis: two-way repeated measures ANOVA; test: the Tukey's post hoc test. Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary; FTr—female trained (69).

Phe-induced contractions were repeated in the presence of L-NAME (10^{-5} mol/L), INDO (10^{-4} mol/L), and NS398 (10^{-5} mol/L) to investigate the functional vascular effects associated with cyclooxygenases and endothelial oxide synthase (eNOS). The presence of INDO was observed to significantly decrease Phe-induced contraction in both the male and female sedentary groups **(Figure 10A and 10C)**. After the swimming training period, both trained groups showed an increase in Phe-induced contraction in the presence of L-NAME **(Figure 10B and 10D).** A sex difference was observed in a specific COX-2 vasoconstriction effect found only in the FSed group **(Figure 10C)**. This specific COX-2 inhibition (NS398) response did not persist in FTr rats after exercise training **(Figure**

10D). Furthermore, the INDO aforementioned effect in the sedentary groups persisted in trained female rats, but not in their male counterparts. **(Figure 10B and 10D)**. Comparing the trained groups for L-NAME effects, no statistically significant differences were observed.

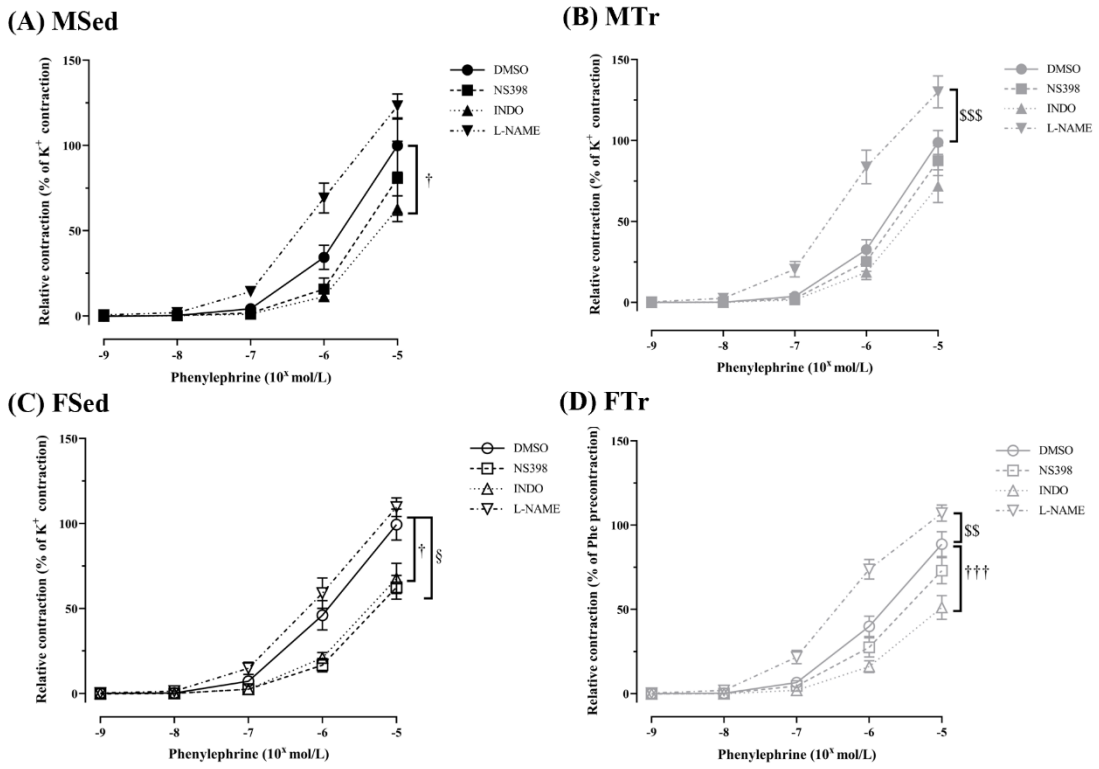


Figure 10. Phenylephrine induced contraction of femoral arteries in the presence of NS398, INDO, L-NAME, or DMSO in sedentary male rats (**A**), in trained male rats (**B**), in sedentary female rats (**C**), and in trained female rats (**D**). Data are shown as means ± SEM; n = 5–17 in each group; analysis: two-way repeated measures ANOVA; test: the Tukey's post hoc test. † $p < 0.05$, ††† $p < 0.001$: DMSO vs. INDO; $$ $p < 0.01$, $$$ $p < 0.001$ DMSO vs. L-NAME; § $p < 0.05$ DMSO vs. NS398. Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary; FTr—female trained; DMSO—diluted dimethyl-sulfoxide; NS398—the cyclooxygenase-2 specific inhibitor; L-NAME—nitro-L-arginine methyl ester; INDO—indomethacin (69).

