BLOOD FLOW PATTERNS, ARTERIAL REYNOLDS NUMBER, EXERCISE, AND ENDOTHELIAL FUNCTION

By

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To my children Nicolás, Joaquín, Fernanda, Benjamín and Sebastián; one day they will read this and they will understand what this was about.
To my soul mate Carolina, without her this journey would have been impossible.

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BLOOD FLOW PATTERNS, ARTERIAL REYNOLDS NUMBER, EXERCISE, AND ENDOTHELIAL FUNCTION

By

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Endothelial dysfunction is the first pathophysiological step in atherosclerosis. It has been shown that exercise training improves endothelial function via direct effects on cardiovascular risk factors. However, direct effects of exercise-induced blood flow on endothelial function are controversial. We hypothesized that exercise training-induced endothelial function improvement is 1) systemic and not only localized in exercising vascular beds, and 2) that improved endothelial function is mediated by retrograde turbulent and antegrade laminar blood flow patterns. To test our central hypotheses, we designed three in-vivo experiments 1) to characterize beneficial blood flow patterns, 2) to characterize blood flow patterns during different exercise types and intensities, and 3) to determine possible mechanisms involved on endothelial function regulation produced by exercise-induced blood flow as a mechanical stimulus.

We recruited 53 apparently healthy, young men for the three studies (18 on the first, 8 on the second, and 27 for the third one) and the most relevant results showed 1) beneficial blood flows include two opposite patterns: antegrade laminar and retrograde turbulent; 2) retrograde turbulent blood flow is increased during cycling exercise in a dose-dependent manner; and 3) exercise-induced blood flow regulates endothelial

function via a decrease on endothelial oxidative stress and increase on endothelial nitric oxide synthase.

These results provide, for the first time, evidence in support of the hypothesis that exercise-induced blood flow regulation of endothelial function is dependent upon blood flow pattern. In addition, these results provide further evidence of the close relationship between endothelial function, endothelial nitric oxide synthase, and endothelial cell oxidative stress *in vivo*.

CHAPTER 1
INTRODUCTION

Cardiovascular diseases (CVD), including coronary artery disease (CAD) and stroke, are the leading cause of death in the Western World, with a direct and indirect cost of more than $430 billion in the United States alone.(160) Several cardiovascular risk factors are associated with the development of ischemic CVD, such as dyslipidemia, obesity, high blood pressure, and physical inactivity. However, it is the decrease of blood flow to noble tissues, due atherosclerotic lesions, that produces mortality and morbidity outcomes. Atherosclerotic plaque formation is the pathophysiologic event responsible for either a chronic decrease in arterial lumen size or an acute thrombi formation, which produces an acute ischemic event.(204) According to current medical theory, endothelial dysfunction is the primary cause of atherogenesis.(113, 117, 204) The endothelial cell monolayer helps to regulate homeostasis of the cardiovascular system by producing anti-thrombotic, anti-inflammatory, and anti-adhesion molecules where Nitric Oxide (NO) is the most important.(101, 117, 135) Endothelial dysfunction, or decreased NO bioavailability, produces a cascade of events including oxidation of LDL-cholesterol, leukocyte recruitment, and foam cell formation, which becomes the basis of atherosclerotic plaque formation.(101, 113, 117, 204)

Multiple factors affect endothelial function, but oxidative stress and low endothelial shear stress (ESS) may be the most relevant.(24, 41, 92) For example, increased superoxide anion ($O_2^{\bullet -}$) concentration in the endothelial cell decreases NO bioavailability via peroxynitrite ($ONOO^-$) production.(130) Whereas low ESS is associated with down regulation of endothelial NO synthase (eNOS) thereby decreasing NO production and

bioavailability.(207) Furthermore, low ESS promotes pro-inflammatory/oxidative stress pathways that produce endothelial dysfunction.(57)

Normal endothelial function mainly depends on hemodynamic forces within the vessel, i.e. blood flow-generated ESS, and ESS modulates endothelial regulation via an integrin/cytoskeleton mechano-transduction pathway.(24, 41) Even though blood flow follows Ohm's principle, where flow is directly proportional to the system's pressure and indirectly proportional to the system's resistance, flow is not always unidirectional. Indeed, blood flow is normally bidirectional in large elastic and conduit muscular arteries.(73, 138) Bidirectional flow consists of both antegrade (downstream) and retrograde (upstream) flows, where both have the capacity to produce ESS. However, recent studies have shown contradictory results when testing endothelial function after different ESS perturbations.(57, 78, 181, 183, 207) For example, Green *et al.*(78) have shown that brachial artery flow is bidirectional during cycling and this pattern improves brachial artery endothelial function. In contrast, the same group of investigators (181) reported that brachial artery flow mediated dilation was impaired after the brachial artery was exposed to increased retrograde flow. If both antegrade and retrograde flows can produce ESS but endothelial function is not always improved, blood flow direction might not be the only hemodynamic factor affecting endothelial function.

It is generally accepted that undisturbed laminar flow improves endothelial function, while disturbed laminar and turbulent flows are detrimental to endothelial function.(24, 41) Presence or absence of blood flow turbulence depends upon three main factors 1) flow speed, 2) vessel diameter, and 3) blood viscosity. These three factors can be quantified by the use of a unitless variable called Reynolds number (Re).

In human circulation, due to vessel geometry and blood viscosity, it is accepted that Re over 2000 produces turbulent flow.(24, 138) Therefore, four distinct patterns of blood flow can contribute to ESS: i.e. 1) antegrade laminar, 2) retrograde laminar, 3) antegrade turbulent, and 4) retrograde turbulent. In this dissertation, I propose that these different blood flow patterns will potentially affect endothelial function in different ways based upon the activation/deactivation of the integrin/cytoskeleton mechano-transduction pathway.

Mechanical blood flow-generated shear stress stimulus is transduced into the endothelial cell via the integrin/cytoskeleton mechano-transduction pathway.(24, 41) This mechano-transduction pathway has four components that elicit mechanical and biochemical-signaling processes within the endothelial cell; 1) flow receptors or glycocalyx, 2) transmembrane proteins called integrins, 3) cytoskeletal filaments such as actin filaments, and 4) mechanical force conversion to chemical activity.(37, 41, 118, 178, 201) Each component will be described, in brief, in the following paragraphs.

The glycolcalyx, first known as an apical membrane integrin, is a heparan sulfate proteoglycan anchored to the apical membrane of endothelial cells. It is a fluid flow mechanosensor that is positively associated with NO upregulation and strongly anchored to the endothelial apical membrane and cytoskeleton. Recent studies have suggested that shear stress mechanically "pushes" the glycocalyx thereby producing cell deformation, in the direction of blood flow. This deformation appears to open calcium (Ca^{2+}) ion channels increasing intracellular Ca^{2+} which activates eNOS.(63, 178) In addition, cell deformation produces mechanical force transductions via integrins and the cytoskeleton to cytoplasmatic second messengers and directly to the nucleus,

where transduction factors can be activated.(41, 63, 178)

Integrins are transmembrane proteins ubiquitously located on the endothelial cell membrane. On the baso-lateral surface, integrins provide endothelium tightness and connection with the extracellular matrix. On the apical surface, integrins are able to "sense" flow.(41, 178) In the absence of the glycocalyx, apical membrane integrins may produce some cell deformation during fluid flow. However, this deformation is not enough to upregulate NO.(63, 178) Therefore, integrin's main role is to link extracellular domains, i.e.: arterial lumen and extracellular matrix, to the cytoskeleton and nucleus via cytoskeletal filaments.(41, 118)

Cytoskeletal filaments provide elastic stiffness and maintain the shape and structure of the cell. Cytoskeletal deformation and displacement, such as actin filament deformation, are linked to force transmission to 'remote' cellular sites.(41) In fact, shear stress sensed by the glycocalyx at the apical membrane is mechanically transmitted to the basolateral membrane via integrin/cytoskeletal filament-complex deformation. Furthermore, this cytoskeletal deformation can be extended to adjacent cells as a type of 'chain reaction' via the same mechanism.(41, 178)

Mechanical force conversion to chemical activity, or 'true' mechano-transduction, happens simultaneously at several levels during the mechano-transduction pathway. First, the glycocalyx and apical integrin deformation produces activation of ion channels, e.g. Ca^{2+} channels, and G-protein receptors. Second, basolateral integrin deformation produced via cytoskeletal filament force transmission causes kinase phosphorylation, e.g. Focal Adhesion Kinase (FAK) and Mitogen-activated protein (MAP) kinases. Finally, the same cytoskeletal filament force transmission produces nuclear

deformation, which can activate transcription factors.(41, 118, 178)

Low ESS has been shown to down regulate glycocalyx expression.(201) In addition, it is well documented that arterial regions exposed to low ESS, such as bifurcations or atherosclerotic lesions, have an increased endothelial oxidative stress.(24, 41, 130) The relationships between low ESS and endothelial mechano-transduction and between low ESS and oxidative stress foster speculations of a potential "mechanical" link between glycocalyx and endothelial NAD(P)H oxidase.(41, 130) This link could explain why *in vitro* retrograde laminar flow increases endothelial oxidative stress, despite presence of ESS (63, 178, 201) and *in vivo* findings of impaired endothelial function after retrograde flow stimulus.(181) Retrograde laminar flow could be "pushing" the glycocalyx upstream, thereby causing decreased eNOS activation, while simultaneously increasing $O_2^{\bullet -}$ production via NAD(P)H oxidase activation, with a further decrease on NO bioavailability.

In summary, endothelial function depends upon two main factors, 1) blood flow-mediated mechano-transduction and 2) endothelial oxidative stress, which could be caused by intrinsic endothelial redox imbalance or produced by non-physiological ESS.(24, 41, 130, 178) Non-physiological ESS includes, but is not limited to, antegrade low laminar ESS and antegrade turbulent ESS. Evidence suggests that ESS effects on endothelial function depend on how the 'bush-like' glycocalyx responds to blood flow. Antegrade high laminar flow would push the glycocalyx downstream producing structural adaptations to endothelial cells and the mechano-transduction system, consequently improving endothelial function. In contrast, antegrade turbulent flow would not push the glycocalyx downstream. Moreover, if some glycocalyx deformation

did occur during antegrade turbulent flow, it would be 'upstream' and in the wrong direction, thereby impairing endothelial function. With respect to retrograde flow, as mentioned earlier, contradictory endothelial function behavior has been reported when retrograde flow-induced shear rate was applied. Unfortunately, to date turbulence has not been assessed in studies designed to assess the effects of flow patterns on endothelial function. We speculate that turbulence could have a major effect on endothelial function outcomes. We further speculate that retrograde laminar flow would impair endothelial function and retrograde turbulent flow would enhance it, showing a 'mirror' effect when compare with antegrade flow.

In conclusion, exercise induced-blood flow stimulates endothelial cells via endothelial mechano-transduction and, depending on the pattern of the flow, this stimulation would be beneficial or detrimental to endothelial function. However, blood flow patterns are not well characterized. Flow direction, but not turbulence, has been used in blood flow classification schemes to date. I hypothesize that exercise training-induced endothelial function improvement is 1) systemic and not only localized in exercising vascular beds, and 2) that improved endothelial function is mediated by retrograde turbulent and antegrade laminar blood flow patterns. Blood flow pattern classification should consist of two anti-atherosclerotic patterns: 1) retrograde turbulent and 2) antegrade laminar; and two pro-atherosclerotic patterns: 1) antegrade turbulent and 2) retrograde laminar. This classification "system" would help to characterize different endothelial mechano-transduction pathways, where endothelial cell activation/deactivation would function in a manner similar to on/off mechanical switch controlled by exercise-induced blood flow.

To test my central hypotheses I designed three different experiments. These *in vivo*-human experiments characterized, for the first time, different blood flow patterns based on direction and turbulence, and their effects on endothelial function and endothelial oxidative stress. In addition, these experiments determined which blood flow patterns are antioxidative, anti-atherogenic, and endothelial-protective; and they established the relationship between exercise training-induced flood flow pattern and endothelial health.

Experiment 1

Specific Aim 1

To determine arterial blood flow patterns in the upper and lower extremities during a single session of enhanced external counter-pulsations (EECP).

Hypothesis 1

Blood flow in the brachial artery during EECP will be antegrade and laminar with Re<2000.

Hypothesis 2

Blood flow in the femoral artery during EECP will be retrograde and turbulent with Re>2000.

Rationale

Enhanced external counter-pulsation (EECP) is a FDA approved treatment for coronary artery disease patients. EECP is a non-invasive treatment for angina that uses the inflation of three sets of pneumatic cuffs wrapped around the lower extremities. These cuffs are placed on calves, thighs, and buttocks and are sequentially inflated, from calf to buttocks, to 250 millimeters of mercury at the onset of diastole and they are rapidly deflated at the onset of systole. These counter-pulsations produce a pneumatic

massage and a retrograde blood flow from the legs to the heart. This pneumatic external massage increases diastolic pressure and venous return, which is known to improve endothelial function of both femoral and brachial arteries.(13, 61, 137) Although invasive central blood pressure measurements have been recorded during a single session of EECP, non-invasive assessment of aortic pulse wave and Re number have not been described.(124, 126) We speculated that EECP will produce high-velocity retrograde flow in the femoral arteries and moderate-velocity antegrade flow in the brachial arteries.(61, 140)

Specific Aim 2

To determine if endothelial function is improved after a single session of EECP in regions of retrograde or antegrade flow.

Hypothesis

Both brachial and femoral flow mediated dilation (FMD) will be improved after a single session of EECP.

Rationale

It is generally accepted that laminar ESS improves endothelial function and flow mediated dilation (FMD).(24, 78, 183) However, recent studies have shown contradictory results when flow is retrograde.(14, 180, 181) For example, Thijssen *et al.* (181) reported impaired brachial artery FMD after acutely producing retrograde blood flow by inflating a pneumatic cuff placed on the forearm. In contrast, Braith *et al.* (14) showed that chronic exposure to retrograde blood flow via 35-1 hr sessions of EECP improves FMD in the femoral artery. During a single session of EECP, femoral and brachial artery blood flows will be retrograde and antegrade, respectively. Comparing femoral and brachial FMD before and after a single session of EECP would determine

how blood flow direction is associated with endothelial function. We hypothesize that both brachial and femoral FMD will improve after a single session of EECP. We speculated that blood flow direction is not the pivotal variable that can modulate endothelial function.

Experiment 2

Specific Aim

To determine which type of exercise and exercise intensity mimics EECP blood flow patterns.

Hypothesis

Moderate intensity resistance exercise, but not low intensity resistance exercise or low and moderate intensity aerobic exercise, will produce blood flow patterns in brachial and femoral arteries that mimic EECP.

Rationale

It is generally accepted that physical activity improves endothelial function.(15, 59, 68, 69, 76) However, there are conflicting opinions whether exercise induces vascular adaptations systemically or only in the active muscle beds. (78, 183) EECP therapy induces both local and systemic arterial adaptations. (6, 13, 14, 21, 137) We aimed to identify the exercise type and intensity that elicits blood flow patterns similar to EECP. The overall objective is to design specific exercise prescriptions based on endothelial blood flow patterns.

Experiment 3

Specific Aim 1

To determine changes in endothelial cell oxidative stress and endothelial function after 4 weeks of moderate intensity exercise training.

Hypothesis 1

Four weeks of moderate intensity resistance exercise training (RXT) will improve brachial and femoral FMD and will increase plasma NO metabolites (NOx) levels compared to moderate intensity aerobic exercise training (AXT) and non-exercising controls.

Hypothesis 2

Four weeks of RXT will increase endothelial cell eNOS content compared to AXT and non-exercising controls.

Rationale

Exercise training improves endothelial function.(15, 59, 68, 69, 76) In healthy subjects, endothelial NO bioavailability depends upon endothelial eNOS content. After exercise training, plasma NOx levels are highly correlated with FMD.(69, 116) To the best of our knowledge, however, human endothelial cell eNOS content before and after exercise training has not been studied. We measured eNOS expression in harvested venous endothelial cells before and after 4 weeks of moderate intensity exercise training.

Specific Aim 2

To determine changes in endothelial oxidative stress after 4 weeks of moderate intensity exercise training.

Hypothesis

Four weeks of RXT will decrease endothelial oxidative stress, measured via endothelial nitrotyrosine and eNOS, compared to AXT and non-exercising controls.

Rationale

NO bioavailability depends on two main factors, i.e.: NOS content and oxidative stress.(24, 41, 92, 130, 207) Increased superoxide anion ($O_2^{\bullet -}$) production in the endothelial cell will decrease NO bioavailability via peroxynitrite ($ONOO^-$) formation. A close relationship between FMD, plasma indices of oxidative stress, and NO has been reported.(8, 59) To the best of our knowledge, however, changes in oxidative stress after exercise training have not been studied in harvested human endothelial cells. We speculated that four weeks of moderate intensity resistance exercise training (RXT) will decrease endothelial nitrotyrosine, measured via immunofluorescence, when compared to moderate intensity aerobic exercise (AXT) and non-exercising controls.(31, 130, 148)

CHAPTER 2
MATERIAL AND METHODS

To achieve the proposed specific aims, we designed three experiments. The three experiments recruited subjects of similar age, gender, and fitness level. The three experiments utilized some common methods that will be described below. The specific description of each experiment follows.

Subjects

Young healthy men, between 18 and 35 years of age, were recruited for the experiments. Exclusion criteria included currently exercising three times per week or more, orthopedic limitations that impair normal physical activity, known cardiovascular disease including cardiac arrhythmias, prescription medication medicines, 'over-the-counter' painkillers, such as NSAIDs or aspirin, or nutritional supplements containing antioxidants. Women were excluded due to significant variations in vascular function during the 4 phases of the menstrual cycle.(2)

Experiment 1

Figure 2-1 shows experiment 1 study design that consider, in general, two groups undergoing a single 45-minutes session of EECP where several hemodynamic and vascular function variables were measured. Experiment 1 protocol details follow:

- 18 subjects were randomly assigned to one of two groups: Group 1: active EECP with cuffs inflated to 250 mmHg; Group 2: sham-EECP with cuffs inflated to 50 mmHg. All subjects received a single, 45–minute session of EECP.
- Brachial and femoral artery Flow Mediated Dilation were performed before and within 10 minutes after the 45-min session.
- To assess changes in aortic pressures, peripheral (radial) and central (aorta) pulse wave analysis, via applanation tonometry, was performed before and during the EECP session.

- Brachial and femoral artery diameters and blood velocity were assessed via high-resolution ultrasound and Doppler during the EECP session.

- Two blood samples from the earlobe, using a micro-hematocrit capillary, were drawn to measure duplicate hematocrit before the EECP session.

- Reynolds number (Re) in femoral and brachial arteries was calculated using $\mathrm{Re} = \frac{\overline{V} D \rho}{\mu}$, where $\overline{V}$ =blood flow velocity; D=blood vessel diameter; ρ=blood density; and μ=blood dynamic viscosity.(138) D and $\overline{V}$ were obtained via high-resolution ultrasound vascular imaging and Doppler, respectively. Blood density and dynamic viscosity were calculated using the following formulas (23, 55, 85)

$$\mu_{plasma} = \frac{\exp\left[-5.64 + \frac{1800}{T + 273}\right]}{SR}$$

$$\mu = \mu_{plasma} * \exp(2.31 Hct)$$

$$\rho = \left[1.09 Hct + 1.035 * (1 - Hct)\right],$$

where μ_{plasma}= plasma dynamic viscosity (N/m^2•s); *T*=temperature (°C); *SR*=shear rate, (if SR<100 s^{-1}; if SR≥100 s^{-1}, then SR=100); and *Hct*=Hematocrit (%).

- Due to multiple factors affecting blood flow velocity acquisition, such as isonation angle, Doppler steer angle and width, and Doppler position within the vessel (155-157) Reynolds number was normalized (nRe) to resting steady-state values of 1800 and 600 for antegrade and retrograde flows, respectively.(18, 35, 41, 60, 102, 105, 138, 156, 157, 164)

- Shear Rate (SR) was determined in brachial and femoral arteries during antegrade and retrograde blood flows using SR=peak blood flow velocity/artery diameter, and expressed in s^{-1}.

- Shear Stress (SS) was determined in brachial and femoral arteries during antegrade and retrograde blood flows using SS=4μQ/D, where μ is blood viscosity, Q is peak blood flow velocity, and D is artery diameter. SS is expressed in dynes/cm^2.

- Blood flow pattern was defined as the combination of three dimensions 1) blood flow direction (antegrade/retrograde), 2) shear stress (increased/decreased), and 3) presence of turbulence (Re > |2000| is turbulent flow).

Experiment 2

Figure 2-2 shows experiment 2 study design that consider, in general, one group of subjects undergoing three exercise sessions 1) baseline measurements, 2) resistance exercise at 2 different intensities, and 3) aerobic exercise at 2 different intensities. Blood flow patterns were assessed during both aerobic and resistance exercise sessions. Experiment 2 protocol details follow:

- 8 subjects were tested for one repetition maximum strength (1RM) on the bilateral knee extension machine and VO_2max on a cycle ergometer.
- Subjects performed two types of exercise at two different intensities. The order of exercise was randomly assigned.
- 2 sets of 10 to 12 repetitions of bilateral knee extension at 40% of 1RM (mild) and 2 sets of 10 to 12 repetitions of bilateral knee extension at 70% of 1RM (moderate), and 5 to 10-minute resting period between sets.
- 2 bouts of 10 minutes at 40% of their cycle VO_2max (mild) and 2 bouts of 5 minutes at 70% of their cycle VO_2max (moderate), and 5 to 10-minute resting period between sets.
- Femoral artery diameters and blood velocity were assessed via high-resolution ultrasound and Doppler before and during the first set or bout of exercise.
- Brachial artery diameters and blood velocity were assessed via high-resolution ultrasound and Doppler before and during the second set or bout of exercise.
- Two blood samples from the earlobe, using a micro-hematocrit capillary, were drawn to measure duplicate hematocrit before every testing session.
- Reynolds number and nRe in femoral and brachial arteries were calculated as described above (Page 27).
- Shear rate, shear stress, and blood flow pattern in femoral and brachial arteries were calculated as described above (Page 27).

Experiment 3

Figure 2-3 shows experiment 3 study design that consider, in general, three groups undergoing three different exercise regimens 1) no-exercise control, 2)

resistance exercise training, and 3) aerobic exercise training. Endothelial function assessments, endothelial oxidative stress, eNOS content, and maximal exercise capacity tests were performed before and after 4 weeks of training. Experiment 3 protocol details follow:

- 27 subjects were randomly assigned to one of three groups: Group 1 non-exercise control (n=9); Group 2 4 weeks of resistance training (RXT) (n=9); and Group 3 4 weeks of aerobic training (AXT) (n=9)
- All subjects were tested for 1 RM strength on the bilateral knee extension machine and VO_2max on a cycle ergometer at baseline and after 4-week training period
- Group 2 and 3 trained for 4 weeks at 70% of 1RM and 70% of their cycle VO_2max, respectively. Group 1 did not exercise and was encouraged to maintain their normal diet and activity habits.
- RXT was performed 3 times per week with a workload of 3 sets of 10 to 15 repetitions on a bilateral knee extension machine (MedX, Ocala, FL), and 5-minute resting periods between sets. To avoid muscle recruitment from the upper body, subjects wore a seat belt and hands were placed on opposite shoulders.
- AXT was performed 3 times per week, one 30-minute continuous bout per session on a isokinetic stationary bicycle (Cybex 750C, Medway, MA). To avoid muscle recruitment from the upper body, subjects were encouraged not to use the bicycle handle-bars, keeping their trunk in a straight-up position.
- Brachial and femoral Flow Mediated Dilation was performed before and after the 4-week training period in all subjects.
- Plasma NOx levels were assessed before and after the 4-week training period in all subjects using commercially available kits (Cayman Chemical Company, Ann Harbor, MI).
- Resting plasma norepinephrine concentration was assessed before and after the 4-week training period in all subjects using commercially available kits (ALPCO Diagnostics, Salem, NH). Blood samples were drawn from an intravenous catheter 20-30 minutes after veno-puncture.
- Venous Endothelial cells, for endothelial molecular studies, were harvested using a J-hook catheter from an antecubital vein before and after the 4-week training period.

EECP Protocol

EECP (Angio New-IV, Vasomedical, Inc., NY) treatment was performed in Dr. Braith's Cardiovascular Laboratory at the University of Florida, Gainesville, FL. Subjects in the active-EECP group will receive one 45-minute EECP session. All subjects were monitored clinically and hemodynamically, by finger plethysmography and electrocardiography, during EECP session. EECP involved sequential inflation and deflation of compressible cuffs wrapped around the patients' calves, lower thighs, and upper thighs. Compressed air pressure was applied via the cuffs to the lower extremities in a sequence synchronized with the cardiac cycle via microprocessor-interpreted ECG signals. The diastolic augmentation pressure was progressively increased by increasing external compression until the finger plethysmography systolic/diastolic index reached a value of one, indicative of optimum diastolic augmentation. In this study, the pressure applied to the cuffs during EECP was set at ~250 mm Hg; equivalent to ~300 mm Hg previously reported using older EECP devices.(97) Study subjects randomized to the sham-EECP group were scheduled separately from subjects randomized to the EECP group. Subjects in the sham-EECP group received modified EECP (50 vs. 250 mm Hg cuff pressure) for one 45-minute session. During sham-EECP the pressure applied to the cuffs during inflation was set at 50 mm Hg. Our rationale for selecting 50 mm Hg inflation pressure for sham-EECP is based upon previous research experience showing that 50 mmHg gives the 'sensation' of EECP without eliciting hemodynamic changes.(7)

Endothelial Function Testing

Brachial Artery Flow-Mediated Dilation

Brachial artery reactivity testing was performed using high-resolution ultrasound (HDI 3000, ATL, Inc), and following international guidelines.(34) Brachial artery reactivity measurements was made with the subjects in a supine position following a fast of at least 8 hours and abstaining from caffeine for at least 12 hours prior to the measurements. After lying quietly for 15 minutes, a 10.5 MHz linear phase array ultrasound transducer was used to image the right brachial artery longitudinally and recorded directly to a digital massive storage device via a super video interface (Pinnacle System GmbH, Avid Technology Inc, Tewksbury, MA). After obtaining resting baseline end diastolic diameters and blood flow velocity, a blood pressure cuff placed on the upper forearm, 1-2 cm below the elbow, was inflated to 200 mmHg for 5 minutes. The transducer was held in the same position for the duration of cuff inflation to ensure the same section of the brachial artery was be measured before and after cuff inflation. Additionally, distal cuff placement has been suggested to serve as a more accurate bioassay of endothelial nitric oxide (NO) availability.(152) Immediately following cuff release, brachial artery diameter and brachial artery peak blood flow velocity were recorded continuously for 2 minutes.

