INTRINSIC EXERCISE CAPACITY ON HIND LIMB ARTERIAL LIGATION: DIFFERENTIAL IMPACT OF ACTIVE EXERCISE AND ANDROGEN SIGNALING ON POST-ISCHEMIC RECOVERY

by

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ABSTRACT

Peripheral arterial disease (PAD) is a common disease that is frequently caused by atherosclerosis and associated thrombosis, leading to a decrease in the blood supply to the lower extremities. PAD has various treatment options due to different physiological responses in each patient. One such option includes exercise therapy, since it benefits patients with intermittent claudication by improving their overall quality of life. However, advising exercise therapy for treatment of PAD is not clearly supported, due to the heterogeneity of responses and outcomes found in the scientific literature. This heterogeneous response elaborates the importance of assessing the differential changes resulted from the interaction of active exercise capacity with PAD between high and low intrinsic aerobic capacity phenotypes. High intrinsic aerobic phenotype rat (HCR) reported to have prolonged dopamine availability in the brain. Recently, Dopamine 3 receptor (D3R) was reported to be associated with decrease dopamine reuptake centrally. Despite evidence indicating a role for androgen in dopamine receptors interaction centrally, this interaction remains not studied at the level of skeletal muscle. The overall

goal of this dissertation is to determine the vascular adaptive response in relation to active exercise capacity and intrinsic aerobic running capacity phenotypes, HCR and LCR, before and after femoral artery ligation. Additionally, this study will test the hypothesis that exercise after ligation is harmful for the LCR phenotype, while exercise before ligation is protective to ischemic injury, and that post-ischemic repair in skeletal muscle cells (SKMC) associates androgen receptor (AR) and dopamine 3 receptor (D3R) ***In vivo* study**: We found that mild exercise intensity after arterial occlusion in rats of low intrinsic aerobic running capacity phenotype (LCR) produces a chronic, ischemia-like vascular adaptive response, with more hypoxia-induced angiogenesis than inflammogenic-induced vasculogenesis. Furthermore, rats of high intrinsic aerobic running capacity phenotype (HCR) showed better muscle-capillary adaptive response to ischemia with more inflammogenic-induced vasculogenesis. However, two weeks of treadmill exercise before femoral arterial ligation was found to precondition the ischemic limb in low intrinsic aerobic running capacity phenotype through remodeling the musculovascular adaptive response. Although, this same procedure did not cause a significant effect in HCR rats. ***In vitro* study**: It was found that HCR and LCR have different androgen/dopamine receptor interaction systems, with a stronger protective mechanism in HCR than LCR. In LCR, there is crosstalk between androgen and dopamine receptor signaling during satellite cell proliferation in response to D3R and AR activation respectively. While in HCR, the signaling effect of D3R stimulation does not depend on androgen receptor signaling.

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DEDICATION

This dissertation is lovingly dedicated to my parents, Omar and Norah Zakari; my children, Adnan and Noran Kinany; their father, Mazen Kinany; and my sisters and brother. Without their support, this work would not have been possible.

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Problem and Approach:

Peripheral arterial disease (PAD) is a common disease that is most commonly caused by atherosclerosis and associated thrombosis, leading to a decreased blood supply to the lower extremities (234). PAD has varying treatment options, such as exercise therapy, medical therapy, endovascular therapy, and surgical intervention, due to different physiological responses in each patient (136). Exercise training is one of the important modalities that is frequently advised for PAD patients, since it benefits patients with intermittent claudication by decreasing disease symptoms and improves both physical activity and quality of life (243). However, advising exercise therapy for PAD patients not clearly supports the different responses seen in the outcomes of patients in the scientific literature. The aerobic capacity, which depends on the maximum O2 consumption (VO2max), is one of the important physiological factors that contributes to in endurance running performance (123), as it accounts for 70% of variations in exercise capacity (57). Thus, studying the differential effects of exercise capacity on hind limb ischemia between the intrinsic aerobic phenotype rats bred for low and high aerobic running capacity, would provide a better platform for understanding the differences in the effect of exercise pre- and post-ligation on vascular recovery, and angiogenic and inflammatory adaptive responses in these phenotypes.

Therefore, studying the interaction of genetic factors with active exercise will clarify the differences in active exercise outcomes, and elude to the possible roles of androgen and dopamine receptors in this relationship. For this reason, we are going to use rats with inherently different running capacity that are artificially selected over generations to produce strains with genetically determined high and low intrinsic capacity (HCR and LCR).

Exercise Physiology:

Exercise is a set of physical activities applied to the skeletal muscles in different ways to keep them fit. It is a form of stressful stimuli that leads to specific changes in the muscle physiology. When the internal homeostasis is imbalanced by any physical activity, the cellular response to that effect will be activated to deal with that change. During exercise, the cellular response to the physical activity will be to limit the internal stress in order to maintain normal homeostasis. Therefore, taking part in regular exercise will produce chronic physiological adaptations. Exercise physiology is a study of how physical activity influences the cardiovascular, endocrine, and musculoskeletal systems. There are two reasons for people to exercise; exercising for fitness or rehabilitation. "Fitness" is the category encompassing those performing exercise for athletic improvement or for individuals looking to promote body health or decrease body weight. On the other hand, exercise for rehabilitation is used for individuals to help them recover from current injury to improve their livelihoods in the face of chronic medical conditions (181). In general, the health benefits that occur as a result from exercise are innumerable.

General Exercise and Disease:

Studies have revealed that regular exercise is extremely favorable in the promotion of health and treatment of diseases. This reality has persuade some researchers and scientists to consider exercise as a medicine (227). A previous study showed that exercise leads to an infinite number of health benefits, including the longevity and enhancement of one's lifespan and health, respectively (227). In healthy individuals, exercise reduces the risk of death by 20-35% (14,138). A 2004 study showed that middle-aged women who participate in less than one hour of exercise per week face a 52% increase in all causes of mortality, including cardiovascular- and cancer-related mortality, in comparison with physically active women (90). Exercise is also beneficial in patients either as a preventive or therapeutic modality. For example, studies showed that regular exercise has been used to prevent cardiovascular and pulmonary diseases such as, coronary heart disease, chronic obstructive pulmonary disease, hypertension, and intermittent claudication, metabolic syndromes (type 2 diabetes, obesity, and hyperlipidemia), cancer, depression, and musculoskeletal diseases such as osteoporosis, rheumatoid arthritis, fibromyalgia, chronic fatigue syndrome (175, 12), and gout (38). These studies indicate that exercise can be thought of as having the property of a drug when thinking of its optimal or effective dose response. It also suggests the importance of understanding the molecular mechanism of exercise in healthy and diseased conditions. Owing to these uses, exercise is now prescribed in dosage form, just like any other drug (7, 86, 177, 66, 102, 208). The reason for the dosing is that one only achieves optimal health from exercise when they observe it in a particular manner, pattern, and extent.

Peripheral Arterial Disease (PAD) and Exercise:

PAD is a common disease in the elderly, usually resulting from atherosclerosis and associated thrombosis that prevent blood supply to the lower limbs (176). It affects 8.5 million people in the US and around 202 million people worldwide (174). If left untreated, this disease can cause critical limb ischemia. Critical limb ischemia is responsible for more than 150,000 lower limb amputations per year in the United States alone (196). Patients' intolerance to their treatments, such as exercise, due to pain, medication, or their incompatibility for surgery due to comorbidity, causes further progression of the disease and increased mortality. PAD has various treatment options due to the different body responses, including: exercise therapy, medical therapy, endovascular therapy, and surgical intervention (136). However, exercise therapy is more cost effective, safer, and is associated with improved walking distance, claudication symptoms, and most importantly, improved quality of life (243). The mechanisms underlying the beneficial effect of exercise include: vasodilation of the collateral vessels or arterial remodeling, improved glucose and fat metabolism of ischemic muscles, improved energy production, decreased oxidative stress, stimulation of angiogenesis, improved peripheral nerve conductance, and increased pain threshold (212). A meta-analysis study (2) showed that exercise has better outcomes compared with revascularization alone in intermittent claudication. This is because revascularization and reversal arterial occlusion, while improving blood flow, do not reverse muscle function impairment. On the other hand, supervised exercise has the ability to improve muscle functional performance in addition to stimulating shear stress response and angiogenesis,

therefore improving quality of life (72, 81,146). Although it has robust outcomes and is widely available as a treatment to PAD patients, exercise as a treatment modality is not adaptable to some patients, for example some patients with asymptomatic PAD.

Therefore, it is not surprising they are not adaptable to exercise as a treatment if they do not have symptoms! Even studies on exercise have various effects in subjects, which could be due to differences in protocols or differences in individual intrinsic aerobic phenotype. It has been clear that endurance exercise is mainly genetic in origin. It accounts for 70% of variations in exercise capacity (86). Thus, studying the different effects of exercise after hind limb ischemia between the intrinsic aerobic phenotype rats bred for low and high aerobic running capacity would provide a better platform for understanding the various effects of exercise, pre- and post-ligation, on vascular adaptive response in these phenotype.

Active Exercise and Genetic Predisposition:

Aerobic exercise capacity is a complex trait of an individual that is impacted by both genetic and environmental factors, such as training, and has been reported as being a predictor of mortality (160). Studies on animals and humans have shown that genes may be a driving force for someone being physiologically active (79). In animals, rodent strains played a role in the divergence of spontaneous physical activity levels (126,131,132). Selective breeding studies of rodents have produced generations of high and low active animals that differ clearly in their predisposition for voluntary physical activity (114, 95). This activity was associated with adaptive physiological changes such as increased cardiac contractility and cardiac hypertrophy (126), suggesting the

importance of physiological adaptation in driving the physical activity (79). Studies on rodent strain breeding through backcrosses and intercrosses between low and high active animals identified multiple quantitative trait loci (QTL) specific for running distance, speed, and duration (120,132). In humans, physical activity has been compared as a hereditary factor. Studies showed that physically active parents produced more active children than inactive parents, even after controlling the environmental factors (154). The variability in the activity among population is related to the variation in genes and the type of instrument used in active exercise (79). Hereditary factor was found to be responsible for 9%-80% of physical activity depending on the intensity of the physical training (11, 39, 50, 141, 151, 179), suggesting the significance of genes in influencing the physical activity. Several genes have been reported to be associated with exercise performance and response to exercise activity (19). These genes responsible for influencing the physiological outcomes of exercise or physical training include: body size or mass, leanness, lipidemia, glucosemia, and hemodynamics (19). These biological traits and phenotypes are consistent with animal models of physical activity, which will help scientists to discover the physiological mechanisms that regulate the capacity of the physical training.

HCR/LCR Rats: A Model of Intrinsic Aerobic Capacity:

The factors involved in regulating aerobic capacity is very complex. Nevertheless, we can limit the main influential factors into intrinsic, or genetic, and extrinsic, or environmental. When genes are influence the aerobic capacity, we call it intrinsic aerobic capacity. This accounts for up to 70% of the variation seen between humans (16)

However, when the environment or active exercise influence the aerobic capacity, we call it extrinsic aerobic capacity or active exercise capacity. This is maximally accounted for (10-20%) and influences the intrinsic aerobic capacity (45, 237). This suggests the importance of the intrinsic factor on the overall aerobic capacity, as well as its dominance in producing the outcomes of diseases that can be prevented or treated by active exercise, such as in cardiovascular diseases. Therefore, genetic predisposition and intrinsic aerobic capacity are likely to contribute to peripheral arterial disease and mortality. Hence, studying this element as an important pathophysiologic mediator in peripheral arterial disease outcomes should be investigated. Thus, we used rats with two different intrinsic aerobic capacity phenotypes. The animals are artificially selected over generations to get strains with phenotypically determined high and low intrinsic aerobic capacity with a 30% difference in maximum oxygen uptake.

Koch and Britton in 2001 generated a rat model with differences in running capacity by using genetically heterogeneous outbreed stock of N:NIH rats from each sex. These rats were artificially selected by using a treadmill running test (111-112). At 12 weeks, two-way artificial selective breeding was used to create low capacity runner (LCR) and high capacity runner (HCR) strains that were divergent for treadmill running capacity (run time for five days to exhaustion on a graded treadmill exercise test). The thirteen lowest and the thirteen highest running capacity rats of each sex were randomly paired for mating. This pattern was repeated over subsequent generations to produce the divergent strains using a rotational breading scheme. This model is very important in studying the interaction of the intrinsic aerobic capacity with the active exercise capacity and how that complex shapes the overall end results in treating or preventing the

peripheral arterial disease. Early studies using HCR and LCR rat models revealed that cardiovascular risk factors emerge after artificial selection for low aerobic capacity (239). LCR rats have higher random glucose, fasting glucose, insulin levels, visceral adiposity, and free fatty acids when compared to HCR (239).

Our lab has been working with this model for about ten years. We utilized generation thirteen rats to determine the phenotypic differences between HCR and LCR in fatty acid disposal as a function of intrinsic oxidation capacity by skeletal muscles under normal and high fat diet protocols. This study confirmed what has been reported by others, i.e. that the LCR phenotype appears to have insulin resistance. The study also revealed the phenotypic differences in the adaptive response to the stress of a high fat diet between HCR and LCR. These results will aid further investigations into the phenotypic differences in adaptive response to chronic stress between HCR and LCR.

Additional work from our lab used this model to investigate the effect of intrinsic aerobic capacity on acute ischemic reperfusion injury. We found that there were phenotypic differences in infarct area to ischemic reperfusion injury after fifteen minutes of ischemia. However, after thirty minutes the differences vanished. This data suggest the significance of the influence of intrinsic aerobic capacity phenotype on the outcome of acute ischemic injury although severe injury cannot be protected by high capacity runner phenotype.

HCR and LCR also were tested in our lab to determine the vascular and metabolic adaptive response to acute peripheral artery ligation. This study showed that LCR vascular and metabolic responses deteriorated after acute ischemic injury and that HCR

was more resistant to the ischemic injury. From this study we developed an interest in the effect of the interaction between intrinsic aerobic capacity and active exercise capacity and their overall outcome on vascular adaptive response before and after the acute peripheral artery ligation.

A study has been shown that exercise attenuates improvements in walking performance for PAD patients with diabetes, while it improves the walking performance in PAD patients without diabetes (137). These studies suggest that genetic susceptibility might be responsible for the negative outcomes resulting from exercise training after the development of peripheral arterial obstructive disease in diabetic patients. The studies also demonstrate that different intrinsic aerobic capacity phenotypic backgrounds might be an important factor in shaping the response to exercise therapy in PAD patients. Using this animal model is essential in studying the link between intrinsic aerobic capacity and active exercise before and after arterial occlusive disease.

Arteriogenesis:

Arteriogenesis, or arterial remodeling, occurs when acute arterial occlusion causes a sudden pressure gradient between proximal arteries (within non-ischemic region) and distal arteries (within ischemic region). This pressure gradient increases blood flow through collaterals, activating shear stress in these collaterals, and subsequently causing alterations in the gene expressions in both calf (ischemic) and thigh (non-ischemic) muscles through shear stress response elements (59, 219). This leads to collateral remodeling to ensure sufficient blood flow to ischemic tissue. During acute ischemia, inflammatory cytokines produced from injured cells potentiate an infiltration of

inflammatory cells into ischemic tissue to remove the debris of necrotic tissue. Monocytes and macrophages also play a crucial role in angio/arteriogenesis by releasing Ccl2/MCP-1 cytokines (5, 52, 77, 223, 224). Also, invading endothelial progenitor cells, which originate from bone marrow mononuclear progenitor cells, a type of stem cell, have shown to be involved in arteriogenesis and blood flow recovery (107) in animal models of peripheral arterial occlusive disease (246). Variations of collateral arteries between animals species (26, 78, 108, 197, 198, 203) and among healthy humans (148) have been recorded. However, the effect of exercise before and after ligation on angio/inflammogenic responses due to hypoxia and shear stress in intrinsic aerobic capacity phenotype, can predict the presence of differential angio/arteriogenesis that stem from the different phenotype backgrounds.

Angiogenesis:

Angiogenesis is the growth of blood vessels from the existing vasculature. It occurs throughout life in both health and disease, beginning in utero and continuing on through old age. *Vascular endothelial growth factor* (Vegf) plays an important role in physiological angiogenesis, such as during exercise, in pathological conditions such as in PAD, and during development (96). Vegfa can produce its signal through Fms-like tyrosine kinase 1(Flt1), which is important receptor in angiogenesis during development. The other receptor, kinase insert domain-containing receptor (Kdr), mediates angiogenesis in the adult. It is important to investigate the impact of exercise-post ligation on angiogenesis signaling between rats that differ in their intrinsic exercise capacity following acute hind limb arterial occlusion. *Angiopoietin 1* (Angpt1) stimulates

and stabilizes angiogenesis while angiopoietin 2 (Angpt2) prevents vascularization. However, when Angpt2 is associated with Vegfa, it stimulates angiogenesis (51). *Nuclear factor kappa-light-chain-enhancer of activated B cells* (Nfkb) is an upstream mediator for Vegfa expression in acute arterial ligation in response to hypoxia (241). *Intercellular adhesion molecule* (Icam) and *vascular cell adhesion molecule* (Vcam) are well-known markers for endothelial activation and dysfunction (220). Their elevated levels indicate leukocyte recruitment from injured endothelial layers to ischemic tissue (127,173). *Platelets derived growth factor alpha* (Pdgfa) plays an important role in early development and in adult. During development, Pdgfa drives the proliferation of undifferentiated mesenchymal cells, but in the adult, it also plays a fundamental role in tissue remodeling, mesenchymal differentiation, and migration (84). Additionally, Pdgfa stimulates fibroblast proliferation for tissue remodeling by recruiting macrophage and fibroblast cells and stimulating their gene expression toward proliferation. Thus, it is responsible for the induction of an inflammatory environment to expedite the healing process (178). *Bone morphogen protein receptor 2* (Bmpr2) is a receptor for BMPs, ligands which are members of the TGF-β superfamily, and is involved in several process of embryonic development and differentiation (29, 109, 242). Its reduced expressions in pulmonary artery hypertension has been associated with increased perivascular macrophages, muscularized distal arteries (195), and endothelial apoptosis (155). The influence of exercise before and after ligation on the vascular adaptive response in intrinsic exercise capacity phenotypes has not been studied.

The Dosage of Exercise:

Dosage is critical in clinical medicine, and drugs need data supporting their safety and efficacy (122). Vina et al. have reported that every organism needs a minimum amount of exercise and performing an amount below which will not be beneficial to their health (227). The advantages accumulating from physical activities increases from an accompanying rise in the amount of physical activities. However, beyond a particular threshold, the negative impacts due to exercise become apparent and begin to replace the advantages. On the other hand, unlike medical drugs, the minimal dose, maximal safe dose, and dose response of exercise vary greatly from one organism to another, and thus the standard of each is unclear (122). This variation has since elicited a non-stop debate on how much, how often, what type, the duration, and with what intensity exercise should be observed (122) due to the different methods in measuring exercise intensity, different classification of dose schemes (122), and different phenotypes within similar species. According to Warburton et al. (232), the levels of intensity of physical training can be measured based on oxygen consumption (VO2) or heart rate. For instance, moderate-intensity exercise is when both heart and respiratory rates are increased without interfering with one's ability to talk, as is observed with brisk walking at a speed of 3.0 mph (80.4 m/min). However, vigorous-intensity exercise is when the heart and respiratory rates are increased to the point of causing difficulty in conversing, as is observed during and after running.