4.3.2. Relaxation ability of femoral arteries

The Ach induced relaxation itself did not show any significant differences between the four groups studied **(Figure 11).**

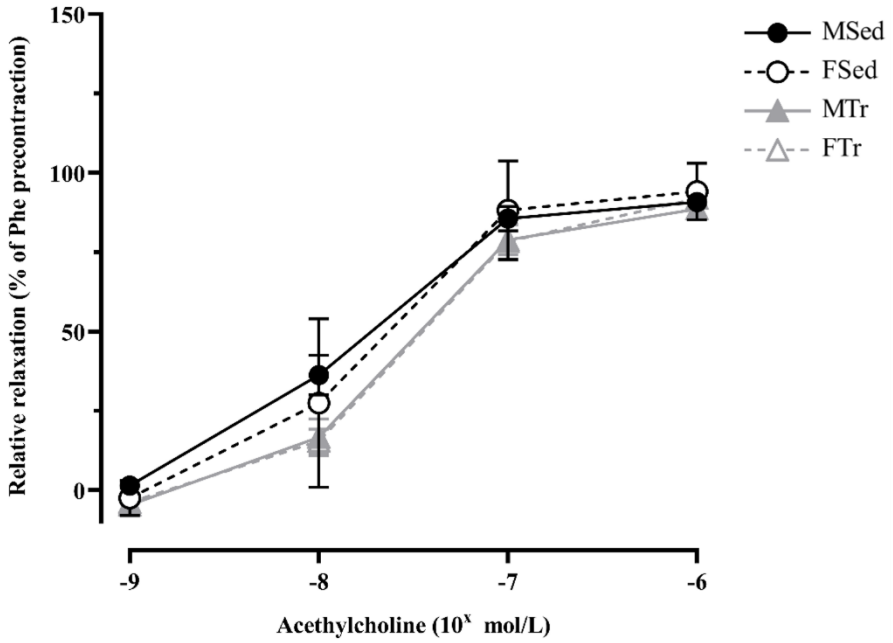


Figure 11. Acetylcholine induced relaxation of femoral arteries. Data are shown as means ± SEM; *n* = 15–19 in each group; analysis: two-way repeated measures ANOVA; test: the Tukey's post hoc test. Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary; FTr—female trained (69).

In line with expectations, the introduction of L-NAME, resulted in a significant decrease in Ach-induced relaxation, observed in all animal groups **(Figure 12).** Furthermore, introduction of L-NAME in the trained male rats group resulted in a significant decrease in Ach-related relaxation compared to that observed among the female rats **(Figure 12).**