Reactive hyperemia blood flow results in flow-mediated dilation (FMD) of the brachial artery due to shear stress-induced NO release from the endothelial wall. Peak brachial artery diameter has been reported to occur ~ 60 seconds after cuff deflation in healthy subjects and is a valid measure of endothelial- mediated artery reactivity and time to peak dilation was reported.(11, 34) Brachial FMD was calculated as absolute (Δmm) and relative change (%) in brachial artery diameter in response to the forearm

hyperemic stimulus. Because the main stimulus for FMD is an acute increase in vascular shear stress or blood flow, peak FMD values were normalized for the magnitude of the hyperemic stimulus using area under the curve of the shear stimulus until the time of peak diameter measurement.(153) End-diastole electrocardiogram synchronized images of the brachial artery were transferred from digital files to an uncompressed AVI file using a digital movie editor (Pinnacle Studios, Avid Technology Inc, Tewksbury, MA). Brachial artery diameters were determined using automated edge-detection FMD software (Vascular Research Tools, Medical Imaging Applications LLC, Coralville, IA).

Femoral Artery Flow-Mediated Dilation

Femoral artery FMD testing was performed using a protocol similar to the one described for brachial FMD testing. The right common femoral artery proximal to the bifurcation was imaged longitudinally. The occlusion cuff will be placed ~5cm above the patella. Blood velocity and artery diameter measurements were recorded and analyzed off-line using the same protocol previously described for the brachial artery.

Artery Diameter and Blood Flow Velocity During EECP and Exercise

During EECP sessions and exercise sets or bouts, femoral and brachial artery diameters and blood flow velocities were measured. These measurements were performed in a similar way to those described for flow mediated dilation testing (pages 31 and 32). The difference is that we did not use reactive hyperemia to assess them. They were performed using high-resolution ultrasound and Doppler during the execution of the EECP session or exercise set or bout. Electronic acquisition and analysis of images followed the same methodology described above.

Peripheral and Central Pulse Wave Analysis (PWA)

The assessment of aortic pressures was performed non-invasively using the SphygmoCor *Pulse Wave Analysis Px* system and SCOR-2000 Version 6.31 software (AtCor Medical, Sydney, Australia). High-fidelity radial artery pressure waveforms were recorded by applanation tonometry of the radial pulse in the left wrist using a "pencil type" micromanometer (Millar Instruments, Houston, TX). The aortic pressure waveform was derived noninvasively from the radial pulse using applanation tonometry and application of a generalized transfer function, which corrects for pressure wave amplification in the upper limb.(138) At least five consecutive measurements were performed per subject, and the average of the best three high-quality recordings, defined as an in-device quality index of over 80% (derived from an algorithm including average pulse height variation, diastolic variation, and the maximum rate of rise of the peripheral waveform), will be used for analysis. The following PWA parameters, related to the aortic blood pressures, were used as independent variables in the present study:

- Peripheral (Radial) pressures: systolic, diastolic, and pulse pressures (Br SP, Br DP, and Br PP, respectively) were directly determined by applanation tonometry measurements.
- Central (Aortic) Pressures: systolic, maximum diastolic, minimum diastolic and pulse pressures (AoSP, AoDPmax, AoDPmin, and AoPP, respectively) were determined by the generalized transfer function after applanation tonometry was performed.
- Central (Aortic) mean arterial pressure(AoMAP) was calculated using the following time-weighted equation: AoMAP= [(AoSP+AoDPmax+AoDPmin)/3]
- Central (Aortic) diastolic augmentation (AoDa) was calculated using the following equation: AoDa= AoDPmax+AoDPmin.

Exercise Testing

Cycle VO_2max

A cycling graded exercise (GXTc) test was performed on an electro-mechanic stationary bicycle (Hudson EC-400, The Hudson Fitness Inc., Dallas, TX) with respiratory gas analysis using a calibrated metabolic cart (Parvomedics Inc., Sandy, UT). Subjects performed an incremental-step protocol starting with 50 watts and increasing 50 watts every two minutes, until failure. GXTc was performed using the American College of Sports Medicine (ACSM) guidelines for fitness testing for young, healthy subjects.(4)

One Repetition Maximum (1RM)

Bilateral knee extension machine (MedX, Ocala, FL) was used to performed strength testing and interventions. 1-RM testing sessions were performed following ACSM guidelines for resistance exercise testing.(4) Briefly, a testing session begins with the subject performing a warm-up set of six to eight repetitions with a light weight. Two rest minutes were given between the warm-up and the start of the 1-RM test. The initial 1-RM weights were standardized among subjects and represented 75% of body weight. When the weight can be successfully lifted through the Range of Motion (ROM), the weight for the next trial will be incremented by 2-5 kg. The increments in weight are dependent upon the effort required for the lift and will become progressively smaller as the subject approaches the 1-RM. Two minutes of recovery time will be allowed between 1-RM trials. The last weight successfully lifted through the full ROM was considered the 1-RM.(16)

Tissue Sampling

Earlobe Blood Collection

To determine hematocrit, necessary for the estimation of blood density and viscosity, a blood micro-sample was collected from subjects' earlobe. Earlobe puncture was performed using a blood lancet (Microlance, Becton-Dickinson, Rutherford, NJ). Two blood samples were collected using a micro-hematocrit capillary tube (Fisher Scientific, Pittsburgh, PA).

Antecubital Blood Collection

To determine plasma catecholamine concentration and NOx levels, a blood sample was collected from an antecubital vein via an intra venous catheter (Insyte, Becton-Dickinson, Rutherford, NJ). First, the catheter is placed in an antecubital vein. Then, after at least 20-minutes pause, blood is collected in tubes containing ethylenediaminetetraacetic acid (EDTA), placed on ice for 15 minutes, and centrifuged immediately at 3,000 rpm for 15 minutes at 4°C. All samples were aliquoted into 1.5 ml eppendorf tubes and immediately stored at -80°C until batch analysis at the end of the study.

Venous Endothelial Cell Harvesting

The following procedure was used for collection and preparation of venous endothelial cells and measurement of protein expression using quantitative immunofluorescence, as previously described.(31, 62, 148) Two sterile J wires (Daig Corp, Minnetonka, Minn) were advanced into an antecubital vein (≈4 cm beyond the tip of the catheter) and retracted through an 18-gauge catheter. The wires were then transferred to a dissociation buffer solution, and endothelial cells were recovered via a washing and centrifugation protocol. Collected cells were fixed with 3.7%

formaldehyde, plated on poly-L-lysine–coated slides (Polysciences Inc, Warrington, PA) and then frozen at -80°C until analysis.

Molecular Analysis

Cells were rehydrated, and nonspecific binding sites were blocked with 5% donkey serum. Cells were incubated with monoclonal antibodies for eNOS (from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and nitrotyrosine (from Millipore, Billerica, MA). Cells were then incubated with an Alexafluor 555 fluorescent secondary antibody (Invitrogen Corp, Carlsbad, CA).

For analysis, slides were viewed with a fluorescence conofocal microscope system (Visitech Infinity^3 2D Array Laser Confocal system, Sunderland, UK; and Olympus BX51WI, New York, USA), and cell images were captured digitally by a ImagEM electron multiplier CCD camera (Hamamatsu, Japan). Endothelial cells were identified by staining for von Willebrandd factor (Dako, Glostrop, Denmark, and Jackson ImmunoResearch Labs., West Grove, PA) and nuclear integrity was confirmed with DAPI (4_,6_-diamidino-2-phenylindole hydrochloride) (Invitrogen Corp, Carlsbad, CA). Once endothelial cells with intact nuclei are identified, they were analyzed with Image-J software (National Institutes of Health, USA). Values for each protein were reported as a ratio of endothelial cell to human umbilical vein endothelial cell average pixel intensity.

Statistical Analysis

Descriptive statistics, including mean, standard deviations, standard error of the means, and minimum and maximum values were obtained. Normal distribution for all dependent variables was confirmed using Shapiro-Wilkins and Smirnoff tests (at least one test $p>0.05$). In general, *t*- tests and analyses of variance (ANOVA) will be performed for baseline comparisons. For experiment 1, 2-way repeated

measurements-ANOVAs comparing dependent variables before and after EECP session (time), and between active and sham groups (group) were performed. For experiment 2, two-way repeated measurements-ANOVAs were performed comparing dependent variables within exercise intensities and between exercise types. For experiment 3, 3x2-way repeated measurements-ANOVAs comparing dependent variables before and after exercise intervention (time) and between the three study groups (group) was performed. Fisher's Least Significant Difference (LSD) was used as *post-hoc* analysis. All statistical analyses were performed using SPSS (version 16.0, Chicago, IL), and statistical significance was considered when $p<0.05$.

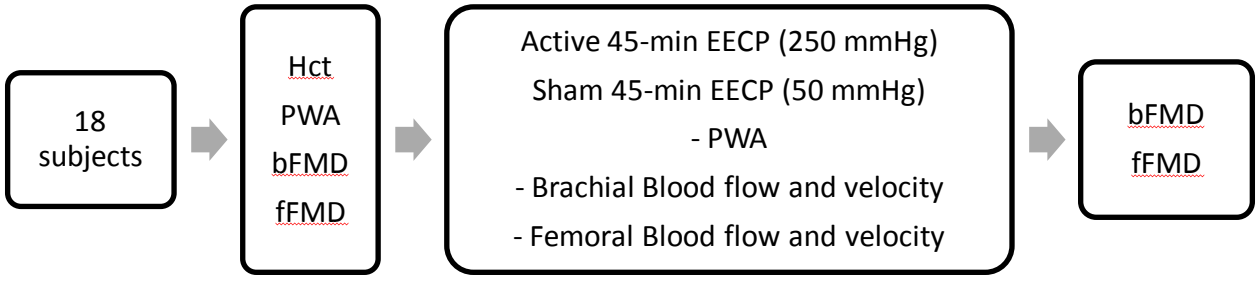


Figure 2-1. Experiment 1 study design. Hct=hematocrit; PWA=pulse wave analysis; bFMD=brachial flow mediated dilation; fFMD=femoral flow mediated dilation

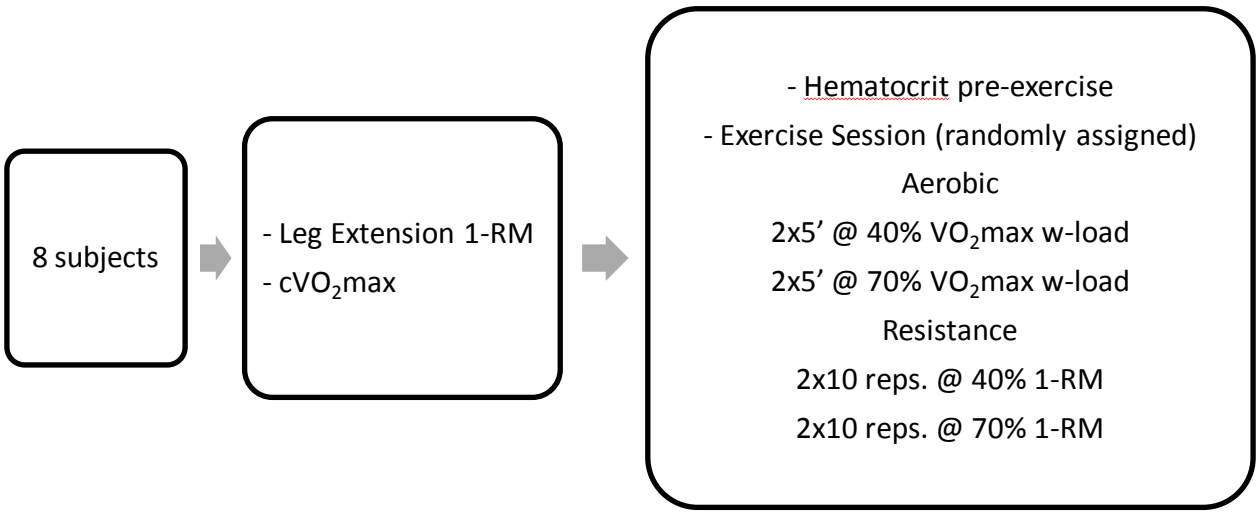


Figure 2-2. Experiment 2 study design. 1-RM=one repetition maximum test; cVO_2max=cycle maximal oxygen uptake test

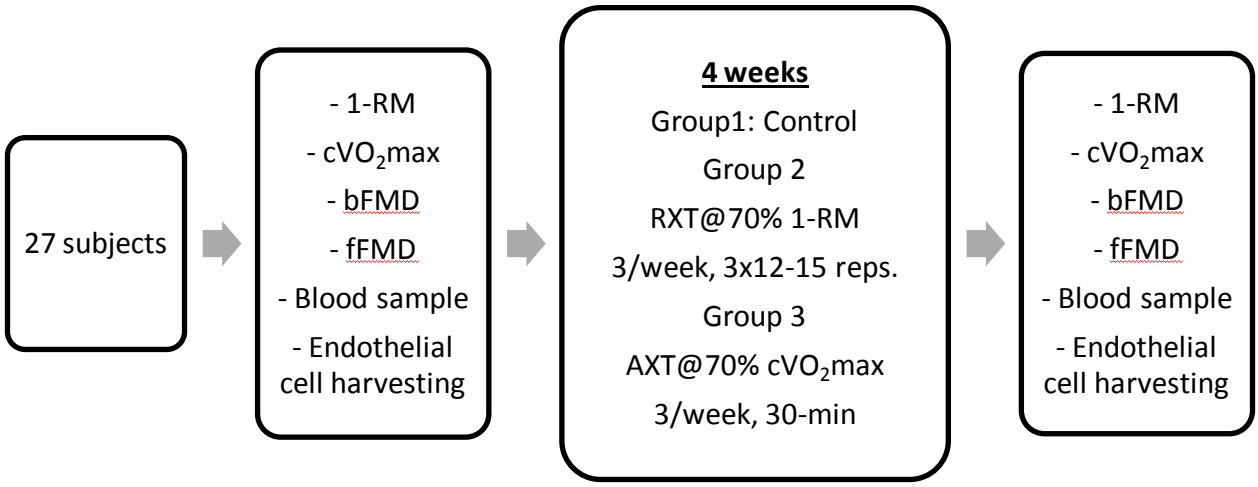


Figure 2-3. Experiment 3 study design. 1-RM=one repetition maximum test; cVO_2max=cycle maximal oxygen uptake test; bFMD=brachial flow mediated dilation; fFMD=femoral flow mediated dilation

CHAPTER 3
LITERATURE REVIEW

Cardiovascular diseases (CVD), including coronary artery disease (CAD) and stroke, are responsible for over 19 million deaths annually worlwide, with a direct and indirect cost of more than $430 billion in the United States alone.(160) Several cardiovascular risk factors (CVRF) are associated with the development of ischemic CVD, such as dyslipidemia, obesity, high blood pressure, and physical inactivity. However, it is the decrease of blood flow to noble tissues, due atherosclerotic lesions, that produces mortality and morbidity outcomes.(133, 134) Although atherosclerotic plaque formation is the pathophysiologic event responsible for acute ischemic events, endothelial dysfunction is the primary cause of atherogenesis.(113, 117, 204) Exercise training decreases traditional CVRF associated to endothelial dysfunction. However, the benefits of exercise training on traditional CVRF do not explain the overall decrease in CVD incidence, leaving ~40% of this improvement to unknown factors.(128, 184) Therefore, exercise could have a direct regulatory effect on endothelial function via blood flow patterns, exercise-induced shear stress, and endothelial mechano-transduction.

The following literature review will be focused on three major topics 1) endothelial (dys)function and endothelial oxidative stress; 2) blood flow patterns and shear stress; and 3) endothelial mechano-transduction and its relationship with blood flow patterns and endothelial health.

Endothelial (Dys)Function

Endothelial Function

The arterial wall consists of three layers: the intima, the media, and the adventitia. The vascular endothelium is a single cell layer that linen the intima and it is in direct contact with the blood.(204) In the past, the endothelium was considered only as an anatomical barrier between circulating blood and the vessel wall. Currently, and after Furchgott and Zawadzki studies (64), the endothelium acts as a metabolically active barrier that regulates vascular tone, platelet aggregation, and vascular smooth muscle cell (VSMC) proliferation.(92, 108, 130) The endothelium releases several vasodilators, including nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF), as well as vasoconstrictors, such as endothelin-1 and angiotensin-II.(115, 193) Although NO is a potent vasodilator; it also regulates endothelial permeability (132), platelet aggregation (190), immune system activation (113), smooth muscle cell proliferation (208), and vascular remodeling (163) making NO a key player on the pathophysiology of atherosclerosis.(29, 204)

NO is a reactive nitrate species (RNS), although it could be also considered a reactive oxygen species (ROS), which will directly affect endothelial redox/oxidative balance.(29, 38, 186) As a short half-life gas (<10 seconds), NO rapidly diffuses endothelial cell membrane towards the vascular lumen, the subendothelial space and the media. As proposed by Matsushita, Lowenstein *et al.* (121), NO acts via S-nitrosylation of first or second messengers. On the endothelial surface, NO diffuses platelet membrane and activates soluble guanylate cyclase (sGC) to decrease platelet aggregation (65), and exerts anti-inflammatory properties limiting the expression of vascular cell adhesion molecule-1 (VCAM-1).(46) In the media, NO inactivates RhoA

decreasing VSMC proliferation.(208) From the subendothelial space, NO diffuses into VSMC and activates sGC. Once activated, sGC dephosphorilates guanosine triphosphate (GTP) to cyclic guanosine-3',5-monophosphate (cGMP). Increased levels of cGMP lead to an increase in protein kinase G (PKG), which activates SERCA pump decreasing intracellular Ca^{2+}, and dephosphorilates VSMC myosin light chains eliciting VSMC relaxation.(127, 194)

Endothelial function is normally focused on NO bioavailability, thus a decrease in NO bioavailability is considered endothelial dysfunction.(136, 204) NO bioavailability will depend on two basic events: NO synthesis and NO consumption. NO is synthesized from the amino acid L-arginine by a family of enzymes, the NO synthases (NOS), which can be constitutively found within the endothelial cell (endothelial (e)NOS or NOS-3) or cytokine-induced (i)NOS (or NOS-2).(3, 127, 130)

Even though NO synthesis from L-arginine is a straightforward process, where L-arginine and oxygen (O_2) are the substrates, NO and citrulline are the products, and NOS is the enzyme, there are multiple factors and co-factors that are involved in NO production:

- **Stimulus:** Two primary stimuli activate NO-production pathway 1) chemical and 2) mechanical. Chemical stimuli include G-protein receptor-dependent agonists such as acetylcholine (Ach), bradykinin, and histamine. Once the G-protein receptor is activated, the α_q subunit increases cytosolic inositol 1,4,5-triphosphate (IP_3), which activates endoplasmic reticulum Ca^{2+} channels increasing cytosolic Ca^{2+} concentration. In addition, β and γ subunits activate several kinases, such as protein kinase A (PKA) and Akt/PKB, which will phosphorylate eNOS.(165, 186) Blood flow-induced shear stress is the main mechanical stimulus and it represents the major physiological factor for the release of NO. In general, shear stress opens transmembrane receptor-independent Ca^{2+} channels, increasing cytosolic Ca^{2+} concentration, and activates an intracellular mechano-transduction complex, glycocalyx-integrins/cytoskeleton, that will increase eNOS phosphorylation and activate eNOS transcription factors increasing eNOS

expression. The endothelial cell mechano-transduction pathways is described in detail on page 53.(24, 41, 186)

- **eNOS activation:** eNOS activation requires two events 1) phosphorylation and 2) binding of Ca^{2+}-calmodulin complex (CaM). Increased cytosolic Ca^{2+} binds to cytosolic calmodulin to create CaM. CaM will bind eNOS reductase domain and, with Akt/PKB-mediated phosphorylation at Ser-1177 and PKA-mediated dephosphorylation at Thr-497. eNOS reductase domain will accept NADPH electrons (e^-) and start a redox potential-induced electron flow. This electron flow will transport e^- to eNOS oxigenase domain where they will interact with two cofactors to complete the overall reaction.(3, 165)

- **Cofactors:** the final step of the reaction needs two cofactors 1) haem-iron group (Fe^{3+}) and 2) tetrahydrobiopterin (BH_4). While Fe^{3+} will be the final e^- receptor, BH_4 has a more complicated function. BH_4 promotes NADPH coupling and L-arginine binding, and its absence or oxidation by ROS leads to uncoupling of eNOS, thereby inducing further superoxide ($O_2^{\bullet-}$) formation.(3, 87, 106)

- **L-arginine supply:** L-arginine is synthesized from L-citrulline (produced in the gut) in a two-step reaction involving the enzymes arginosuccinate synthase and lyase (ASS and ASL, respectively). The L-arginine synthesized in the proximal tubules of the kidney cortex provides the major endogenous supply distributed throughout the body. Although, total L-arginine synthesis is preserved in chronic kidney disease (CKD), it is thought that endothelial L-arginine production could be compensatory in CKD because vascular endothelium has ASS and ASL necessary for arginine synthesis.(9, 127)

- **eNOS competitive inhibitor:** Methylated protein degradation, normally mediated by oxidative stress, increases dimethylarginines concentration.(12, 87, 123) Among these dimethylarginines, asymmetric-dimethyl-arginine (ADMA) is an eNOS competitive inhibitor. Increased concentration of ADMA inhibits eNOS and increases risk of CVD.(12) However, it is ADMA regulation by dimethylaminohydrolase (DDAH) that is responsible for altered ADMA up-regulation.(9, 12) DDAH metabolizes ADMA to citrulline and dimethylamine, and its activity is decreased by oxidative stress.(9, 12, 33, 186)

- **Others:** Several other factors can influence NO synthesis. ***1) eNOS location:*** it has been shown that eNOS associated with the caveolae portion of the plasma membrane is more active than non-caveolae or cytoplasmic eNOS.(165) ***2) L-arginine transporter:*** as the majority of L-arginine comes from renal synthesis, it has to be transported into the endothelial cells.(9) The cationic amino acid transporter (CAT)-1 is the primary and most functionally significant in endothelial cell L-arginine transport. CAT-1 also transports ADMA, symmetric-DMA, lysine, and ornithine, which are competitive inhibitors of L-arginine.(9, 51) ***3) Heat shock protein-90 (HSP 90):*** The molecular chaperone HSP 90 has been identified as a regulator of eNOS activity, possibly as an allosteric modulator. Even though the mechanism of action is not well known, HSP 90 presence increases eNOS activity

by 3-fold. In addition, studies show that HSP 90 is up-regulated after shear stress and down-regulated after oxidative stress.(3, 67, 165, 186)

NO consumption depends upon physiological needs. While some NO is consumed in physiological events, physiological-inactive NO catabolic pathway is ROS-dependent.(131, 185) If endothelial cell oxidative stress is low, NO is metabolized to nitrite (NO_2^-) and then to nitrate (NO_3^-).(9, 119) Moreover, blood nitrate/nitrite ratio (NOx) after a 24-hour low nitrate diet is considered a good estimate of overall NO bioavailability.(142) In contrast, an increased endothelial cell oxidative stress will increase superoxide ($O_2^{\bullet-}$) production, which has high NO affinity producing peroxinitrite ($ONOO^-$), an even more active ROS. $O_2^{\bullet-}$ presence will decrease NO bioavailability, decreasing NOx with a further increase in endothelial cell oxidative stress.(9, 87, 119, 131, 185, 186)

Although plasma NOx is an overall measurement of NO bioavailability, it does not assess endothelial function per se. Flow mediated dilation (FMD), blood flow-induced arterial dilation after 5-min ischemia (reactive hyperemia), is considered a biomarker for endothelial function.(8, 22, 34) Moreover, evidence shows that brachial FMD is well correlated with coronary artery endothelial function (5, 176) and that reactive hyperemia produces the same effects that acetylcholine infusion.(93) In addition, clinical studies have shown an improvement in coronary endothelial function and brachial FMD after exercise training in CAD patients (59, 82, 84), and recent studies have proven that brachial FMD can predict cardiovascular events.(167, 202, 203)

As previously described, there are multiple factors that influence eNOS activity, NO synthesis, and NO consumption affecting NO bioavailability. Consequently, endothelial dysfunction can be established via five primary mechanisms: 1) L-arginine

deficit, 2) presence of eNOS and CAT-1 competitive inhibitors, 3) eNOS cofactors (BH_4 and HSP 90) deficit, 4) decreased eNOS expression, and 5) increased NO oxidation. All five mechanisms are directly or indirectly associated with endothelial cell oxidative stress.(3, 87, 119, 136, 186) For this reason, and not surprisingly, the first step in endothelial dysfunction is endothelial cell oxidative stress.(29, 87, 131, 136, 165, 185) However, new theories focused on endothelial dysfunction origin and sustainability, based on endothelial mitochondrial dysfunction, have been lately raised.(1) A brief description of endothelial cell oxidative stress origin follows.

Endothelial Cell Oxidative Stress

Although endothelial cell oxidative stress has been associated with the overall process of atherosclerosis and unstable plaque formation (27, 28, 116, 130, 133-135, 204), it is oxidative stress-induced endothelial dysfunction that is the first step toward cardiovascular diseases. Endothelial oxidative stress results from an increase ROS production or a decreased cell anti-oxidant capacity.(92, 130) Endothelial ROS production has two major sources.

- Nicotamine adenine dinucleotide phosphate (NADPH) oxidases are transmembrane complexes that can transfer electrons across membranes to oxygen and produce $O_2^{\bullet-}$.(92, 130, 135) Traditional cardiovascular risk factors, such as tobacco, hypercholesterolemia, obesity, and hypertension, upregulate endothelial NADPH oxidase via activation of Angiotensin II and Tumor Necrosis Factor-alpha cascades increasing $O_2^{\bullet-}$ production.(92, 130, 135) In addition, endothelial NADPH oxidases react to oscillatory shear stress and short-term vascular stretch increasing $O_2^{\bullet-}$ production in vitro, which is inhibited by NO.(58, 94)

- As described earlier, a decreased BH_4 content produces uncoupled eNOS which increases $O_2^{\bullet-}$ production.(3, 87, 106)

Concerning the endothelial anti-oxidant system, NO itself is a major component. When NO production largely exceed $O_2^{\bullet-}$ production, produced $ONOO^-$ is metabolized

into nitrogen trioxide (N_2O_3) and nitrite ion (NO_2^-) decreasing overall oxidative stress.(100, 185) However, Dai *et al.* (36) have showed that ESS activates nuclear factor-like 2 (Nrf2) via the PI3-K/Akt pathway, decreasing ROS production independently of NO concentration. In addition, and regardless NO- or non-NO-mediated anti-oxidant balance, the three isoforms of superoxide dismutase (SOD) (ie: extracellular (EC), cytosolic copper and zinc (CuZn), and mitochondrial manganese (Mn)) are present on vascular walls, where EC-SOD appears to be the predominant endothelial anti-oxidant.(92, 173) The facts that endothelial MnSOD content is 10 times lower than in other human tissues (173), and that thioredoxin is the main endothelial mitochondrial anti-oxidant (205) could explain new theories regarding the role of mitochondrial dysfunction on the onset of endothelial dysfunction. A decrease on mitochondrial function would increase mitochondrial $O_2^{\bullet-}$ production and a more anaerobic metabolism would alter redox balance increasing oxidative stress.(1, 17, 149)

In summary, endothelial function depends upon a delicate balance between NO production and oxidative stress, both mediated by the presence of blood flow-induced shear stress and its transduction into the endothelial cell (Figure 3-1).