In order to determine the minimal effective dose of exercise for each individual, the exercise dose should be handled clinically, as a pharmacological dose. A physician

begins administrating a drug at its minimal effective dose, and if the patient dose not respond effectively, then the physician begins titrating the dose upwards, towards the maximal effective dose. Exercise should be treated the same way, starting with minimal effort and then titrated towards the maximal effort the individual can tolerate, depending on the current condition (122). That means those who are untrained may require the minimal effective dose of exercise while athletic individuals would requires the maximal effective dose (202). Thus, a person who has a fitness level as recommended for all healthy young to middle-age adults, would be required to train, during a six-month time period, for 300 min or more of moderate-intensity aerobic exercise per week, or 150 min or greater of vigorous-intensity aerobic exercise per week (121,169).

Caution is required when adjusting the exercise dose prescribed for specific populations such as elderly, children, pregnant women, and patients with comorbidities (232). For example, elderly men over 60 do not require vigorous exercise to prevent cardiovascular risks. Instead, regular active exercise is sufficient to significantly decrease mortality rate in this age group (227). For type 2 diabetic patients, they might benefit initially from the training levels that are recommended for healthy adults, followed by a gradual increased toward the recommended doses for specific condition of each individual (169). Overall, according to Wen et al. (235), 15 minutes per day or 90 minutes per week of moderate-intensity physical activity is valuable to an individual's health and life expectancy, and even for persons with high cardiovascular risks (122). This suggests that exercise might be harmful if used in wrong dose in specific condition.

Role of Androgen in Cardiovascular Protection:

Clinical and basic experimental studies provided huge evidences about the atheroprotective effect of estrogen and testosterone on blood vessels. While studies regarding androgen's role on vasculature have been controversial for a long time, in recent years a great amount of work has suggested that androgens exert protective effects against the development of atherosclerosis in both genders in human and in animals. Among the female population, cardiovascular diseases have been known to be more detrimental than in the male population (9, 74, 240, 252). Although the effect of testosterone on cardiovascular events and mortality in women has not yet been evaluated in large cohort studies, a prospective cohort study in more than 2000 women, that were followed up after more than four years from the start of the study, found that women with the lowest levels of testosterone had the greatest risk of a cardiovascular event independent of traditional risk factors (148). In men, several studies showed the inverse relationship between testosterone levels and cardiovascular events (200, 152, 104). In animal model, studies on hind limb occlusive model found androgen receptors are essential for revascularization response in ischemia in both male and female mice (103). Moreover, the testosterone hormone also has been found to increase numerical density of capillaries in left ventricle after injection into aging male rats for 4 months (64). The mechanisms underlying the associations of low testosterone with CVD are complex and poorly understood. However, estradiol and dihydrotestosterone administration in gonadectomized male mice has been reported to increase angiogenesis through increasing vascular branching and vascular diameter, respectively (213).

CVD incidence among women is low before menopause, but progressively elevates upon beginning menopause. This increase is thought to result partially from the

loss of endogenous estrogen. (149) However, androgens also decrease with increasing age (35). Whether the ratio of sex hormone receptors is playing a protective role in preventing the onset of CVD in the female population, and whether it is phenotypically dependent, has yet to be known.

HCR Rats and Possibility of Androgen Oriented-Phenotype:

HCR rats are characterized as having a higher maximum oxygen uptake and resting metabolic rate, lean body mass, increased longevity, and resistance to developing dyslipidemia, peripheral insulin resistance, diet induced-liver steatosis, depression, and metabolic syndrome in comparison with LCR. These differences are possibly due to different underlying endocrine hormonal profile between these phenotypes. Several studies on testosterone prove the relationship between these biological characters and testosterone levels in human and animals. Studies in humans showed that men who had prostatic cancer and who were under androgen deprivation therapy for more than 3 months, developed lower VO2max and lower resting metabolic rate when compared to the group that received the therapy for less than three months (228). Moreover, studies have showed that testosterone decreased visceral fat mass (143), improved insulin resistance (143), and increase lean body mass (99). Additionally, studies revealed that long-term testosterone replacement therapy in men with late onset hypogonadism had decreased obesity parameters and improved metabolic syndrome and health-related quality of life (143). Testosterone replacement therapy for hypogonadal and aging men caused decrease in total cholesterol and LDL levels with no change in HDL levels (250). Testosterone has also been linked to the onset of depression in old men. A low free

testosterone level in elderly men over 70 years of age was associated with a higher prevalence of depression (3). Hormonal replacement therapy has been found to increase the longevity in both genders. Testosterone replacement therapy for men with late onset hypogonadism increased their survival rate by 9-10% in 5 years, which is similar to that of eugonadal men with normal endogenous testosterone level (32). Also, in menopausal women with estrogen replacement therapy, their survival rate increased by 2.6% in 5 years (32). Fatty liver is also associated with low testosterone concentration in both human and animal studies. In 495 men diagnosed with non-alcoholic fatty liver disease, 251 were observed to have a low serum testosterone level with absence of an alternative cause (76). In animal studies, mice with a liver-specific androgen receptor deletion have higher liver steatosis than obese controls (134). On the other hand, estrogen signaling has been shown to be associated with metabolic syndrome and its risk factors (106). It is also reported that LCR is known as a genetic model of metabolic syndrome (183). Whether LCR rats, as compared to HCR rats, have a different activation of sex hormone receptors that are responsible for the differential expression of receptor genes, and whether that difference plays a role in remodeling skeletal muscle injury following ischemic stress response, is not clear.

Some epidemiological studies provide strong evidence that hormone replacement therapy contributes toward cardioprotection in postmenopausal women (100), while other studies do not (91). It has been suggested that the inconsistency in these results is due to the heterogeneity of responses to hormonal replacement therapy. LCR and HCR rats are generated from heterogeneous N:NIH founder population. Whether AR/ERα ratio in gastrocnemius muscle is differentially associated with acute hind limb ischemia between

the female HCR and LCR rats, and whether AR differentially regulates skeletal muscle repair during ischemia has yet to be studied.

Skeletal Muscle Satellite Cells and Ischemia:

Satellite cells are the myogenic precursor cells and are responsible for the repair and regeneration of muscle fiber in adult tissue, either by fusing together or by integration into injured myocytes and their nuclei (75). During ischemia, these cells play a critical role in muscle preconditioning, remodeling, and repairing of damaged muscle fibers. This process involves several inflammatory cells, cytokines, growth factors, neurotransmitters, and hormones that play synergistically to activate quiescent satellite cells, see (Figure 1.1) (75). Activation of cells was reported at around 6 hr post-injury and within 24 hr the cells display significant growth (75). It remains unclear whether intrinsic exercise capacity phenotypes differentially activate the cells at these time points in response to the interaction between androgen and dopamine receptor D3R signaling pathways.

HCR Phenotype and Possible Association with D3R Activation:

HCR and LCR rats are animals models generated to examine the mechanism by which their phenotypes influence the development of metabolic disease and life span (111-112). Several studies were conducted using this unique model, one of them was to explore whether the intrinsic aerobic capacity phenotype influences the central

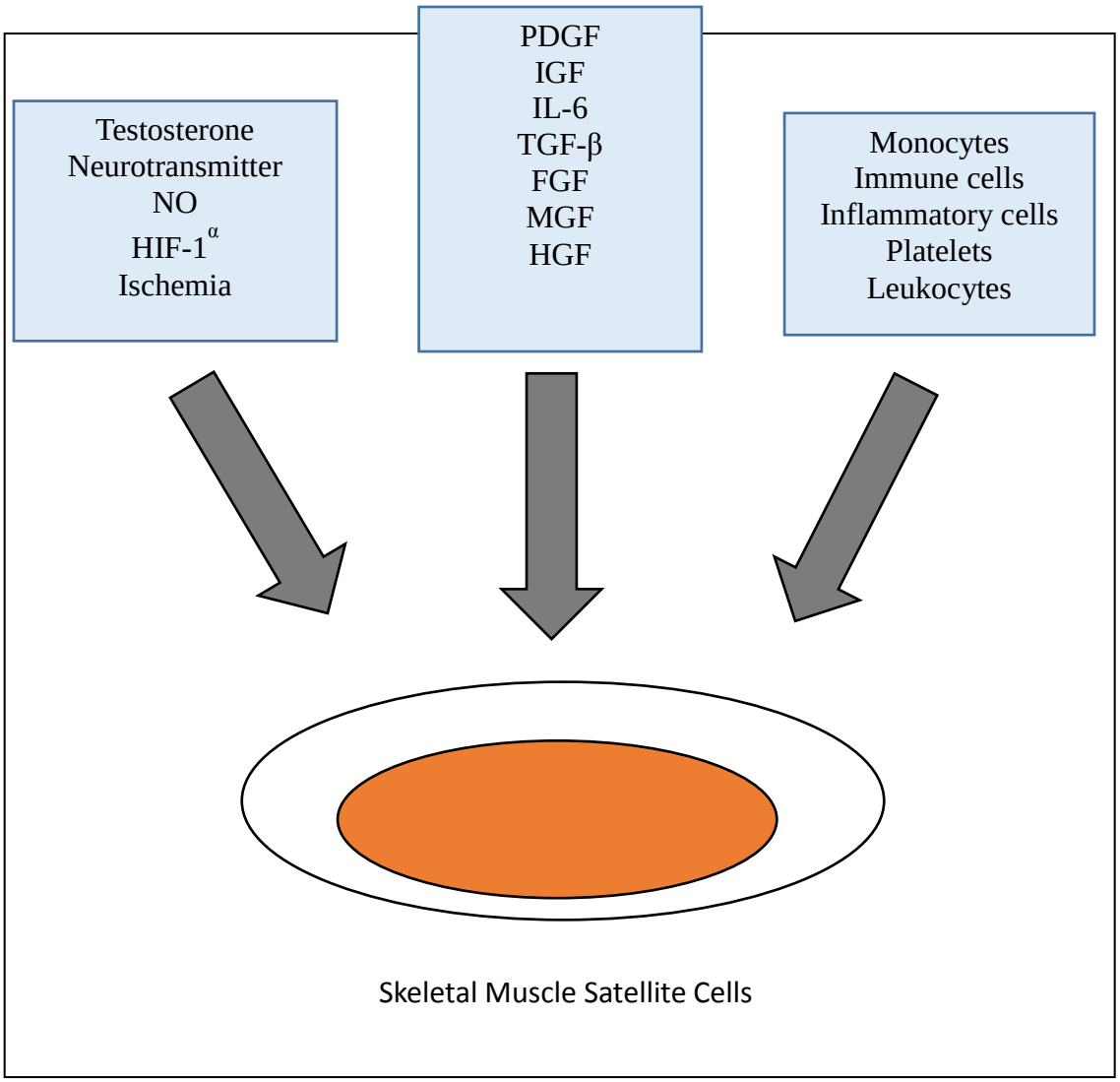

Figure 1.1: Multiple factors are responsible for satellite cells activation during muscle injury, including cells, growth factors, hormones, and neurotransmitters. Insulin-like growth factor, IGF; mechano growth factors, MGF; tissue growth factors, TGF; platelets derived growth factors, PDGF; hepatocyte growth factor, HGF; fibroblast growth factor, FGF; hypoxia inducible factor, HIF-1α; and nitric oxide, NO. (75).

monoamine adaptation by active exercise (53). Interestingly, they found that dopaminergic neuroplasticity was influenced by intrinsic exercise capacity phenotype, and HCR have prolonged dopamine availability compared to LCR (53). They reported that HCR phenotype was associated with increased gene expression of D2R, but not D1R. D2R is an inhibitory while D1R is a stimulatory receptors. Although D3R is another inhibitory dopaminergic receptor, to date no study has reported an association between D3R stimulation and high intrinsic aerobic capacity phenotype. It could be possible to show the association of D3R gene expression in this study to understand the mechanisms by which high intrinsic aerobic capacity phenotype regulate pathological and physiological stresses. Nevertheless, D3R has higher possibility to be associate with the high capacity runner phenotype. It has been reported that D3R was associated with the reduction in dopamine reuptake in the brain during prolonged administration of Pramipexole (D3R Agonist) (24). In addition to the central role, D3R stimulation was associated with renal function following renal ischemia reperfusion injury (230). Our lab also showed that D3R knockout mice have low life span and associated with adverse remodeling in cardiac tissue compared to wild type mice. We also shown that HCR has more recovery following hind limb ischemia as compared to LCR. Thus, association of D3R stimulation with prolonged dopamine bioavailability, longer life span, and protection from cardiovascular disease will open a question to explore the association of HCR phenotype, which shows similar biological findings of D3R stimulation, with D3R stimulation in ischemic repair such as in PAD model is novel.

Dopamine and androgen physiologically and pathologically was shown modifying each other centrally (1, 49,116). It is unclear whether HCR phenotype, in response to

active exercise with hind limb ischemia, is associated with differential AR and D3R expressions as compared to LCR, and whether the interaction between the D3R and AR in skeletal muscle satellite cells explains the mechanism of that association is novel.

Role of Dopamine Receptor in Cardiovascular Protection:

Dopamine receptors are members of the G-protein-coupled receptor family. The primary endogenous ligand of theses receptors is the neurotransmitter dopamine. Dopamine receptor D1 (D1R) and D5R are stimulatory receptors responsible for the synthesis, storage, and release of dopamine, and the activation of the production of the second messenger cAMP (165). On the other hand, D2R, D3R and D4R are inhibitory receptors that directly inhibit formation of cAMP by inhibiting adenylyl cyclase (166). D1R has been reported to be expressed in the pulmonary artery and thought to cause vasodilation (189). It is also found in the epicardium, myocardium, and endocardium of the heart (25), and in vascular smooth muscle of major organs (92). D1R dysfunction was associated with hypertension in animal models of hypertension and in humans with essential hypertension (92, 99).

Dopamine Receptor and Ischemia:

Several studies have showed that dopamine receptors play a role in ischemia. An *In vivo* study Araki et al. found that D1R and dopamine uptake sites, 7 days after the initial 10 minutes of cerebral ischemia, are susceptible to ischemia while D2R are resistant (4). In cultured neonatal rat cardiomyocytes, activation of D2R inhibited the release of cytochrome C, accumulation of intracellular Ca^{+2} ion concentration and

apoptosis, while inhibition of D2R did not significantly affect apoptosis of cardiomyocyte (128). A similar study was conducted to determine the role of D1R in ischemia-mimetic solution for 2 hours. It was found that activation of D1R induces apoptosis by ischemia/reperfusion, and its inhibition has no effect on apoptotic myocytes during ischemia reperfusion injury (129). The role of D3R in ischemia was also recorded. Its activation was found to improve the neurological function, learning, and memory of rats after global cerebral ischemia/reperfusion (229).

In peripheral arterial ligation, D2R was found to prolong the post-ischemia recovery. In a mouse model of D2 knockout, a unilateral hind limb ischemia showed faster recovery rate and stimulation of angiogenesis in the D2 knockout mice compared to the wild-type (194). For more roles of dopamine receptors in ischemia refer to (Table 1.A). This data lead to questions about whether intrinsic differences in endurance capacity could be due to differences in dopamine/receptors systems that modulate ischemic remodeling in femoral arterial ligation, the answer of which is unknown.

Dopamine and Exercise:

Several studies have linked the role of the central nervous system in skeletal muscle fatigue. This is referred to central fatigue (105). Dopamine is one of the neurotransmitters that has been focused on regarding the mechanisms of exercise-induced fatigue, such as the motivational state during physical activity (36). Studies on human and animals showed that increases in the dopamine concentration delay the onset of fatigue (12, 33, 61, 236). However, reducing dopamine activity accelerated the time to fatigue (37, 40, 80, 101). An important question arose to Teresa E. Foley et al. regarding whether intrinsic differences in endurance capacity could be due to differences in the

Table 1.A: Summary of Some Roles of Different Dopamine Receptors in Ischemia

Receptor	Receptor Status	Organ/Vessel model	Effect	Source
D1	S	Gut ischemia	Attenuate vasoconstrictive response to Gut ischemia (Favorable effect).	(70)
D1/D5	S	Femoral arterial ligation	Enhanced bone marrow-derived cell mobilization & post-ischemic angiogenes	(187)
D1	S	Vascular hypertension	Smooth muscle cell relaxation and vasodilation	(204)
D1/D2	S	Coronary artery occlusion	Reduce ischemia severity canine	(226)
D2/D3	S	Global cerebral I/R	Improve neurological function, learning and memory	(229)
D3	S	Renal I/R	Attenuates renal I/R injury	(230)
D1	I	Global cerebral ischemia	Neuroprotective effect	(71)
D2/D3	S	Brain I/R	Neuroprotective effect	(71)

Dopamine 1 (D1); dopamine 2 (D2); dopamine 3 (D3); dopamine 5 (D5); ischemia/reperfusion (I/R); stimulation (S); inhibition (I).

dopamine system (53). Using high capacity (HCR) and low capacity runners (LCR), they hypothesized that HCR have prolonged dopamine availability compared to LCR. So, upon studying various brain areas involved in movement, motivation, and fatigue, they found that D2R (and not D1R) in HCR was higher than LCR. These data direct to explore more about the intrinsic differences in endurance capacity and especially the role of dopamine peripherally at the level of skeletal muscle. This data is important in order to find whether the HCR phenotype also has a higher dopamine receptor expression at the

level of the skeletal muscle than LCR, and if that difference is responsible for hind limb ischemia remodeling.

Dopamine and its Relationship with Androgen and Estrogen Receptors:

Sex steroid receptors commonly function as initiators of transcription genes and subsequent synthesis of several proteins to regulate cell function during physiological and pathological responses. In regard to the synthesis of dopamine, the rate limiting enzyme is tyrosine hydroxylase. A study has explored the androgen responsive element in the tyrosine hydroxylase region, base pairs 1562 to 1328, upstream of the promotor (98). Studies have shown that testosterone treatment increased or maintained tyrosine hydroxylase activity, which decreases after castration (1). Moreover, sex hormones such as testosterone and estrogen have been shown to regulate tyrosine hydroxylase gene expression (1, 73, 207, 217). Another study done by Ely D. et al. showed that androgen alters brain catecholamine content and blood pressure in the testicular feminized male rats. They found testosterone manipulation increases blood pressure and correlates with dopamine content in the different brain regions through non-genomic or non-classic androgen receptor signaling (49). An additional study recorded that the ability of nandrolone (anabolic androgen steroid) to modulate motivation and reward-related effects of psychostimulant drugs, such as amphetamine, is dependent on the activation of androgen or estrogen receptors (116). However, since this research has mostly been performed in the brain, we do not know whether androgen and estrogen receptors can modulate dopamine receptor signaling in skeletal muscle, and whether intrinsic

phenotype differences in endurance capacity differentially modulate dopamine receptors by sex steroid receptors in a model of hind limb ischemia.