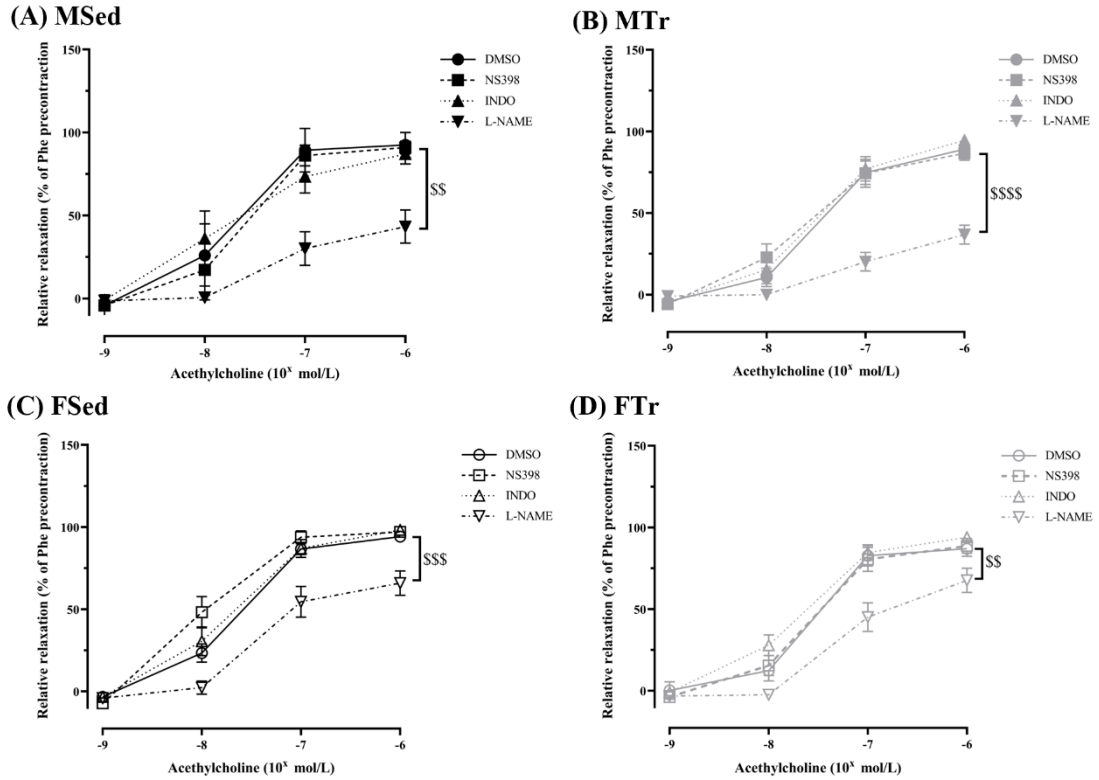


Figure 12. Acetylcholine induced relaxation of femoral arteries the presence of NS398, INDO, L-NAME, or DMSO in sedentary male rats (**A**), in trained male rats (**B**), in sedentary female rats (**C**), and in trained female rats (**D**). Data are shown as means ± SEM; n = 5–19 in each group; analysis: two-way repeated measures ANOVA; test: the Tukey's post hoc test. $$ $p < 0.01$, $$$ $p < 0.001$, $$$$ $p < 0.0001$ DMSO vs. L-NAME. Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary; FTr—female trained; DMSO—diluted dimethyl sulfoxide; NS398—the cyclooxygenase-2 specific inhibitor; L-NAME—nitro-L-arginine methyl ester; INDO—indomethacin (69).

4.3.3. Histological alterations of femoral arteries

Following a 12-week training period, there was an increase in the optical density (OD) of eNOS protein in the MTr groups. In the MTr group, the optical density was greater than in the FTr group **(Figure 13A and 13B)**. Upon examination of COX-2 staining following exercise training, no significant differences were observed (26.35 ± 5.299, 77.16 ± 9.001, 38.77 ± 8.580, and 32.93 ± 12.130 arbitrary units for the MSed, MTr, FSed, and FTr groups, respectively (n.s.)). The OD measured using NT staining

likewise showed nodifferences (0.08 ± 0.003, 0.07 ± 0.007, 0.08 ± 0.004, and 0.07 ± 0.003 arbitrary units for the MSed, MTr, FSed, and FTr groups, respectively (n.s.)).

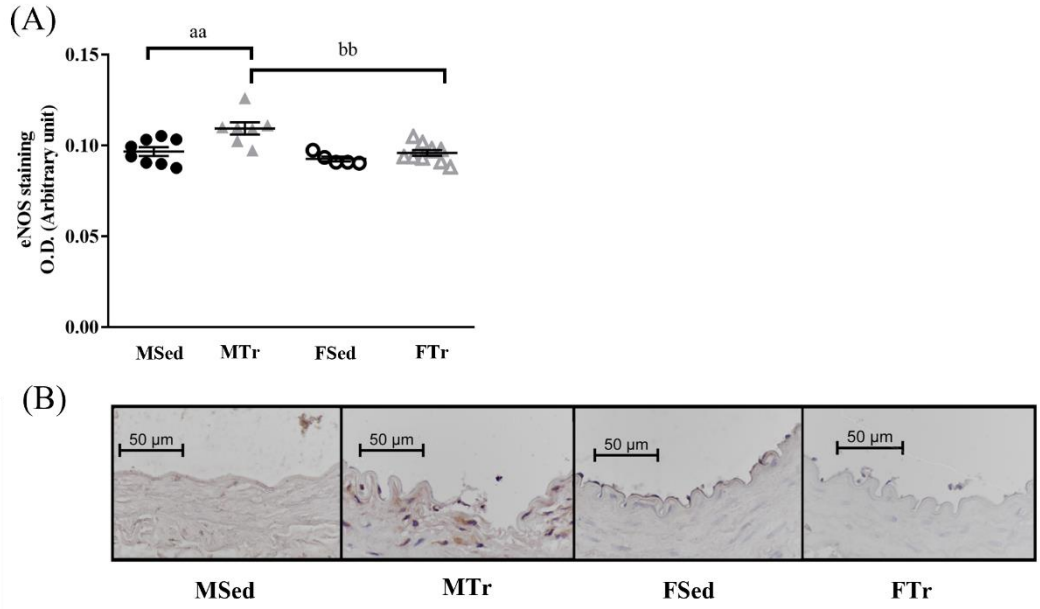
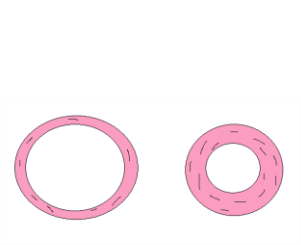
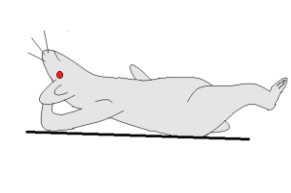


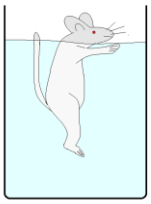
Figure 13. (**A**) Optical density of eNOS labeling in the intimal layer of femoral arteries. Data are presented as individual data points, and lines represent means ± SEM; *n*= 5–10 in each group; analysis: two-way ANOVA; test: the Tukey's post hoc test. (**B**) Representative images of vessels labeled with an anti-eNOS antibody. Scale bar, 50 µm. aa, $p < 0.01$ MSed vs. MTr; bb, $p < 0.01$ MTr vs. FTr. Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary; FTr—female trained; OD—optical density (69).