Blood Flow Patterns and Shear Stress

As previously described, blood flow-induced shear stress is an important, if not the most important, regulator factor of endothelial function. Before describing the physiological interactions between blood flow, shear stress, and endothelial function, it is important to define several arterial hemodynamic concepts.(24, 41, 56)

- **ENDOTHELIAL SHEAR STRESS (ESS OR T):** The tangential force derived by the friction of the flowing blood on the endothelial surface. It is the product of the shear rate at the wall and the blood viscosity (μ). ESS is expressed in units of force/unit area (N/m^2 or Pascal), and it can be calculated using $\tau = 4\mu Q/(\pi \bullet r^3)$,

where *Q* is blood flow rate or velocity and *r* is arterial radius, derived from Poiseuille's law.(143)

- **SHEAR RATE (SR):** The spatial gradient of blood velocity, which is normally used to estimate ESS.(20, 144, 187) SR is expressed in units of time-rate (s^{-1}), and it can be calculating using $SR = 4\overline{Q}/d$, where $\overline{Q}$ is the average blood flow and *d* is arterial diameter that is used to normalize FMD; while the equation $SR = Q/d$ is used to determine SR in a specific point time. Physiologically, the shear rate decreases at the center of the lumen and gradually increases toward the wall.

- **BLOOD VISCOSITY (μ):** A principal property of blood related to its internal friction that causes blood to resist flow. Hematocrit is the major determinant of blood viscosity.

- **NEWTONIAN BLOOD BEHAVIOR:** Constant blood viscosity independent of shear rate. Blood behaves as a Newtonian fluid when SR is greater than 100 s^{-1}. In large-sized arteries (e.g., aorta and brachial) blood behaves largely in a Newtonian fashion.(172)

- **NON-NEWTONIAN BLOOD BEHAVIOR:** Non-constant blood viscosity is inversely related to shear rate. Blood has non-Newtonian properties, especially in veins, small-sized arteries, and in the microcirculation.(172)

- **LAMINAR BLOOD FLOW:** Smooth, streamlined blood flow where viscous forces prevail against inertial forces. Laminar flow can be undisturbed, as observed primarily in relatively straight arterial segments, or disturbed characterized by reversed flow (i.e., flow separation, recirculation, and reattachment to forward flow). Due to the vascular tree characteristics in humans, it is highly probable that disturbed laminar blood flow is the primary blood flow type.

- **TURBULENT BLOOD FLOW:** Flow in which the blood velocity at any given point varies continuously over time, even though the overall flow is steady. In turbulent flow the inertial forces are more significant than viscous forces. Turbulent blood flow rarely occurs but has been described in human aorta at peak systole, during heavy exercise in much of the central arterial system, distal to severe stenoses (>75%), and in aneurysms.

- **REYNOLDS NUMBER (RE):** Dimensionless ratio of blood inertial forces to viscous forces. For a given geometry, whether the flow will be laminar or turbulent is determined by its Reynolds number. For low Re values blood flow is laminar, whereas for high Re values (typically, above 2,000) blood flow is turbulent. Re can be calculated using $\mathrm{Re} = \frac{\overline{V} D \rho}{\mu}$, where $\overline{V}$ =blood flow velocity; *D*=blood vessel diameter; *ρ*=blood density; and *μ*=blood viscosity.(138)

- **STEADY BLOOD FLOW:** Blood flow in which velocity does not vary with time. This type of flow does not occur *in vivo*; however, it has been largely used on *in vitro* and computational fluid dynamic studies.

- **PULSATILE (UNSTEADY) BLOOD FLOW:** Blood flow with periodically changing velocity during the cardiac cycle. This is the primary blood flow type in small-sized muscular arteries and arterioles.

- **OSCILLATORY (BIDIRECTIONAL) BLOOD FLOW:** Blood flow with periodically changing direction (i.e. downstream or antegrade and upstream or retrograde). This is the primary blood flow type in conduit and large- and mid-sized muscular arteries (e.g. descending aorta, brachial, femoral, coronary, radial, and popliteal arteries).

- **STEADY ESS:** ESS that does not vary with time (i.e., constant direction and magnitude). This type of ESS does not occur in vivo.

- **PULSATILE ESS:** Unidirectional ESS with a magnitude varying, typically, within a range of 15 to 70 dyne/cm^2 over the cardiac cycle, yielding a positive time-average. This type of ESS occurs in small-sized muscular and large-sized elastic arteries.

- **LOW ESS:** Unidirectional ESS with a periodically varying magnitude over the cardiac cycle, yielding a significantly low time-average (<10 to 12 dyne/cm^2). This type of ESS occurs in the microcirculation.

- **OSCILLATORY ESS:** Bidirectional ESS with a periodically varying magnitude over the cardiac cycle, yielding a very low time-average, usually close to 0 dyne/cm^2. This type of ESS occurs in conduits and large- and mid-sized muscular arteries; however, time-average is >0 dyne/cm^2.(77, 200)

These definitions are a general convention on resting blood flow and its applications to *in vitro* and computational models. However, there is no convention on how blood flow behaves during exercise.(18, 35, 41, 60, 102, 105, 138, 156, 157, 164) Although it is known that exercise training reduces risk of cardiovascular (CV) events, ~40% of this reduction has no relationship with traditional CV risk factors.(74, 128) Considering the relevance of ESS on endothelial function and its direct relationship with atherosclerosis, research focused on exercise-induced blood flow as a direct modulator of endothelial function has grown during the last 10 years.(54, 71, 72, 75, 78, 79, 177, 181-183, 187)

Several human studies have shown improved endothelial function following exercise training, either in clinical or healthy populations.(20, 59, 68, 71, 77, 82-84, 158, 188, 198) However, it has not been established whether this endothelial function improvement is the direct result of exercise-induced blood flow or to the result of hormonal-induced systemic effects of exercise on endothelial cell phenotype.(78, 79, 108, 109, 183) *In vitro* and animal studies suggest that improved endothelial function is associated with upregulation of eNOS, which would be directly connected with blood flow-induced ESS and the endothelial mechano-transduction system (reviewed on page 53).(24, 41, 47, 48, 107, 110, 122, 189, 207) Regardless hormonal-induced systemic effects, blood flow redistribution during exercise might play an important role in systemic endothelial adaptations. During exercise, cardiac output and blood pressure increase and there is a vasoconstriction in non-exercising vascular beds.(10, 161) Although blood flow-induced shear stress in exercising vascular beds increases, there is no agreement on what happen in non-exercising vascular beds. There is a decrease in blood flow derived from peripheral vasoconstriction and lack of vasodilatory metabolites, which would decrease shear stress. However, based on the endothelial shear stress equation (page 46); a decreased vessel diameter would significantly increase shear stress.

These contradictory outcomes are reflected when trying to associate exercise-induce blood flow with local or systemic effects in humans. Hopman *et al.* (182) showed vascular adaptations to electrical stimulation-exercise only in “active” vascular beds in lower extremities of patients with spinal cord injury. In addition, Gokce *et al.* (71) observed improved femoral artery endothelial function, assessed via FMD, after 10

weeks of leg exercise in patients with CAD, while no significant difference was observed on brachial artery endothelial function. These studies support the idea that exercise induces local rather than systemic vascular changes. On the other hand, Green *et al.* (73) characterized brachial artery blood flow during cycle ergometry showing that 1) brachial artery blood flow was altered during leg exercise compared to resting conditions, and 2) brachial artery blood flow during cycle ergometry was oscillatory (i.e. antegrade and retrograde). In addition, Tanaka *et al.* (177) showed an increased shear stress in the brachial artery during leg-cycle exercise and in the femoral artery during arm-cycle exercise. Furthermore, Thijssen *et al.* (180) described that 1) brachial blood flow and shear rate increase in a dose-dependent manner during different intensities in three different leg-exercise modalities, and 2) there is an increased brachial retrograde blood flow during aerobic exercise while no brachial retrograde blood flow is observed during leg-resistance exercise. Although these studies used low intensity single exercise bouts, they confirm that changes in exercise-induced blood flow are systemic rather than only local (Figure 3-2).

In addition to the controversy surrounding exercise-induced local versus systemic effects on endothelial function, exercise-induced blood flow patterns and endothelial function studies in humans have also shown conflicting results. Using a one cuffed-arm/one non-cuffed-arm model, Tinken *et al.* (188) observed an increased brachial artery FMD after 4 weeks of handgrip training only in the non-cuffed arm, confirming that shear stress is needed to improve endothelial function *in vivo*. In addition, Tinken *et al.* (187) designed an interesting study including three different interventions 1) arm skin heating, 2) handgrip exercise, and 3) cycling exercise. Brachial artery shear rate

during both exercise types was matched to the brachial shear rate observed during arm skin heating. In addition, while one arm had normal blood flow, the other arm was cuffed to decrease it. They observed three important outcomes 1) both cycling and handgrip exercise produces brachial retrograde blood flow, 2) all interventions improve brachial artery FMD in the non-cuff arm and 3) brachial artery FMD is not improved in the cuffed arm. The authors conclude that shear stress is needed to improve endothelial function and that retrograde flow could impair it. Moreover, Thijssen *et al.* (181) observed a dose-dependent decrease on brachial artery FMD when resting arm blood flow was externally blocked using a pressure cuff inflated to three different pressures (i.e. 25, 50, and 75 mmHg). In addition, the authors observed a positive relationship between blood flow blockage and retrograde shear rate, which was highly correlated with decreased brachial artery FMD.

Although these results appear to be conclusive evidence that retrograde flow is detrimental for endothelial function, there is contrasting evidence from studies that used enhanced-external counter pulsation (EECP).(6, 21, 111, 125, 206) EECP is a non-invasive FDA approved treatment for coronary artery disease patients. It consists of three-300 mmHg pneumatic compression cuffs applied to each of the patient's legs, buttocks, and lower abdomen. The mechanism is synchronized with the patient's electrocardiogram such that with each cardiac cycle pressure is sequentially applied distally to proximally in early diastole, resulting in an increase in diastolic blood pressure (diastolic augmentation) and retrograde aortic diastolic blood flow.(13, 166) There are three basic hypotheses to explain EECP's mechanism of action (i.e. increased shear stress, increased vascular reactivity, and increased peripheral vascular function) and all

of them appear to be associated with beneficial effects of retrograde flow.(61, 124, 126, 140, 175) For example, Braith *et al.* (14, 21) have shown an improvement in endothelial function measured using the brachial and femoral artery FMD technique, and an increased plasma NOx and a decreased plasma endothelin-1. In addition, they showed an improvement in arterial stiffness measured using central and peripheral pulse wave velocity, and decreased inflammatory cytokines and adhesion molecules, after 35 sessions of EECP in patients with angiographic coronary artery disease. Furthermore, Nichols *et al.* (137) observed an improvement in peripheral arterial wall properties and wave reflection characteristics in a similar group of patients. Moreover, Zhang *et al.* (206), using a hypercholesterolemic pigs model, showed that EECP reduces hypercholesterolemia-induced endothelial damage and arrests vascular smooth muscle cell proliferation and migration by increasing aortic wall retrograde shear stress, which in turn activates the eNOS/NO pathway. In general, EECP outcomes mimic the ones observed after exercise training what could describe EECP as a "passive-exercise" intervention.

Thus, there is strong evidence that retrograde blood flow can be either beneficial or detrimental. The explanation for these seemingly contradictory outcomes is presently unclear. With respect to exercise and retrograde flow, previous studies have focused on exercise intensity and oscillatory blood flow. However, turbulence has not been carefully investigated. It is generally accepted that undisturbed laminar flow improves endothelial function, while disturbed laminar and turbulent flows are detrimental to endothelial function.(24, 41) Recent evidence from animal models of CAD (27, 28) has shown that turbulent flow produces less endothelial dysfunction and more stable

plaques than low laminar flow.

Presence or absence of blood flow turbulence depends upon three main factors 1) flow speed, 2) vessel diameter, and 3) blood viscosity. These three factors can be quantified by the use of a dimentionless variable called Reynolds number (Re). Although there is no convention on Re in the human circulation, it is assumed that Re ranges between 400 and 3000 in small-sized muscular and large elastic arteries, respectively.(18, 35, 41, 60, 102, 105, 138, 156, 157, 164) As described earlier, during exercise retrograde blood flow and shear rate are increased. In addition, flow velocity and vessel diameter are increased as well, thereby increasing Re. Thus presence or absence of turbulence will depend on which artery is studied. It is highly probable that the aorta will increase turbulent blood flow in an oscillatory way, while small-sized muscular arteries will increase laminar ESS. It is unknown what would happen in conduit arteries (e.g. coronary and carotid arteries) and large- and mid-sized muscular arteries (e.g. brachial, femoral, radial, and popliteal arteries), although a trend toward turbulent oscillatory flow is expected.(24, 138) The presence, or absence, of turbulent blood flow could be the unexplained factor responsible contradictory results observed during retrograde blood flow.

In summary, we could state that 1) ESS is needed to upregulate endothelial function; 2) blood flow in conduit arteries is oscillatory, showing antegrade and retrograde shear rate; 3) exercise increases overall shear rate and retrograde blood flow; and 4) blood flow during exercise is assumed to be oscillatory and laminar, despite the fact that there is no *in vivo* evidence to support the assumption. Therefore, in this dissertation project I propose to characterize blood flow in upper and lower extremities

during a single 45-minute EECP session and during exercise. I anticipate that blood flow patterns will depend on direction and turbulence. Thus, we are proposing a new blood flow classification scheme including: 1) antegrade laminar, 2) retrograde laminar, 3) antegrade turbulent, and 4) retrograde turbulent. In addition, I determined the effects of the various blood flow patterns on endothelial function. We anticipate that knowledge gained from this new blood flow classification scheme will improve our understanding regarding blood fluid mechanics interaction with the vascular endothelium. This fluid mechanics interaction would induce different type of ESS interacting in different ways with the endothelial mechano-transduction system.

Endothelial Mechano-Transduction

Although *in vitro* studies have shown that ESS can affect endothelial cells in several different ways, such as ion channel openings, G-protein receptor activation, or tyrosine kinase receptor activation, mechanical blood flow-generated shear stress stimulus *in vivo* is transduced into the endothelial cell via the integrin/cytoskeleton mechano-transduction pathway.(24, 26-28, 41, 63, 67, 89) This mechano-transduction pathway has three "mechanical" components that can elicit biochemical-signaling processes within the endothelial cell; 1) flow receptors or the glycocalyx, 2) transmembrane proteins called integrins, and 3) cytoskeletal filaments such as actin filaments.(37, 41, 118, 150, 168, 178, 195, 201) Each of these components will be described in the following paragraphs.

The glycolcalyx, first described as an immobile sheet of plasma and macromolecules (195), got vascular attention when Ryan *et al.* (162) showed an increased vascular binding of immune complexes, complement activation, and intravascular coagulation when the glycocalyx was damaged. Due to glycocalyx's

association with ESS effects, it was first assume that it was an apical membrane integrin. However, glycocalyx is a heparan sulfate proteoglycan fluid flow mechanosensor anchored to the apical membrane of endothelial cells.(63, 150, 195)

Three different glycocalyx functions have been described 1) modulation of permeability in the transcapillary exchange of water, 2) regulation of red and white blood cell interactions, with emphasis on the inflammatory response, and 3) mechano-transduction of fluid shear stress to the endothelial cytoskeleton.(104, 162, 195) Although the first function has an important impact on the microvasculature, this review will focus on the glycocalyx as a flow receptor and its interactions with endothelial mechano-transduction and endothelial function.

Pries *et al.* (151) presented the first piece of evidence that glycocalyx was a flow receptor when they were able to demonstrate that glycocalyx significantly increases vascular resistance, implying that mechanical forces were involved. Although the glycocalyx cannot produce any biochemical reaction itself, due to its molecular configuration (150, 195), integrins activation, NO production, signaling pathways, and endothelial function are impaired when the glycocalyx are damaged or not present.(50, 63, 67, 104, 201) This has been recently confirmed by Paszeck *et al.*(147) Using a chemo-mechanical model, they were able to determine that integrin-flow activation is largely mediated by the glycocalyx, where a lower integrin clustering activation is seen when the effective glycocalyx length is increased.

Currently, Thi *et al.* (178) have proposed a glycocalyx working model that is the best accepted so far. This 'bumper-car' model shows that the glycocalyx is connected to integrins, gap junctions, vinculins, and syndecans via an actin cortical web and stress

fibers, which are structural filaments within the endothelial cell cytoskeleton. When the glycocalyx is present, ESS mechanically 'pushes' against it pulling the actin cortical web, thereby producing an integrated torque and cell deformation in the direction of blood flow. This cell deformation produces mechanical force transductions via cytoeskeletal stress fibers activating integrins (Figure 3-3).(41, 63, 67, 147, 178, 201) In addition, cell deformation appears to open calcium (Ca^{2+}) ion channels increasing intracellular Ca^{2+}which binds calmodulin and activates eNOS.(63, 178)

When glycocalyx is removed, endothelial cell deformation is absent and endothelial dysfunction occurs.(50, 147, 150, 162, 178, 195) For example, Devaraj *et al.* (50) were able to link C-reactive protein with glycocalyx presence and endothelial dysfunction. In that study, using human aortic endothelial cells incubated in different C-reactive protein concentration and an *in vivo* rat model, the authors showed that C-reactive protein damaged the glycocalyx and that the extent of damage was inversely related with eNOS activity. These results give a better understanding of C-reactive protein as an endothelial dysfunction risk factor, indirectly via mechanical factors rather than directly as an inhibitor of eNOS activity.

Integrins are transmembrane glycoproteins ubiquitously located on the endothelial cell membrane. The extracellular domain binds directly to extracellular matrix (ECM) proteins, such as vitronectin, fibronectin, laminin, and collagen; while the cytoplasmic domain interacts with signaling molecules and cytoskeletal proteins to regulate cellular events, such as signal transduction, cytoskeletal organization, and cell motility via the modulation of integrin affinity and/or avidity.(24, 168) Structural effects, such as cell motility and cytoskeletal organization, are elicited via small GTPases (e.g.

RhoA, Cdc42, and Rac) when baso-lateral integrins are mechanically activated via ECM shear. In addition, integrin cytoplasmic domains regulates several shear-activated kinases, such as focal adhesion kinase, which could synergistically activate eNOS.(168)

Interestingly, Tzima *et al.* (191) showed an ESS-induced integrin upregulation on the basal membrane and not on the apical one, although ESS was applied directly to the apical membrane. In addition, Thi *et al.* (178) showed that apical integrins are able to "sense" flow and may produce some cell deformation during ESS; however, this deformation is not enough to upregulate NO.(63, 178) Moreover, Paszek *et al.* (147), as described earlier, showed that integrins activation depends on glycocalyx structure, where effective glycocalyx thickness modulates integrin clustering and activation. Finally, Maniotis *et al.* (118), using a very elegant approach pulling integrins with micropipettes, were able to show that integrins, cytoskeletal filaments, and nucleoplasm are mechanically connected. These findings suggest that integrins are part of the endothelial mechano-transduction system but 1) they need the glycocalyx and cytoskeletal filaments to be activated, 2) the main effect is seen on the basal membrane associated with ECM and cell organization, and 3) integrins activation via endothelial mechano-transduction may have a direct impact on gene expression.(44, 146)

Cytoskeletal filaments, mainly α- and β-actin, provide elastic stiffness and maintain the shape and structure of the cell. However, there is convincing evidence showing that cytoskeletal filaments function is more active than that.(40, 41, 44, 89, 90, 96, 118, 129, 146, 178, 192) Davies *et al.* (43) first observed structural endothelial cell changes after ESS, suggesting changes in the cytoskeleton. Moreover, DePaola *et al.* (49) showed endothelial cell density differences when different flow patterns were used

to induce ESS. Although these early studies were focused to show ESS-induced changes in the overall cell function and turnover, they describe structural changes that are associated with the cytoskeleton. In addition, Maniotis *et al.* (118), as described earlier, showed that cytoskeletal filaments and nucleoplasm are mechanically connected, and Danuser (37) observed a dynamic coupling of the actin network during cell protrusion. These findings support an active rather than just structural function of the cytoskeletal filaments.

Cytoskeletal deformation and displacement, such as actin filament deformation, are linked to force transmission to 'remote' cellular sites.(41, 96) In fact, Helmke and Davies (89) created the 'decentralized model' of endothelial mechano-transduction, where cytoskeletal deformation plays a major role 'transmitting' ESS, sensed by the glycocalyx at the apical membrane, to the basolateral membrane and nucleus to activate integrins and transcription factors, respectively. This theory has been tested using computational mapping systems, showing that cytoskeletal filaments move at the onset of ESS, before integrins and transcription factors are activated.(90, 96, 129) Furthermore, this cytoskeletal deformation can be extended to adjacent cells as a type of 'chain reaction' via the same mechanism, activating other mechano-transducers, such as platelet endothelial cell adhesion molecule (PECAM)-1.(41, 178, 192)

This mechanical interaction between glycocalyx, cytoskeletal filaments, and integrins will be converted to chemical activity, or 'true' mechano-transduction, in several ways along the mechano-transduction pathway. Following a time-line from the onset of ESS, glycocalyx deformation produces activation of ion channels (e.g. Ca^{2+} channels), G-protein receptors, and triggers cytoskeletal filaments deformation.

Transmembrane receptor-independent Ca^{2+} channels open and induce an increase in cytosolic Ca^{2+} concentration.(24, 41) In addition, once G-protein receptors are activated, cytosolic Ca^{2+} increases via IP_3-induced endoplasmic reticulum Ca^{2+} channels activation, and eNOS is activated and phosphorylated via CaM and PKA - Akt/PKB pathways, respectively.(165, 186) Then, cytoskeletal filament deformation would produce nucleus deformation and integrins activation.(41, 44, 66, 146, 168, 191) Nucleus deformation could directly activate gene expression, such as eNOS and SOD genes.(36, 44, 66, 146) Finally, integrins activation could modulate expression of transcription factors, such as Nerf2 and protein-rich tyrosine kinase (PYK2) (36, 42, 44, 146), and will regulate some shear-induced kinases, such as focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK). FAK activation would activate mitogen-activated protein kinase (MAPK), increasing endothelial cell migration and adhesion to ECM, and RhoA, promoting the formation of cytoskeletal stress fibers.(112, 168) It is also thought that integrins could mediate eNOS phosporylation via PI3K-Akt and PKA pathways (Figure 3-4).(165, 168, 186)

Summary

In summary, endothelial function depends upon two main factors, 1) blood flow mediated mechano-transduction and 2) endothelial oxidative stress, which, in general, is ESS/mechano-transduction-dependent.(24, 36, 41, 130, 178) Although endothelial mechano-transduction is a well organized and synergistic system, it appears that the cytoeskeltal filaments (i.e. actin cytoskeleton) are necessary for many, if not all, mechano-transduction processes.(41, 89, 168) Based on the decentralized model of endothelial mechano-transduction, cytoskeletal deformation and endothelial cell migration would depend upon flow-dependent glycocalyx shift.(40, 41, 89) When ESS

is physiological and laminar, the glycocalyx are ‘oriented’ downstream pushing cytoskeletal filaments toward blood flow and ‘pulling’ actin-stress fiber ‘levers’ activating anti-atherogenic processes (Figures 3-3 and 3-4).(146, 178) If ESS is lower-than-physiological, glycocalyx is downregulated and no mechano-transduction is developed. Consequently there is increased oxidative stress and endothelial phenotype becomes pro-atherogenic. Similar to what is observed if the glycocalyx are damaged.(50, 201) When ESS is higher-than-physiological and/or antegrade-turbulent, endothelial cell displacement decreases, endothelial oxidative stress increases, and atherogenic lesions shift to stable lesions.(26-28, 36, 96) These different outcomes confirm different mechano-transduction signaling pathways, all of them leaded by the glycocalyx/blood flow interaction.

Even though retrograde flow significantly increases during exercise, exercise-induced retrograde blood flow relationship with endothelial function has never been studied. If every blood flow pattern activates the endothelial mechano-transduction system in a unique way, retrograde-laminar and retrograde-turbulent patterns should have a particular blood flow/glycocalyx interaction. Studying the blood flow patterns/endothelial function/endothelial oxidative stress interaction will allow a better understanding of exercise as an endothelial function regulator.