Thesis Overview:

The purpose of this project is to gain a better understanding about the differential changes in vascular adaptive response in ischemic skeletal muscle following exercise training between HCR and LCR. Our previous studies in this model without exercise showed that LCR rats have an attenuated and less effective angiogenic response compared to HCR rats. We predict that exercise will also deteriorate vascular adaptive response in LCR as compare with HCR. Previous studies have found that the LCR phenotype is a genetic model of metabolic syndrome (183) and presence of diabetes attenuates the improvement in walking performance in PAD patients who received exercise therapy (137). Studies also have showed that testosterone decreased visceral fat mass (143), improved insulin resistance (143), and increased lean body mass (144). This suggests that the LCR phenotype will have a decreased tolerance to a peripheral ischemic injury and demonstrate maladaptive responses from exercise during vascular ischemia. It also suggests that this can be due to the low androgen receptor activation in LCR as compared to HCR. The overall hypothesis is that intrinsic exercise capacity regulates sex steroid receptor expression, which affects the remodeling response to PAD with or without exercise. In chapter two we describe how exercise before peripheral artery ligation in LCR rats protects against vascular adaptive response to ischemia due to sufficient angio/inflammogenic response as compared to HCR rats. In chapter three we investigate how exercise after peripheral artery ligation in LCR exacerbates vascular

adaptive response to ischemia due to an insufficient angio/inflammogenic response as compared to HCR rats. Lastly, in chapter four we examine how the intrinsic aerobic capacity phenotype modifies the dopamine receptors through the androgen receptor during skeletal muscle satellite cell proliferation.

CHAPTER 2: INTRINSIC AEROBIC CAPACITY INFLUENCES THE VASCULAR ADAPTIVE RESPONSE TO ACTIVE EXERCISE FOLLOWING LOWER LIMB ISCHEMIA

Abstract:

The genotype associated with an inducible response to dynamic exercise is different from the genotype associated with the intrinsic capacity for aerobic exercise. The inducible response is an established component of rehab protocols after ischemic events, but the influence of the intrinsic capacity for exercise on the vascular response to active exercise following ischemic injury is unknown. **Method**: HCR and LCR, Female rats, 40 weeks, n = 16 each, treated with unilateral femoral artery ligation. Three days after the occlusion, half of the animals began treadmill running protocol for 10 days. The 2nd half served as sedentary post-ischemic controls. Skeletal muscle was harvested from both hind limbs. Samples from the non-occluded contralateral limb were used as internal controls. Samples were assessed histologically for capillarization using Rosenblatt staining, and genetically using PCR. Significant results were then supported by protein expression using ELISA to identify the difference in vascular adaptive response between the phenotypes following ischemia with/without exercise. **Results**: LCR rats were about 80g heavier while HCR had about 500% greater intrinsic running capacity (both $p < 0.01$). HCR rats showed 26% better preservation of muscle fiber area in the post-ischemic limb, but LCR rats showed at least a 2 fold increase in fiber area in response to active exercise ($p < 0.05$). HCR samples showed a greater increase (80%) in capillary density and perfusion exchange ratio (40%) in response to exercise. PCR and ELISA assays for multiple angiogenic factors showed expression levels that were consistently higher in the

LCR samples, consistent with increased ongoing ischemic stress. **Conclusion**: Combining occlusion with exercise after occlusion in LCR appeared to overwhelm the early angiogenic remodeling responses.

NEW & NOTEWORTHY: Exercise is commonly used as a treatment for those with peripheral arterial disease. This paper shows that the intrinsic aerobic capacity phenotype is an important internal factor that influences the outcomes from active exercise in lower limb ischemia remodeling. We demonstrated that combining occlusion with exercise after occlusion in LCR appeared to overwhelm the early angiogenic remodeling responses due to imbalanced muscle-capillary relationship and attenuated the vasculogenic adaptive response to ischemia.

Key words: Ischemia, HCR, LCR, exercise, PAD, intrinsic aerobic capacity.

INTRODUCTION

Peripheral arterial disease (PAD) is a common disease in the elderly, usually resulting from atherosclerosis and associated thrombosis that prevent blood supply to the lower limbs (176). It affects 8.5 million people in the US and around 202 million people worldwide (174). If left untreated, this disease can cause critical limb ischemia. It is responsible for more than 150,000 lower limb amputations per year in the United States alone (196). Patients' intolerance to treatments such as exercise due to pain, or patients not suitable for surgery due to comorbidity causes advanced progressions of the disease and increased mortality. PAD has various treatment options due to different body responses. These options include: exercise therapy, medical therapy, endovascular therapy, and surgical intervention (136). However, exercise therapy is more cost

effective, safer, and associated with improved walking distance, claudication symptoms, and most importantly quality of life (243). The mechanisms underlying the beneficial effects of exercise include: vasodilation of the collateral vessels or arterial remodeling, improved glucose and fat metabolism of ischemic muscles, improved energy production, decreased oxidative stress, stimulation of angiogenesis, improved peripheral nerve conductance, and improved pain threshold (212). A meta-analysis study (2) showed that exercise has better outcomes compared with revascularization alone in intermittent claudication. This is because revascularization and reversal arterial occlusion doesn't reverse muscle function impairment as it improves blood flow. On the other hand, supervised exercise has the ability to improve muscle functional performance and stimulate the shear stress responses and angiogenesis, therefore improving quality of life (72, 81,146). Although it has robust outcomes and is applicable to a majority of PAD patients, exercise as a treatment modality is not adaptable to some patients, for example patients with asymptomatic PAD. Therefore, it is not surprising to not be adaptable if they don't have symptoms! Even studies on exercise have different effects in subjects which could be due to differences in protocols or differences in individual intrinsic aerobic phenotype. It has been clear that endurance exercise is mainly genetic in origin. It accounts for 70% of variations in exercise capacity (57). Thus, studying the differential effects of exercise after hind limb ischemia between the intrinsic aerobic phenotype rats bred for low and high aerobic running capacity would provide a better platform for understanding the differences in the effects of exercise post-ligation on vascular adaptive response in these phenotypes.

Arteriogenesis, or arterial remodeling, occurs when acute arterial occlusion causes a sudden pressure gradient between proximal arteries (within non-ischemic region) and distal arteries (within ischemic region). This pressure gradient increases blood flow through the collateral circulation and activates shear stress in these collaterals, causing alterations in the gene expression in both calf (ischemic) and thigh (non-ischemic) muscles through shear stress response elements (59, 219), and leading to collateral remodeling to ensure sufficient blood flow to ischemic tissue. During acute ischemia, inflammatory cytokines produced from injured cells causes infiltration of inflammatory cells in ischemic tissue to remove debris of necrotic tissue. Monocytes and macrophages also play a crucial role in angio/arteriogenesis through the release of Ccl2/MCP-1 cytokines (5, 22, 77, 223, 224). Also, invading endothelial progenitor cells, which originate from bone marrow mononuclear progenitor cells have been shown to be involved in arteriogenesis and blood flow recovery (107) in animal models of peripheral arterial occlusive disease (246). Variations of collateral arteries between animal species (26, 77, 108, 197, 198, 203) and among healthy humans (148) have been recorded. However, the effect of exercise post-ligation on angio/inflammogenic responses due to hypoxia and shear stress in intrinsic aerobic capacity phenotype can predict the presence of differential angio/arteriogenesis that stem from the different phenotype background.

Vascular endothelial growth factor (Vegf) plays an important role in physiological angiogenesis, such as during exercise, in pathological conditions such as PAD, and during development (96). Vegfa can produce its signaling through Fms-like tyrosine kinase 1(Flt1), which is important in angiogenesis during development. The other receptor, kinase insert domain-containing receptor (Kdr), mediates angiogenesis in

the adult. It is important to investigate the impact of exercise post-ligation on angiogenesis signaling between rats that differ in their intrinsic exercise capacity following acute hind limb arterial occlusion. *Angiopoietin 1* (Angpt1) stimulates and stabilizes angiogenesis while angiopoietin 2 (Angpt2) prevents vascularization. However, when Angpt2 is associated with Vegfa, it stimulates angiogenesis (51). *Nuclear factor kappa-light-chain-enhancer of activated B cells* (Nfkb) is an upstream mediator for Vegfa expression in acute arterial ligation due to hypoxia (241). *Intercellular adhesion molecule* (Icam) and *vascular cell adhesion molecule* (Vcam) are well-known markers for endothelial activation and dysfunction (220). Their elevated levels indicate leukocyte recruitment from injured endothelial layers to ischemic tissue (127,173). *Platelet derived growth factor alpha* (Pdgfa) plays an important role in early development and in adult physiology. During development, Pdgfa drives the proliferation of undifferentiated mesenchymal cells, while in adults it plays a fundamental role in tissue remodeling, mesenchymal differentiation, and migration (84). Pdgfa also stimulates fibroblast proliferation for tissue remodeling by recruiting macrophage and fibroblast cells and stimulating the expression of genes necessary for proliferation. Thus, it is responsible for the induction of an inflammatory environment for faster healing (178). *Bone morphogen protein receptor 2* (Bmpr2) is a receptor for BMP ligands which are members of the TGF-β superfamily and are involved in several process of embryonic development and differentiation (29, 109, 242). The expression of Bmpr2 is reduced in pulmonary artery hypertension is associated with increased perivascular macrophages, mascularized distal arteries (195), and endothelial apoptosis (155). The influence of exercise post-ligation on the vascular adaptive response in intrinsic exercise capacity phenotypes has not been

studied. Using a rat model to determine how intrinsic aerobic exercise capacity responds and adapts to several diseases has been developed by Koch and Britton (20). The low aerobic capacity rats are a genetic model for metabolic syndrome that contribute to an increased risk of diabetes and cardiovascular disease. A study has been shown that exercise for PAD patients with diabetes attenuates the improvement in walking performance while it improves the walking performance of PAD patients without diabetes (137). These studies suggest genetic susceptibility might responsible for the negative outcomes from exercise training after the development of peripheral arterial obstructive disease in diabetic patients, and demonstrate that different intrinsic aerobic capacity phenotypic background might be an important factor in shaping the response to exercise therapy in PAD patients. Using this animal model is essential in studying the link between intrinsic aerobic capacity and active exercise after arterial occlusive disease.

We are interested in understanding differential changes in vascular adaptive response in ischemic skeletal muscle following exercise training between HCR and LCR. *Our hypothesis is that LCR rat's vascular adaptive response is deteriorated by exercise post-ligation due to an insufficient angio/inflammogenic response compared to the HCR rats.* Our model utilizes a unilateral hind limb femoral artery ligation followed by a mild intensity of progressive treadmill exercise. Our previous studies in this model without exercise showed that LCR rats have an attenuated and less effective angiogenic response compared to HCR rats. We predict that exercise will also deteriorate vascular adaptive response in LCR as compared with HCR.

MATERIAL AND METHODS

Animal Strains and Experimental Groups. HCR and LCR, female rats, 40 weeks, n= 16 each, generation 32. Eight HCR and eight LCR rats were exercised post-ligation for 10 days, beginning at day 3 post-ligation. The remaining 16 animals served as sedentary post-ischemic control. These animals were obtained from Drs. Lauren Koch and Steven Britton at the University of Michigan. They have previously described the artificial selection used for the generation of the HCR and LCR strains (111-112,22). Briefly, each sex was selected from the founder population (N: NIH stock). Two-way artificial selective breeding was used to create low capacity runner (LCR) and high capacity runner (HCR) strains that were divergent for treadmill running capacity (run time until exhaustion on a graded treadmill exercise test). The 13 lowest and 13 highest running capacity rats of each sex were randomly paired for mating. This pattern was repeated over subsequent generations to produce the divergent strains using a rotational breeding scheme. In the present study animals from generation 32 were used. All the animals were exposed to treadmill exercise for the 5 days at 11 weeks of age when the animals were phenotyped for treadmill running capacity. Upon verifying the phenotypes of the animals, animals were prepared for shipping at 14 weeks of age, or soon after as weather conditions (airport tarmac temperatures < 85°F) permitted. Once received by the Department of Comparative Medicine at ECU, the animals were maintained under mandatory quarantine for 10 weeks before they were released for study. Rats were provided standard rat chow and water *ad libitum* and were kept on a 12 h light/ 12 h dark time schedule until sacrifice. Animal procedures were conducted in accordance with American Physiological Society guidelines for the humane and safe use of animals, and

all protocols involving animals used for these experiments were approved by the East Carolina University Animal Care and Use Committee.

Exercise Training. Motorized treadmill running exercise was used for Ex-post ligation groups as a type of mild intensity supervised exercise training. Animals were subjected to 5 days on the treadmill at a very low speed (10 m/min) to condition them to the treadmill environment. Animals that were not running on the treadmill were removed and returned to their cages. Significant attention was devoted to minimizing exposure to the shock grid by using blasts of air or gentle prodding with a brush. Animals began exercising for 5 minutes at 14-16 m/min for 5 days a week for two weeks; the duration of exercise was gradually increased 5 min per day, until they were training for 60 minutes. Ex post-ligation protocol was generally 2 weeks in duration, beginning on post-op day 3 (8 events). The Total number of sessions was: 5 conditioning + 8 protocol = 12 events/animal (174).

Hind Limb Femoral Artery Occlusion. Femoral artery occlusion was produced as outlined by others (135, 136,184). Briefly, rats were anesthetized with ketamine xylazine (0.1ml/100g body weight IP). The fur was shaved from the inner thigh and the surgical sight was cleaned with Betadine and 70% alcohol. Utilizing a small incision, the left femoral artery was isolated and three ligatures of 6-0 surgical silk were placed to cause an ischemic condition in the downstream tissue. One ligature was placed 5-6mm distal to the inguinal ligament, a second was placed on a collateral artery, and the third was placed 5-6 mm distal to the first ligature. When all three ligatures were in place, the incision was closed, and the animal was given a Buprenex injection (0.1ml/100g body weight IP) and placed in its cage to recover. Animals were allowed to wake up and have unlimited

access to food and water. Animals were sacrificed at day 14 following placement of the ligature. This procedure produces a uniform occlusion of the femoral arteries that reduces blood flow reserve capacity to 10–20% of normal, but also remains sufficient to meet resting blood flow requirements (243, 244, 245).

Skeletal Muscle Morphology: Rosenblatt Staining and Analysis.

Gastrocnemius muscle for each animal was excised and quickly frozen by placing them in optimal cutting temperature (O.C.T.) compound. The muscle in the OCT was cut into transverse sections (thickness 8µm) and then was underwent capillary staining as originally defined by Rosenblatt *et al* (26, 60). Briefly, Rosenblatt staining allows visualization of capillaries for subsequent photographs under 20 X magnification. Capillaries were quantified manually from the image on individual fibers. The following six indexes were measured: 1. Share factor (SF), 2. Average cross-sectional myofiber perimeter (AP), 3. Average cross-sectional myofiber area (AA),. 4. Capillary per fiber perimeter exchange (CFPE), 5. Number of capillary attached per fiber (NCAF), 6. Capillary density (CD). Fiber cross-sectional average area and perimeter were measured with the ImageJ-analysis system calibrated to transform the number of pixels (viewed on a computer monitor) into micrometers from an image of the myosin ATPase stain.

RNA Isolation, Reverse Transcriptase-PCR, and Real-Time PCR. Total RNA was extracted from harvested gastrocnemius muscle using TRIzol reagents (Invitrogen) and cDNA was generated using the High-Capacity cDNA Reverse Transcription kit from Applied Biosystems (Thermofisher) following the manufacturer's protocol. qReal-time PCR was performed using 5̀ fluorescent labeled primers for Vegfa, Angpt1, Angpt2, Hif1a, Icam, Vcam, Pdgfa, Kdr, Flt1, Nfkb, Ccl2, Il6, and Bmpr2 with TaqMan Fast

Advanced Master Mix (Life Technology) following the manufacturer's protocol. Actin β was used as the reference gene since CT values for this gene did not change with treatment. The qReal-Time PCR detection system used was the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). LCR and HCR internal legs samples were used for all data to be normalized with treated samples of other legs using the ratio of $2^{-\Delta}$CT of treated sample/$2^{-\Delta}$CT of controlled sample method.

ELISA. The levels of several cytokines were detected by ELISA kits from harvested gastrocnemius muscle using 1x phosphate buffered saline (PBS 1X, pH 7.4) (Thermofisher) containing 2% Triton X-100 (Sigma) and an electronic homogenizer. Vegfa and Mcp-1 ELISA kits (Abcam), Nfkb and Angiop 2 ELISA kits (Aviva System Biology); and Pdgfa and Bmpr2 ELISA kits (LSBio) were utilized according to the manufacturer's instruction. The tissue lysate was diluted with the sample dilution buffer at a ratio of 1:5. Results were calculated using computer software to generate a four-parameter logistic (4-PL) curve fit.

Statistics. Statistical analysis was performed using data analysis software in Microsoft Excel worksheet (Windows 10). Data is expressed as means ± SEM, and a *p* value <0.05 was considered statistically significant. Single factor ANOVA tests were used to compare between HCR and LCR to determine statistical significance among the groups (two groups at a time) comparing three different ways: internal control data of HCR with the counterpart of LCR group, ischemic limb data of HCR with its counterpart of LCR, and finally the ratio of ischemic/non-ischemic limb of HCR with the LCR counterpart. Similarly, relative gene expression levels were calculated by comparing q-

RT PCR data (after normalizing them to the internal standard housekeeping gene, Actin-β) of non-ischemic limb of HCR with its counterpart of LCR and comparing ischemic limb and the ratio of ischemic to non-ischemic limb of HCR with LCR counterpart groups as explained above. Linear regression was used to analyze the interrelationship between the mean muscle fiber cross-sectional area and capillary density in exercised post-ligation groups. Significant differences were accepted in all circumstances where $p < 0.05$.

RESULTS

The Differential Changes in Muscle-Capillary Relationship in the Calf Gastrocnemius Muscle 14 Days Following the Ligation Without EX between LCR and HCR:

In the ratio of ischemic over non-ischemic limb: The ratio of capillaries to average muscle fiber area or CD did not significantly increase ($p \geq 0.05$) between the phenotypes (figure 2.1A). The amount of capillary surface available for exchange per muscle fiber surface area, or capillary-to-fiber perimeter exchange (CFPE), is an accurate indicator of muscle capillary interference. CFPE was not significantly changed between the phenotypes ($p \geq 0.05$) (figure 2.1B). The number of capillaries attached per fiber (NCAF) is another important parameter in muscle-capillary relationship and is affected by ischemia and exercise. NCAF was 26% higher in the HCR than in LCR group ($p < 0.05$) (figure 2.1C). The number of fibers sharing a single capillary, or the share factor (SF), was also significantly ($p < 0.05$) altered in HCR compared to LCR, as it was 27% higher in HCR than LCR (figure 2.1D). The average area of muscle fiber (AA) is a very important

parameter in measuring muscle functionality and a good indicator of CD state in the skeletal myocyte. HCR muscle fiber average area was 110% higher as compared to LCR ($p<0.05$) after ischemia without exercise (figure 2.1E). The average perimeter of muscle fiber also followed the similar change of AA. Ligation without exercise affected the average perimeter of muscles, and it was not significantly ($p>0.05$) higher in HCR than LCR due to the increased muscle fiber average area at that point in time (figure 2.1F).