A summary of femoral artery function results is shown in **Figure 14.**





Sedentary



Trained

	♂	♀	♂	♀
Alpha1 adrenerg contraction	Yes, similar	Yes, similar	Yes, similar	Yes, similar
NO relaxation ability	Yes, similar	Yes, similar	Yes, similar	Yes, similar
NO relaxation moderating alpha1 adrenerg contraction	Limited	Limited	Elevated	Elevated
Constrictor prostanoids enhancing alpha1 adrenerg contraction	Yes	Yes (COX2 product)	Diminished	Yes (But diminished COX2 product)
Vasoactive prostanoids modifying cholinergic relaxation	No	No	No	No

Figure 14. Summary of changes in femoral vascular function during the 12-week sports adaptation of the rat femoral artery (69).

5. DISCUSSION

The cardiovascular benefits of regular sports are well known. However, gender-specific differences in long-term sports adaptation of blood vessels remain subject to question.

During aerobic exercise, alterations in hemodynamics are observed. While blood flow in skeletal muscle arteries is seen to increase, blood flow is seen to decrease in visceral vessels due to mesenterial vasoconstriction. Given the priority of maintaining renal blood flow during intensive sport, we made it our aim investigate and identify possible gender differences in respect of sport adaptation, specific to the renal and femoral arteries. For experimentation, Wistar rats were grouped accordingly: male sedentary (MSed), male trained (MTr), female sedentary (FSed), and female trained (FTr). The trained groups were subjected to a 12-week program of intensive swimming. Wire myography was used to examine vascular function of the isolated renal and femoral artery segments. Vascular reactivity and histology were compared after conclusion of the exercise program. The present study proves the existence of gender differences in respect of sports adaptation mechanisms of renal arteries and musculocutaneous arteries.

5.1. Renal arteries

During exercise, renal vasoconstriction is observed as an effect of increased sympathetic neural outflow. This sympathetically mediated vasoconstriction of the renal arteries is a crucial reflex mechanism for redistribution of renal blood flow (70, 71). This moderation of renal vasoconstriction was only observed in trained male rodents in our study. Furthermore, Phe-induced vasoconstriction was observed to be higher in sedentary male animals than in their female counterparts and this difference was seen to disappear following the swim-training period. There are contradictory findings regarding the degree of renal vasoconstriction in trained and sedentary individuals (43-46). Kocer et al. in a study conducted with forty adult female rats, Kocer et al. discovered that the renal resistance arteries of rats that were subjected to exercise exhibited a greater contractile response to sympathetic agonists (norepinephrine). There was no difference in the contractile response to the other vasoconstrictor agents (thromboxane A2, KCL, endothelin-1, and vasopressin) between the sedentary and exercised groups. This modification could be the result of an increase in the number of receptors, Ca2+ efflux,

or a heightened sensitivity of the post-receptor signaling pathway (43). Other studies have, like ours, observed a decrease in renal vasoconstriction in trained male animals in contrast sedentary ones (44-46). A possible explanation might concern the attenuated response to norepinephrine in renal arteries resulting from the 12-week training period (72). In human studies, static training resulted in no observable difference in the vasoconstriction of the renal vessels in men and women (51). However, when subjected to 9–12-weeks of intense aerobic exercise, human subjects have responded with results comparable to those in our study: Following a 9 to 12 week exercise program, the observed reduction in splanchnic circulation before and during training is substantially smaller in men than in women. The same effect was noticeably absent in women, for whom the degree of splanchnic vasoconstriction remains unchanged after following the same training program (52).

In our study, the prostanoid pathway (INDO – COX related vasoconstriction) plays a role in the magnitude of phenylephrine-induced vasoconstriction in MSed, MTr, and FSed rats, but not in FTr rats. In a mouse model, Liu et al. demonstrated that endogenous COX-1-mediated PGI2 synthesis could play a crucial role in the regulation of renal vascular reactivity (73). Meanwhile, in both male and female exercised animals we observed a NO-related relaxation that 'restricted' the maximum degree of vasoconstriction. Relatedly, a previous study observed a significant role played by constrictor prostanoids in sedentary coronary arteries (49).