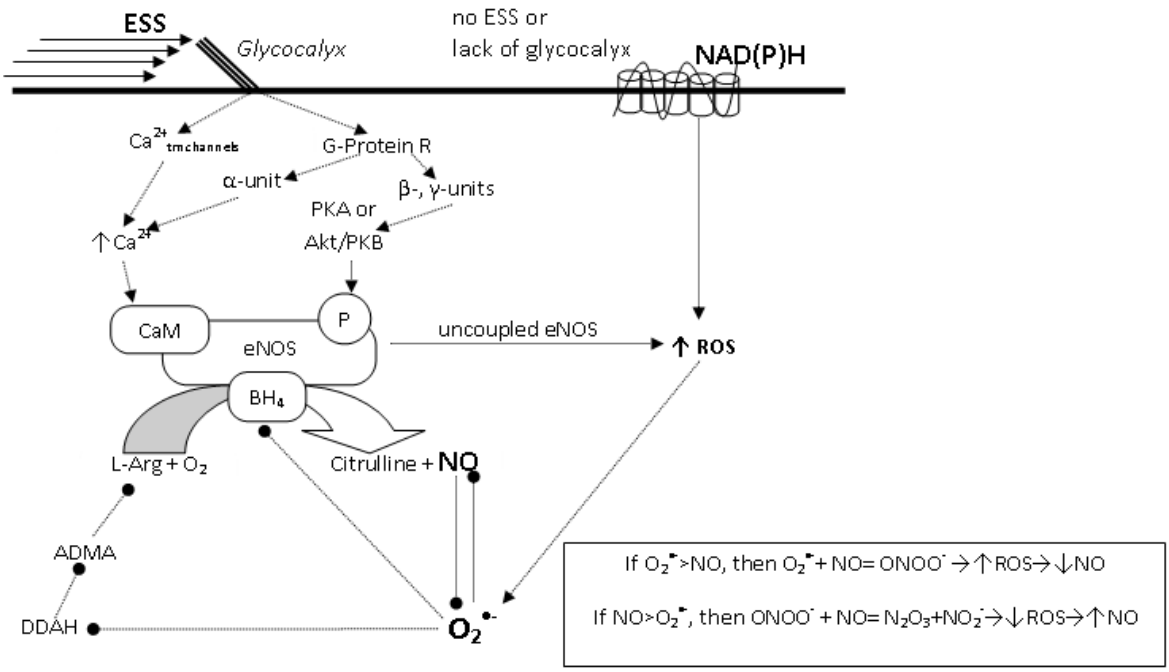


Figure 3-1. Delicate balance between NO production and oxidative stress (ROS). ESS=endothelial shear stress; NAD(P)H=NADPH oxidases; Ca2+tm channels= transmembrane receptor-independent Ca2+ channels; PKA=protein kinase A; Akt/PKB=Akt/protein kinase B; CaM=Ca2+/calmodulin complex; eNOS=endothelial nitric oxide synthase; L-Arg=L-arginine; BH4=tetrahydrobiopterin; ADMA=asymmetric-dimethyl-arginine; DDAH=dimethylaminohydrolase; NO=nitric oxide; O2•-=ion superoxide. (Adapted from Thuillez and Richard(186))

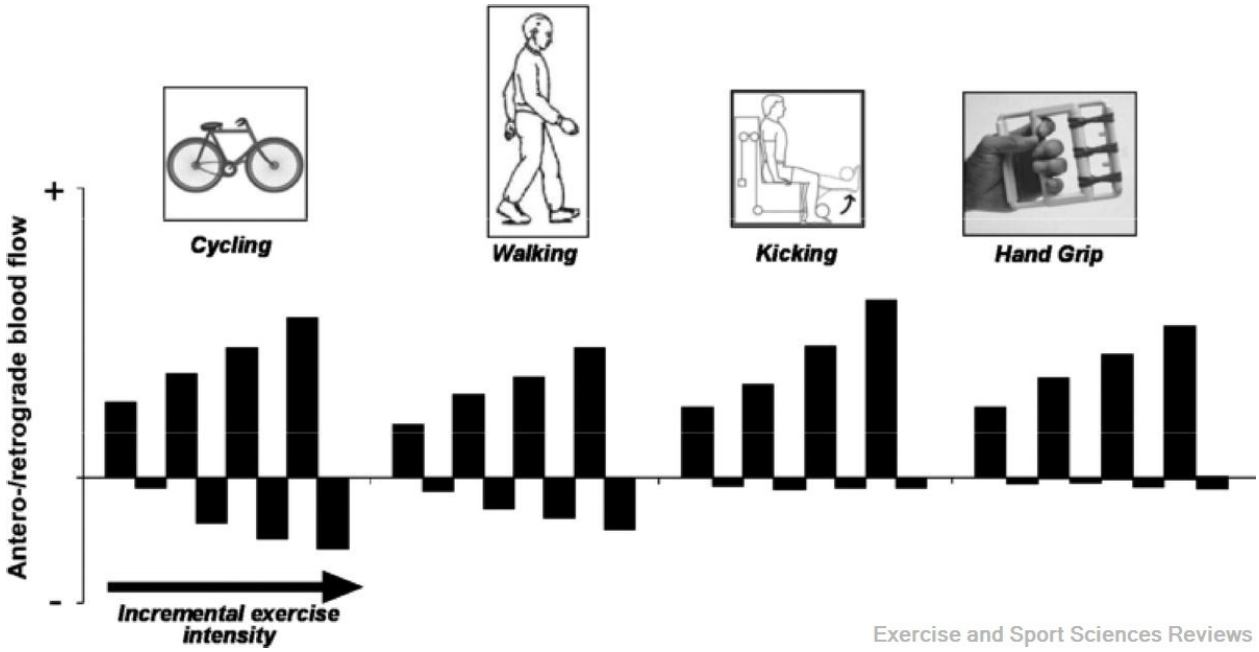


Figure 3-2. Effect of different exercise modalities and intensities on brachial artery blood flow patterns. Antegrade shear rate increases in a dose-dependent manner, while retrograde shear rate increases in aerobic lower-extremities exercises.(74)

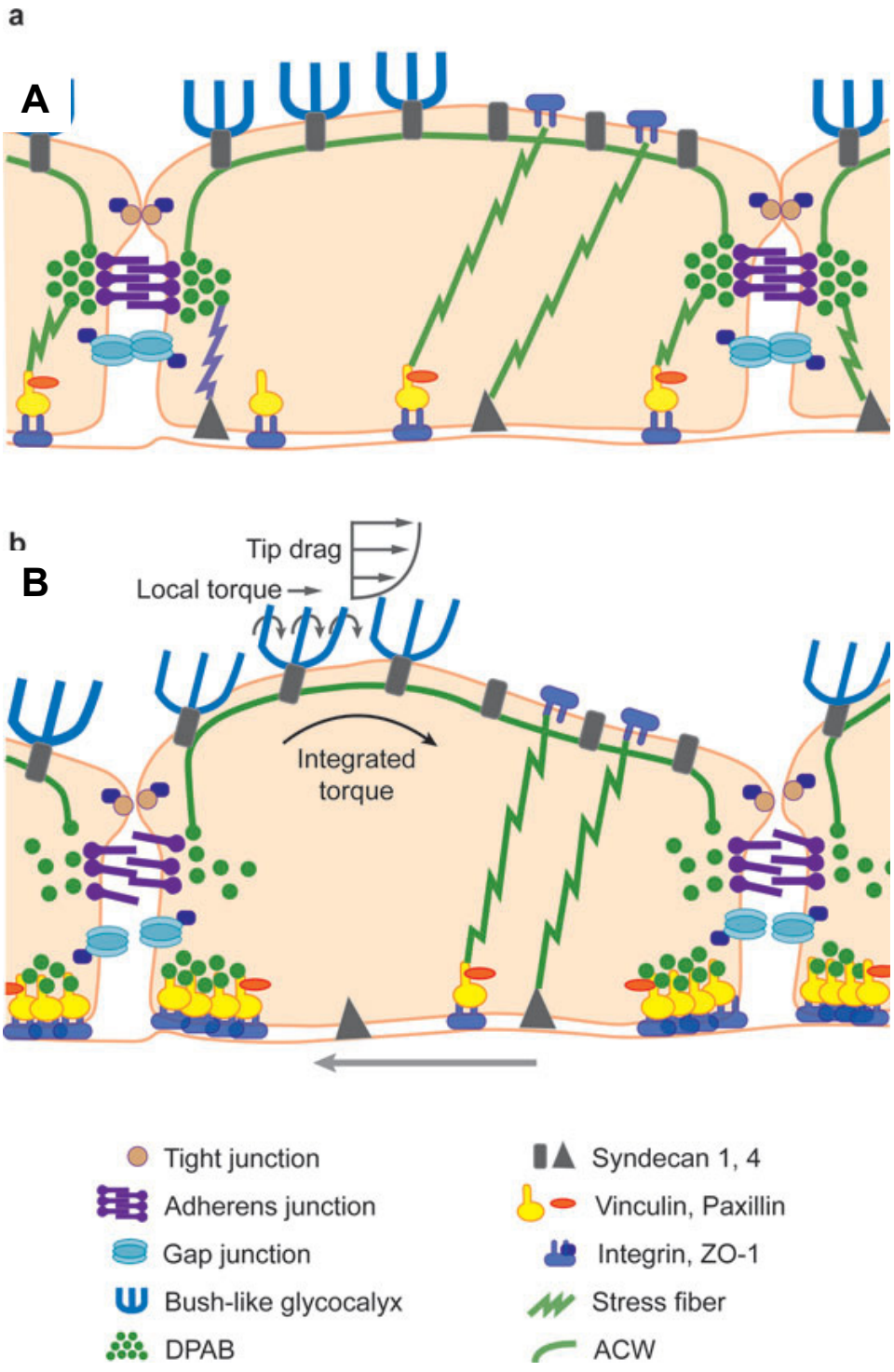


Figure 3-3. The "bumper-car" model of endothelial cell (EC) organization.(178) A) No blood flow or no glycocalyx function. B) Blood flow-induced shear stress is transmitted from glycocalyx to integrins via the actin cortical web (ACW)/dense peripheral actin bands (DPAB)/stress fibers (α-actin) system. Notice the increase in integrins number and activation after EC displacement, which produces shear stress on the extracellular matrix.

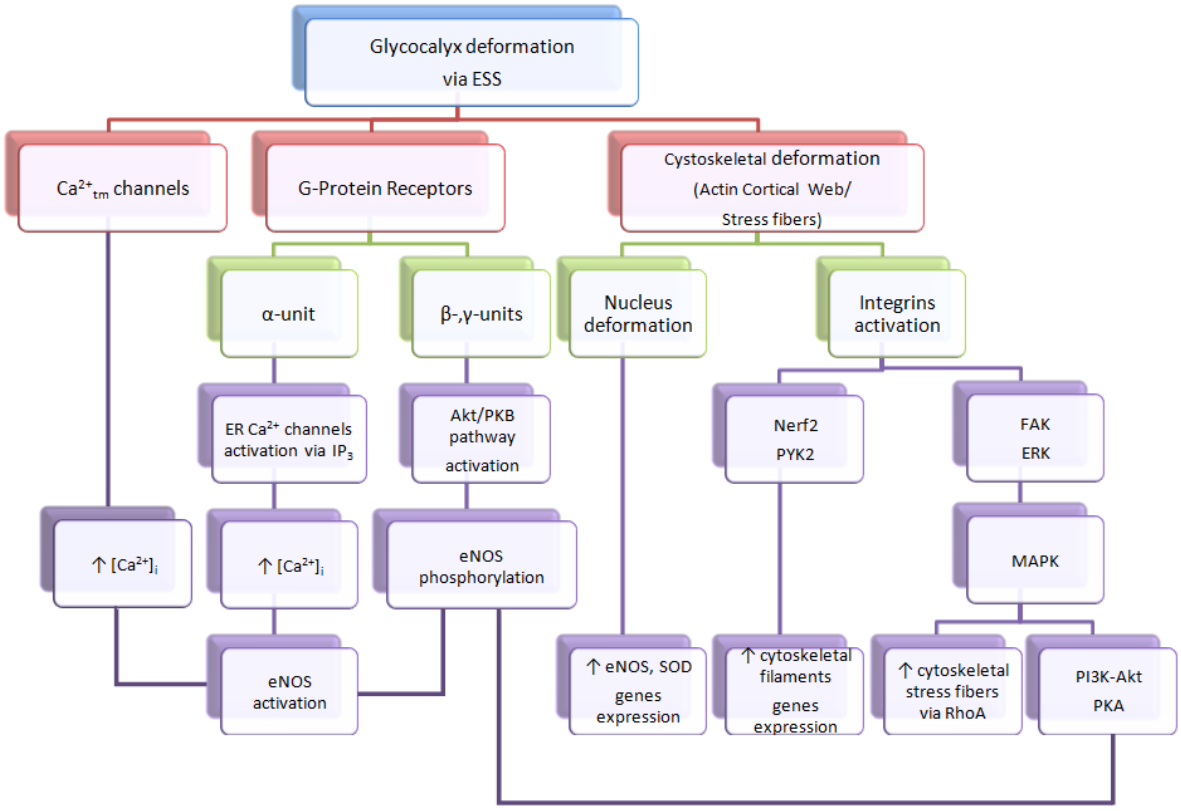


Figure 3-4. Endothelial cell mechano-transduction summary based on the decentralized and "bumper-car" models.(89, 178) Cascade of events following glycocalyx shift. ER=endoplasmic reticulum; IP3=inositol 1,4,5-triphospate; Akt/PKB=protein kinase B; PYK2=protein-rich tyrosine kinase; FAK=focal adhesion kinase; ERK=extracellular signal-regulated kinase; [Ca2+]i=cytosolic Ca2+ concentration; eNOS=endothelial nitric oxide synthase; MAPK=mitogen-activated protein kinase; SOD=superoxide dismutase; RhoA= Ras homolog gene family, member A (small GTPase); PI3K= Phosphoinositide 3-kinases; PKA=protein kinase A

CHAPTER 4
RESULTS

Experiment 1

Table 4-1 shows general characteristics from both groups, sham and EECP, at resting conditions before intervention. There were no significant differences in age, height, weight, body mass index, peripheral blood pressure, hematocrit, and blood density between both groups at baseline.

Figure 4-1 shows peripheral and central aortic pressure waveforms from two subjects (A = sham; B = EECP). The most remarkable difference between waveforms is the increase of both central aortic and peripheral diastolic pressures that generates a bimodal waveform in the EECP group. Central aortic diastolic and mean arterial pressures increased during EECP compared to sham (111±9 vs. 71±8 mm Hg and 98±8 vs. 81±7 mm Hg, $p<0.05$, respectively), but central aortic systolic blood pressure did not change during the 45-min session (Figure 4-2).

High definition ultrasound pictures and Doppler spectrum of brachial and femoral arteries are presented in figures 4-3 and 4-4, respectively. During EECP, brachial artery blood flow velocity shows two peaks of antegrade flow per cardiac cycle compared to sham (Figure 4-3 B, bottom), while femoral artery blood flow velocity is increased and mainly retrograde during late diastole and early systole (Figure 4-4 B, bottom).

Shear rate was increased during EECP compared to resting conditions in both brachial and femoral retrograde flows (61.3±16.9 vs. 48.6±16.1 s^{-1} and 252.4±71.7 vs.48.0±14.5 s^{-1}, $p<0.05$, respectively) (Figure 4-5). However, shear stress was

increased during EECP compared to sham only in brachial antegrade flow (58.1±13.2 vs. 33.3±8.1 dynes/cm^{-2}, $p<0.05$) (Figure 4-6).

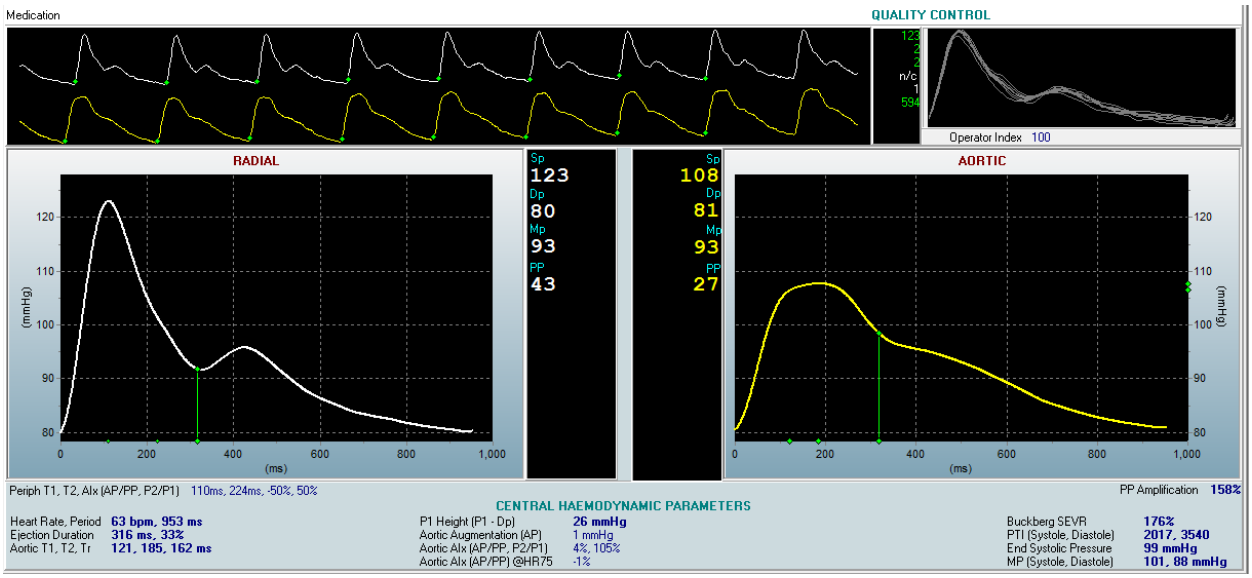
Both absolute and normalized Reynolds numbers (Re) showed a significant increase in brachial and femoral retrograde flows during EECP compared to resting conditions (Figures 4-7 and 4-8). Absolute and normalized Re were turbulent in femoral retrograde flow (Re>2000) during EECP compared to sham (Figure 4-7 D and 4-8 D). There was a trend toward increased nRe in brachial retrograde flow during EECP compared to sham (1710±1317 vs. 789±237, $p<0.08$) showing sub-turbulent or disturbed flow characteristics.

Both brachial and femoral artery flow mediated dilation (FMD) increased after EECP compared to baseline (10.6±4.8 vs. 7.0±3.5% and 13.1±3.7 vs. 7.8±4.5%, $p<0.05$, respectively) (Figure 4-9 A and C). Femoral FMD increased after EECP compared to sham (13.1±3.7 vs. 7.9±4.6%, $p<0.05$), while the time to peak femoral dilation was reduced (48.4±16.0 vs. 76.3±23.4 s, $p<0.05$) (Figure 4-9 C and D).

Table 4-1. Group characteristics at baseline (S.D. = Standard Deviation)

		Sham	EECP	p
Age	Mean	22.6	27.1	0.054
(years)	S.D.	1.9	5.0	
Height	Mean	1.80	1.80	0.107
(m)	S.D.	.05	.05	
Weight	Mean	86.0	80.9	0.509
(kg)	S.D.	17.9	13.8	
Body Mass Index	Mean	26.6	26.2	0.854
(kg/m^2)	S.D.	4.9	4.1	
Systolic Blood Pressure	Mean	124	122	0.540
(mm Hg)	S.D.	5	8	
Diastolic Blood Pressure	Mean	69	73	0.350
(mm Hg)	S.D.	7	7	
Hematocrit	Mean	50.1	50.5	0.820
(%)	S.D.	2.4	3.1	
Blood density	Mean	1062	1062	0.820
(kg/m^3)	S.D.	1	2	

A



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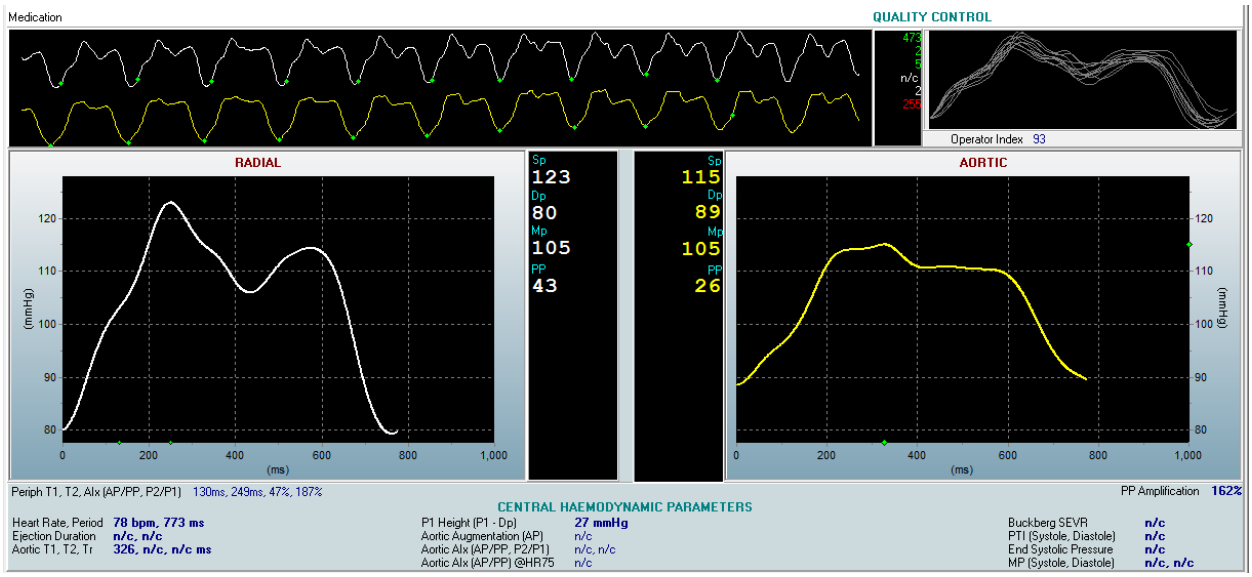

Figure 4-1. Peripheral and central pressure wave forms during sham (A) and EECP (B). Data acquired via applanation tonometry (SphygmoCor, AtCor Medical, Australia)

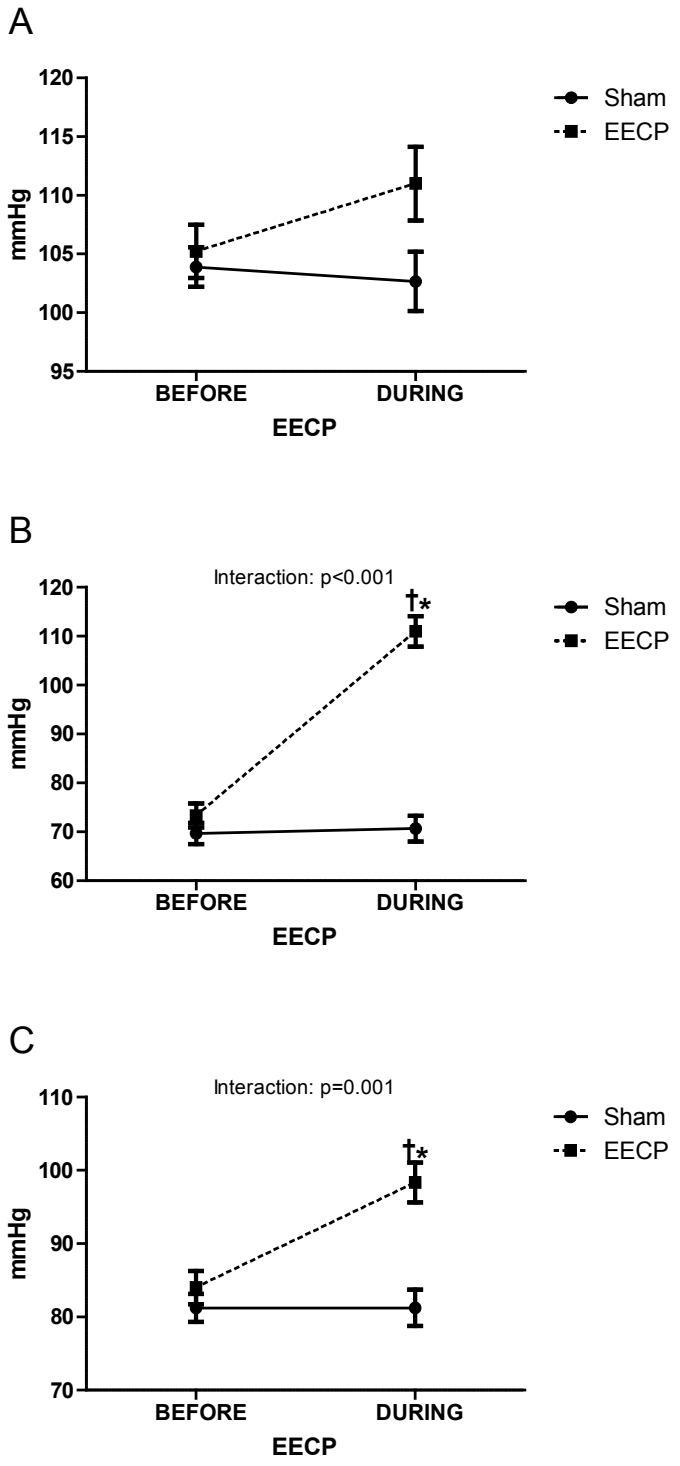

Figure 4-2. Central aortic blood pressure before and during EECP. A) Central systolic blood pressure, B) Central diastolic blood pressure, C) Central mean arterial pressure. Values are mean ± S.E.M. (*=p<0.05 EECP vs. Sham. †=p<0.05 during vs. before)

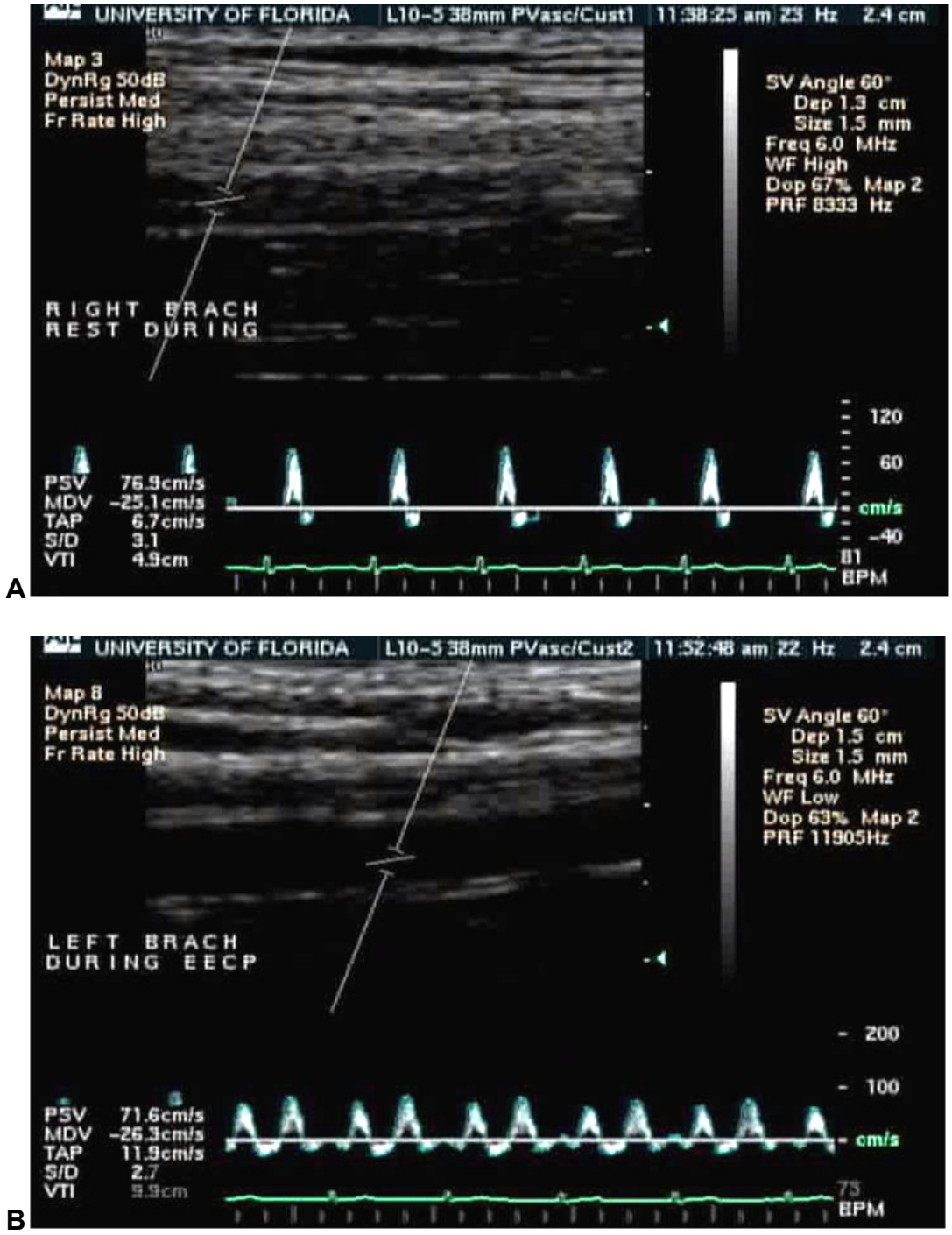

Figure 4-3. High definition ultrasound pictures and Doppler spectrum of the brachial artery during sham (A) and EECP (B).