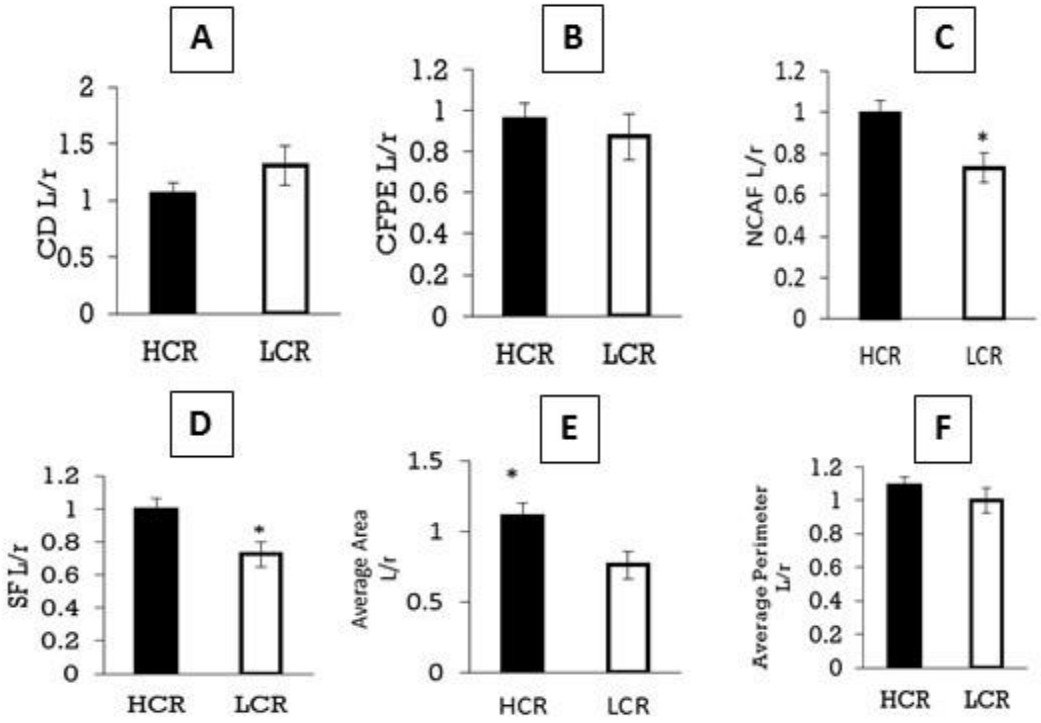

FIGURE 2.1. The effect of intrinsic aerobic capacity phenotype on gastrocnemius muscle-capillary relationship following 14 days of left femoral artery ligation. Capillary density, CD (A), capillary-to-fiber perimeter exchange, CFPE (B), number of capillaries attached to fiber, NCAF (C), sharing factor, SF (D), and muscle fiber average area (E), muscle fiber average perimeter (F). Data is expressed by LCR and HCR left leg to right leg ratio, LCRL/r and HCRL/r, respectively, n=15. Single factor ANOVA test was used to analyze the data, * indicates $p<0.05$. The error bars represent standard error.

In the non-ischemic (right) limb, only the average area (AA) of muscle fiber was significantly higher (~30%) in the LCR right limb (LCRr) than HCRr, while the other muscle-capillary parameters were not statistically significant (figure 2.2A-F).

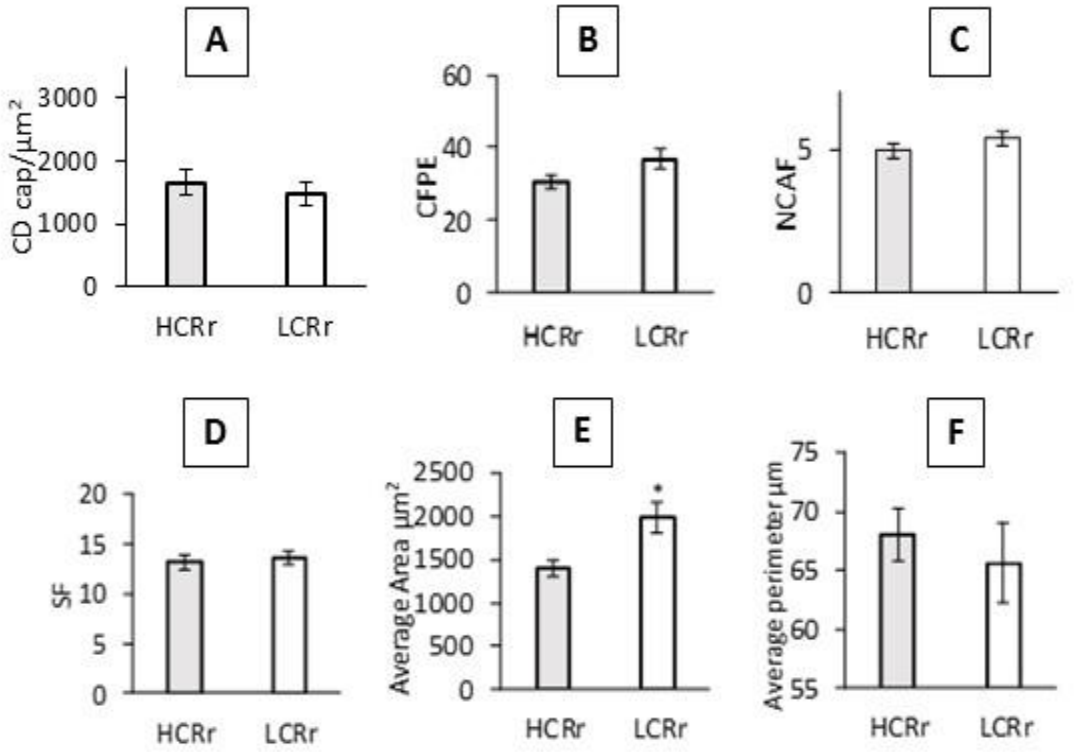

FIGURE 2.2. The effect of intrinsic aerobic capacity phenotype on non-ischemic (right leg) gastrocnemius muscle-capillary relationship following 14 days of left femoral artery ligation. Capillary density, CD (A), capillary-to-fiber perimeter exchange, CFPE (B), number of capillaries attached to fiber, NCAF (C), sharing factor, SF (D), muscle fiber average area (E), and muscle fiber average perimeter (F). Data is expressed by LCR and HCR right leg, LCRr and HCRr, respectively, n=15. Single factor ANOVA test was used to analyze the data, * indicates $p<0.05$. The error bars represent standard error.

The Differential Changes in Muscle-Capillary Relationship in the Calf Gastrocnemius Muscle Between LCR and HCR Following Exercise Post-Ligation:

In the ratio of ischemic over non-ischemic limb: CD was approximately 0.8-fold higher in HCR as compared to LCR ($p<0.05$) after 14 days of treadmill exercise starting at day 3 of the ligation (figure 2.3A). It was approximately 0.4-fold higher in HCR as compared to LCR ($p<0.05$) at day 14 when the animals exercised post ligation (figure 2.3B). Exercise did not significantly affect ($p\geq0.05$) NCAF between phenotypes (figure 2.3C). However, there was no significant difference ($p\geq0.05$) in SF between the phenotypes after training (figure 2.3D). AA was significantly higher (~8 fold) in LCR than HCR when ligation was followed by exercise (figure 2.3E). The average perimeter was significantly ($p<0.05$) greater (16%) in LCR as compared to HCR after the training (figure 2.3F).

In the non-ischemic (right) limb, The CD was significantly lower (33%) in the HCRr due to an approximately 37% larger average area (AA) as compared with LCRr. However, the other muscle-capillary parameters were not statistically significant (figure 2.4A-F).

Sample images of the histological sections are provided in figure 2.5, A-H.

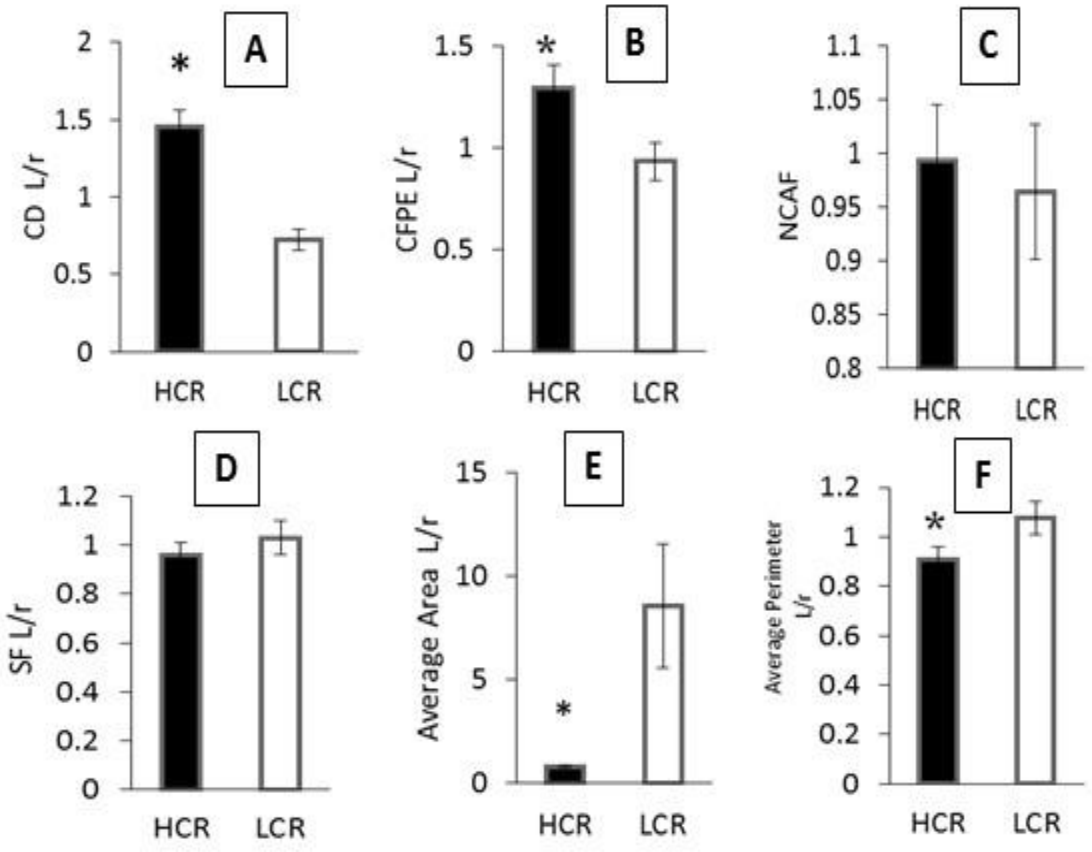

FIGURE 2.3. The effect of active exercise following left femoral artery ligation, exercise began on day 3 until day 14, on gastrocnemius muscle-capillary relationship in high and low aerobic capacity phenotype rats (HCR and LCR) respectively. Capillary density, CD (A), capillary-to-fiber perimeter exchange, CFPE (B), number of capillaries attached to fiber, NCAF (C), sharing factor, SF (D), muscle fiber average area (E), and muscle fiber average perimeter (F). Data is expressed by LCR and HCR left leg (ischemic) over right leg (non-ischemic) ratio, LCRL/r and HCRL/r, respectively, n=15. Single factor ANOVA test was used to analyze the data, * indicates $p<0.05$. The error bars represent standard error.

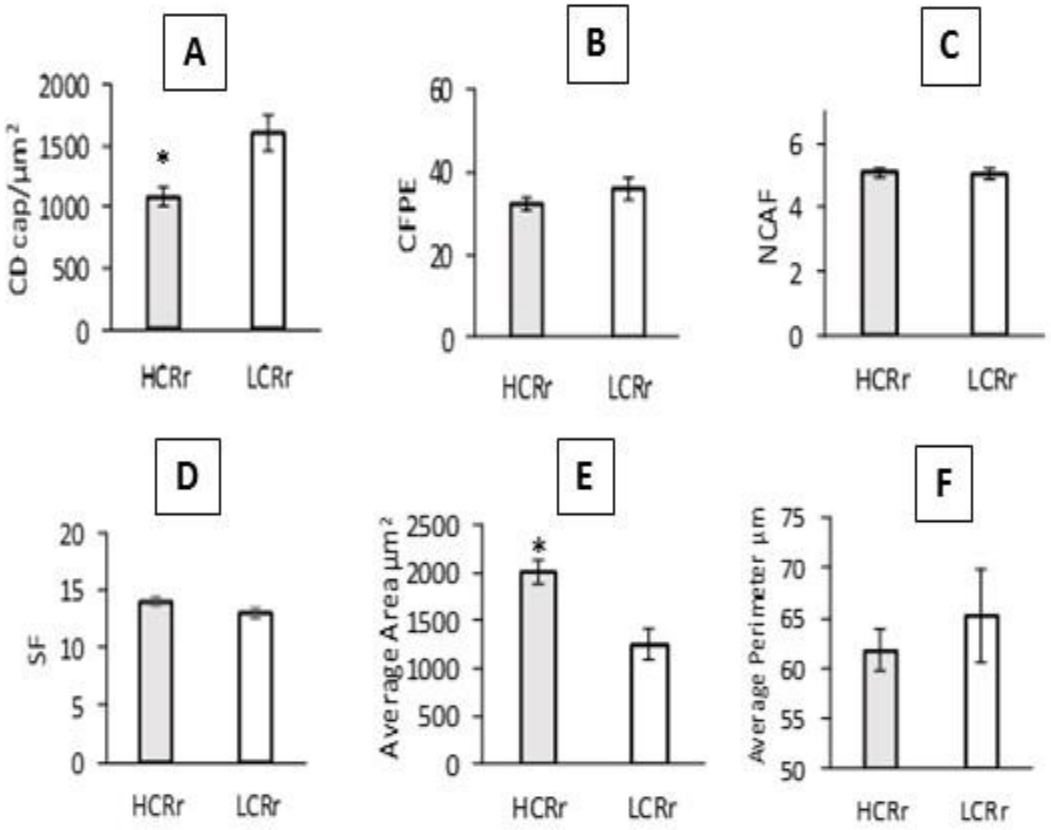

FIGURE 2.4. The effect of active exercise following left femoral artery ligation, exercise began on day 3 until day 14, on gastrocnemius muscle-capillary relationship in the non-ischemic right leg of high aerobic capacity rat (HCRr) and low aerobic capacity rats (LCRr), n=15. Capillary density, CD (A), capillary-to-fiber perimeter exchange, CFPE (B), number of capillaries attached to fiber, NCAF (C), sharing factor, SF (D), muscle fiber average area (E), and muscle fiber average perimeter (F). Single factor ANOVA test was used to analyze the data, * indicates $p<0.05$. The error bars represent standard error.

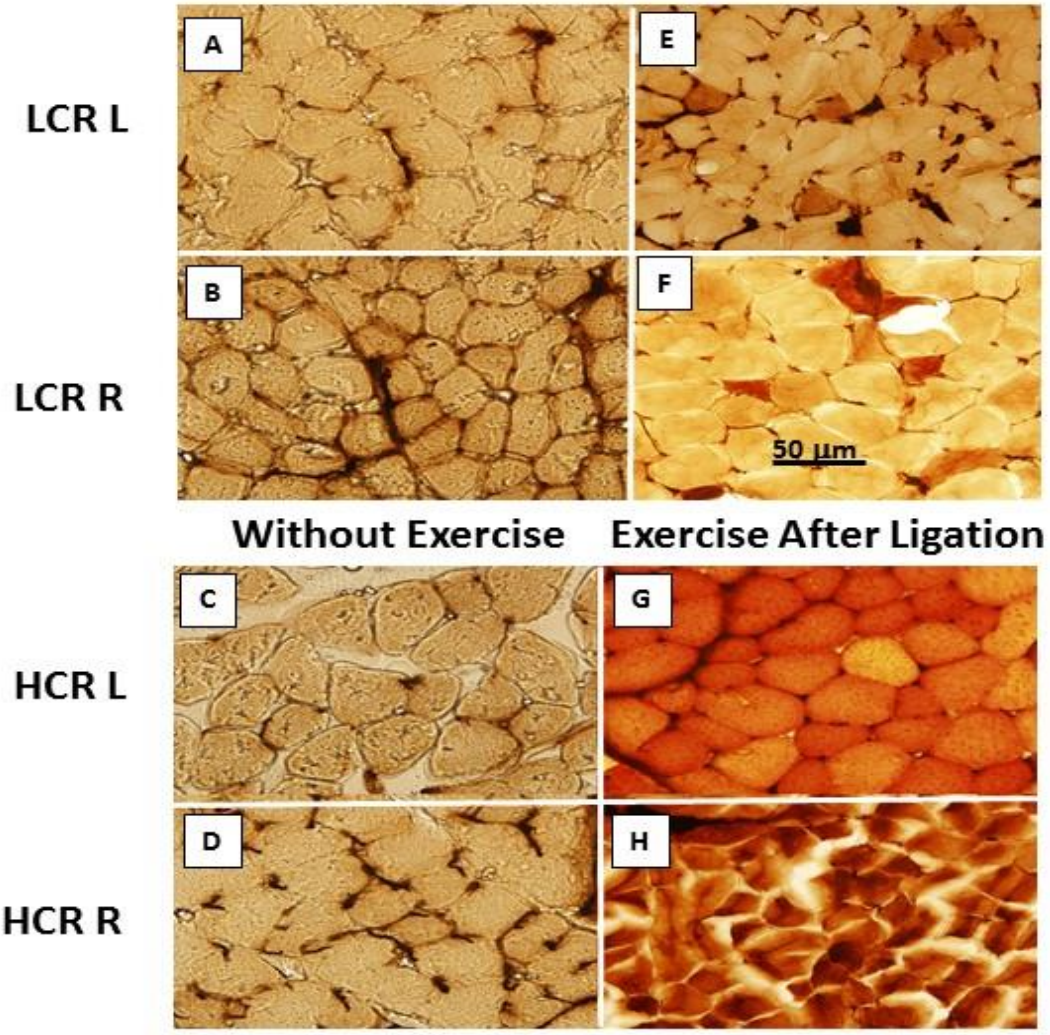

FIGURE 2.5. A histological representation of Gastrocnemius muscle cross-section following left femoral artery ligation in trained and untrained groups, using 8 micron sectioning and the myosin ATPase staining "Rosenblatt staining", showing cross section of the muscle fiber and capillaries. In all animals, the left leg (LCR-L and HCR-L) was ischemic, and the right leg (LCR-R and HCR-R). Images A-D show the effects of occlusion without any exercise after ligation. Images E-H show sections from the corresponding limb muscle with an exercise regimen after occlusion. As noted elsewhere LCR = low intrinsic aerobic capacity rat; HCR = high intrinsic aerobic capacity rat. Fiber cross-sectional average area and perimeter were measured using NIH imageJ software calibrated to transform the number of pixels (viewed on a computer monitor) into micrometers from an image of the myosin ATPase stain.