The gender differences in renal vasoconstrictor response indicate that while renal vasoconstriction (as a feature of visceral circulation) doesn't alter in females as a consequence of swim-training, it does in males, i.e., sport decreases renal vasoconstriction. These results suggest that when sympathetic activation is increased – for instance during exercise – in trained males, the vasoconstrictor response of the renal artery is less pronounced, with a likely beneficial result in terms of blood flow maintenance.

In our study, training did not effect a change in vasorelaxation. Ach-induced relaxation was predominantly NO-related with the degree of relaxation uniform across the groups. In contrast to the literature on the vasoconstrictor response, the literature on renal artery vasodilation is more uniform (43, 46, 72). The majority of studies observed no distinction between the trained and sedentary groups in terms of vasorelaxation groups. The

explanation for this is that the control animals were also healthy, with no impairment to their vasorelaxations. Consequently, no improvement was observed in the vasorelaxation response. We can infer that vasorelaxation is a beneficial adaptation response for skeletal muscle vessels, where an increase in visceral vascular perfusion due to physical activity is not anticipated.

Vessels respond differently to training according to type, size, and location. This is because various forms of hemodynamic forces influence them during physical activity. Previous studies have investigated the structural adaptation of mesenteric arteries to swimming in male rats: mesenteric vessel weight was observed to decrease significantly with exercise, while no difference in relative mesenteric vessel weight (relative to body weight, mg/g) was observed between the groups (74). Following exercise, Portal vein diameter, cross-section area, and blood flow in rats was observed to decrease (75). While the present study precluded the opportunity to examine renal artery diameter, we did examine elastic fiber density (with RF staining) and smooth muscle actin density in renal vessels. Consequently, we were able to show that elastic fiber density and alpha smooth muscle actin exhibited lower levels in the renal arteries of female trained rats. It may be that these early structural alterations in female rats contribute to their sport adaptation by changing the compliance of renal arteries. In male animals, the reduction in elastic fiber density resulting from exercise was less pronounced at all stages.

It may be that increased oxidative-nitrative stress also changes vascular functions. The formation of peroxynitrite from NO and superoxide can decrease NO bioavailability, thereby reducing NO-related endothelium relaxation. Additionally, peroxynitrite can cause tissue injury by reacting with various cellular components. 3-Nitootyrosine formation is a signature reaction of peroxynitrite with proteins (76, 77). Chronic oxidative-nitrative stress, it is known, can be reduced with regular exercise (78). The present study, however, showed no difference between groups in terms of NT staining. Rodrguez et al. investigated sex differences in nitrative stress in female and male Sprague-Dawley rats and found no difference in endothelial nitric oxide synthase and neuronal nitric oxide synthase expression, nitric oxide or 3-nitrotyrosine levels in nonischemic kidneys (79).

Through our investigation of the role of COX-2 in renal circulation, we observed that COX-2 mediated pathway is a factor in the contraction of the renal artery in sedentary

male animals, but not in females or trained rats. However, we observed no significant difference in respect of COX-2 density with immunohistochemistry. Within the literature related to COX-2 activity and sex difference, data conflict. In spontaneously hypertensive rats, females exhibited higher COX-2 expression in the internal renal medulla, as well as higher urinary PGE2 concentration (80). Moreover, in the renal macula densa of female rats, COX-2 expression was observed to be higher in comparison to males (81). Dihydrotestosterone treatment in male rats was observed to increase COX-2 level on cerebral arteries (82). Despite the observed functional difference, however, our investigation did not enable us to show the changes of COX-2 expression as a response to sex or physical exercise.

An accepted limitation of our study concerns the lack of confirmation of the effects of sex hormones, meaning that any attribution of sex differences to sex hormones must remain in the realm of theory.

5.2. Femoral arteries

The present study did not identify any difference between the groups in respect of Phe-induced contraction in the presence of Phenylephrine alone in the organ chambers. However, when phenylephrine was introduced alongside specific inhibitors (INDO, NS398, L-NAME), significant differences were observed, firstly, in the effects of training and secondly, between male and female groups.

In male and female sedentary animals, INDO-COX-related vasoconstrictive activity, also referred to as the prostanoid pathway was seen to be instrumental in determining the extent of Phe-induced vasoconstriction. Furthermore, this endothelium-related vasoconstriction was cancelled when males were subjected to exercise. It is accepted in the literature that as a consequence of shear force, the equilibrium between endothelium-derived vasoconstrictor (TXA2) and vasodilator substances (PGI2, 11,12-eicosatrienoic acids) tends to vasodilation (62, 83-85). Moreover, when aortas are treated with indomethacin, the impaired endothelium-related relaxation in males is restored, with the implication that cyclooxygenase (COX)-derived vasoconstrictors are elevated in aged males (86). However, the current lack of agreement in the literature,, means there are opposing observations: it was found that vasopressin-induced contraction in the thoracic aorta of Sprague-Dawley rats was attenuated in females by the non-selective COX