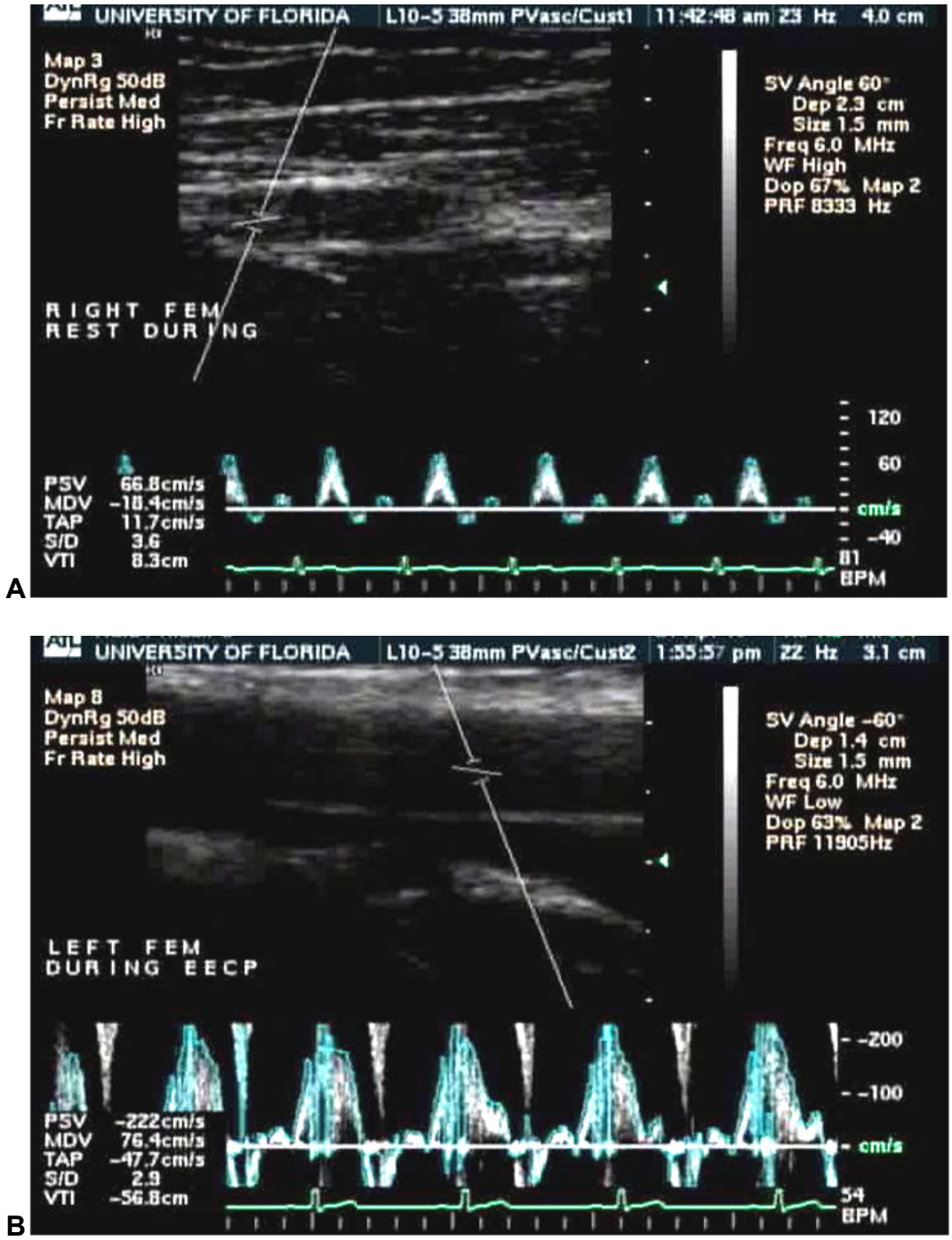

Figure 4-4. High definition ultrasound pictures and Doppler spectrum of the femoral artery during sham (A) and EECP (B).

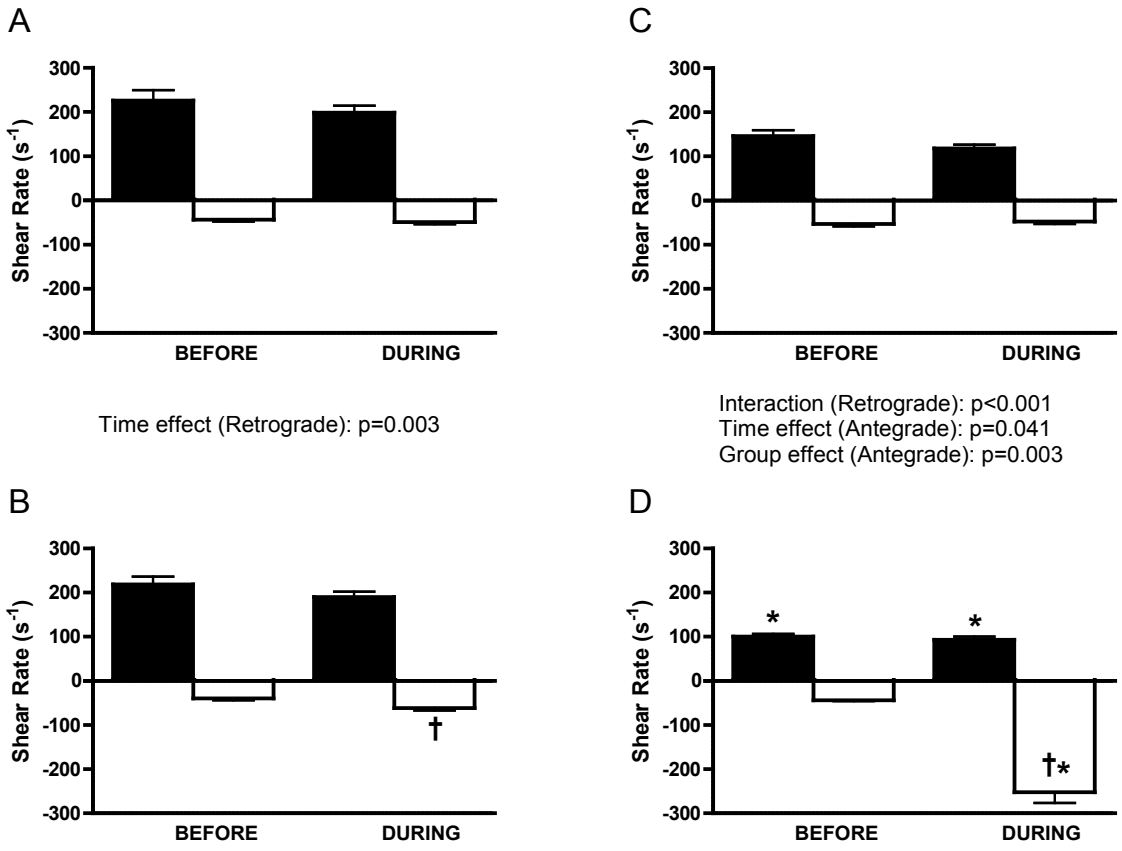

Figure 4-5. Shear rates (s^{-1}) before and during EECP. A) Brachial artery, sham group; B) Brachial artery, EECP group; C) Femoral artery, sham group; D) Femoral artery, EECP group. Closed boxes (■) = Antegrade flow; open boxes (□) = Retrograde flow. Values are mean ± S.E.M. (*=p<0.05 EECP vs. Sham. †=p<0.05 during vs. before)

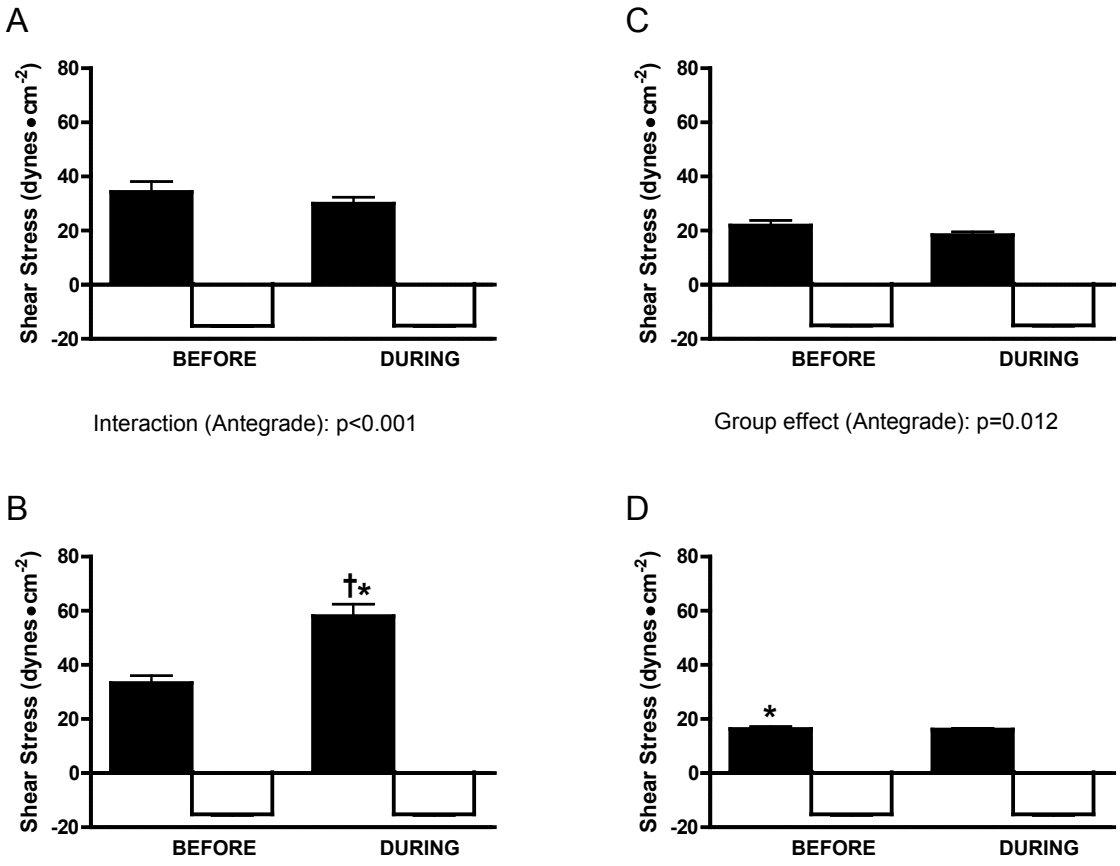

Figure 4-6. Shear stress (dynes/cm^2) before and during EECP. A) Brachial artery, sham group; B) Brachial artery, EECP group; C) Femoral artery, sham group; D) Femoral artery, EECP group. Closed boxes (■) = Antegrade flow; open boxes (□) = Retrograde flow. Values are mean ± S.E.M. (*=p<0.05 EECP vs. Sham. †=p<0.05 during vs. before)

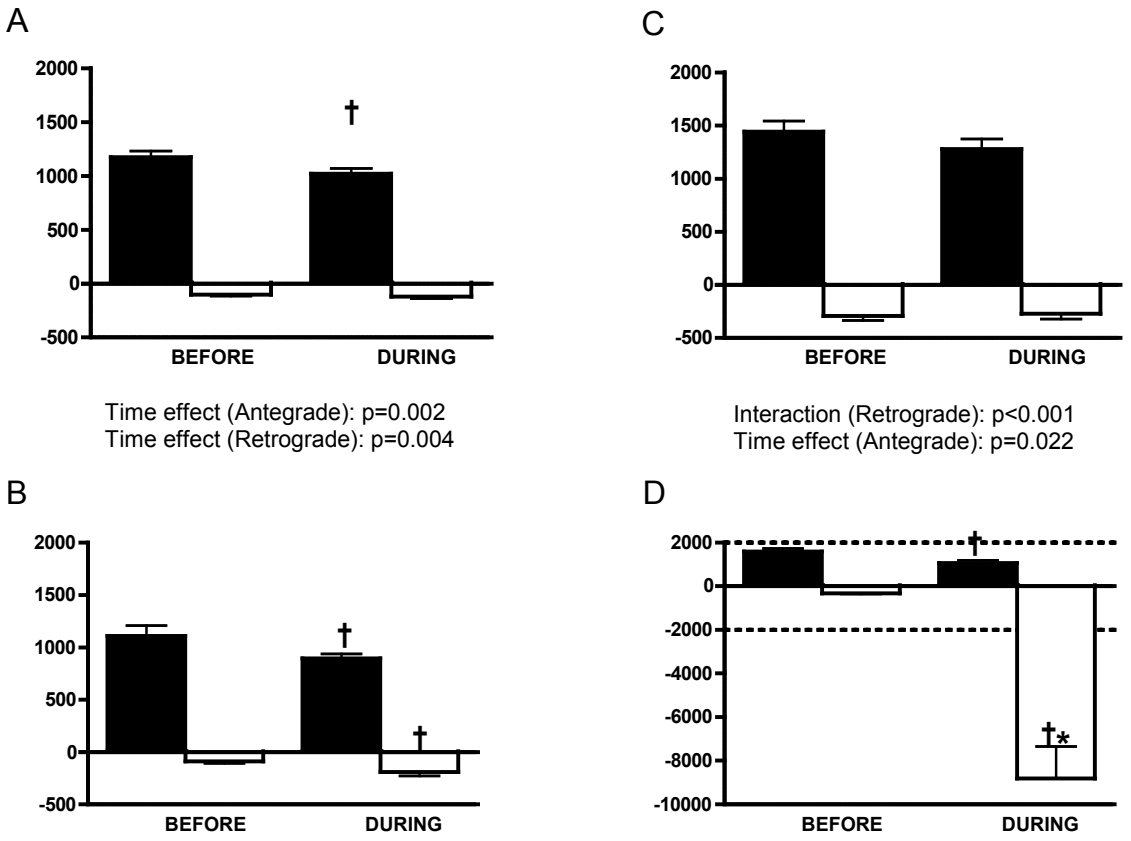

Figure 4-7. Reynolds number before and during EECP. A) Brachial artery, sham group; B) Brachial artery, EECP group; C) Femoral artery, sham group; D) Femoral artery, EECP group. Closed boxes (■) = Antegrade flow; open boxes (□) = Retrograde flow. Re≤-2000 = Retrograde turbulent flow; Re≥2000 = Antegrade turbulent flow. Values are mean ± S.E.M. (*=p<0.05 EECP vs. Sham. †=p<0.05 during vs. before)

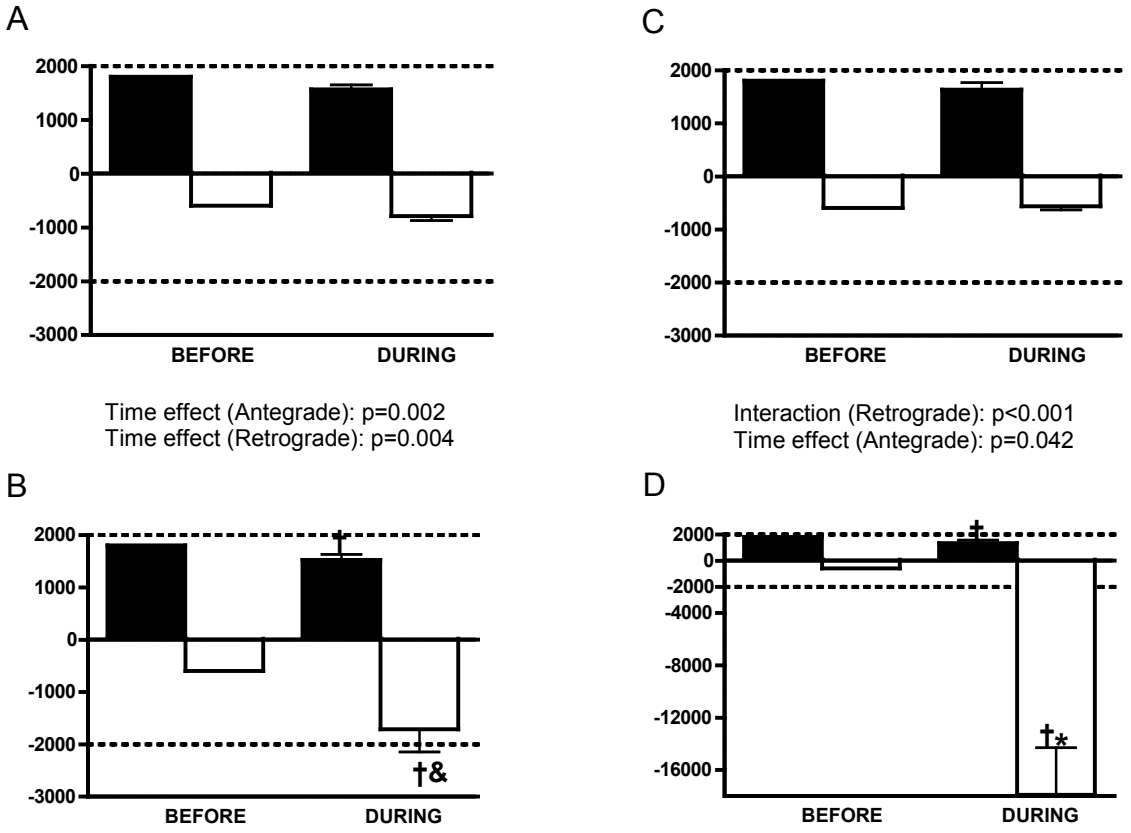

Figure 4-8. Normalized Reynolds number before and during EECP. A) Brachial artery, sham group; B) Brachial artery, EECP group; C) Femoral artery, sham group; D) Femoral artery, EECP group. Closed boxes (■) = Antegrade flow; open boxes (□) = Retrograde flow. Re≤-2000 = Retrograde turbulent flow; Re≥2000 = Antegrade turbulent flow. Values are mean ± S.E.M. (*=p<0.05, &=p<0.08 EECP vs. Sham. †=p<0.05 during vs. before)

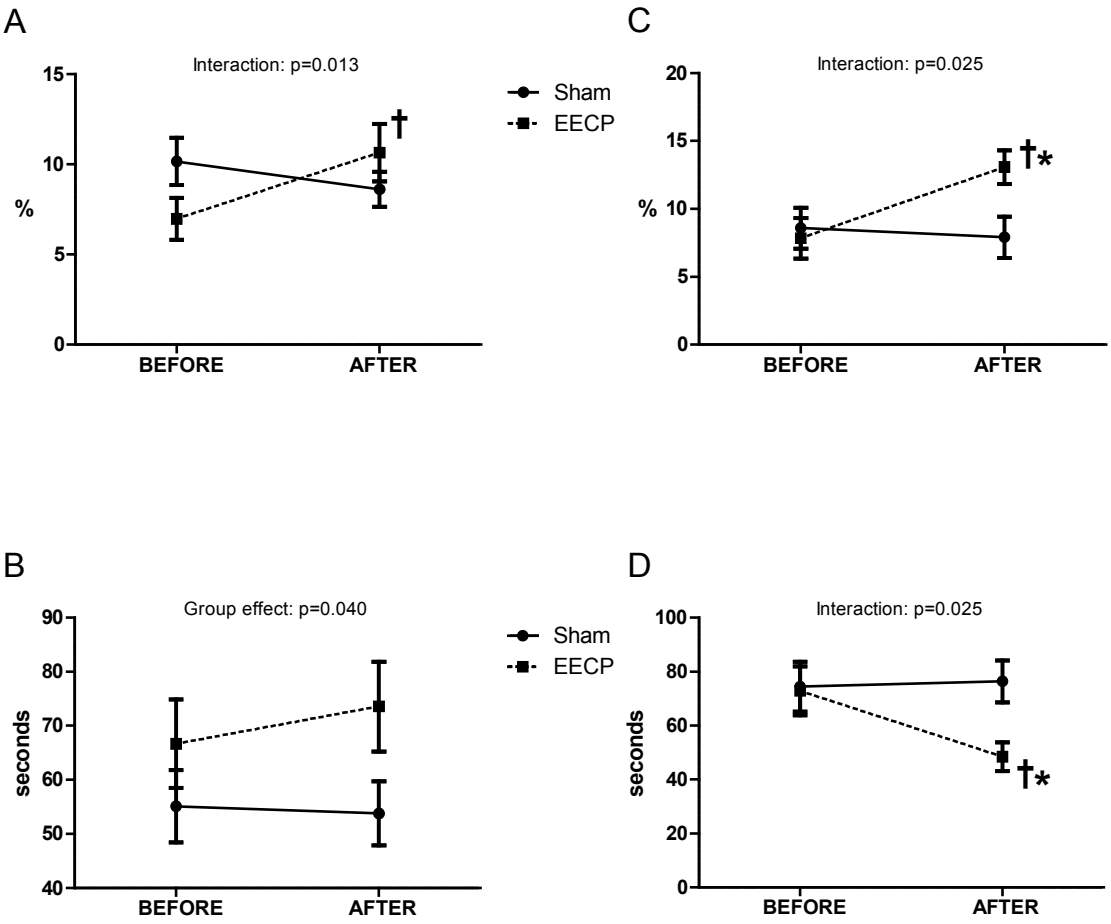

Figure 4-9. Flow mediated dilatation (FMD) and time to peak dilation before and after EECP. A) Brachial FMD; B) Time to peak brachial FMD; C) Femoral FMS; D) Time to peak femoral FMD. Values are mean ± S.E.M. (*=p<0.05 EECP vs. Sham. †=p<0.05 during vs. before)

Experiment 2

Table 4-2 shows means, standard deviations, and ranges of general characteristics, exercise tests, and hematocrits of the sample (n=8).

Heart rate and systolic blood pressure increased with exercise intensity in a dose dependent manner in both resistance and aerobic exercise intervention (Table 4-3). Diastolic blood pressure decreased during aerobic exercise compared to resistance exercise for both exercise intensities (40%=70±10 vs. 81±9 mm Hg; 70%=68±13 vs. 80±9 mm Hg, $p<0.05$).

Antegrade and retrograde shear rates increased in a dose dependent manner in the femoral artery with resistance and aerobic exercise and in the brachial artery with aerobic exercise (Figure 4-10). Aerobic exercise at 70% increased antegrade femoral and retrograde brachial shear rates compared to resistance exercise at 70% (186±86 vs. 125±33 s^{-1}; 135±35 vs. 101±35 s^{-1}, $p<0.05$, respectively).

Antegrade shear stress increased in a dose dependent manner in the femoral and brachial arteries only during aerobic exercise (Figure 4-11). Aerobic exercise at 70% VO_2max increased antegrade femoral shear stress compared to resistance exercise at 70% 1-RM (27.7±11.1 vs. 19.0±4.8 dynes/cm^{-2}, $p<0.05$).

Absolute Reynolds numbers (Re) showed a significant increase in brachial and femoral antegrade and retrograde flows during both exercise types compared to resting conditions (Figures 4-12). Normalized Reynolds number (nRe) indicates that blood flow was clearly turbulent in retrograde flows in both femoral and brachial arteries during aerobic exercise and in antegrade flows in femoral artery during both exercise types (nRe [95% CI] >2000) (Figure 4-13). During resistance exercise, femoral artery retrograde flow and brachial artery antegrade and retrograde flows were disturbed or

sub-turbulent (nRe [95% CI] higher than 1500, but lower than 2000) but not clearly turbulent (nRe [95% CI] >2000) (Figure 4-13 A and C). During aerobic exercise, femoral artery antegrade and retrograde flows and brachial artery retrograde flow were clearly turbulent (nRe [95% CI] >2000) however brachial artery antegrade flow was disturbed or sub-turbulent (1500< nRe [95% CI] <2000) (Figure 4-13 B and D). In general, turbulence increases with exercise intensity in a dose-dependent manner in both retrograde and antegrade flows and both exercise types.