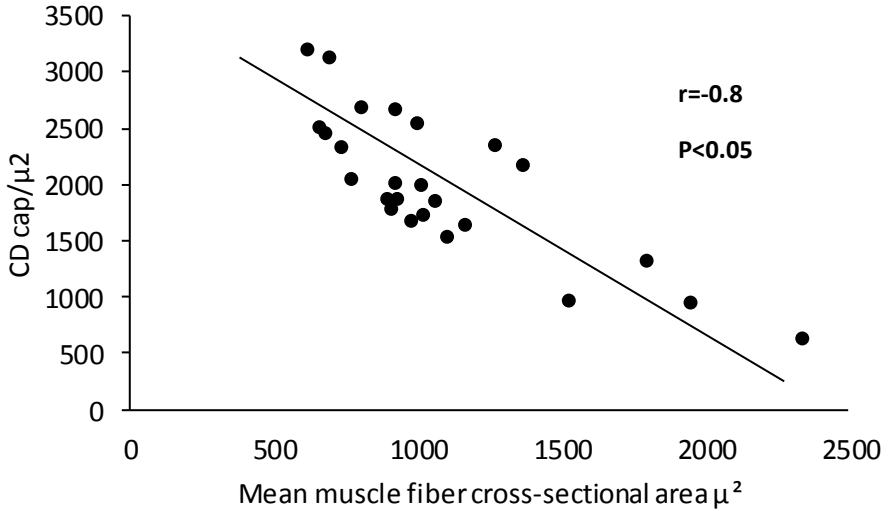

FIGURE 2.6. The relationship between capillary densities (CD) and mean muscle fiber cross-sectional area of the gastrocnemius muscle in trained HCR rats. Linear regression test was used to analyze the data, n=10.

The Differential Changes in Gene Expression in the Calf Gastrocnemius Muscle Following Exercise Post-Ligation in HCR vs. LCR:

The patterns in expression of multiple genes are known to be essential for the understanding of the vascular adaptive response of the hind limb to arterial occlusion. These patterns were significantly different after exercise post-ligation in ischemic, nonischemic, and the ratio of ischemic to the non-ischemic hind limbs (I/N).

Vegfa is a strong indicator of angiogenesis in response to physiological and pathological hypoxia. Its mRNA expressions were doubled in both non-ischemic and ischemic muscle of untrained LCR as compared to HCR, although the trend was only

significant in the non-ischemic side (p=0.003 in the non-ischemic side and p=0.1 in ischemic side). However, there was no change in Vegfa expression in I/N ratio between the phenotypes (Table 2.A; 1, 14 and 27). The expression of Vegfa receptors, Kdr and Flt1, were not significantly changed (p>0.05) in untrained groups, although they were generally higher in LCR than HCR (Table 2.A; 2-3, 15-16, and 28-29). Vegfa levels were almost doubled on the ischemic side of LCR as compared to HCR (p=0.02 after exercise), and I/N ratio was also higher (32%) in LCR, but the trend was near significant (p=0.07). There was no change of Vegfa expression in non-ischemic sides of both phenotypes after exercise (Table 2.B; 1, 11, and 21). The expression of Kdr and Flt1 were not altered significantly between the phenotypes. Although, the Flt1 in I/N ratio was near to significantly higher (p=0.07) in HCR than LCR (Table 2.B; 2-3, 12-13, and 22-23).

Hif1a is the sensitive mediator for hypoxia that mediates the activity of Vegf for angiogenesis. The mRNA expression in untrained and trained phenotypes was not significantly elevated at the 14th day after femoral artery ligation. However, mRNA expression was higher (p>0.05) in LCR than the HCR (Table 2.A; 4, 17, and 30; & Table 2.B; 4, 14, and 24). The expression of other hypoxia-related transcription factors include Nfkb, Angpt1, and Angpt2. Although the gene expression of Nfkb and Angpt1 did not reach significant difference (p>0.05), they were higher in untrained LCR than HCR (Table 2.A; 6-7, 19-20, and 32-33). Angpt2 in untrained groups was 4-fold higher in the non-ischemic side of LCR rats as compared to HCR (P=0.001) (Table 2.A; 8). The ischemic side of LCR (Table 2.A; 21) also had higher Angpt2 but the difference was not statistically significant (p=0.1). The I/N ratio of Angpt2 gene expression was not changed (p>0.05) (Table 2.A; 34).

Table 2.A. Gene Expression in Gastrocnemius Muscle of Non-Exercised HCR and LCR Rats Following Two Weeks of Abrupt Left Femoral Artery Ligation

No.	Genes	LCR	HCR
Non-Ischemic Gastrocnemius Muscle (right leg (r))			
1	Vegfa	0.38± 0.02*	0.20 ± 0.04
2	Flt1	0.02 ± 0.003	0.03 ± 0.01
3	Kdr	0.04 ± 0.004	0.03 ± 0.01
4	Hif1a	0.02 ± 0.003	0.03 ± 0.01
5	Il6	$8.8x10^{-5} \pm 1.5x10^{-5}$	$8.6x10^{-5} \pm 1.6x10^{-5}$
6	Nfkb	0.02 ± 0.001	0.03 ± 0.002
7	Angpt1	0.01 ± 0.001	0.009 ± 0.001
8	Angpt2	0.01 ± 0.001*	0.002 ± 0.001
9	Ccl2	0.003 ± 0.0005	0.004 ± 0.004
10	Vcam	0.0033 ± 0.001	0.003 ± 0.001
11	Icam	0.01 ± 0.001	0.005 ± 0.001
12	Bmpr2	0.035 ± 0.003	0.03 ± 0.003
13	Pdgfa	0.02 ± 0.001	0.023 ± 0.002
Ischemic Gastrocnemius Muscle (Left leg (L))			
14	Vegfa	0.383 ± 0.1	0.20 ± 0.1
15	Flt1	0.03 ± 0.01	0.02 ± 0.01
16	Kdr	0.043 ± 0.01	0.021 ± 0.01
17	Hif1a	0.03 ± 0.01	0.02 ± 0.01
18	Il6	$0.0001 \pm 3.7x10^{-5}$	$9.9x10^{-5} \pm 3x10^{-5}$
19	Nfkb	0.02 ± 0.006	0.02 ± 0.003
20	Angpt1	0.013 ± 0.005	0.004 ± 0.001
21	Angpt2	0.01 ± 0.002	0.004 ± 0.001
22	Ccl2	0.007 ± 0.004	0.009 ± 0.001
23	Vcam	0.04 ± 0.001	0.004 ± 0.001
24	Icam	0.01 ± 0.002	0.004 ± 0.001
25	Bmpr2	0.04 ± 0.02	0.03 ± 0.003
26	Pdgfa	0.22 ± 0.003	0.025 ± 0.003
Ratio of Ischemic Over Non-Ischemic Muscle (L/r)			
27	Vegfa	1.03 ± 0.2	1.05 ± 0.3
28	Flt1	1.13 ± 0.2	0.8 ± 0.3
29	Kdr	1.2 ± 0.3	0.8 ± 0.2
30	Hif1a	1.13 ± 0.2	0.8 ± 0.3
31	Il6	1.5 ± 0.3	1.2 ± 0.2
32	Nfkb	1.2 ± 0.5	0.8 ± 0.13
33	Angpt1	1.9 ± 1.05	1.1 ± 0.2
34	Angpt2	1.2 ± 0.43	1.4 ± 0.5
35	Ccl2	2.45 ± 1.13	0.91 ± 0.11
36	Vcam	1.45 ± 0.4	1.1 ± 0.2
37	Icam	1.004 ± 0.3	0.84 ± 0.1
38	Bmpr2	0.8 ± 0.1	0.9 ± 0.1
39	Pdgfa	0.97 ± 0.2	1.03 ± 0.1

*Significant differences between phenotypes at corresponding side.

Table 2.B. Gene Expression in Gastrocnemius Muscle of Two Weeks Exercise Following Day 3 Post-Abrupt Left Femoral Artery Ligation in HCR and LCR Rats

No.	Genes	LCR	HCR
Exercised-Non-Ischemic Gastrocnemius Muscle (right leg (r))			
1	Vegfa	0.07± 0.01	0.073 ± 0.01
2	Flt1	0.02 ± 0.001	0.02 ± 0.003
3	Kdr	0.03 ± 0.002	0.022 ± 0.01
4	Hif1a	0.005 ± 0.0004	0.006 ± 0.0005
5	Nfkb	0.022 ± 0.002*	0.031 ± 0.002
6	Angpt1	0.014 ± 0.001	0.02 ± 0.004
7	Angpt2	0.01 ± 0.001*	0.004 ± 0.0004
8	Ccl2	$0.001 \pm 9.1x10^{-5}$	0.003 ± 0.001*
9	Bmpr2	0.18 ± 0.011*	0.24 ± 0.02
10	Pdgfa	0.02 ± 0.002	0.03 ± 0.004*
Exercised-Ischemic Gastrocnemius Muscle (Left leg (L))			
11	Vegfa	0.09 ± 0.01*	0.05 ± 0.01
12	Flt1	0.02 ± 0.003	0.015 ± 0.003
13	Kdr	0.024 ± 0.01	0.02 ± 0.003
14	Hif1a	0.006 ± 0.001	0.007 ± 0.001
15	Nfkb	0.02 ± 0.002	0.028 ± 0.002
16	Angpt1	0.023 ± 0.01	0.02 ± 0.002
17	Angpt2	0.01 ± 0.0011*	0.005 ± 0.001
18	Ccl2	0.001 ± 0.0002	0.003 ± 0.001*
19	Bmpr2	0.221 ± 0.02	0.211 ± 0.010
20	Pdgfa	0.022 ± 0.002	0.03 ± 0.001
Ratio of Ex-Ischemic Over Ex-Non-Ischemic Muscle (L/r)			
21	Vegfa	1.09 ± 0.12	0.77 ± 0.1
22	Flt1	0.6 ± 0.12	0.9 ± 0.1
23	Kdr	0.92 ± 0.2	1.1 ± 0.2
24	Hif1a	1.3 ± 0.2	1.14 ± 0.14
25	Nfkb	1.24 ± 0.12	0.96 ± 0.11
26	Angpt1	1.71 ± 0.55	1.4 ± 0.5
27	Angpt2	1.42 ± 0.3	1.4 ± 0.2
28	Ccl2	1.32 ± 0.3	1.24 ± 0.4
29	Bmpr2	1.3 ± 0.12*	0.9 ± 0.08
30	Pdgfa	1.3 ± 0.2	1.1 ± 0.2

*Significant differences between phenotypes at corresponding side.

After exercise post-ligation, Nfkb was only significantly higher (33%) in the non-ischemic side of HCR as compared to LCR ($p<0.05$) (Table 2.B; 5, 15, and 25). Angpt2 gene expression significantly was higher (25-30%) at the phenotypic baseline of LCR

than HCR, which does not make the I/N ratio statistically significant (Table 2.B; 7, 17, and 27). Angpt1 in trained animals followed the similar pattern as in untrained animals (Table 2.B; 6, 16, and 26).

Other genes related to an inflammatory response such as Ccl2, Icam, Vcam, Il6, Bmpr2, and Pdgfa showed distinct patterns in the HCR compared to LCR. The phenotype did not show any significant increase ($p>0.05$) for Ccl2 (Table 2.A; 9, 22, and 35), Vcam (Table 2.A; 10, 23, and 36), Icam (Table 2.A; 11, 24, and 37), Il6 (Table 2.A; 5, 18, and 31), Bmpr2 (Table 2.A; 12, 25, 38), and Pdgfa (Table 2.A; 13, 26, 39) under the influence of ischemia alone without exercise. However, the pattern of expression was different between the phenotypes under the influence of exercise post-ligation. Ccl2 gene expression was significantly greater ($p<0.05$) in non-ischemic and ischemic sides of HCR (60% and 55%, respectively) as compared to LCR (Table 2.B; 8 and 18), but no difference in I/N ratio ($p>0.05$) (Table 2.B; 28). Similarly, in Pdgfa, the expression was approximately 48% and 24% higher in non-ischemic and ischemic sides of HCR rats, respectively, as compared to LCR rats (Table 2.B; 10 and 20), but no changes were observed in I/N ratio between the phenotypes (Table 2.B; 30). Bmpr2 gene expression between the phenotypes under the influence of exercise post-ligation was very interesting. The non-ischemic side of HCR had significantly ($p=0.01$) higher levels of Bmpr2 (26%) compared to the non-ischemic side of LCR (Table 2.B; 9). However, these levels were not significantly higher in LCR ($p= 0.6$) in ischemic limb (Table 2.B; 19). The final percentage change of I/N limbs was 28% higher in LCR than HCR ($P=0.03$) (Table 2.B; 29). Il6, Vcam, and Icam were not significantly altered ($p>0.05$) among the phenotypes after exercise (Table 2.C).

Table 2.C. List of the genes in Gastrocnemius muscle that didn't show significant differences between the HCR and LCR rats following two weeks exercise beginning day 3 post-left femoral artery ligation.

Genes	LCR	HCR
Exercised-Non-Ischemic Gastrocnemius Muscle (right leg (r))		
Il6	$6.41x10^{-5} \pm 4.6x10^{-6}$	$0.0002 \pm 5.5x10^{-5}$
Vcam	$0.001 \pm 9.5x10^{-5}$	0.0011 ± 0.0003
Icam	0.01 ± 0.001	0.005 ± 0.001
Exercised-Ischemic Gastrocnemius Muscle (Left leg (L))		
Il6	$4.5x10^{-5} \pm 1.25x10^{-5}$	$6.1x10^{-5} \pm 6.2x10^{-6}$
Vcam	0.001 ± 0.0002	$0.0007 \pm 8.6x10^{-5}$
Icam	0.006 ± 0.0005	0.005 ± 0.001
Ratio of Ex-ischemic over Ex-non-ischemic muscle (L/r)		
Il6	0.7 ± 0.2	0.5 ± 0.1
Vcam	1.1 ± 0.234	0.81 ± 0.2
Icam	0.93 ± 0.14	0.89 ± 0.1

The Differential Changes in Protein Expression in the Calf Gastrocnemius Muscle Following Exercise Post-Ligation in HCR vs. LCR:

Since protein levels in a particular cell dictate its function, here we conducted experiments to measure the concentrations of cytokines that were significantly expressed at the genetic levels in the non-ischemic and ischemic gastrocnemius muscles of both untrained and EX post-ligation groups between the phenotypes.

Vegfa protein levels were almost doubled in both non-ischemic and ischemic legs of untrained LCR as compared to HCR (P=0.03) (Figure 2.7 A and B). After exercise, Vegfa levels were doubled in non-ischemic legs of LCR (Figure 2.7C), but did not differ significantly in ischemic leg as compared to HCR (Figure 2.7D).

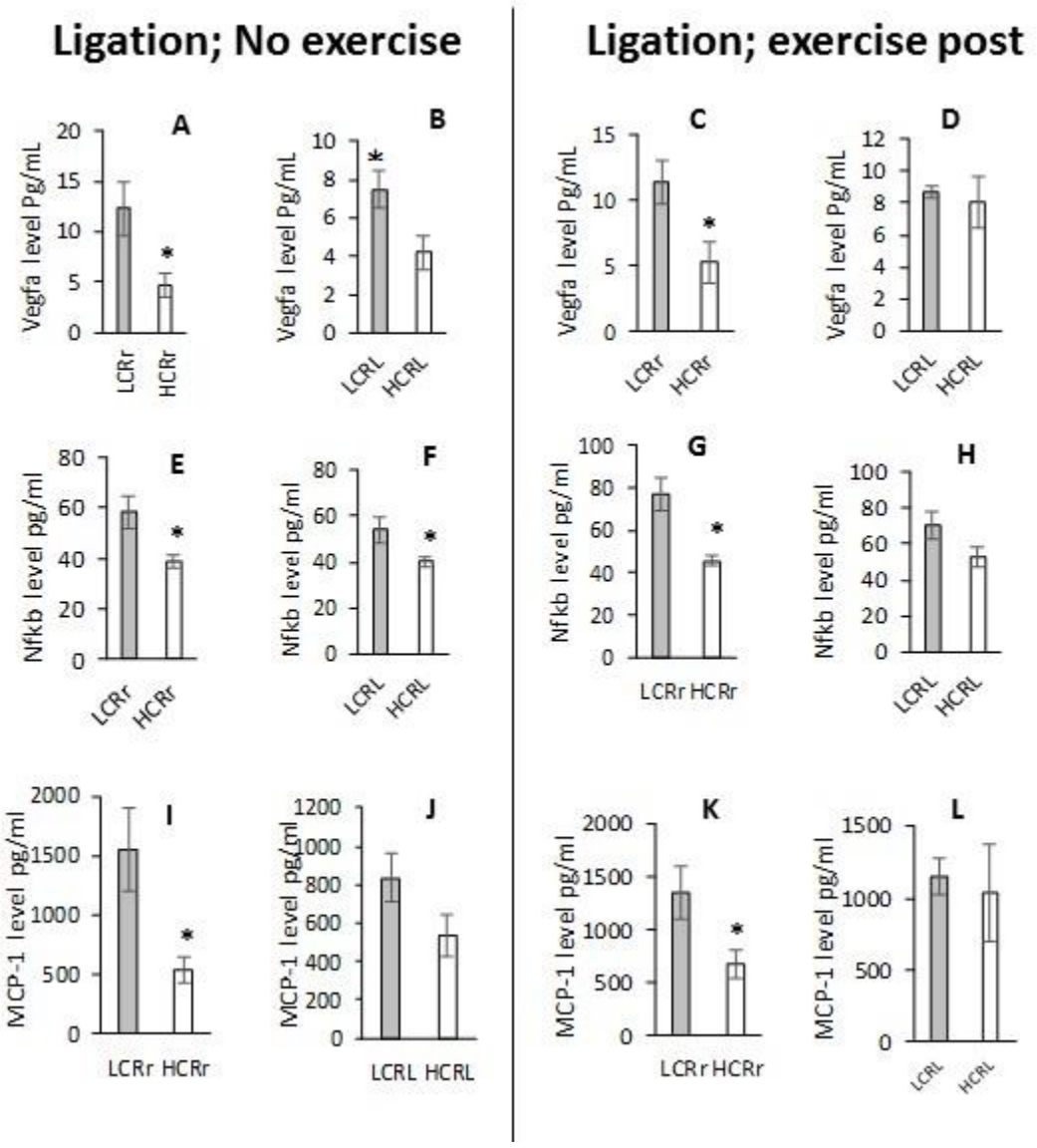

FIGURE 2.7. Protein expression in calf muscle 2 weeks after acute femoral arterial lygation in non-ischemic (LCRr/HCRr) LCR/HCR limbs and ischemic limbs (LCRL/HCRL) of LCR and HCR rats in untrained and EX-post ligation groups, n=10. ELISA was used to determine the protein expression level of mediators that showed already statistical difference in their genes expressions including Vegfa in untrained (A and B) and trained (C and D), Nfkb in untrained (E and F) and trained (G and H), MCP-1 in untrained (I and J) and trained (K and L). Single factor ANOVA test was used to analyze the data, * indicates $p<0.05$. The error bars represent standard error.