inhibitor but the same effect was not observed in males (87). Previous studies have identified gender differences in respect of the level of prostaglandins produced by COX-2 in the kidneys of spontaneously hypertensive rats (SHR) (80). In particular, the urine of female SHR rats contained higher concentrations of PGE2 metabolite and thromboxane B2 than that of male rats. Moreover, female SHR rats exhibited higher expression of microsomal PGE2 synthase protein in the renal internal medulla, while a significant increase in cyclooxygenase-2 (COX-2) expression was observed in the outer renal medulla of the same group. In respect of COX-2, and in parallel to the results of our study, other reports demonstrate that COX-2 is a determining factor of the extent of contraction in female rat aorta segments to vasopressin via the prostanoid pathway, and that COX-1 does not produce the same effect (88). Age-dependent changes were observed in respect of cerebrovascular activity to vasopressin following a selective blockade of COX-2 only. It was observed that this phenomenon was more prominent in the female group compared to the male group (89). However, in the case of the latter, the underlying cause of this difference may be the difference in contraction agonist, in connection with different activation pathways. In our present study, the non-specific COX inhibitory effect was seen to be cancelled in males as a result of exercise. This effect enhanced contraction in the control animals. Accordingly, the balance moved toward vasodilation as a consequence of training. As observed, COX-related vasoconstriction saw no change among trained females, while COX-2-related vasoconstriction vanished. Hence, the balance was observed to move towards vasodilation in female rats as a consequence of swimming.

The effects of eNOS on vascular function were investigated by repeating Phe-induced contraction following the introduction of L-NAME. Phe-induced contraction with L-NAME was enhanced in both trained groups, with the implication that in trained animals, the strength of Phe-induced contraction is counteracted by NO. Sex differences in respect of the L-NAME effect were not observed between the trained groups, that is, the counterbalancing of the degree of Phe-induced contraction by NO release was observed to be similar in both trained groups. Fabrício N Macedo et al. also observed that L-NAME significantly enhances the vasoconstriction response of Phe in trained rats (90). The observed blunting effect of chronic exercise on phenylephrine-induced vasoconstriction in isolated rat aorta, probably resulted from an increase in NO release through activation

of inducible and endothelial NOS. On this basis, it is reasonable to speculate that, as a result of long-term exercise training, there is an increase in the gene expression of both inducible and endothelial NOS in isolated rat aortic endothelium and endothelial NOS and neuronal NOS in the mesenteric arteries (90, 91).

In the present study, no difference was observed between the groups in respect of Ach-induced relaxation in the presence of acethylcholine alone in the organ chambers. Yet, when acethylcholine was introduced to the organ chambers in combination with the specific inhibitors (INDO, NS398, L-NAME), significant differences were observed both in the effects of exercise and between the males and females.

Introduction of L-NAME resulted in a decrease in Ach-induced contraction, observed in all four of the studied groups in our model. Therefore, in all groups, NO played a predominant role in mediating the relaxation activity described. Ach-induced relaxation ocurred predominantly by means of the NO-related pathway. Marchio et al. studied the effect of long-term exercise training on femoral arteries in male New Zealand white rabbits, producing results similar to ours, that is, no differences were observed between trained and sedentary groups during the relaxation test with Ach (92). Likewise, no changes were observed in response to Ach in a previous study of male Sprague-Dawley rats (93). Once again, in a study of the femoral arteries of mini pigs, no differences were observed between trained and sedentary groups in respect of Ach-induced relaxation ability (72, 94-96). The literature, however, does present findings to the contrary, with studies of rat abdominal aorta showing increased relaxation to induced vasodilation after exercise training (97). Notwithstanding conflicts, data from the literature suggests we should expect an association between the presence of estrogen in females and increased NO release and/or activity in various types of blood vessels (98). Laughlin et al. studied the femoral and brachial arteries of mini pigs. Results showed that, in the case of the brachial artery, males exhibited greater acetylcholine- and bradykinin-related relaxation compared with females. In respect of femoral arteries, meanwhile, opposite results were recorded in control sedentary animals (99). No differences were observed in respect of eNOS protein expression in analysis of control group data, although there could be a difference in terms of function. This difference in function may imply a sex related difference in the phosphorylation of eNOS or possibly another component of the cascade. Moreover, it is accepted, for instance, that women exhibit a higher eNOS dimer-monomer

ratio. A lower dimer-to-monomer ratio may indicate the decoupling of eNOS and it could affect the generation of reactive oxygen species (ROS). Cattaneo et al. (98) however, observed no differences between sexes in respect of the amount of ROS released from human endothelial cells.