Table 4-2. Group characteristics (S.D. = Standard Deviation)

n=8	Mean (±S.D.)	Range
Age (years)	24.0 (±3.4)	20.5 – 31.5
Height (m)	1.82 (±0.07)	1.72 – 1.91
Weight (kg)	81.1 (±11.8)	62.7 – 102.0
BMI (kg/m^2)	24.7 (±4.2)	20.8 – 34.1
1RM (kg)	146 (±27)	105 – 173
VO2max (ml/kg/min)	42.8 (±6.0)	34.8 – 51.8
VO2max (Watts)	282 (±39)	238 – 330
Heart Rate max (bpm)	183 (±3)	180 – 190
Heart Rate (%max)	93.5 (±2.1)	91.2 – 97.1
Hematocrit @ resistance session (%)	49.5 (±3.4)	46.2 – 56.6
Hematocrit @ aerobic session (%)	50.3 (±3.6)	45.5 – 56.3

Table 4-3. Systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) before and during resistance at 40 and 70% of 1-RM and aerobic exercise at 40 and 70% of VO_2max. Mean±S.D. (*=p<0.05 AX vs. RX; &=p<0.07 AX vs. RX. †=p<0.05 vs. rest; ††=p<0.05 vs. 40%)

	Resistance Exercise			Aerobic Exercise		
	Rest	40%	70%	Rest	40%	70%
SBP (mm Hg)	125±12	133±13	147±15 † ††	121±10	139±12 †	168±11 * † †††
DBP (mm Hg)	78±6	81±9	80±9	75±4	70±10 *	68±13 &
HR (bpm)	72±12	117±13 †	134±12 † ††	77±12	115±14 †	139±18 † ††

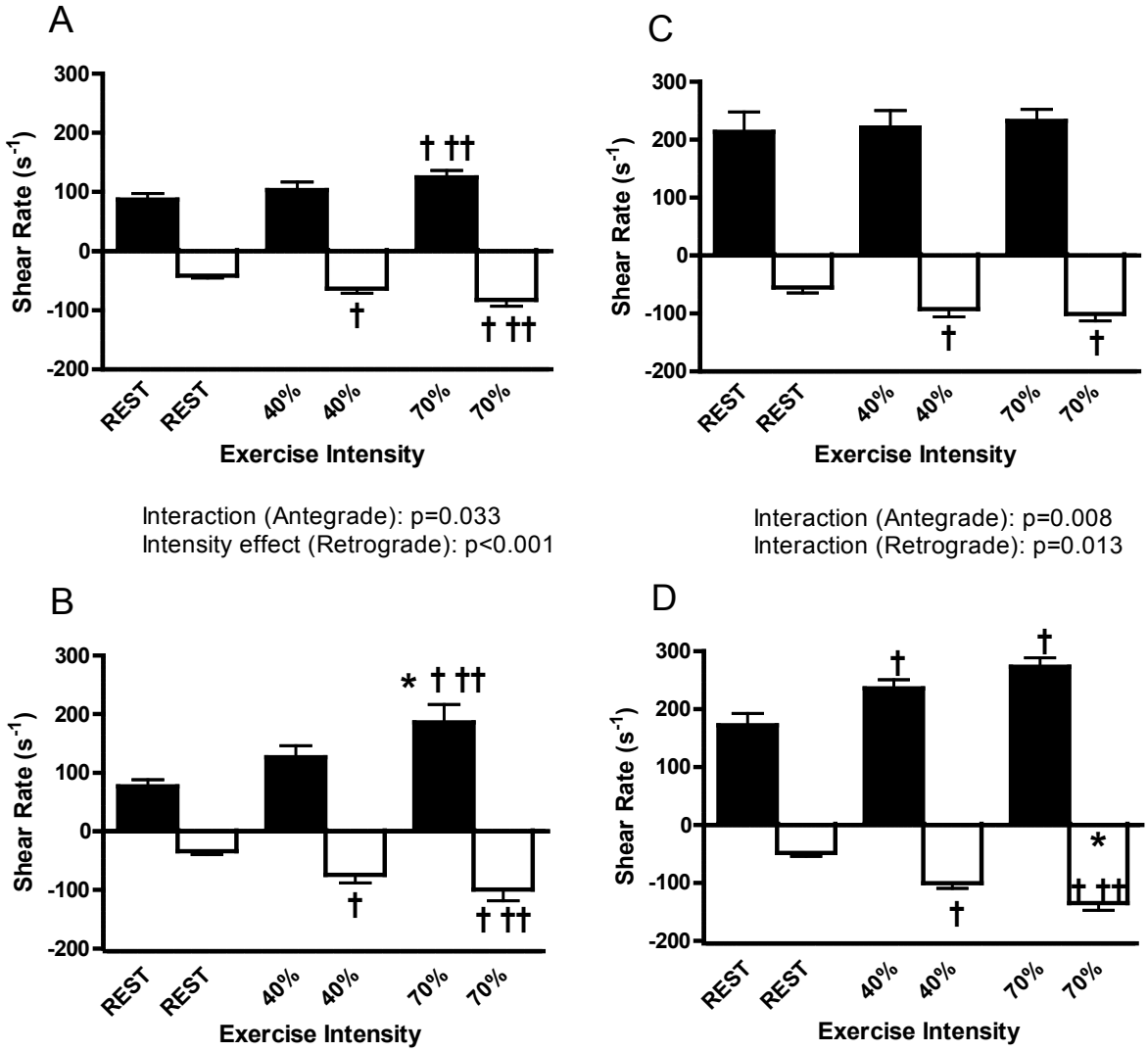

Figure 4-10. Shear rates (s^{-1}) at rest and during resistance (RX) and aerobic exercise (AX). A) Femoral artery during RX; B) Femoral artery during AX; C) Brachial artery during RX; D) Brachial artery during AX. Closed boxes (■) = Antegrade flow; open boxes (□) = Retrograde flow. Values are mean ± S.E.M. (*=p<0.05 AX vs. RX. †=p<0.05 vs. rest; ††=p<0.05 vs. 40%)

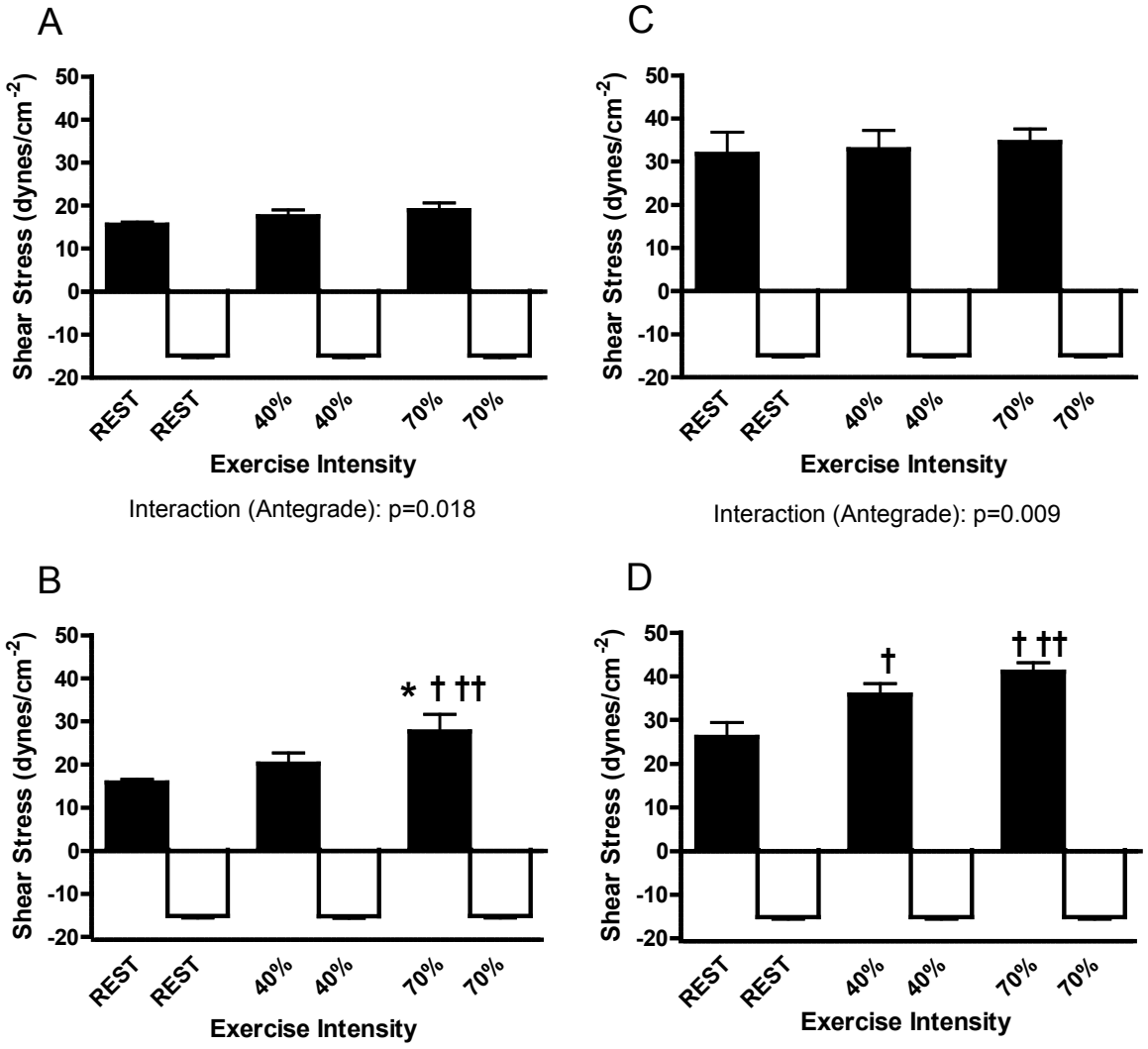

Figure 4-11. Shear stress (dynes/cm^2) at rest and during resistance (RX) and aerobic exercise (AX). A) Femoral artery during RX; B) Femoral artery during AX; C) Brachial artery during RX; D) Brachial artery during AX. Closed boxes (■) = Antegrade flow; open boxes (□) = Retrograde flow. Values are mean ±S.E.M. (*=p<0.05 AX vs. RX. †=p<0.05 vs. rest; ††=p<0.05 vs. 40%)

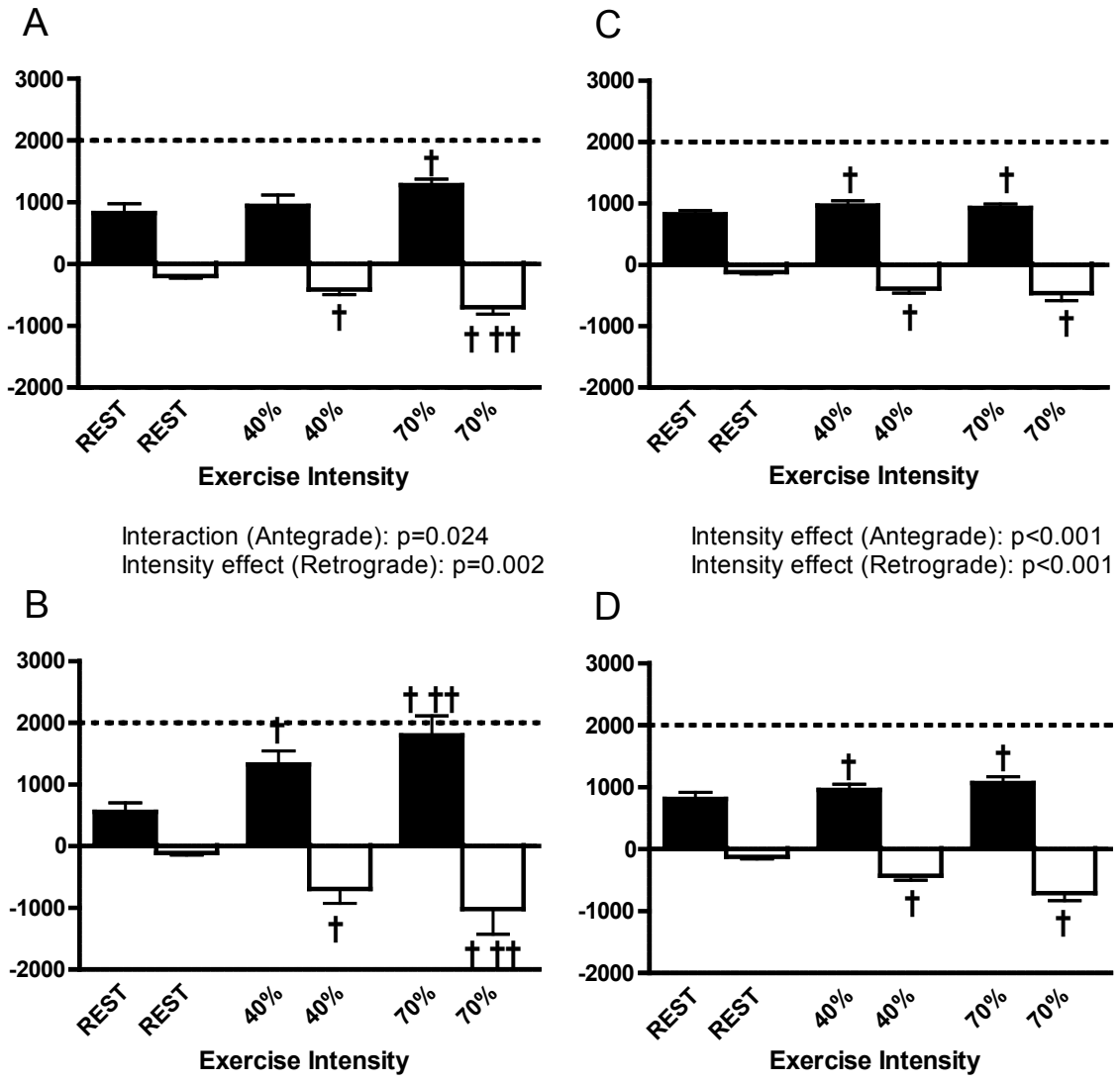

Figure 4-12. Reynolds number at rest and during resistance (RX) and aerobic exercise (AX). A) Femoral artery during RX; B) Femoral artery during AX; C) Brachial artery during RX; D) Brachial artery during AX. Closed boxes (■) = Antegrade flow; open boxes (□) = Retrograde flow. Re≤-2000 = Retrograde turbulent flow; Re≥2000 = Antegrade turbulent flow. Values are mean ± S.E.M. (†=p<0.05 vs. rest; ††=p<0.05 vs. 40%)

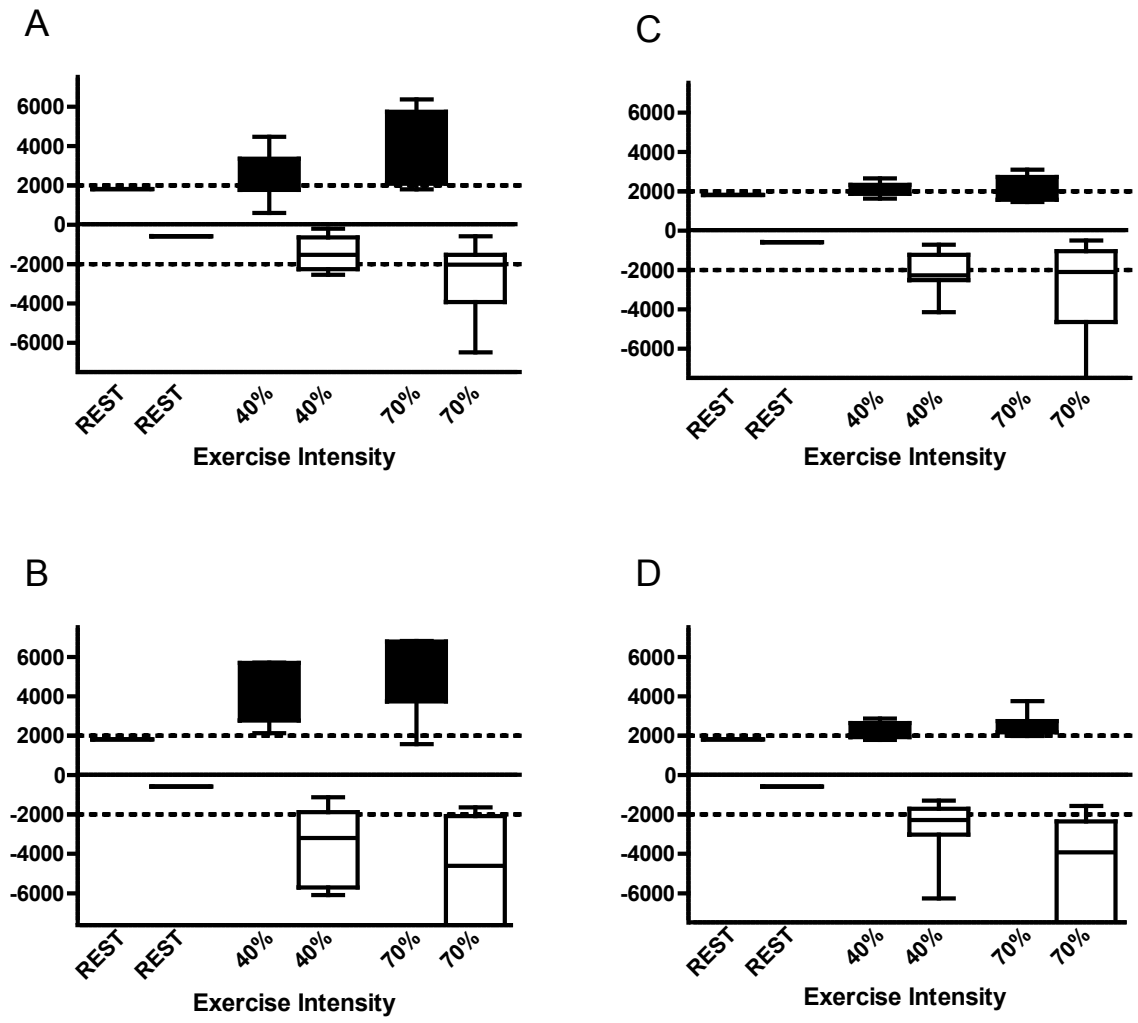

Figure 4-13. Normalized Reynolds number at rest and during resistance (RX) and aerobic exercise (AX). A) Femoral artery during RX; B) Femoral artery during AX; C) Brachial artery during RX; D) Brachial artery during AX. Closed boxes (■) = Antegrade flow; open boxes (□) = Retrograde flow. Box = [nRe 95% CI], error bars = range. [nRe 95% CI] ≤-2000 = Retrograde turbulent flow, [nRe 95% CI] ≥2000 = Antegrade turbulent flow.

Experiment 3

Table 4-4 shows general characteristics from the control, aerobic exercise training (AXT), and resistance exercise training (RXT) groups at baseline conditions. There were no significant differences in age, height, weight, body mass index, peripheral blood pressure, and VO_2max between groups at baseline. Compliance to the exercise training was 99%.

Although there was an increase in leg extension 1-RM in all groups, RXT had the highest change after 4 weeks of intervention (Figure 4-14 A). AXT group had a small but significant change in VO_2max (3.0±3.0 ml/Kg/min) while VO_2max in control and RXT groups did not change (Figure 4-14 B). Plasma norepinephrine levels did not change in any group (Figure 4-14 C), suggesting no change in resting hormonal state after exercise training. Norepinephrine data was analyzed using Friedman's non parametric two-way ANOVA due to lack of data's normal distribution.

There was a significant increase in brachial FMD after training in both exercising groups (AXT=12.5±5.7% vs. 8.7±3.0%, $p<0.05$; RXT=17.1±7.9% vs. 13.1±5.8%, $p<0.05$) (Figure 4-15). Similar results were found in femoral FMD (AXT=10.9±4.7% vs. 6.0±2.7%, $p<0.05$; RXT=10.6±5.7% vs. 7.3±2.5%, $p<0.05$), although absolute diameter changes reached significance only in AXT (Figure 4-16).

There was an increase in NOx after training in AXT (15.7±1.8 vs. 21.7±8.7 µmol/L, $p<0.05$) while a trend to increase was observed in RXT (17.3±3.8 vs. 21.3±7.7 µmol/L, $p=0.164$) (Figure 4-17).

Von Willebrand factor, used as housekeeping protein, did not change after 4-week intervention in any group (Figure 4-18), confirming stability of the immunofluorescent method. There was an increased eNOS pixel intensity ratio after training in RXT when

compared to control (112±30% vs. 84±22%, $p<0.05$) while a trend to increase was observed in AXT (105±27% vs.84±22%, $p=0.114$) (Figure 4-19). There was a significant decrease in nitrotyrosine pixel intensity ratio after training in both exercising groups when compared to baseline (AXT=95±9% vs. 122±14%, $p<0.05$; RXT=86±21% vs. 121±12%, $p<0.05$) (Figure 4-20).

Table 4-4. Group characteristics at baseline (AXT = Aerobic exercise training group, RXT = resistance exercise training group, S.D. = Standard Deviation)

		Control	AXT	RXT	p
Age	Mean	25.7	27.1	26.7	0.876
(years)	S.D.	6.3	5.9	6.2	
Height	Mean	1.78	1.75	1.78	0.285
(m)	S.D.	0.05	0.04	0.04	
Weight	Mean	76.5	73.9	76.7	0.313
(kg)	S.D.	8.0	7.9	8.0	
Body Mass Index	Mean	24.1	24.1	25.2	0.406
(kg/m^2)	S.D.	1.8	1.9	2.4	
Systolic Blood Pressure	Mean	123	123	129	0.620
(mm Hg)	S.D.	10	12	20	
Diastolic Blood Pressure	Mean	75	73	77	0.645
(mm Hg)	S.D.	8	6	12	
VO_2max	Mean	39.5	40.3	38.2	0.849
(ml/Kg/min)	S.D.	9.1	7.2	6.3	

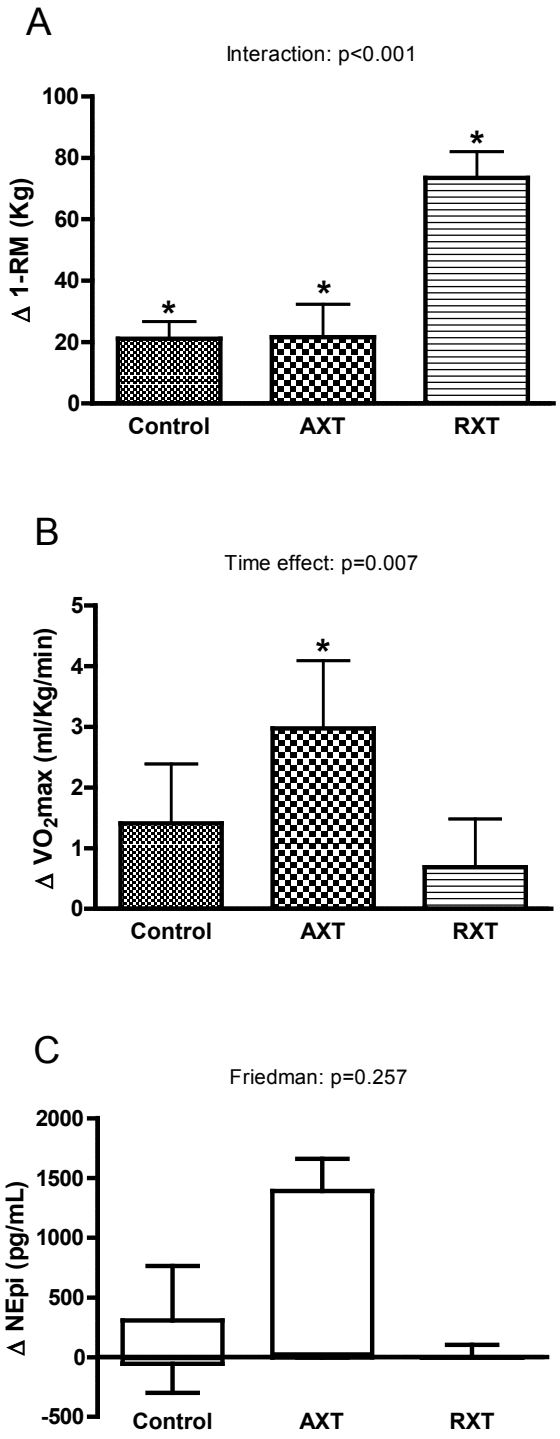

Figure 4-14. Changes in leg extension 1-RM (A), VO_2max (B), and Norepinephrine (C) after 4 weeks of intervention. AXT = Aerobic Exercise Training, RXT = Resistance Exercise Training. Values are mean ± S.E.M., except for C where box=[95% CI] and error bars=range. (*=p<0.05 before vs. after intervention)

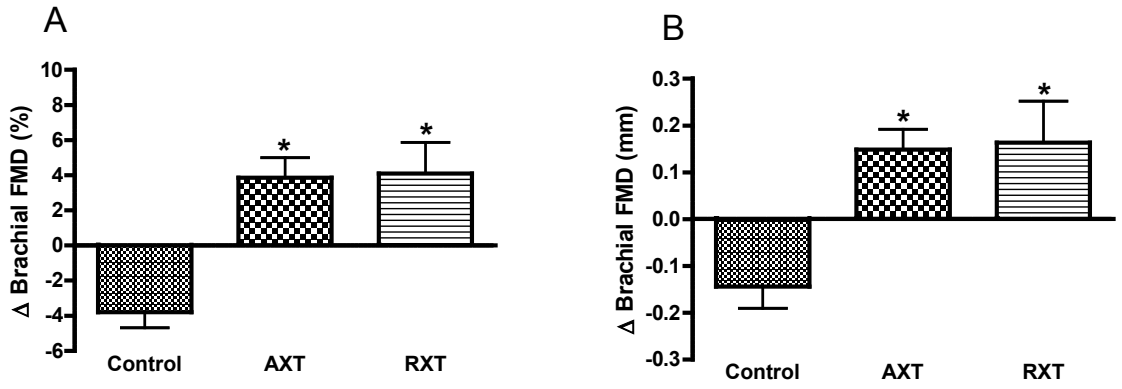

Figure 4-15. Changes in brachial FMD, percent change (A) and absolute change (B), after 4 weeks of intervention. AXT = Aerobic Exercise Training, RXT = Resistance Exercise Training. Values are mean ± S.E.M. (*=p<0.05 before vs. after intervention)

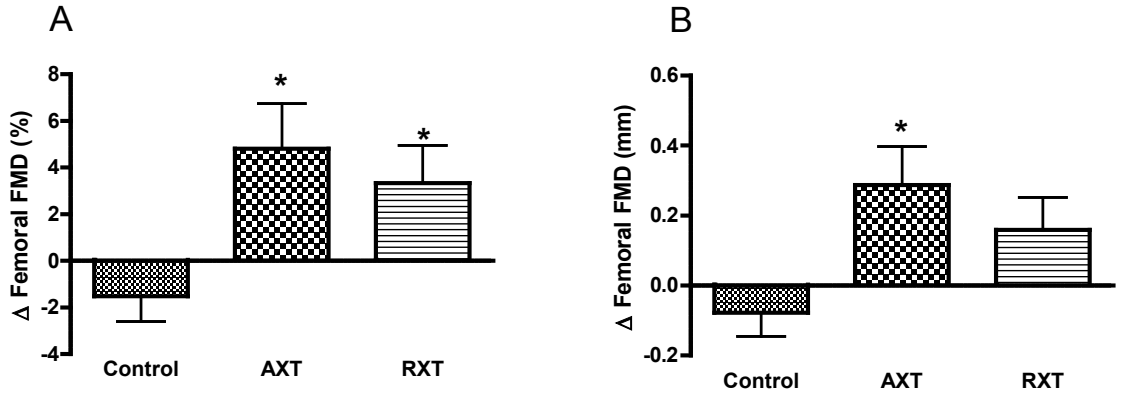

Figure 4-16. Changes in femoral FMD, percent change (A) and absolute change (B), after 4 weeks of intervention. AXT = Aerobic Exercise Training, RXT = Resistance Exercise Training. Values are mean ± S.E.M. (*=p<0.05 before vs. after intervention)

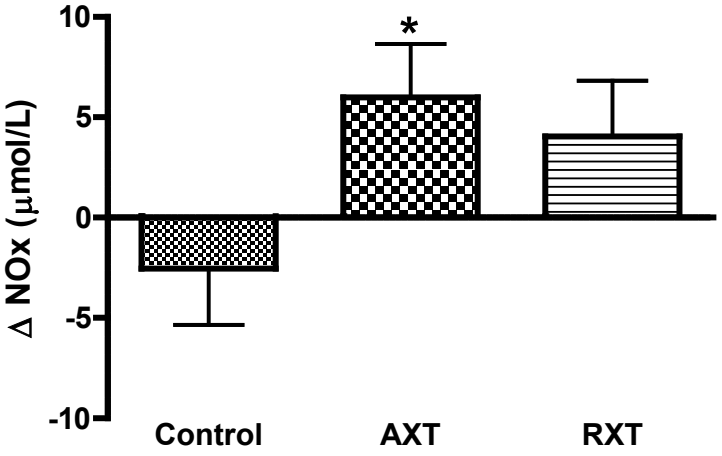

Figure 4-17. Changes in Nitrite/Nitrate (NOx) after 4 weeks of intervention. AXT = Aerobic Exercise Training, RXT = Resistance Exercise Training. Values are mean ± S.E.M. (*=p<0.05 before vs. after intervention)

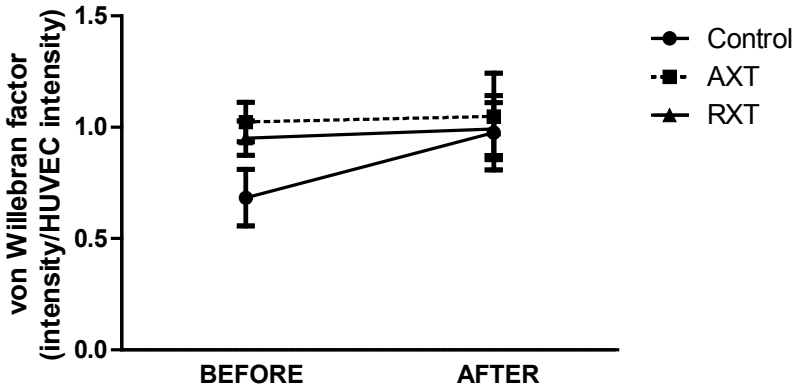

Figure 4-18. von Willebrand factor pixel intensity before and after 4 weeks of intervention. AXT = Aerobic Exercise Training, RXT = Resistance Exercise Training. Values are mean ± S.E.M.