The protein levels of Nfkb and Ccl2 (MCP-1) were higher in non-ischemic limb of LCR as compared to HCR in untrained groups. The levels of Nfkb and Ccl2 in non-ischemic untrained LCR (34% and 66%, respectively) were significantly higher in LCR as compared to HCR (Figure 2.7E and 2.7I). In the ischemic limbs, Nfkb levels were near significantly higher in LCR (25%) as compared to HCR (P=0.05) (Figure 2.7F), whereas there was no significant change in Ccl2 levels in ischemic limbs between the phenotypes (Figure 2.7J). In exercise post-ligation, only non-ischemic limbs showed significant difference between the phenotypes. Nfkb and Ccl2 were 40% and 50% higher, respectively, in LCR as compared to HCR (Figure 2.7G and 2.7K), while no significant changes occurred in ischemic limbs after the exercise training between the phenotypes (Figure 2.7H and 2.7L).

The protein levels of Angiop2 did not significantly change between the untrained phenotypes. However, after exercise training they were significantly altered (P=0.0009), as non-ischemic limb of LCR was ~70% higher than HCR. In the ischemic limbs, Angiop2 was 50% higher and near significant in LCR than HCR.

BMPR2 protein levels in untrained and trained groups were higher in LCR than HCR. In non-ischemic limb of untrained LCR, BMPR2 was 90% higher and near significant in LCR as compared to HCR, while in ischemic limb of trained LCR the trend was ~90% higher in LCR as compared to HCR. However, in EX post-ligation groups Bmpr2 levels were 85% higher in non-ischemic limb of LCR as compared to HCR, but there is no significant difference between the phenotypes in the Bmpr2 levels after the exercise post-ligation. Interestingly, Pdgfa protein levels were not significantly different between the phenotypes in untrained and trained groups.

DISCUSSION

The most common symptom of PAD is the painful cramping of muscle, which commonly occurs in the calf muscle during walking. This pain is a major interference in the quality of life (145, 150, 211, 225, 233) because it limits the ability to exercise and perform many activities of daily living, such as walking. Exercise therapy increase pain threshold, improve exercise capacity and avoid surgical revascularization and other invasive therapies (212, 150, 225). Most studies focused on the mechanisms of how exercise can produce its beneficial effects on vasculature and blood flow to the ischemic limb (47, 96, 136). However, the inability of some asymptomatic PAD patients to exercise may be due to their intrinsic aerobic capacity phenotype background. Therefore, it is not surprising if those patients can not finish their exercise therapy after developing symptomatic disease. The underlying genetic susceptibility for aerobic endurance capacity might mechanistically affect the genes that are responsible for developing the intrinsic risk factors for PAD and cardiovascular diseases. This could include developing metabolic syndrome or other conditions that would affect the genes responsible for vascular adaptation after arterial occlusion. It suggests that exercise post-peripheral arterial occlusion might not always be beneficial due to different vascular adaptive responses at the cellular or molecular levels to exercise after acute arterial occlusion between responsive and unresponsive patients.

The model of intrinsic aerobic endurance capacity helped to better understand the differential effect of exercise post-ligation. This model infers that genetic susceptibility is an effect of active exercise post-ligation on vascular remodeling in response to femoral

arterial ligation and helps to better understand the benefit from the exercise therapy for patients with PAD. This is especially relevant for those who require exercise therapy because of their medication intolerance, asymptomatic PAD, they would be at a high risk for complications during surgical interventions. This study also may provide more mechanistic insights for developing a novel molecular therapy for patients who cannot perform the active exercise therapy. The goal of this study was to determine the differential vascular adaptive responses between rats with low intrinsic running capacity and rats with high intrinsic running capacity. The results of this study showed that rats selectively bred for high endurance running capacity (HCR) and low endurance running capacity (LCR) have different responses to exercise post-ligation in a model of peripheral arterial obstructive disease. The results also provide a distinct corroboration that the low endurance running capacity phenotype has a higher risk for developing exercise post-ligation injury due to an insufficient priming in the anatomical structure for adapting to ischemic stress.

Differential Changes After Acute Ligation Without Exercise

The muscle-capillary relationship after acute arterial ligation was distinctly different between the two phenotypes. HCR rats' muscle-capillary relationship was not affected by acute arterial ligation. The number of capillaries attached per muscle fiber (NCAF) and share factor (SF) did not change from the non-ischemic side, whereas these parameters in LCR rats prominently decreased (Figure 2.1, C and D). The average muscle fiber cross-sectional area was higher in HCR, while it was decreased in LCR. The changes in mean muscle fiber area indicate the severity of ischemic muscle injury in LCR

rather than HCR. Additionally, capillary density (CD) changes are negatively correlated with muscle fiber area. Therefore, CD was lower in HCR rats due to increase in their muscle fiber area, while CD increased in LCR rats due to the reduction in their muscle fiber area. These results indicate that HCR rats have an underlying intrinsic resistance mechanism against acute arterial ligation while the vascular response of LCR rats is less primed to adapt to arterial occlusion. One possible mechanism behind the different adaptations to ischemia is that HCR muscle fiber area is inherently and significantly lower than muscle fiber area in LCR rats (Figure 2.2E). This difference is very important in maintaining the muscle capillary relationship intact and in resisting a reduction in blood flow. Our linear regression data, which is consistent with other studies (191, 205), showed that mean muscle cross-sectional area is negatively, and significantly, correlated with the CD (Figure 2.6). It suggests that having a smaller muscle cross-sectional area, before the development of acute arterial occlusion, is protective against severe injury of ischemic muscle by decreasing diffusion surface area between capillary and muscle fiber. These results are consistent with our previous (unpublished data) and others who also found that cross-sectional area of HCR muscle fiber was 35% smaller as compared to LCR (88), and consistent with those who reported that lower muscle cross-sectional area in HCR rats contributes to higher intrinsic capillary density (87). In this study, only mean muscle cross-sectional area was significantly different in non-ischemic sides between the phenotypes. The capillary density was not significantly higher in HCR when compared to LCR, which was possibly due to low sample size (figure 2.2A). However, our previous study (unpublished data) and others (89) showed significantly higher CD in non-ischemic control HCR vs. LCR. The significant increase of mean

muscle cross-sectional area in HCR as compared to LCR after acute arterial ligation indicates the severity of the injury was less in HCR, and we infer that their physical activity was not affected. This is consistent with a human study which showed resistance training at low intensity leads to an increase in muscle size and strength when combined with blood flow restriction, and that without muscle training hypertrophic promoting factors, muscle cannot be hypertrophied (139). Although HCR rats in this case did not receive exercise training except for their spontaneous cage activity, their cage activity may resemble mild aerobic exercise in its effect. In LCR rats, mean muscle fiber area significantly decreased after arterial ligation, with a related increase in CD. This suggests a different vascular adaptive response to ischemia by increasing both capillary surface area and muscle atrophy to compensate with low blood supply to the ischemic tissue, therefore, decreasing muscle necrosis.

HCR rats at this time point did not show a significant increase in any angiogenic and inflammatory gene expression. This suggests that HCR rats resist the arterial ligation injury by possibly depending on native collateral circulation, which conducts few blood flow under normal circumstances, as the influence of share stress stimulus was below the threshold. However, LCR rats showed an increase in both Vegfa and Angiop2 mRNA, but only in Vegfa protein expression at this time point. Protein levels of Vegfa has been reported by Brandao et al. to be increased in muscle biopsy of patients with critical limb ischemia, and they suggested effective angiogenic drive (18). Increasing Nfkb, Ccl2, and Bmpr2 protein expressions in non-ischemic limbs of LCR as compared to HCR indicate an ongoing stress response to ischemia. The increase in the protein levels without an increase in their gene expressions might suggest a decreased rate of protein degradation

and/or an increase mRNAs degradation, which mediate these proteins, degradation (209). Nfkb was found to play a crucial role in Ccl2 expression in pancreatic beta cells (117). Ccl2 expression in cardiovascular system during hypoxia has also been reported. Rat Ccl2 was found to be expressed in chronic intermittent hypoxia in the carotid body (118). Increased protein levels in LCR could be due to their higher weight versus HCR. Only Bmpr2 protein expression was higher in both limbs after ischemia without exercise in LCR than HCR. Low protein expression of Bmpr2 has been reported to recruit macrophages in pulmonary hypertension (203) and has a role in endothelial injury (210). Here, the high level of Bmpr2 might suggest negative feedback inhibition of macrophage infiltration as indicated by high Ccl2 levels in LCR versus HCR. However, whether Bmpr2 is regulating Ccl2, or vice versa, is not clear. On the other hand, HCR rats seemed reacted differently from the LCR pathway, with more resistant phenotype. These results indicate that LCR rats respond to acute arterial ligation by initial muscle fiber atrophy, functional gene expression of Vegfa, and lately, by angiogenesis. Since the number of capillaries did not increase here, this confirms that active angiogenesis or vascular sprouting will take place later on to bring closer muscle-capillary relationship. Taken together, these results clearly show the presence of differential baseline phenotypic changes in muscle-capillary relationship that further regulate differential vascular adaptive response under the influence of arterial ligation.

Differential Changes After Exercise Post-Ligation

HCR and LCR intrinsically differ in their muscle-capillary relationship which is regulated, to some extent, by genes controlling systemic and local metabolism. For

example, in a human study by Lillioja et al., CD in skeletal muscle has been found to negatively correlate with fasting plasma glucose and insulin concentrations (133). This report also found improvement in muscle-capillary relationship in HCR due to the effect of exercise post-ligation. HCR skeletal muscle is strong, genetically well primed, and resistant to exercise post-ligation stress. However, this relationship in LCR rats seems to be deteriorated.

CD in the HCR rats was higher than the LCR rats due to a decrease in mean muscle cross-sectional area regulated by muscle atrophy, indicating less muscle necrosis and adequate blood flow. Other researchers also reported that CD is increased following acute arterial occlusion in a mouse model (246). On the other hand, CD in LCR was 25% lower than CD of the non-ischemic side. This decrease in CD was associated with around an 8-fold increase in their mean muscle cross-sectional area (Figure 2.3A), suggesting the presence of a large necrotic area and blood flow restriction. Another study also reported there is an association of occlusive exercise with muscle hypertrophy of the occlusive leg (139). Occlusive exercise is a type of resistance exercise training with moderate blood flow restriction in human using a thigh compressor elastic belt. The absence of muscle necrosis suggests that LCR muscle hypertrophy may be due to a compensation of live muscle fibers in replacement of necrotic cells in order to maintain body support during exercise. The non-ischemic side of LCR had significantly lower mean muscle cross-sectional area than HCR. This is not consistent with the same human study (139), as they found that the cross-sectional area of trained arm muscles of an individual with occlusive exercise in the leg was increased due to strong systemic endocrine activation. However, the study is consistent with the development of non-ischemic muscle hypertrophy in HCR

rats, suggesting an activation of systemic neuroendocrine mediators. The results are in line with our findings of increased gene expressions of androgen and androgen/estrogen ratio, in both ischemic and non-ischemic gastrocnemius muscles of HCR rats as compare to LCR (see chapter 4). These results suggest HCR and LCR have different underlying baseline phenotypic and ischemic responses to the same stimulus.

Huge muscle hypertrophy in the ischemic side of LCR also had a negative impact on the other muscle-capillary parameters, such as CFPE and NCAF. CFPE was lower in LCR due to decrease in both CD and number of fibers, which in turn may be due to necrosis and increased mean muscle cross-sectional area. NCAF also decreased in LCR compared to HCR, and also compared to the non-ischemic side of LCR. CFPE was higher in HCR due to higher CD and better perfusion of atrophic muscle, despite lower Vegfa gene expression. However, protein levels in the ischemic HCR were higher than non-ischemic HCR, although there was no significant difference at the protein levels in ischemic limbs between the HCR and LCR phenotypes at that time point. This may suggest that collateral artery engagement is the initial vascular adaptive response or fasting angiogenic response. Taken together, these results show that HCR responds to exercise post-ligation by their intrinsic CD, muscle atrophy and collateral artery involvement, or faster angiogenic response. Therefore, it is expected to have accelerated muscle regeneration and normotrophy. In this study, since the model was not observed at additional extended time points, the data showed no increase in angiogenic gene expression, it is not known yet when angiogenesis was stimulated in this phenotype. In contrast, LCR may have responded to exercise post-ligation by early muscle necrosis with compensatory hypertrophy, increased expression of angiogenic factors, late capillary

formation, and insufficient early collateral artery involvement, as explained by the inflammatory and angiogenic genes and proteins expressions studied in this phenotype (see below).

The screening of angiogenic and inflammatory genes and proteins in the gastrocnemius muscle was done to obtain a complete picture of the differential molecular mechanisms and functions between the LCR and HCR phenotypes, as well as to support the histological results. Vegfa gene expression was higher in the ischemic limb of LCR while HCR showed lower Vegfa. Interestingly, the protein levels did not match gene expression. In ischemic limbs, the protein levels of Vegfa were similar between the phenotypes ($P>0.05$). Decreased levels of Vegfa gene expressions were consistent with another study on rats (215), which showed decreased Vegfa gene expression in ischemic muscle after acute arterial occlusion, but not in mice (246), which showed increased Vegfa expression. The difference between our study and their study is that our animals were exposed to exercise post-ligation. Similar results in exercised human with restricted leg has been recorded, showing increased gene expression and protein levels of Vegfa, which has been suggested to be due to short mRNA bursts induced by exercise bout (69). These data indicate that HCR requires both ischemia and exercise to induce functional Vegfa gene expression, while in LCR each of these stimuli separately is able to induce Vegfa protein expression effectively. The data also suggests that our exercise protocol was not the effective dose for HCR and therefor, a dose titration required for each phenotype is recommended in order to reach optimal effective dose of exercise response. However, Hif1a, Kdr, and Flt1 were not significantly changed in LCR, which is not in line with the findings of others who reported increased Hif1a with Vegfa expression to

stimulate angiogenesis (246). This is due to a difference in time points where Hif1a expression increased after in 6 hours, and then decreased 24 hours after of the occlusion (246). Therefore, it is possible that Hif1a, after inducing Vegfa gene expression, returned to normal levels at this time point in LCR. However, this expression was not associated with capillary development or angiogenesis, which these rats depended on to resupply blood flow to their ischemic fibers, and was consistent with decreased CFPE, NCAF, and CD. In HCR, the pattern of Hif1a mRNA expression followed the low expression of Vegfa, which suggests either HCR did not depend on angiogenesis in increasing capillary parameters in non-ischemic conditions, or the angiogenesis took place prior to 2 weeks, as is seen by the increased protein expression in ischemic limb. Angiogenic genes such as Angpt1 and Angpt2 are known to play roles in angiogenesis, but only Angpt2 gene expression and protein levels were increased in both ischemic and nonischemic limbs of LCR. These findings suggest the underlying differential phenotypic responses to ischemia alone, or with exercise, between HCR and LCR.

As angiogenic gene expression did not support the idea of their playing a role in capillary formation in the HCR, we hypothesized that, in addition to possible angiogenesis occurrence, there might be early collateral artery involvement that senses the large pressure gradient between the collateral arteries in the thigh and the arteries in the ischemic hind limb after exercise-post ligation, activating inflammatory gene expression in gastrocnemius muscle as compared to LCR. It is well known that acute shear stress, both in vitro (246) and in vivo (8) elicits rapid endothelial cytoskeletal remodeling that activates transduction signaling, through the acute release of NO in the

proximal non-ischemic region, of several transcription factors, such as AP-1, Nfkb, Egr-1, SP1, and serum responsive elements. These transcription factors and responsive elements in turn regulate the transcription of genes such as Icam, Vcam, Ccl2, Pdgfa, Pdgfb, TGF-β, and Egr-1 (17). Egr-1and nitric oxide are known in the regulation of shear stress-mediated induction of Pdgfa (30), Ccl2 /MCP-1 (7), and Icam (163).Therefore, we investigated the genes, Nfkb, Pdgfa, Ccl2, Icam, and Vcam in non-ischemic, ischemic, and the ratio of ischemic/non-ischemic in trained and untrained phenotypes. We also looked at the Bmpr2 gene expression to understand its differential regulation after exercise post-ligation between the phenotypes.

Nfkb is the upstream mediator to Ccl2/MCP-1 (17). The gene expression of Nfkb was higher in the non-ischemic limb of HCR when compared to LCR, However, the protein level of Nfkb was opposite to that which was observed in its gene expression, i.e. it was significantly higher in LCR than HCR. However, there was no difference in the gene expression and protein levels between the phenotypes in ischemic limb. This was possibly due to the upregulation of Nfkb in the early few hours after acute arterial ligation, followed by its subsequent return to baseline levels within 24 hours of the ligation (246). Additionally, increased Nfkb gene expression in HCR was associated with an increased trend of Il6, but did not reach statistical difference ($p=0.1$) when compared to LCR. This finding might also interpret the early muscle hypertrophy of non-ischemic side in the HCR due to the cross-transfer effect that was reported by Haruhiko et al. (139), since Nfkb is also an upstream mediator of Il6. Il6 is secreted locally by skeletal muscle during exercise-induce muscle hypertrophy (159). Expression of Il6 was observed to be greater in the ischemic side of HCR, but the result did not achieve statistical

significance (Table 2.C). Another study (215) that also used a rat model of acute arterial occlusion reported nonsignificant changes in Il6 as well.

Although the Ccl2 gene expression in ischemic and non-ischemic limbs of HCR gastrocnemius muscle was higher in HCR than LCR. The protein level was significantly higher in the non-ischemic limb of LCR than HCR. However, the protein level increased in the ischemic limb of HCR while it decreased in LCR. There was no statistical difference at the protein level in the ischemic limb between the phenotypes. Ccl2/MCP-1 in ischemic limb plays a role in monocyte and macrophage recruitment, which is expressed mainly by these cells to induce an inflammatory environment in the ischemic muscle. Macrophages play crucial roles in acute ischemic injury. They are phagocytic cells for necrotic debris and stimulate arteriogenesis (26, 77, 223, 224). Ccl2 is considered pro-arteriogenic because it increases collateral blood flow in acute arterial ligation (184) through the upregulation of monocyte adhesion molecules. This suggests that the possible reason of increased capillarization parameters in the HCR histology is due to increase blood flow in the collateral arteries and not due to angiogenesis. Although we did not measure blood flow in this study, we had measured it in the previous study, where it was shown that blood flow was decreased slightly in HCR after 1 hour and 7 days of acute ligation without exercise, while greater reduction was found in the LCR (unpublished data).

Low Ccl2 expression in LCR rats in this study also suggests that the LCR phenotype either has an insufficiency in activation of the collateral artery and small pressure gradients developed between the collateral arteries and arteries in ischemic limb

(51, 164, 220), or a low number of collateral arteries is not sufficient to adapt to ischemia. An increase in Ccl2 expression in the non-ischemic limb of HCR, but not LCR rats, may suggest that the non-ischemic limb was the dominant limb used by the HCR rats during daily spontaneous activity in the cage, which is consistent with the hypertrophy. It has been reported that an increase Ccl2 expression after exercise was suggested to be due to the influence of exercise intensity rather than the presence of exercise induced-muscle damage, but its role was not clear in exercise condition (67). In LCR, lower levels of Ccl2 with decreased mean muscle cross-sectional area in non-ischemic muscle as compared to HCR suggested that LCR rats were more sedentary between the exercise regimens than HCR. These results suggested differential inflammatory phenotypic background between HCR and LCR.