A sex difference may be observed in the decreasing role of NO in female rats following exercise, compared to that in males: that is, the relaxation-reducing effect of L-NAME in Ach-induced relaxation is more marked in male trained than in female trained groups. Data in the literature, once again, are not in full agreement in respect of training and NO-related relaxation. While some papers point to an increase in NO-related relaxation as a result of exercise only in women (100, 101), others report an increase in both men and women (102), while in some others no sex difference is observed (102-104). Moreover, other studies report an increase in brachial artery dilatation in response to exercise training in men but not in women (105, 106). Dietz et al.'s human study compared the forearm vasodilator responses of women to those of men in the presence of compounds promoting the release of nitric oxide, NO donor compounds, and NO-independent mechanisms. Results showed a decrease in blood flow in women (107). In another human study, Nishiyama et al implemented a novel approach for investigating vascular reactivity in men and women, aimed at excluding mathematical distortions by normalizing the flow mediated dilatation to shear rate. Results showed greater endothelium-dependent vascular reactivity in the lower limbs in men, and especially in the case of the popliteal artery (108). Based on the data in the literature, we may assert that there is a greater increase in muscle mass in males as a consequence of exercise. Accordingly, the extent of increase in respect of perfusion was observed to be greater in male rats than in females (109). This could be as a result of training-induced increased eNOS-related relaxation in males (48).

A portion of the increased relaxation seen in males may reflect a greater increase in the amount of eNOS, observable in our experiment in the increase of eNOS OD recorded in the male trained group. It should be noted that the extent of eNOS activity is not uniform across the vascular system. For instance, in the coronary artery microcirculation, the amount of eNOS increases in a nonuniform fashion following exercise, and regional differences occur as a result of the effects of shear stress and intraluminal pressure (110).

To summarize the histological results in respect of vascular function, the exercise-induced increased NO release/bioavailability is observed to counteract vasoconstriction

and improve relaxation in femoral arteries. In terms of sex difference, NO release/bioavailability following exercise is likely to be more beneficial in males than in females.

5.3. Comparison of renal artery and femoral artery sports adaptation

Studying the literature, there is no known sports adaptation experiment on a rat model that takes gender differences into account and discusses the differences and similarities between a visceral vessel - the renal artery - and the femoral artery, the main artery supplying the lower limb. We would like to highlight the similarities and differences between these two types of vessels.

Regarding the renal artery, Phe induced vasoconstriction was observed to decrease in the sedentary females. In male groups vasoconstriction was observed to decrease as a consequence of training. Regarding the femoral artery in contrast to the renal artery, the femoral artery showed no significant difference between the four groups. General Cox inhibition (INDO) led to a reduction in Phe-induced vasoconstriction in both the renal and femoral arteries in the FSed and MSed groups. Selective COX-2 inhibition with NS398 led to decreased Phe-related vasoconstriction in the MSed group in respect of the renal artery, while this effect was observed in the femoral artery only in the FSed group. Examining both types of blood vessels, it can be concluded that blocking NO synthase with L-NAME in the sedentary groups did not, however, lead to an increase in Phe-induced vasoconstriction in both trained groups. Examining the COX and COX-2 signaling pathways in the renal artery and the femoral artery, opposite processes took place depending on sexes. In the case of the renal artery, the selective COX-2 effect observed in males disappeared after training, while the general COX effect remained. In the case of the femoral artery, the same process took place, however, in females and not in males.

Regarding both the renal artery and the femoral artery, no significant difference was observed in terms of Ach-induced vasodilatation between the 4 investigated groups. When administered together with L-NAME, Ach-induced vasodilatation was observed to decrease in all groups. In the trained groups, with regard to the renal artery, the NO effect was more pronounced in females, while in the case of the femoral artery, this effect was more pronounced in males.

Regarding the renal artery, the OD of resorcin-fuchsin staining was markedly lower in the MSed group in comparison to the FSed group. After exercise, the OD of elastic fibers decreased in females. A decrease in smooth muscle actin OD was observed in females after training. Regarding the femoral artery, the OD of eNOS increased in male animals after 12 weeks of training. This value was noticeably higher in the MTr group in comparison to the FTr group. In respect of both the renal artery and the femoral artery, it can be stated that COX2 and NT staining showed no variance between the 4 groups.

Table 1. Comparison of renal and femoral artery functions after 12 weeks of swimming training in Wistar rats.