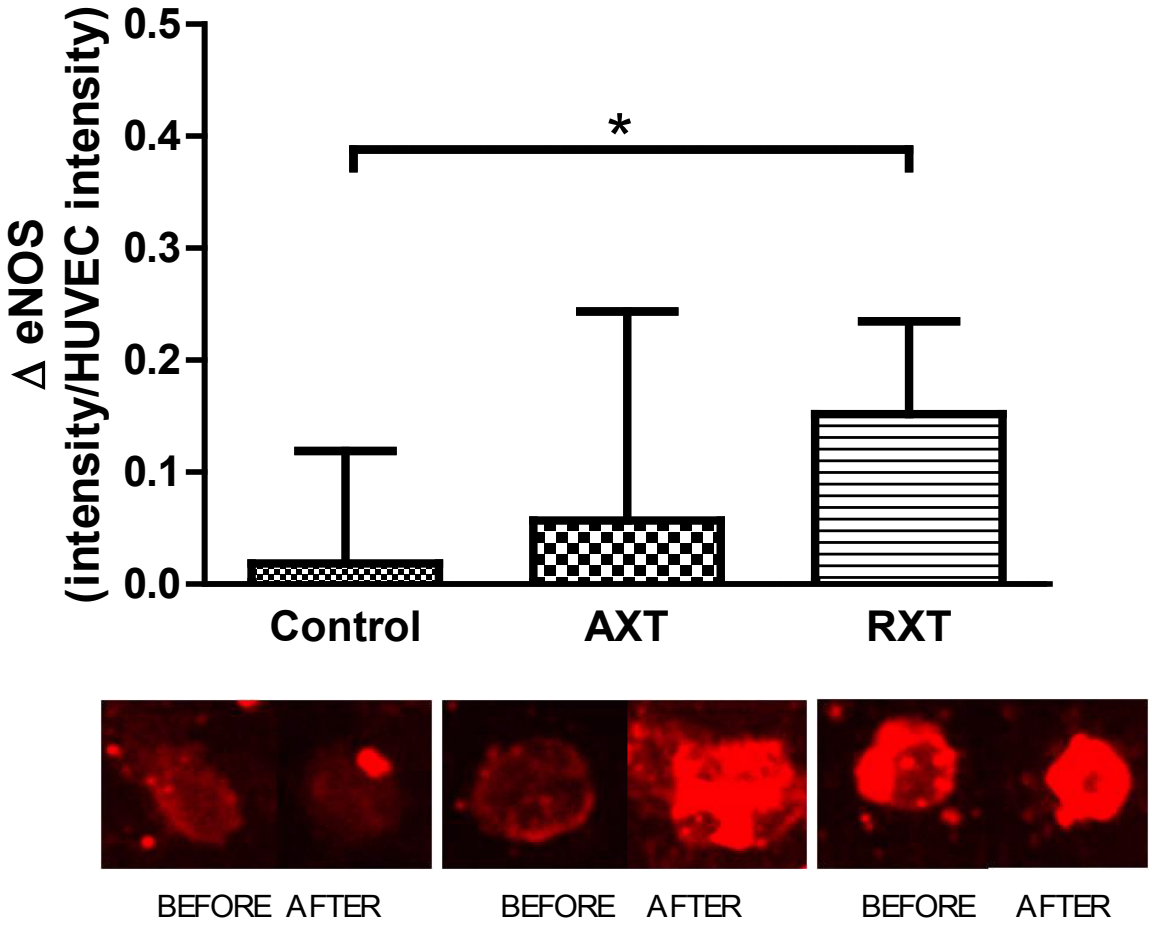

Figure 4-19. Changes in endothelial nitric oxide synthase expression after 4 weeks of intervention. AXT = Aerobic Exercise Training, RXT = Resistance Exercise Training. Values are mean ± S.E.M. (*=$p<0.05$ RXT vs. Control after intervention)

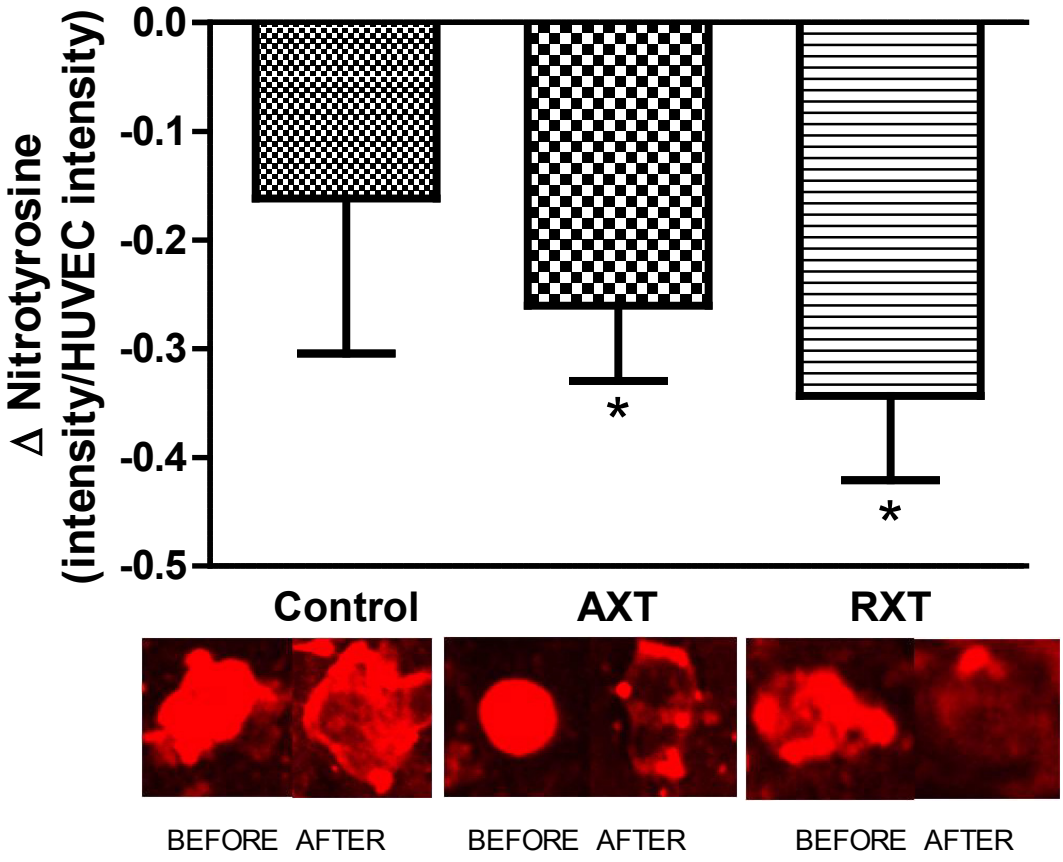

Figure 4-20. Changes in endothelial nitrotyrosine expression after 4 weeks of intervention. AXT = Aerobic Exercise Training, RXT = Resistance Exercise Training. Values are mean ± S.E.M. (*=$p<0.05$ before vs. after intervention)

CHAPTER 5
CONCLUSIONS

Experiment 1

Based on the results observed on Experiment 1, we can conclude that:

- During a single 45-minute session of EECP, the primary brachial blood flow pattern is antegrade, laminar, and shear stress is increased.
- During a single 45-minute session of EECP, the primary femoral blood flow pattern is retrograde and turbulent; however, shear stress does not change.
- During a single 45-minute session of EECP, central diastolic and mean arterial pressures are increased.
- After a single 45-minute session of EECP, brachial flow mediated dilation is improved.
- After a single 45-minute session of EECP, femoral flow mediated dilation is improved and time to peak dilation is reduced.
- Opposite blood flow patterns, antegrade-laminar and retrograde-turbulent, produce brachial and femoral flow mediated dilation improvements, respectively.
- It appears that, to improve endothelial function after EECP, brachial artery blood flow pattern has to be antegrade, laminar, and shear stress increased, while femoral artery blood flow pattern has to be retrograde and turbulent and increased shear stress is not necessary.

Experiment 2

Based on the results observed on Experiment 2, we can conclude that:

- Aerobic exercise increases antegrade and retrograde shear rate in a dose dependent manner in both femoral and brachial arteries.
- Resistance exercise increases antegrade and retrograde shear rate in a dependent manner only in the femoral artery.
- Aerobic exercise increases antegrade shear stress in a dose dependent manner in both femoral and brachial arteries. However, retrograde shear stress did not change.
- Resistance exercise did not change antegrade or retrograde shear stress in femoral and brachial arteries.

- Both resistance and aerobic exercises increase retrograde turbulence in a dose dependent manner in both femoral and brachial arteries. However, retrograde-turbulent flow occurs only in aerobic exercise at 70%.

- Both resistance and aerobic exercises increase antegrade turbulence in both femoral and brachial arteries. However, antegrade-turbulent flow occurs only in the femoral artery in aerobic exercise and in resistance exercise at 70%.

- None of the exercise modalities or intensities mimics blood flow patterns observed during a session of EECP. However, aerobic exercise at 70% created flow patterns approximating EECP where antegrade shear stress and retrograde turbulence are increased in both femoral and brachial arteries.

Experiment 3

Based on the results observed on Experiment 2, we can conclude that:

- Four weeks of exercise training, either aerobic or resistance, did not produce significant changes in resting norepinephrine, suggesting no changes in the hormonal resting state of the subjects.

- Exercise training, either aerobic or resistance, increases brachial flow mediated dilation.

- Exercise training, either aerobic or resistance, increases femoral flow mediated dilation.

- Aerobic exercise training increases nitric oxide overall body bioavailability (NOx).

- Resistance exercise training upregulates endothelial nitric oxide synthase when compared to control after 4 weeks of intervention.

- Exercise training, either aerobic or resistance, decreases nytrotirosine expression, a marker of endothelial oxidative stress.

Summary

Based on the results observed on all three experiments, we can conclude that:

- Exercise-induced NO-dependent arterial vasodilation improvement is systemic and not limited to exercising vascular beds.

- Turbulent blood flow, when retrograde, produces the same beneficial effects on NO-dependent arterial vasodilation than antegrade laminar blood flow.

- Exercise-induced endothelial function improvement is associated with upregulation in endothelial oxide nitric synthase and a decrease in endothelial oxidative stress.

- Exercise-induced blood flow is an importamt stimulus for NO-dependent arterial vasodilation improvement. However, improvements in NO-dependent arterial vasodilation depend on exercise intensity and blood flow pattern created by the exercise.

CHAPTER 6
DISCUSSION

This is the first *in vivo* study designed to evaluate the effects of exercise-induced blood flow patterns on endothelial function, endothelial cell phenotype, and endothelial cell oxidative stress. The major findings in this study are as follows: 1) antegrade-laminar with increased shear stress blood flow pattern as well as retrograde-turbulent without increased shear stress blood flow pattern improve flow mediated dilation in brachial and femoral arteries, respectively, 2) aerobic exercise increases antegrade shear stress in both femoral and brachial arteries, and both aerobic and resistance exercise increase retrograde-turbulent blood flow in both femoral and brachial arteries, and 3) short term lower body exercise training, either aerobic or resistance, improves brachial and femoral artery flow mediated dilation and the improvement is related to upregulation of endothelial nitric oxide synthase and a decrease in endothelial oxidative stress.

Blood (Fluid) Mechanics

Fluid mechanics refers to all mechanical factors that affect and are affected by the flow of a liquid.(56) As a non-Newtonian fluid, blood changes its viscosity according to blood flow's shear rate.(23, 55, 85) Moreover, these changes in blood viscosity, added to blood flow velocity, will affect blood flow turbulence. The present study showed novel findings associated with blood mechanics, such as shear stress vs. shear rate differences and presence of turbulence, which should be considered when interpreting blood flow pattern data.

Shear Stress vs. Shear Rate

Blood flow-induced vascular shear stress is the primary physiological stimulus that regulates vascular endothelial function.(24, 35, 41) Although the definition of shear stress refers to the tangential force produced by blood flow, blood flow has multiple factors that can affect shear stress such as blood viscosity, flow direction, and flow turbulence. Due to the complex multifactorial nature of blood flow, shear stress has been typically studied *in vitro* while considering only one factor at a time (43, 45, 49, 52, 159, 207), or simplifying shear stress to shear rate, which is a function of blood flow velocity and vessel diameter, in *in vivo* studies.(72, 159, 180, 181, 187, 188)

Results from experiments 1 and 2 of the present study show that shear stress and shear rate do not change in parallel during enhanced external counterpulsation (EECP) and during resistance and aerobic exercise (Figures 4-5, 4-6, 4-10, and 4-11). During EECP, shear rate was significantly increased in both brachial and femoral arteries during retrograde blood flow, while shear stress was significantly increased only in brachial artery during antegrade blood flow (Figures 4-5 and 4-6). Similar results were observed during exercise where shear rate increases with exercise intensity, in a dose dependent manner, in brachial and femoral arteries during both antegrade and retrograde blood flows, while shear stress only increases in brachial and femoral arteries during antegrade blood flow.

The best explanation for these contradictory results is related to blood's non-Newtonian fluid characteristics. In a Newtonian fluid, the relationship between fluid's viscosity and shear rate is constant. Blood viscosity, an important factor in shear stress determination, increases exponentially when shear rate decreases below 100 s^{-1} and it is constant when shear rate is over 100 s^{-1}.(23, 55, 56, 85, 114, 172) This blood's non-

Newtonian fluid characteristic allows shear stress to stay constant and work as a mechanical buffer against shear rate variations under 100 s^{-1}. For example, during EECP, the observed increase in retrograde shear rate (blood flow velocity/vessel diameter) is lost when multiplied per blood viscosity to obtain shear stress (Figures 4-5 and 4-6, B and D, open boxes). The fact that brachial artery antegrade shear stress during EECP increases significantly, despite no change in shear rate, is caused by an increase in time-averaged blood flow due to the double antegrade blood flow peak observed during EECP (Figure 4-3B).

Blood's non-Newtonian fluid characteristic can explain differences between shear rate and shear stress during exercise. Retrograde shear rate in brachial and femoral arteries is' buffered' when transformed to shear stress, because shear stress is lower than 100 s^{-1} and viscosity is no longer a constant.(Figures 4-10 and 4-11, B and D, open boxes). In contrast, both antegrade shear stress and shear rate increase in brachial and femoral arteries during aerobic exercise because blood viscosity is constant when shear rate is over 100 s^{-1} (Figures 4-10 and 4-11, B and D, closed boxes).

In vivo vascular studies in humans normally report shear rate values, assuming that blood is a Newtonian fluid or processing constant blood viscosity.(152, 177, 179-181, 187, 188) However, results from the present study clearly show that this is an incorrect assumption that could lead to incorrect conclusions. We suggest that in human vascular studies shear stress should be reported, rather than shear rate. Hematocrit should be measured or estimated at 50% in healthy subjects.

Laminar vs. Turbulent Flow

Another assumption in vascular human studies is that blood flow in straight conduit arteries is always laminar in healthy individuals.(159) Although several previous *in vitro* and animal studies were designed to determine effects of flow turbulence on endothelial function and atherosclerotic plaque evolution (27, 28, 45, 49, 98, 105), the present study was the first to investigate *in vivo* blood flow turbulence, in humans. We calculated Reynolds number (Re) using blood flow velocity from Doppler acquisition, artery diameter from high-resolution ultrasound imaging, and kinematic viscosity from hematocrit and shear rate (Pages 31 and 32). Due to multiple factors that affect blood flow velocity acquisition, we normalized Re to resting theoretical Re values.

During EECP, turbulent blood flow is present only in the femoral artery during retrograde blood flow (Re and nRe > 2000, Figures 4-7D and 4-8D, open boxes). This finding can be explained by the significant increase in femoral artery retrograde blood flow velocity produced by EECP sequential cuff inflation (Figure 4-4B, bottom).(32, 61, 126, 140, 175) Dramatic differences between Re and nRe values observed during exercise in the present study (Figure 4-12 and 4-13) may be due to Doppler acquisition that was made while subjects were in movement.(155-157) Due to this limitation, the use of the 95% confidence interval of nRe, as the point of reference to determine turbulence, is statistically and clinically appropriate (Figure 4-13). Our results show a trend toward increased turbulence in both antegrade and retrograde flows with exercise intensity in a dose-dependent manner. The pattern of femoral artery retrograde turbulent blood flow observed during EECP is observed during aerobic exercise but only when intensity is 70% of VO_2max (Figures 4-8 and 4-13).

Based upon the shear stress and turbulence data from the present study, we conclude that blood flow patterns should be characterized by three different dimensions: 1) blood flow direction (antegrade/retrograde), 2) shear stress (vs. shear rate), and 3) presence of turbulence. Combining these three dimensions will allow a better understanding of the mechanical events involved in blood flow-induced endothelial function regulation.

Blood Flow Patterns and Endothelial Function

Previous blood flow and endothelial function studies focused in the effects of only one or two blood flow dimensions. For example, Ziegler *et al.* (207) showed an eNOS mRNA downregulation when oscillatory blood flow was applied *in vitro* to bovine aortic endothelial cells (BAEC), DePaola *et al.* (49) showed that low endothelial shear stress is associated with a decrease of cultured BAEC density, and Davies *et al.* (45) showed an increased endothelial cell turnover when turbulent flow was applied *in vitro* to BAEC. Although these classic studies all controlled shear stress, none of them measured time-averaged retrograde flow, which is an important factor especially during exercise and EECP interventions.(61, 73, 74, 188)

The present study shows, for the first time, the interaction of all three blood flow dimensions with endothelial function, as assessed by flow mediated dilation (FMD). Our results show that one 45-minute session of EECP improves FMD in the brachial and femoral arteries. EECP also improves FMD kinetics, assessed by time-to-peak FMD, in the femoral artery (Figure 4-9). Blood flow patterns observed during EECP were laminar-antegrade with increased shear stress in the brachial artery and retrograde-turbulent without increased shear stress in the femoral artery (Figures 4-6 and 4-7, B and D). The blood flow patterns observed during an acute session of EECP may have

an additive effect when used chronically, as shown in our Laboratory with 35 1-hour sessions in patients with coronary artery disease.(14)

Our results are consistent, at least in part, with *in vitro* and animal studies.(27, 28, 45, 57) Davies *at al.* (45) observed that changes in BAEC, produced by turbulent flow *in vitro*, were independent from shear stress, as observed in our femoral artery blood flow pattern results during EECP. In addition, Duchene *et al.* (57) observed that beneficial changes in human umbilical vein endothelial cells, produced by laminar flow *in vitro*, depended on shear stress, as observed in our brachial artery blood flow pattern results during EECP. Moreover, *Cheng at al.* (27, 28) have shown that turbulent flow could promote atherosclerotic plaque stability rather than the often-reported endothelial dysfunction, confirming than turbulent blood flow is not always detrimental to vascular health, as observed in our femoral artery blood flow pattern results.

Despite some agreement with *in vitro* and animal studies, results of the present study conflict with *in vivo* human studies.(181, 187) Using similar approaches, Thijssen *et al.* (181) and Tinken *et al.* (187) used the 'bioassay' FMD to determine the effects of blood flow restriction (181), forearm heating, and two types of exercise on endothelial function.(187) In general, they found a decrease in brachial artery FMD with increased brachial artery retrograde blood flow, in a dose dependent manner. However, there are several problems associated with the design and conclusions in those studies. For instance, retrograde shear rate actually increases during blood flow restriction, but shear stress (not reported) should have remained constant due to low shear rate values, lower than 100 s^{-1}. In this case, blood viscosity acts as shear stress buffer, as previously discussed (Pages 96 and 97). If brachial artery retrograde shear stress was

not increased during the blood flow restriction model (181), changes in brachial FMD could be explained by the third blood flow pattern dimension: presence of turbulence. Assuming an average brachial artery diameter of 4 mm (187), peak retrograde velocity from reported shear rate (181, 187), and an average hematocrit of 50%in healthy, young men (Tables 4-1 and 4-2), peak retrograde Re calculated for both studies should be lower than ~500, indicating laminar flow. Including the three dimensions of blood flow patterns to these studies could elicit a different interpretation. Based upon the three dimensions blood flow pattern classification, the decrease in brachial FMD was not produce by an increase in retrograde shear stress, as state by the authors (181, 187), but by a retrograde-laminar blood flow, without increased shear stress.

Based upon previous and present study results (27, 28, 45, 57, 181, 187), we could characterize blood flow patterns as either beneficial for endothelial health, such as 1) retrograde-turbulent without increased shear stress and 2) antegrade-laminar with increased shear stress, or detrimental for endothelial health, such as 1) antegrade-turbulent without increased shear stress and 2) retrograde-laminar without increased shear stress. Three dimensions blood flow pattern classification allows a better understanding of blood mechanics and endothelial mechano-transduction, which regulates endothelial function.(24, 41)

Endothelial Mechano-Transduction

According to the 'bumper-car' (178) and 'decentralized' (89) models of endothelial mechano-transduction, blood flow-induced shear stress will 'push' the glycocalyx activating a cascade of events that promotes beneficial endothelial cell activation (Figure 3-4). However, the new blood flow pattern classification scheme with three dimensions as outlined in the present study, illustrates how this mechanical stimulus

could either activate or de-activate endothelial cells depending on the blood flow pattern. For example, both antegrade-laminar with increased shear stress and retrograde-turbulent without increased shear stress blood flow patterns would 'push' the glycocalyx downstream, thereby beneficially activating endothelial cells. Both patterns of mechanical stimulation were observed in our brachial and femoral artery blood flow patterns, respectively, during EECP. In contrast, both antegrade-turbulent without increased shear stress and retrograde-laminar without increased shear stress blood flow patterns would 'push' the glycocalyx upstream de-activating endothelial cells, as observed in DePaola *et al.* (49) and Thijssen *et al.* (181) studies, respectively (Figure 6-1).

The majority of the in vitro and animal vascular studies to date were designated to investigate atherosclerosis plaque formation and evolution (26-28, 41-43, 49, 57, 89, 159, 178, 189, 207). Few studies have been designed to investigate the mitigating effects of different blood flow patterns on endothelial function.(177, 181, 187, 188) The new blood flow pattern classification scheme outlined in the present study will allow a better understanding of endothelial changes induced by interventions such as exercise or EECP.(14, 188)

Exercise-Induced Blood Flow Patterns, Endothelial Function, and Endothelial Oxidative Stress

It is generally accepted that exercise training decreases the risk of a cardiovascular event and improves endothelial function, in both normal and clinical populations.(15, 74, 80, 128, 167) However, ~40% of the beneficial effects of exercise training on cardiovascular risk factors is due to unknown factors, where the direct effects of exercise-induced blood flow on endothelial function may play an important

role.(74, 128) Although, both acute and chronic exercise have been shown to improve FMD in apparently healthy (30, 86, 88, 141, 196) and in clinical populations (20, 59, 68, 71, 82-84, 198), the direct effects of exercise-induced blood flow on endothelial function are yet to be determined.

The present *in vivo* study was designed to determine, for the first time, the direct effects of exercise-induced blood flow patterns on endothelial function and endothelial oxidative stress in humans.

Exercise-Induced Blood Flow Patterns and Endothelial Function

Based on the beneficial blood flow patterns described in Figure 6-1A, the present study shows that aerobic exercise at an intensity of 70% VO_2max will produce a retrograde-turbulent without increased shear stress blood flow pattern in both brachial and femoral arteries (Figures 4-11 and 4-13, B and D, open boxes). A similar blood flow pattern in the brachial and femoral arteries is produced by resistance exercise at an intensity of 70% 1-RM (Figures 4-11 and 4-13, A and C, open boxes). Although this is the first time that blood flow patterns during exercise have been characterized with three dimensions, our results agreed in part with previous studies, specifically in the dose-dependent increase of shear rate in antegrade and retrograde flow observed during exercise.(73, 177, 181, 187) However, the lack of three-dimension blood flow characterization in these studies perhaps fostered spurious conclusions, such that all retrograde blood flow is detrimental for endothelial function.(181, 187)

Although neuro-muscular adaptations due to exercise training happen within 4 days, cardiovascular adaptations and changes in traditional and inflammatory cardiovascular risk factors, in general derived from changes in the hormonal state, are observed after 8-10 weeks.(17, 53, 81, 128, 149, 154, 161) To investigate the

mechanical effects of exercise-induced blood flow on endothelial function, we designed a short-term, short-session lower body exercise training protocol that did not affect the resting hormonal axis, but significantly change blood flow pattern during exercise.

There were significant changes in leg extension 1-RM with resistance exercise training (RXT) and VO_2max with aerobic exercise training (AXT), which could be attributed to neuro-muscular adaptations.(17, 149) Although several hormones are related to cardiovascular and metabolic changes after exercise training, e.g. epinephrine, cortisol, and growth hormone, norepinephrine is one of the most representatives.(17, 53, 81, 128, 149, 154, 161) Indeed, there was no significant change in resting plasma norepinephrine levels, indicating that observed vascular exercise-induced adaptations could be attributed to the mechanical effect of exercise-induced blood flow (Figure 4-14).