Pdgfa and Icam are the inflammatory mediators that play positive roles in the shear stress response (17). Pdgfa gene expression was significantly higher in ischemic and non-ischemic limbs of HCR than LCR. Although the protein levels did not significantly differ between the phenotypes. Both phenotypes showed a slight increase in the Pdgfa protein level in ischemic limbs. Pdgfa plays an important role in tissue remodeling and cellular differentiation (84) either in the non-ischemic limb of HCR or ischemic limb of LCR where there was muscle hypertrophy, but still non-significantly lower than HCR (Table 2.B; 20). These data were consistent with Yang et al. (246) who reported increased Pdgfa gene expression in ischemic gastrocnemius muscle after acute arterial occlusion. Pdgfa also has the ability to remodel the tissues by stimulating fibroblast proliferation and macrophage recruitment (178). Although there was no significant increase in the Icam and Vcam gene expressions between phenotypes, their

expression levels were higher in LCR. It has been reported that increased shear stress response in acute arterial ligation causes a decrease in Vcam (17). In chronic arterial occlusion, Vcam increased more than in acute ligation (246). Increased Vcam and Icam are associated with endothelial dysfunction. However, increased Icam was also associated with increase shear stress (17).

Although higher gene expression of Bmpr2 was found in non-ischemic limb of HCR and in ischemic limb of LCR, but lower expression was found in ischemic limb of HCR and in non-ischemic limb of LCR (opposite response between the phenotype!), the protein level was higher in the LCR ischemic and non-ischemic limbs as compared to HCR. Low protein expression of Bmpr2 has been reported to recruit macrophage in pulmonary hypertension (174). Bmps, Bmpr ligands, have been shown to prevent mesenchymal stem cell infiltration in the myogenic lineage (62). Thus, these results suggest the role of Bmpr2 in preventing macrophage and mesenchymal stem cell infiltrations on the ischemic and non-ischemic limbs of LCR, and to a lesser extent in the ischemic limb of HCR. They also suggest an activation of non-ischemic muscle hyperplasia due to muscle hypertrophy during exercise, which is consistent with the lower CFPE and Ccl2 opposite to LCR. Lower protein levels of Bmpr2 after exercise was seen only in HCR non-ischemic-exercised limbs, but not in LCR rats, suggesting there is a phenotypic influence (LCR vs. HCR) on Bmpr2 protein expression after exercise training.

These data suggest that the vascular system of HCR rats is primed to adapt to exercise post-ligation through previously existent and newly activated internal collaterals. This is through the increased effective expression of shear stress-related genes that is

seen in response to exercise post-ligation than in response to ligation without exercise as compared to LCR. In LCR, the vascular adaptive response to exercise post-ligation is directed mainly by angiogenesis and insufficient inflammatory protein expression. However, this can also make LCR ischemic muscle more prone to chronic ischemic stress, because delayed muscle-capillary remodeling, increase the incidence of developing atherosclerosis, and increase the susceptibility to develop recurrent arterial thrombosis because their endothelial cells are prone to increase expression of abnormal adhesion molecules such as Vcam and Icam. Clinically, this is relevant especially after revascularization of arterial occlusion when some patients develop recurring thrombosis.

CONCLUSION

This study showed that mild exercise intensity after arterial occlusion in LCR rats produces chronic ischemia-like vascular adaptive response, with more hypoxia-induced angiogenesis than inflammogenic-induced vasculogenesis. This is possibly due to less toleration to the same temporal and spatial effect of two stimuli on their physiological and pathological responses coming from exercise post-ligation and acute arterial occlusion, respectively. Therefore, exercise post-ligation outcomes depend on the type of intrinsic aerobic phenotype an individual has, with favorable outcomes appearing in individuals with a high intrinsic aerobic capacity phenotype. Those with low intrinsic aerobic capacity phenotype might respond better to exercise pre-conditioning than exercise post-ligation to first allow their body to adapt to the physiological stimulus before they develop lower limb ischemia (a pathological stimulus).

CHAPTER 3: INTRINSIC AEROBIC CAPACITY INFLUENCES THE VASCULAR ADAPTIVE RESPONSE TO EXERCISE PRECONDITIONING AGAINST LOWER LIMB ISCHEMIA

Abstract:

Peripheral arterial disease (PAD) is a common disease affecting the elderly. It affects one in every 20 Americans who are over the age of 50. This causes an increase in the clinical and economic burdens on patients and the government, respectively. Exercise preconditioning has extensively been used to protect heart and skeletal muscle from ischemia reperfusion injury. However, there are heterogeneous responses as effects to exercise in animals and humans. The relationship between exercise preconditioning and its effect on peripheral arterial ligation in low and high intrinsic aerobic running capacity phenotypes (LCR and HCR, respectively) is novel, and will give a clear picture about the mechanistic effect of preventive exercise on PAD in both phenotypes. Our previous work with this model has shown that both untrained and exercised post-ligation LCR rats have impaired vascular adaptive response compared to HCR following peripheral arterial ligation. In this study, we examined the effect of exercise preconditioning on muscle-capillary relationship as well as the levels of angiogenic/inflammogenic factors including Vegfa, Kdr, Flt-1, Angp 1, Angp 2, Hif-1a, Il-6, Pdgfa, Pdgfrb, Icam, and Vcam. These factors were examined 14 days following unilateral hind limb femoral artery occlusion using mild treadmill exercise training for two weeks or without exercise. Trained LCR rats showed closed relationship in the muscle-capillary interface. The number of capillaries attached per fiber increased with decreasing average muscle fiber area in the

ischemic muscle and showed an increase in angiogenic gene expression. On the other hand, the muscle-capillary relationship in HCR rats was neither deteriorated nor improved in the ischemic muscle that was preconditioned for two weeks by treadmill exercise. However, the effect of exercise on the non-ischemic limb of HCR was more favorable than what was found in the ischemic limb. Interestingly, exercise pre-ligation did not decrease adhesion molecules in LCR as it did in HCR. Therefore, it appears that a mild exercise intensity can precondition hind limb occlusive disease in low intrinsic aerobic exercise capacity phenotype through remodeling the musculovascular adaptive response. These findings will help protect the elderly with a high risk of PAD from developing the disease.

NEW & NOTEWORTHY: The protective effect of exercise preconditioning has been reported in several diseases, including peripheral arterial diseases. However, the PAD model that has been used in exercise preconditioning has only been studied in the context of ischemia reperfusion injury. This study reported for the first time the effect of exercise preconditioning on the prevention of peripheral arterial occlusive disease in a model of low intrinsic aerobic capacity rats, or LCR. This paper shows that exercise pre-ligation in LCR encourages musculovascular remodeling in response to ischemia.
Key words: Ischemia, HCR, LCR, exercise preconditioning, PAD, intrinsic aerobic capacity.

INTRODUCTION

Peripheral arterial disease (PAD) is a common but serious disease. The National Heart, Lung, & Blood Institute reported that one in every 20 Americans over the age of

50 has PAD. Those with PAD have an increased risk for developing myocardial infarction and stroke. PAD develops when the arterial vasculature is exposed over time to fatty deposits, causing plaque formations that narrow the lumen and limit blood flow to the legs.

Commonly, this disease is asymptomatic and can be discovered suddenly in the clinic by ankle-brachial index during screening for PAD in elderly people, or it is diagnosed when the patient complains of painful walking that either disappears during rest (intermittent claudication), or less commonly, progresses into severe pain that persists at rest (critical limb ischemia). When the disease gradually increases in its severity, it is diagnosed by ankle-brachial index and is treated by several plans, each of which depends on the disease severity, patient tolerance, and body response. PAD therapies include: exercise therapy, medical therapy, endovascular and surgical therapy, and as a last resort, amputation of the affected leg (136).

PAD is associated with increased morbidity and mortality due to comorbidities with conditions/diseases such as obesity, diabetes, and hypertension; all of which that are also expected to increase significantly with aging of the population (10, 83). Additionally, the current clinical interventions can increase the burden on PAD patients due to recurring hospitalizations and repeated re-vascular surgery. Moreover, this burden extends into the economic domain. It has been estimated that the US total annual costs of hospitalizations for PAD patients in 2004 was more than $21 billion (140), indicating that PAD requires more preventive measures to resolve the debilitating clinical and economic burdens.

Compared to other therapies, exercise therapy is considered cheap, safe, convenient, non-invasive, and more importantly, it improves the quality of life (243). Several mechanisms have been found to be impacted from exercise therapy in PAD such as angiogenesis, arteriogenesis, improved muscle metabolism, decreased oxidative stress, and increase pain threshold (212). Accumulating evidence indicates the protective effect of exercise preconditioning against diverse cardiovascular diseases by decreasing risk factors (216) and promoting muscular metabolic and morphological adaptations to several types of stress (28,31). It has been documented in a mouse model that exercise preconditioning caused resistance to acute myocardial infarction (216). However, since 70% of aerobic exercise capacity is determined by genetic factors, this explains the possible heterogeneous response to active exercise and opens the question: What is the differential effect of exercise preconditioning on protecting peripheral arterial disease between low intrinsic aerobic capacity and high intrinsic aerobic capacity phenotypes? Since the role of exercise preconditioning against peripheral arterial ligation has not yet been studied, it is important to investigate how intrinsic aerobic phenotypes respond to active treadmill exercise preconditioning to promote muscle-capillary adaptive response, both morphologically and genetically, to ischemic and remodeling stressors.

A rat model of intrinsic aerobic exercise capacity has been generated by Koch and Britton at Michigan University. Through the use of artificial selection and based on the endurance treadmill running phenotype, they were able to generate low capacity running rats (LCR) and high capacity running rats (HCR). LCR rats are characterized as having multiple risk factors for cardiovascular diseases including hypertension, insulin

resistance, hyperlipidemia, high levels of fasting blood glucose, obesity, and a decreased exercise running capacity (239). This study could potentially aid the elderly by providing insight into the prevention of PAD, of which there is an increased risk of acquiring during aging, by using a rat model to study the intrinsic aerobic capacity backgrounds and whether or not exercise can act as a preconditioning modality against PAD in both phenotypes.

MATERIALS AND METHODS

Animal Strains and Experimental Groups. HCR and LCR, female rats, 40 weeks, n= 16 each, generation 32. Eight HCR and eight LCR rats were exercised pre-ligation for 10 days, beginning at day -14 before the occlusion. The remaining 16 animals served as sedentary ischemic control. These animals were obtained from Drs. Lauren Koch and Steven Britton at the University of Michigan. They have previously described the artificial selection used for the generation of the HCR and LCR strains (111, 112, 10). Briefly, each sex was selected from the founder population (N:NIH stock). Two-way artificial selective breeding was used to create low capacity runner (LCR) and high capacity runner (HCR) strains that were divergent for treadmill running capacity (run time to exhaustion on a graded treadmill exercise test). The 13 lowest and 13 highest running capacity rats of each sex were randomly paired for mating. This pattern was repeated over subsequent generations, using a rotational breeding scheme, to produce the divergent strains. In the present study animals from generation 32 were used. All animals were exposed to treadmill exercise for 5 days at 11 weeks of age when the animals were phenotyped for treadmill running capacity. Upon verifying the phenotypes of the animals,

they were prepared for shipping at 14 weeks of age, or as soon after as weather conditions (airport tarmac temperatures < 85F) permitted. Once received by the Department of Comparative Medicine at ECU, the animals were maintained under mandatory quarantine for 10 weeks before they were released for study. Rats were provided standard rat chow and water *ad libitum* and were kept on a 12 h light/ 12 h dark time schedule until sacrifice. Animal procedures were conducted in accordance with the American Physiology Society guidelines for the humane and safe use of animals. Additionally, all protocols involving animals were approved by the East Carolina University Animal Care and Use Committee.

Exercise Training. Motorized treadmill running exercise was used for exercise pre-ligation groups as a type of mild intensity supervised graded exercise training. Animals were subjected to 5 days on the treadmill at a very low speed (10 m/min) to condition them to the treadmill environment. Animals that were not running on the treadmill were removed and returned to their cages. Significant attention was devoted to minimizing exposure to the shock grid by using blasts of air or gentle prodding with a brush. Animals began exercising for 5 minutes, at a rate of 14-16 m/min, for 5 days a week for two weeks. The duration of exercise was gradually increased by 5 min per day, until the training sessions lasted 60 minutes at a time. Exercise pre-ligation protocol was generally 2 weeks in duration (10 events). The total number of sessions was: 5 conditioning + 10 protocol = 15 events/animal (174).

Hind Limb Femoral Artery Occlusion. Femoral artery occlusion was produced as outlined by others (136,135,184). Briefly, rats were anesthetized with ketamine xylazine (0.1ml/100g body weight IP). The fur was shaved from the inner thigh and the surgical

sight was cleaned with Betadine and 70% alcohol. Utilizing a small incision, the left femoral artery was isolated and three ligatures of 6-0 surgical silk were placed to cause an ischemic condition in the downstream tissue. One ligature was placed 5-6mm distal to the inguinal ligament, a second was placed on a collateral artery, and the third was placed 5-6 mm distal to the first ligature. When all three ligatures were in place and the incision was closed, the animal was given a Buprenex injection (0.1ml/100g body weight IP) and placed in its cage to recover. Animals were allowed to arouse to consciousness on their own and have unlimited access to food and water. Finally, animals were sacrificed at day 14 following placement of the ligature. This procedure produces a uniform occlusion of the femoral arteries that reduces blood flow reserve capacity to 10–20% of normal levels, while remaining sufficient to meet resting blood flow requirements (243, 244, 245).

Skeletal Muscle Morphology: Rosenblatt Staining and Analysis. Gastrocnemius muscle for each animal was excised and quickly frozen by placing the tissue into an optimal cutting temperature (O.C.T.) compound. The muscle in the OCT was cut into transverse sections (thickness 8um) and underwent capillary staining as originally defined by Rosenblatt et al. (21, 60). Briefly, Rosenblatt staining allows for capillary visualization for subsequent photographs under 20X magnification. Capillaries were quantified manually from the image on individual fibers. The following six indexes were measured: 1. SF; 2. Average perimeter (AP); 3. Fiber cross-sectional average area (AA); 4. CFPE; 5. NCAF; 6. CD. Fiber cross-sectional average area and perimeter measurements were calculated with the ImageJ-analysis system, which was calibrated to transform the number of pixels (viewed on a computer monitor) into micrometers from an image of the myosin ATPase stain.

RNA Isolation, Reverse Transcriptase-PCR, and Real-Time PCR. Total RNA was extracted from harvested gastrocnemius muscle using TRIzol reagents (Invitrogen), and cDNA was generated using the High-Capacity cDNA Reverse Transcription kit from Applied Biosystems (Thermofisher) following the manufacturer's protocol. qReal-time PCR was performed using 5̀ fluorescent labeled primers for Vegfa, Angpt1, Angpt2, Hif1a, Icam, Vcam, Pdgfa, Kdr, Flt1, Nfkb, Ccl2, Il6, and Pdgfrb utilizing TaqMan Fast Advanced Master Mix (Life Technology) and following the manufacturer's protocol. Actinb was used as the reference gene since CT values for this gene did not change with treatment. The qReal-Time PCR detection system used was the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). LCR and HCR internal control right leg samples were used for the normalization of all data points with treated samples of other legs using the ratio of $2^{-\Delta}$ CT of treated sample / $2^{-\Delta}$ CT of controlled sample.

Statistics. Statistical analysis was performed using the data analysis software in the Microsoft Excel worksheet (Windows 10). Data is expressed as mean values ± SEM, and a p value < 0.05 was considered statistically significant. Single factor ANOVA tests were used to compare between LCR and HCR to determine statistical significance among the groups (two groups at a time). Data was compared three different ways: internal control data of LCR with the counterpart of HCR group, ischemic limb data of LCR with its counterpart of HCR, and finally the ratio of Lt ischemic/non-ischemic Rt limb (Lt/Rt) of LCR with the HCR counterpart. Similarly, relative gene expression levels were calculated by comparing q-RT PCR data, after normalizing them to the internal standard

housekeeping gene (Actinb), of non-ischemic Rt limb of LCR with its counterpart of HCR, and comparing ischemic Lt limb and the Lt/Rt ratio of LCR with HCR counterpart groups as explained above. Significant differences were accepted in all circumstances where $p<0.05$.

RESULTS

The Differential Changes in Muscle-Capillary Relationship in The Calf Gastrocnemius Muscle 14 Days Following The Ligation Between Untrained LCR and HCR:

Ratio of Ischemic Over Non-Ischemic Limb: The ratio of capillaries to average muscle fiber area, or CD, did not significantly increase ($p\geq0.05$) between the phenotypes (figure 3.1A). The amount of capillary surface available for exchange per muscle fiber surface area, or capillary-to-fiber perimeter exchange (CFPE), is an accurate indicator of muscle capillary interference. CFPE was not significantly altered between the phenotypes ($p\geq0.05$) (figure 3.1B). The number of capillaries attached per fiber (NCAF) is also another important parameter in muscle-capillary relationship and is affected by ischemia and exercise. NCAF was 26% higher in HCR than in LCR group ($p<0.05$) (figure 3.1C). The number of fibers sharing a single capillary, or the share factor (SF), was also significantly ($p<0.05$) altered in HCR compared to LCR, as it was 27% higher in HCR (figure 3.1D). The average area of muscle fiber (AA) is an important parameter in measuring muscle functionality and is a good indicator of CD state in the skeletal myocyte. HCR muscle fiber average area was 110% higher, after ischemia and without exercise, as compared to LCR ($p < 0.05$) (figure 3.1E). The average perimeter of muscle

fiber also followed the similar change in AA. Ligation without exercise affected the average perimeter of muscles, and it was not found to be significantly ($p > 0.05$) higher in HCR than LCR due to the increased muscle fiber average area at that time instant (figure 3.1F).