Functional reaction	**Arteria Renalis**				**Arteria Femoralis**			
Training factor	Sedentary		Trained		Sedentary		Trained	
Sex	♂	♀	♂	♀	♂	♀	♂	♀
Alpha Adrenerg contraction	Yes	Diminished	Diminished	Yes	Yes, similar			
NO relaxation ability	Yes, similar				Yes, similar			
NO relaxation moderating alpha 1 adrenerg contraction	Limited		Elevated		Limited		Elevated	
Constrictor prostanoids enhancing alpha 1 adrenerg contraction	Yes	INDO (NO COX2)	Diminished		Yes	Yes (Cox2 product)	Diminished	Yes (but diminished Cox2 products)
Vasoactiv prostanoids modifying cholinergic relaxation	No				No			

5.4. Chronic exercise and cardiovascular diseases

Cardiovascular risk factors promote CVD (cardiovascular diseases) in either biological sex, but with different relative importance. Regarding smoking, the incidence of heart attack increases 6 times for women who smoke at least 20 cigarettes per day, while the same effect increases the risk 3 times for men (111, 112). One of the most important risk factors for CVD is systolic hypertension in both men and women (113). Diabetes and an unfavorable lipid profile (low HDL/total cholesterol level ratio) are less favorable in terms of women's cardiovascular risk (114, 115). Obesity is associated with a known atherogenic risk, while physical inactivity also increases the risk of CVD (116, 117). Male sex alone contribute to the risk of CVD (118).

Among the listed risk factors, in present thesis, our studies focused on exercise and lack of exercise as a CHD risk. In their human study, Paffenbarger et al proved that the risk of death was reduced by 23% in men engaged in moderately active sports (119). The

Prospective Urban Rural Epidemiological (PURE) study, which followed adults from 21 low-, middle-, and high-income countries for a period of 10 years, showed the proportion of CVDs related to lifestyle habits (including exercise) attributable to the entire population was higher in men than in women (120).

Our results are supported by numerous examples found in the literature, according to which regular physical exercise has more significant cardiovascular benefits for men compared to women.

6. CONCLUSIONS

Our experiments focused on the following questions:

1. Does the renal artery adapt after long-term exercise, and are there sex differences in terms of sport adaptation?

Our results indicate sex-specific renal arterial adaptation as a consequence of aerobic physical activity in Wistar rats. Exercise in male rats leads to a reduction in phenylephrine-induced contraction, with a possible role played by decreased COX-2 related contraction and increased NO-related compensation. In females, meanwhile, no significant functional change was recorded; at the same time, decreased COX related contraction and NO related compensation was observed, along with reduced elastin and SMA density.

2. Does the femoral artery adapt after long-term exercise, and are there sex differences in terms of sport adaptation?

Swim training had the consequence of shifting the balance between endothelium-derived vasoconstrictor and vasodilator compounds in the clear direction of vasodilation, and this was observed in animals of either sex. In the swim trained males, NO-related relaxation and relaxation reserve was observed to increase. It is our finding that a greater eNOS expression is the underlying cause of this. It may be that sex hormones beneficially affect eNOS, COX and COX-2 signaling. To conclude, increases in training-induced NO release/bioavailability are observed to counteract vasoconstriction and improve relaxation in femoral arteries. Males are likely to benefit more than females from NO release/bioavailability following training.

7. SUMMARY

We set out to investigate the changes of vascular reactivity and histology of isolated renal and femoral arteries in male and female rats as a response to swim-training.

Methods: Male sedentary (MSed), male trained (MTr), female sedentary (FSed), and female trained (FTr) animal groups were set up. Animals in the trained groups were subjected to a 12-week period of intensive swim training. Isolated renal and femoral artery rings were examined via wire myography.

Results: Regarding the renal artery: Phenylephrine (Phe) induced contraction was observed to be lower in FSed than in MSed animals. Training led to a decrease in male animals but not in females. Inhibition of cyclooxygenases by indomethacin (INDO) led to a reduction in contraction in both sedentary groups, but only in MTr and not in FTr animals. Inhibition of nitric oxide (NO) production led to increased contraction observable in both trained groups, male and female. Acetylcholine (Ach) induced relaxation was seen to be alike in all experimental groups, with NO-dependency observed as predominant. Elastin and smooth muscle cell actin density showed a reduction in female rats following aerobic training.

In respect of the femoral arteries: No difference in contraction induced by Phe was observed between the four groups. A decrease in contractile ability in the presence of INDO was observed in both sedentary groups. However, only the FSed animals exhibited a specific cyclooxygenase-2 (COX-2) role. Following exercise training, an increase in vasoconstriction was observed in both sexes, in the presence of nitro-L-arginine methyl ester (L-NAME). The COX-related vasoconstriction effect was found to disappear in MTr rats, while in FTr rats, it was the COX-2-related vasoconstriction effect that disappeared. The presence of L-NAME led to a significant reduction in relaxation in MTr animals in comparison to FTr animals. Exercise was associated with greater expression of endothelial nitric oxide synthase (eNOS) protein in male animals.

Conclusion: The swim training was found to moderate renal artery vasoconstriction in male animals, while in female animals, swim training was found to depress elastic fiber and smooth muscle actin density. Swim training was found to significantly increase the relaxation reserve capacity in the femoral arteries of male animals, in comparison to compared to those of female animals.

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9. BIBLIOGRAPHY OF PUBLICATIONS

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