The present study shows that both brachial and femoral artery flow mediated dilation were improved after 4 weeks of either AXT or RXT (Figures 4-15 and 4-16). Because the upper extremities were not recruited during either exercise modality, these results confirm that lower body exercise-induced blood flow beneficially affects endothelial function and that this improvement is systemic and not localized to exercising vascular beds, as previously reported.(72, 78, 177) Furthermore, this improvement in brachial and femoral FMD with AXT and RXT occurs coincident and in parallel with the increase of plasma Nitrite/Nitrate (NOx), which is a broad indicator of NO biovailability. This FMD/NOx relationship strongly suggests an improvement in NO-mediated vasodilation and endothelial function.(20)

Endothelial Function and Endothelial Oxidative Stress

To elucidate the possible mechanisms involved in this endothelial function improvement, we analyzed eNOS content and oxidative stress, via endothelial nitrityrosine content, in venous endothelial cells collected from AXT, RXT, and control subjects. While eNOS expression was upregulated with exercise training, nitrotyrosine expression was downregulated (Figures 4-19 and 4-20). These results suggest that endothelial cell oxidative stress is decreased after 4 weeks of either AXT or RXT and that there is a direct and inverse relationship between NO bioavailability and endothelial oxidative stress. These findings are in agreement with *in vitro* and animal studies showing that endothelial cell oxidative stress is a key factor responsible for endothelial cell regulation.(29, 87, 92, 135, 186, 204)

As there was no change in resting plasma norepinephrine levels, we could attribute endothelial oxidative stress downregulation to the mechanical effects of exercise-induced blood flow. However, endothelial cell samples were obtained from an antecubital vein and not from the arterial side. Regardless of anatomical differences, there is a direct relationship between arterial and venous eNOS and nitrotyrosine expression.(31, 62, 148) Moreover, the expected venous blood flow pattern would be antegrade-laminar with increased shear stress, due to venous unidirectional flow and increased venous return during exercise, which is considered beneficial. Therefore, the direct mechanical effect of exercise-induced blood flow would activate venous endothelial cells via mechano-transduction, as described in the 'bumper-car' and 'decentralized' models for endothelial cell mechano-transduction (Figure 6-1).(41, 89, 201)

Although aerobic exercise at 70% VO_2max produced a more beneficial blood flow

pattern than resistance exercise at 70% 1-RM, both types of exercise improved brachial and femoral FMD. It is interesting to note that we observed greater changes in eNOS and nitrotyrosine expression after RXT compared to AXT. One possible explanation for these mildly contradictory results would be that determination of blood flow patterns during resistance exercise (Experiment 2) was made at the onset of the quadriceps muscle contraction. The lack of a full dynamic quadriceps contraction during RX in Experiment 2 could have cause underestimation of turbulence and shear stress. It would be interesting to investigate the effects of other RTX regimens on endothelial cell function. Recruiting larger muscle mass, as during leg-press exercise, shifting higher repetition frequency vs. higher workloads, and having longer training sessions may achieve a better approximation of blood flow patterns observed during a therapeutic session of EECP.

Clinical Relevance

Cardiovascular diseases are the leading cause of death in the United States and Western World.(160) Endothelial dysfunction is the first pathophysiological step toward atherosclerosis, which is the cause of 80-90% of cardiovascular deaths.(29, 113, 117, 135, 204) Characterization of blood flow mechanics during cardiovascular interventions, e.g. EECP or exercise training, could explain the ~40% of cardiovascular risk reduction that is not related with traditional CV risk factors.(74, 128) Based upon the results from the present study, the clinical relevance section will focus on the three major ischemic cardiovascular diseases, i.e.1) coronary artery disease (CAD), 2) cerebro vascular disease (CVD), and 3) peripheral artery disease (PAD).

Coronary Artery Disease

The heart is supplied with oxygen and nutrients by the coronary circulation. Three medium-sized conduit epicardial arteries, i.e. right main, left main and circumflex coronary arteries, are the feed arteries for a complex and tortuous arterial net that perfuse the myocardium.(10, 25, 99) These proximal epicardial arteries, in particular those perfusing the left heart, are more likely to form atherosclerotic plaques principally due to the low shear stress observed at bifurcations.(24, 40, 41, 44, 105)

Major left coronary artery (LCA) perfusion occurs during diastole, when myocardial contraction is not compressing the vessel.(10) LCA blood flow pattern is complex and depends upon heart rate and myocardial contractility.(39, 98, 145, 169) As described by Davies *et al.* (39) there are 4 phases to the blood flow pattern in the left coronary circulation during a single cardiac cycle. First, blood flow is retrograde at onset of ventricular systole. There is then a rapid shift, for a short period of time, to antegrade flow due to peak systolic pressure. Blood flow then becomes retrograde for the remainder of systole. Finally, flow shifts back to antegrade during the duration of diastole.(39)

Based upon our results, during an EECP session the coronary blood flow pattern would be antegrade-laminar with increased shear stress due to the peripheral pumping action and the increase in central aortic diastolic pressure produced by the cuffs (Figure 4-2B), which should improve coronary endothelial function. In contrast, exercise interventions could improve coronary endothelial function via an increased retrograde-turbulent flow due to 1) an increase in heart rate that will reduce diastolic time, and 2) an increase in myocardial contractility that will enhance retrograde flow during systole. However, these hypothetical effects need to be investigated.

Cerebro Vascular Disease

Of all strokes, 87% are ischemic.(160) Although embolisms from the heart and periphery are included in this tally, the vast majority of ischemic strokes have their origin in CVD.(19, 95) Although there is increasing evidence that the predominant mechanism for stroke is embolism (19), the primary sources of cerebral emboli are the atherosclerotic plaque rupture and intra-plaque hemorrhage.(19, 204) The internal carotid artery is the principal site where atherosclerotic plaques are found in the cerebral circulation. The development of these atherosclerotic plaques is associated with the lower shear stress observed at the common carotid artery bifurcation.(24, 40, 41, 44, 105)

Despite some controversy about cerebral circulation hemodynamics (70, 91), it seems appropriate to say that the intracraneal blood flow pattern is antegrade-laminar beyond the circle of Willis. In fact, the circle of Willis works as a hub that redistributes blood flow to the brain despite carotid artery stenosis.(139, 170) On the other hand, the extracranial artery blood flow pattern is antegrade and pulsatile, without retrograde flow, and generally laminar except at the bifurcations.(28, 44)

Based upon our results, during an EECP session the extracranial blood flow pattern would be antegrade-laminar with increased shear stress, as observed in the brachial artery during EECP (Figures 4-3B,4-6B, and 4-7B), which should improve coronary endothelial function. This hypothetical cerebral blood flow pattern during EECP is in agreement with recent studies of blood flow velocity in the middle cerebral artery during EECP.(120, 197) In contrast, exercise interventions could stabilize carotid artery atherosclerotic plaque, as proposed by Cheng *et al.* and Koskinas *et al.* (27, 28, 103), via antegrade-turbulent with increased shear stress blood flow pattern due to the

increase in heart rate and blood pressure observed during exercise. However, these hypothetical effects need further investigation.

Peripheral Artery Disease

Pathophysiology of PAD involves atherosclerotic plaque formation below the iliac artery bifurcation from the abdominal aorta and other bifurcation throughout the length of the femoral artery (174, 199, 204), which are associated with low shear stress.(24, 40, 41, 44, 105)

Although normal femoral blood flow pattern is laminar oscillatory or bi-directional, normal iliac artery blood flow is antegrade laminar but pulsatile, similar to what is observed in the common carotid artery.(44, 156, 174)

Based upon our results, the femoral artery blood flow pattern during EECP was retrograde-turbulent without increased shear stress (Figures 4-4B,4-6D, and 4-7D). We could expect a similar blood flow pattern in the iliac artery during EECP due to the anatomical proximity with the femoral artery. Thus, this blood flow pattern would decrease endothelial cell oxidative stress in these arteries and improve PAD.(38, 87, 92, 135) Similarly, exercise interventions could improve iliac and femoral arteries endothelial function via an increased retrograde-turbulent without increased shear stress blood flow pattern (Figures 4-11 and 4-13). However, these hypothetical effects need to be investigated.

Study Limitations and Future Research

Study Limitations

The present study was not without limitations. Although our Laboratory has state-of-the-art equipment to acquire vessel diameters and blood flow velocity for FMD assessment, single determinations of blood vessel diameter, blood flow velocity Doppler

spectrum, and electrocardiogram (EKG) during exercise and EECP would require additional equipment. For example, high-resolution ultrasound, Doppler spectrum, and EKG signals may be synthesize in a single computer screen via LabView (NI Corp, Austin, TX) to improve data accuracy. These changes could improve time-averaged calculations of vessel diameter and blood flow velocity.(73, 171) In addition, data acquisition during exercise was challenging, as also described by others.(155-157) To decrease methodological error, data was continuously recorded during exercise and all frames included for analysis had to meet at least 2 of 3 inclusion criteria: 1) be more than 3 seconds apart from any contiguous selected frame, 2) contain a clean vessel diameter, and 3) contain averaged peak systolic and diastolic velocities calculated by Doppler. Then, data analysis involving blood flow velocity, an important variable for Reynolds number determination, was averaged among at least 10 different time points per bout of exercise. Finally, to declare the presence of turbulent flow during exercise, we use normalized Re 95% C.I. > 2000 to assure that 95% of the subjects had turbulent flow, further decreasing blood flow velocity variability.(155-157)

Another limitation of the present study was that endothelial cells were harvested from the venous side and not from the arterial side. Endothelial cell biopsies from the arterial side would have allowed stronger statements regarding mechanical effects of blood flow patterns on endothelial function, endothelial cell oxidative stress, and endothelial cell mechano-transduction. However, arterial endothelial cell harvesting technique is much more invasive and should perhaps be performed by a general surgeon. Fortunately, previous studies have shown that there is a direct relationship between arterial and venous eNOS and nitrotyrosine expression.(31, 62, 148) Thereby,

our findings on venous eNOS and nitrotyrosine expression could be extrapolated to the arterial side.

Future Research

The present study has revealed controversial results, specifically related to the importance of blood's non-Newtonian fluid characteristics. Previous *in vitro* and *in vivo* studies have neglected to address this factor and assumed a constant blood viscosity.(24, 28, 41, 45, 49, 73, 177, 181, 187) The next research step would be the design of an *in vitro* model that would permit the study of blood flow patterns in arterial preparations, where manipulation of blood flow's three dimensions, i.e. direction, turbulence, and shear stress, would answer the remained questions about blood flow-induced endothelial mechano-transduction.

Once the characterization of anti-atherogenic and pro-atherogenic blood flow patterns is attained *in vitro*, translational studies to clinical populations, such as obese, hypercholesterolemic, the elderly, and patients with coronary artery disease and stroke should follow systematic investigation. Moreover, these clinical studies could investigate the endothelial effects after different exercise intensities. For instance, we observed two different beneficial blood flow patterns during two different aerobic exercise intensities. Aerobic exercise at 40% VO_2max produced an antegrade-laminar with increased shear stress blood flow pattern, while at 70% VO_2max the blood flow pattern was retrograde-turbulent without increased shear stress (Figures 4-11B and 4-13B). However, we studied only the vascular effects of chronic aerobic exercise at 70% VO_2max. Perhaps AXT at 40% VO_2max would also improve endothelial function; however, via a different blood flow pattern.

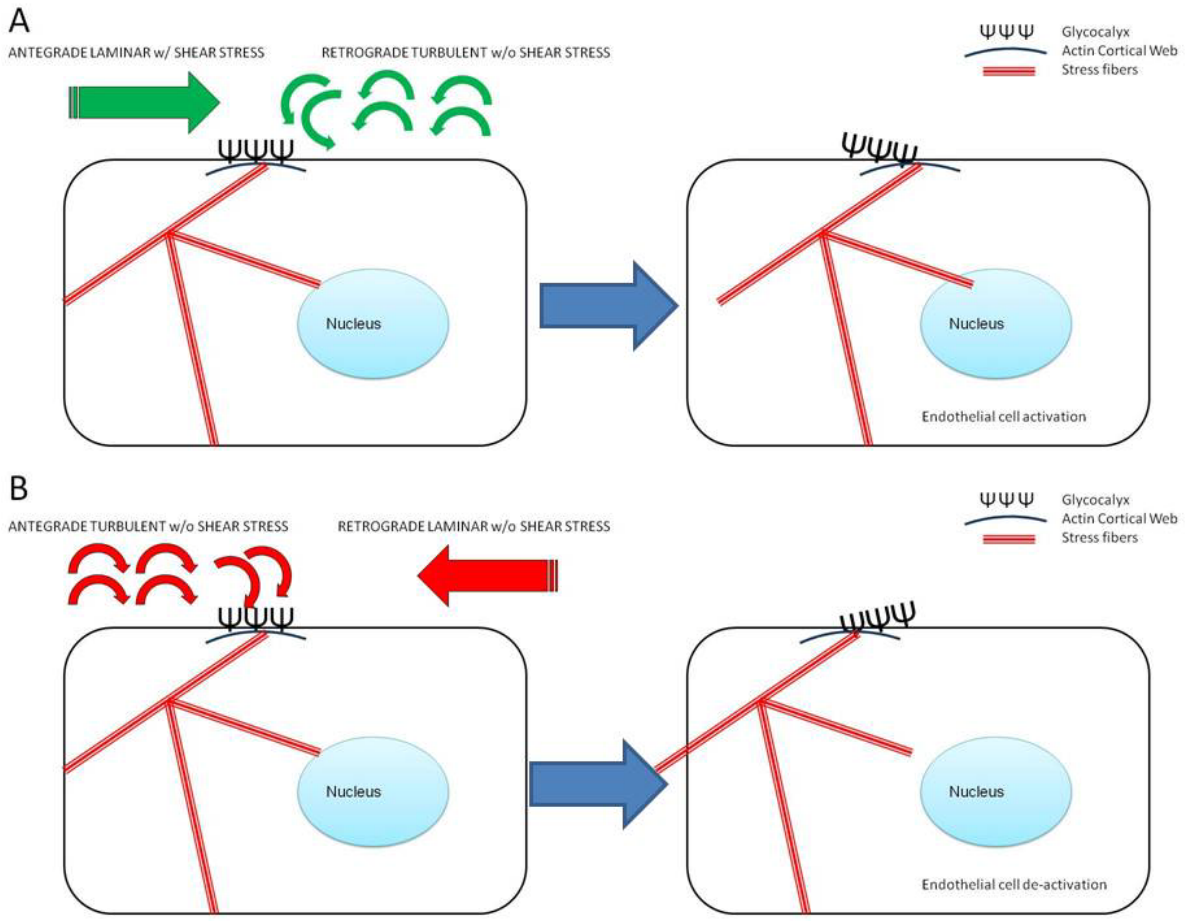

Figure 6-1. New blood flow pattern classification scheme and endothelial activation. A) Antegrade-laminar with increased shear stress and retrograde-turbulent without increased shear rate blood flow patterns activate endothelial cells via mechano-transduction. B) Antegrade-turbulent without increased shear stress and retrograde-laminar without increased shear stress blood flow patterns de-activate endothelial cells via mechano-transduction.

APPENDIX A
ENDOTHELIAL CELL HARVESTING PROTOCOL

The endothelial cell harvesting protocol used in the present study was based upon the literature (31, 62, 148) and personal advice provided by Dr. Gary L. Pierce from the Medical College of Georgia. Basically, the protocol is divided in two parts 1) collecting the sample and 2) processing and fixing the sample.

Endothelial Cell Sample Collection

Before starting with the procedure, be sure to place a paper-towel pad under the designated elbow. The procedure MUST to be sterile. Practitioner and helper needs to keep their hands sterile at any time during the procedure. Be sure to have sterile surgical gloves in the size of practitioner and helper. All material in contact with the subject has to be sterile and accordingly prepared in a sterile field set with a non-fenestrated surgical drape (Object A- 1).

Object A-1. Sterile preparation of surgical material used during endothelial cell harvesting procedure (Preparation.mov, 18.6Mb)

Once the antecubital area of the designated arm is cleaned with ChloraPrep® Sepp® applicator, another non-sterile helper will apply a tourniquet on the upper arm to proceed with phlebotomy. Phlebotomy will be performed with a Becton-Dickinson® Angiocatheter Autoguard® 18GA 1.16 inches, following manufacturer recommendations (Object A- 2). Once the catheter is placed, release tourniquet, put the Baxter® Interlink® Injection Site (cap) on the tip of the catheter, and check catheter permeability with saline (5 ml syringe with 0.9% saline). Fix the catheter with a 3M® Tegaderm® dressing, leaving free cap and catheter tip.

Object A-2. Sterile phlebotomy with intra-venous catheter (Phlebotomy.mov, 21.4Mb)

Then, make surgical field using a fenestrated surgical drape and placing 4 to 5 sterile gauze pads beneath the catheter tip. Grab one sterile J-shaped wire and proceed to open the catheter cap. Leave cap on the sterile field and advance the wire through the catheter. Be sure to advance it 2 to 5 inches "scraping" 2 to 3 times and rotating the wire with the fingers. Take the wire out and, with the free hand, close the catheter tip with a finger until the wire is cut and thrown away into the sharps box (Object A-3). Re-cap the catheter, check for catheter permeability with saline, grab the second J-shaped wire, and re-start the procedure.

Object A-3. Endothelial cell harvesting technique using J-shaped wire (Cell_ harvesting.mov, 19.9Mb)

After finishing with second or third J-shaped wire, sterile conditions are not longer needed and cleaning up process begins. First, grab fenestrated surgical drape and bloody gauze pads and throw them away in the biohazard box. Then, take the 3M® Tegaderm® off and throw it away in the biohazard box. Afterward, take the catheter off, putting pressure with a clean gauze pad with free hand, and throw it away in the sharps box. Finally, clean any blood residue on the antecubital area with alcohol pads and put a Band-Aid® in the catheter mark. Leave the subject on the gurney for 5 to 10 minutes, to prevent arm bleeding.

The wires are collected in a 50 mL Falcon tube with dissociation buffer (pH=7.4; 495 mL PBS; 2 mL EDTA (0.5 M); 1.8 mL Heparin (5000 µ/mL); 2.5 g BSA) by cutting ~3" from the tip of the wire, trying not to get drops of blood in the tube. Keep the tubes on ice until processing and fixing procedures start. Clean the wire-cutters with an alcohol pad immediately, before the next procedure.

Endothelial Cell Sample Processing and Fixing Procedures

Once the wires are in the 50 mL Falcon tube with dissociation buffer, there are several steps until slides with endothelial cells are ready for -80°C storage or analysis.

1. Put gloves on. Get 10 mL serological pipette and forceps.
2. Clasp the wires with a pair of forceps, and hold the wires inside of the tube, but above the solution.
3. Rinse the wires with the dissociation buffer using serological pipette for 10 minutes.
4. After 10-minute period is up, throw the wires away in the sharps box. Clean forceps with alcohol wipes.
5. Centrifuge the tube with a balance for 6 minutes at 400x g and 4°C.
6. Prepare the Formaldehyde solution by first placing 15 mL Corning test tube in foil cover marked "Formaldehyde".
    - 1 collection: 100μL Formaldehyde + 900μL PBS
    - 2 collections: 200μL Formaldehyde + 1800μL PBS
7. Dump ice from cooler into sink, and place cooler back on top of fridge.
8. Leave ~4 mL in the tube and vacuum off the rest without disturbing the pellet.
9. Fix the sample(s) with 1mL Formaldehyde solution (5%) into the sample tube. Do not re-suspend. Incubate for 10 minutes at room temperature.
10. Place 8 slides on a slide tray, and label them with a Sharpie. Subject ID, Visit #, Vein/Artery, Date, Protocol #, your initials.
11. Draw an oval on each slide with pap pen.
12. Add 15mL PBS into the sample tube and re-suspend pellet by mixing up and down in serological (or 1000 mL) pipette until pellet is re-suspended.
13. Centrifuge the tube with a balance for 6 minutes at 400x g and 4°C.
14. Leave ~4 mL in the tube and vacuum off the rest without disturbing the pellet.
15. Add 12mL PBS into the sample tube and re-suspend pellet.
16. Centrifuge the tube with a balance for 6 minutes at 400 x g and 4°C.

17. Leave ~3-4 mL in the tube and vacuum off the rest without disturbing the pellet.

18. If still blood in pellet, do a third wash: add 10mL PBS and re-suspend. Centrifuge for 5 minutes. Leave ~2mL and vacuum off the rest.

19. Re-suspend and then evenly spread onto 6 to 10 slides within the circular area.

20. Place into incubator @ 37°C for 5 hours.

21. Store in -80°C freezer in corresponding slide box.

APPENDIX B
ENDOTHELIAL CELL STAINING PROTOCOL

The endothelial cell staining protocol used in the present study was based upon the literature (31, 62, 148) and personal advice provided by Dr. Gary L. Pierce from the Medical College of Georgia. The protocol is divided in two parts 1) preparation of solutions and 2) staining protocol.

Preparation of Solutions

Before starting the staining protocol, be sure to prepare all the solutions that will be used. Dilutions shown used in the present study were obtain from several pilot staining ran beforehand.

1. Prepare 5% Donkey Serum (Jackson ImmunoResearch Labs., West Grove, PA)
2. Prepare primary antibodies
    a. eNOS (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) dilution 1:500.
    b. Nitrotyrosine (from Millipore, Billerica, MA) dilution 1:250.
    c. Von Willebrand factor (Dako, Glostrop, Denmark) dilution 1:200.
3. Prepare secondary antibodies. Ensure that these secondary antibodies are prepared under foil shields to protect from the light.
    a. Alexafluor 555 fluorescent secondary antibody (Invitrogen Corp, Carlsbad,CA) dilution 1:250.
    b. Prepare secondary VWF in two steps by adding each of Biotin and Streptavidin into 5% Donkey Serum.
        i. Biotin (Jackson ImmunoResearch Labs., West Grove, PA) dilution 1:600.
        ii. Streptavidin (Jackson ImmunoResearch Labs., West Grove, PA) dilution 1:1000
4. DAPI (Invitrogen Corp, Carlsbad, CA) dilution 1:2800.

Place all solutions in the fridge immediately after they are made.

Staining Protocol

Before proceeding with the staining protocol, take slides out of freezer and wait 5 minutes at room temperature. Then, wipe away excess water with kimwipes without

touching center of slide. Finally, re-hydrate the slides by adding PBS to each slide and leaving them for 10 minutes.

1. After 10 minutes of re-hydration, wipe away excess PBS with kimwipes without touching center of slide.
2. Add 300-350 µL of 5% Donkey Serum for 60 minutes. A piece of parafilm could be placed over the circled area to ensure complete coverage of the chemical.
3. Wipe away excess 5% Donkey Serum with kimwipes without touching center of slide.
4. Wash with PBS in columns for 5 minutes on a rocker. Wipe away excess PBS with kimwipes without touching center of slide.
5. Add 300-350 µL of primary antibodies (von Willebrand factor and protein of interest) for 3 hours. A piece of parafilm could be placed over the circled area to ensure complete coverage of the chemical.
6. Wipe away excess primary antibodies with kimwipes without touching center of slide.
7. Wash with PBS in columns for 5 minutes on a rocker. Wipe away excess PBS with kimwipes without touching center of slide.

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8. Add 300-350 µL of Alexafluor 555 (secondary antibody) for 60 minutes. A piece of parafilm could be placed over the circled area to ensure complete coverage of the chemical. Cover from light.
9. Wipe away excess secondary antibody with kimwipes without touching center of slide.
10. Wash with PBS in columns for 5 minutes on a rocker. Wipe away excess PBS with kimwipes without touching center of slide.
11. Add 300-350 µL of von Willebrand factor secondary antibody for 30 minutes. A piece of parafilm could be placed over the circled area to ensure complete coverage of the chemical. Cover from light.
12. Wipe away excess secondary antibody with kimwipes without touching center of slide.
13. Wash with PBS in columns for 5 minutes on a rocker. Wipe away excess PBS with kimwipes without touching center of slide.

14. Add 300-350 µL of DAPI for 30 minutes. A piece of parafilm could be placed over the circled area to ensure complete coverage of the chemical. Cover from light.

15. Wipe away excess DAPI with kimwipes without touching center of slide.

16. Wash with PBS in columns for 5 minutes on a rocker. Wipe away excess PBS with kimwipes without touching center of slide.

17. Allow slides to dry for 20 minutes. Cover from light.

18. Add one drop only of mounting medium to each slide and cover each with a cover slip.

19. Place slides in refrigerator covered with foil overnight.

20. Analyze the next day

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BIOGRAPHICAL SKETCH

Alvaro N. Gurovich was born on 1967, in Santiago, Chile. After making the Chilean swimming national team several years and being the 50 and 100 meters freestyle Chilean record holder, Alvaro moved 300 km south of Santiago to pursue his degree in Physical Therapy and Exercise Science at the most prestigious Exercise Science School at that time, the Pontificia Universidad Católica de Chile. Alvaro graduated in September 1990 and started an Exercise Sciences diploma at Biosystem-Rosario, Argentina, headed by Dr. Juan Carlos Massa, FACSM.

From 1992 to 2005, Alvaro worked as exercise physiology advisor and sports physical therapist for several Chilean national teams and professional athletes including, roller skating, cycling, swimming, and synchronized swimming, the Division-A professional soccer team Cobreloa F.C., 2000 Chile's Davis Cup team, and 2004 Olympic tennis gold medalist Nicolás Mazzú.

Since 1991 Alvaro has been linked to Academia. First, by teaching exercise physiology to Physical Education undergraduates at the Universidad Católica de Valparaiso. Then, Alvaro did research on Physical Anthropology at Prof. Atilio A. Almagià's Life Population Anatomy Laboratory in the Pontifica Universidad Catolica de Valparaiso. In 1995, in collaboration with Prof. Almagià, Alvaro participated in the curricular design of the School of Kinesiology and Physical Therapy of the Pontificia Universidad Católica de Valparaiso. In 2000, the School of Kinesiology and Physical Therapy of the Pontificia Universidad Católica de Valparaiso hired Alvaro as a half-time faculty member. In 2004, Alvaro was promoted to a full-time tenured faculty position and served as Academic Chair until he moved to University of Florida to pursue his Ph.D in August 2006.

Upon completion of his Ph.D., Alvaro will have an appointment as post-doctoral research fellow in Dr. Chris Baylis' Renal Physiology Laboratory, Department of Physiology and Functional Genomics, University of Florida College of Medicine.

Alvaro has been married to Carolina Valencia for 8 years and they have two kids, Benjamin who is 5 years old, and Sebastian who is expected in mid October 2010. Also, Alvaro has three teenage children, Nicolás, Joaquín and Fernanda who live in Chile. They have been very patient while waiting for dad to complete his dream.