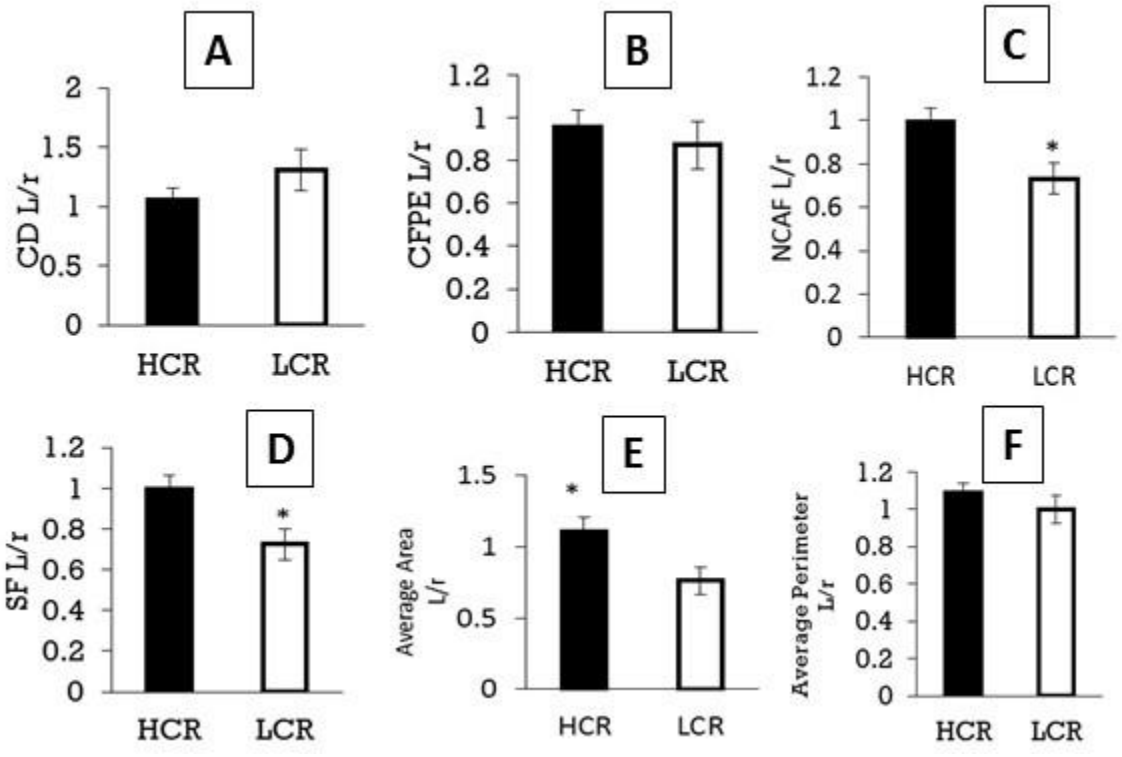

FIGURE 3.1. The effect of intrinsic aerobic capacity phenotype on gastrocnemius muscle-capillary relationship following 14 days of left femoral artery ligation. Capillary density, CD (A), capillary-to-fiber perimeter exchange, CFPE (B), number of capillaries attached to fiber, NCAF (C), sharing factor, SF (D), muscle fiber average area (E), muscle fiber average perimeter (F) are shown. Data is expressed by LCR and HCR left leg to right leg ratio (LCRL/r and HCRL/r) respectively, n=15. Single factor ANOVA test was used to analyze the data, * indicates $p<0.05$. The error bars represent standard error.

In the non-ischemic (right) limb, only the average area (AA) of muscle fiber was found to be significantly higher, by approximately 30%, in the LCR right limb (LCRr) than HCRr. The other muscle-capillary parameters were not statistically significant (figure 3.2A-F).

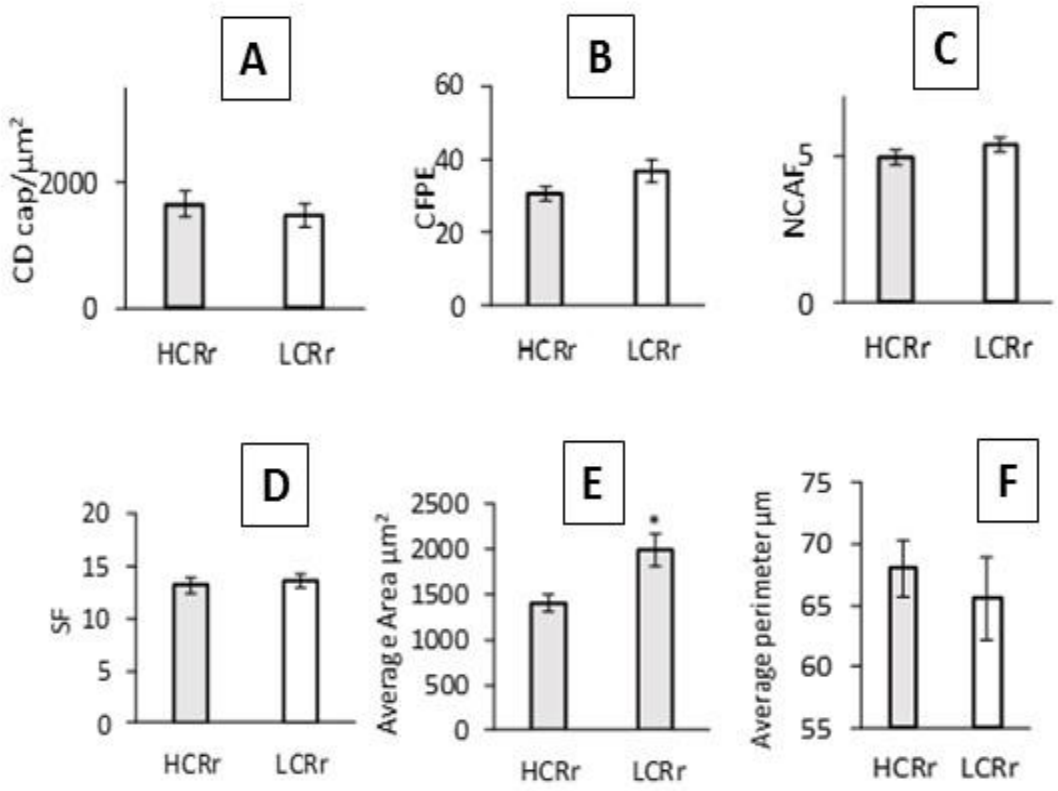

FIGURE 3.2. The effect of intrinsic aerobic capacity phenotype on non-ischemic (right leg) gastrocnemius muscle-capillary relationship following 14 days of left femoral artery ligation. Capillary density, CD (A), capillary-to-fiber perimeter exchange, CFPE (B), number of capillaries attached to fiber, NCAF (C), sharing factor, SF (D), muscle fiber average area (E), and muscle fiber average perimeter (F) are shown. Data is expressed by LCR and HCR right leg. LCRr and HCRr, respectively, n=15. Single factor ANOVA test was used to analyze the data, * indicates $p<0.05$. The error bars represent standard error.

The Differential Changes in Muscle-Capillary Relationship in the Calf Gastrocnemius Muscle Following Exercise Pre-Ligation Between LCR and HCR:

Ratio of Ischemic Over Non-Ischemic Limb: CD in the Lt/Rt ratio decreased slightly, but insignificantly, in both phenotypes to an almost comparable level after 14 days of left femoral artery ligation, which was preceded by 2 weeks of treadmill exercise (figure 3.3A). CFPE was 24% higher in LCR than HCR at day 14 of the ligation when the animals exercised before ligation, but the increase was non-significant ($p>0.05$) (figure 3.3B). However, exercise caused a 14% increase ($p<0.05$) in NCAF in LCR while HCR did not show much change in the Lt leg (figure 3.3C). Although there was no significant difference ($p>0.05$) in SF between the phenotypes in training before ligation, LCR showed a 7% increase in SF than HCR while HCR did not show much change in the Lt Leg (figure 3.3D). AA was increased in both phenotypes, but LCR showed a 48% higher (~2 fold) level as compared with HCR when exercise was preceded by ligation (figure 3.3E). In the average perimeter, both phenotypes showed ~20% increase without statistical significance in the difference ($p>0.05$) (figure 3.3F).

Non-Ischemic Limb: In the non-ischemic (right) limb, the CD was 40% lower, but non-significant, in the HCRr due to a ~30% increased ($p<0.05$) average area (AA) as compared with LCRr. Additionally, NCAF and SF were 15% higher in HCRr as compared with LCRr ($p<0.05$) (Figure 3.4A and C-E). The other muscle-capillary parameters (CFPE and average perimeter) were not statistically significant (figure 3.4B and F). Representative images of histological sections are shown in Figure 3.5.

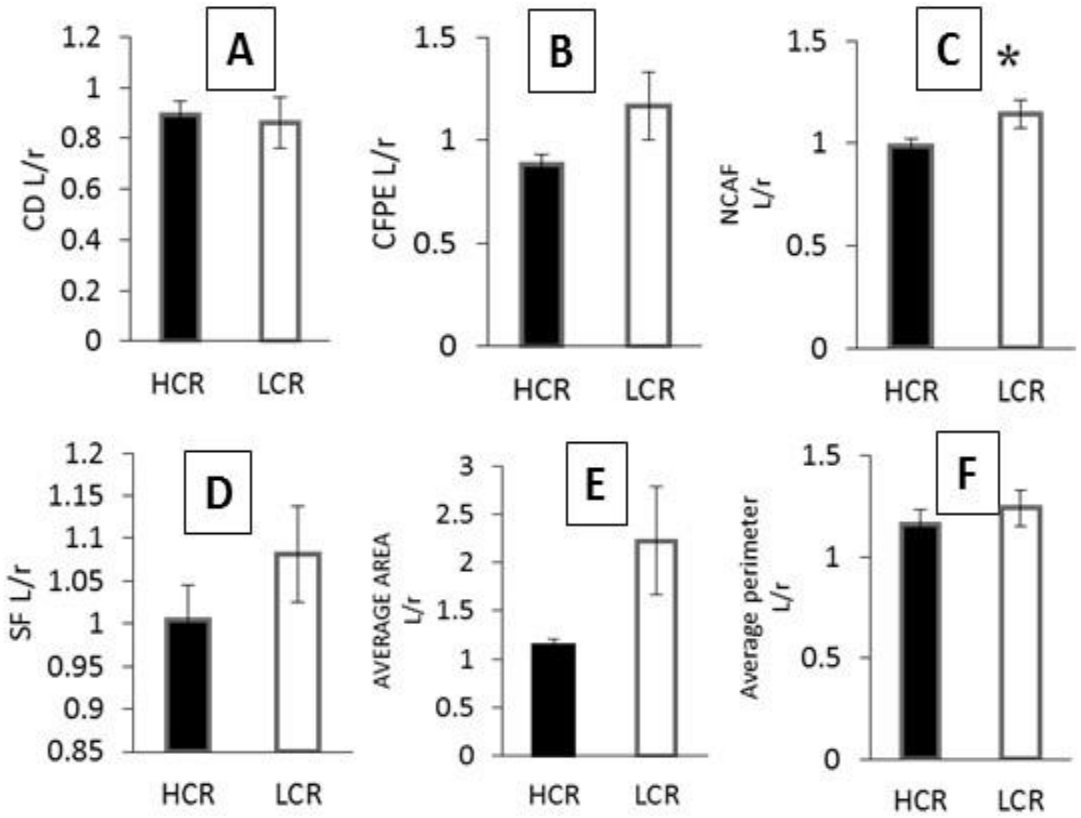

FIGURE 3.3. The effect of exercise preconditioning on gastrocnemius muscle-capillary relationship in high and low aerobic capacity phenotype rats, HCR and LCR, respectively. Rats were exercised for two weeks, followed by left femoral artery ligation for another two weeks before sacrifice. Capillary density, CD (A), capillary-to-fiber perimeter exchange, CFPE (B), number of capillaries attached to fiber, NCAF (C), sharing factor, SF (D), muscle fiber average area (E), and muscle fiber average perimeter (F) are shown. Data is expressed by LCR and HCR left leg (ischemic) over right leg (non-ischemic) ratio, LCRL/r and HCRL/r, respectively, n=15. Single factor ANOVA test was used to analyze the data, * indicates $p<0.05$. The error bars represent standard error.

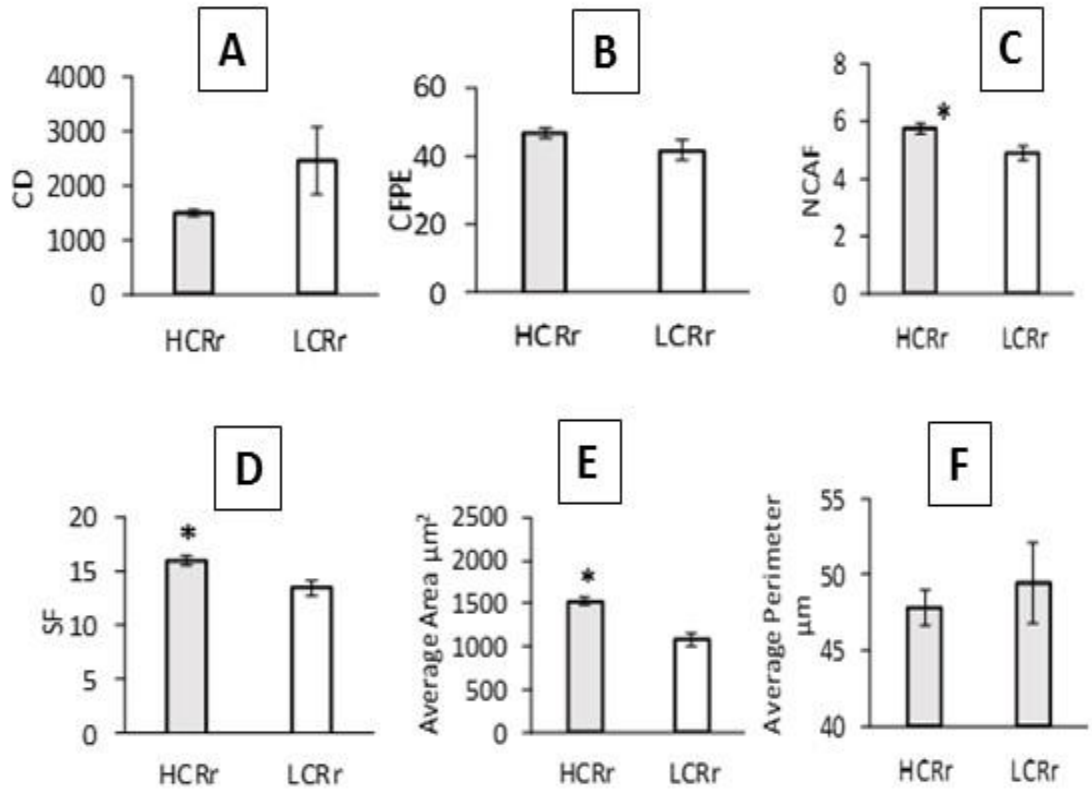

FIGURE 3.4. The effect of exercise preconditioning on gastrocnemius muscle-capillary relationship (Rt non-ischemic limb) in high and low aerobic capacity phenotype rats, HCR and LCR, respectively. Rats were exercised for two weeks, followed by left femoral artery ligation for another two weeks before sacrifice. Capillary density, CD (A), capillary-to-fiber perimeter exchange, CFPE (B), number of capillaries attached to fiber, NCAF (C), sharing factor, SF (D), muscle fiber average area (E), and muscle fiber average perimeter (F) are shown. Data is expressed by LCR and HCR non-ischemic right leg, LCRr and HCRr, respectively, n=15. Single factor ANOVA test was used to analyze the data, * indicates $p<0.05$. The error bars represent standard error.

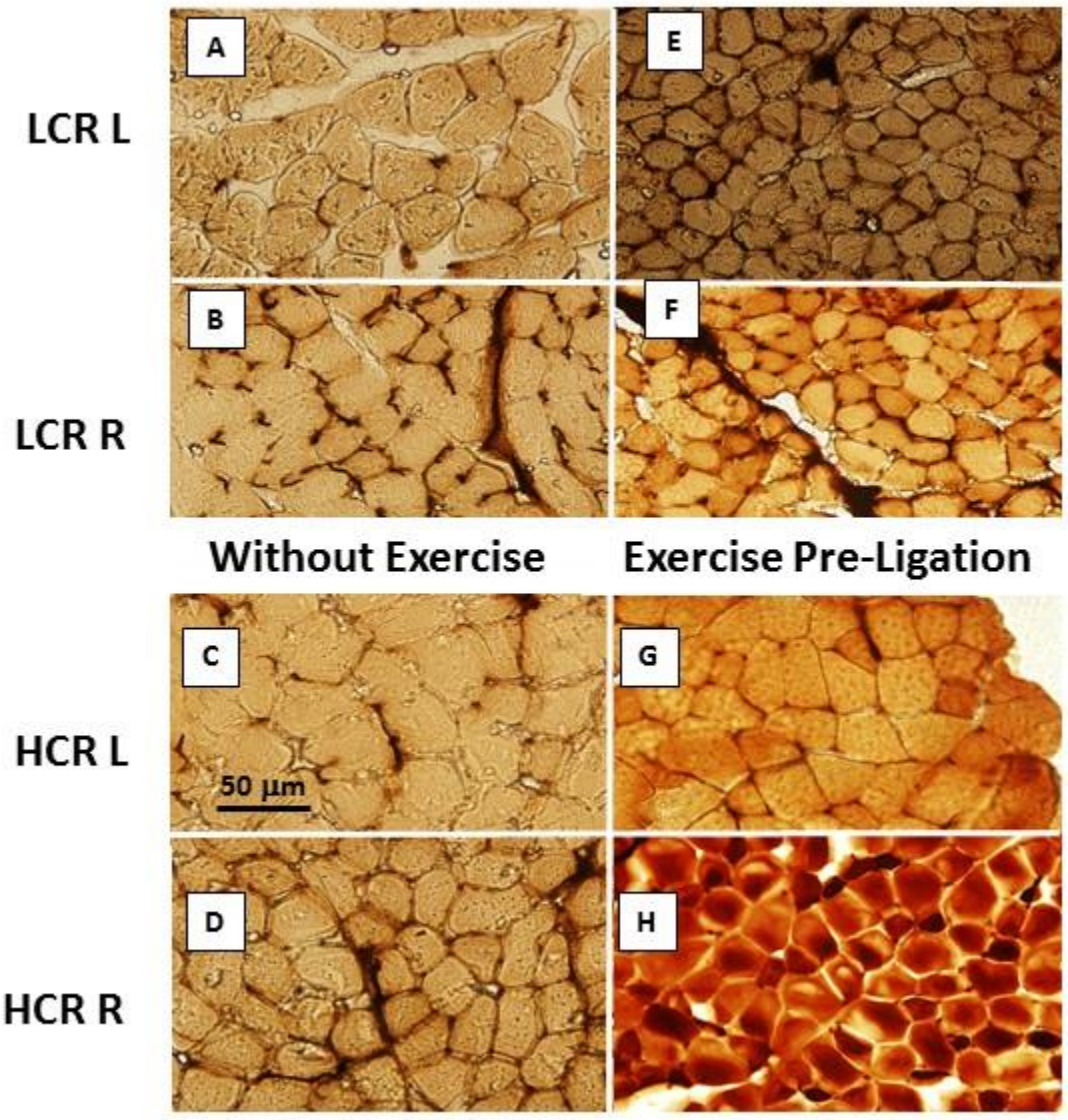

FIGURE 3.5. A histological representation of Gastrocnemius muscle cross-section following left femoral artery ligation in trained and untrained groups, using 8 micron sectioning and the myosin ATPase staining "Rosenblatt staining", showing cross section of the muscle fiber and capillaries. In all animals, the left leg (LCR-L and HCR-L) was ischemic, and the right leg (LCR-R and HCR-R). Images A-D show the effects of occlusion without any exercise before ligation. Images E-H show sections from the corresponding limb muscle with an exercise regimen before occlusion. As noted elsewhere LCR = low intrinsic aerobic capacity rat; HCR = high intrinsic aerobic capacity rat. Fiber cross-sectional average area and perimeter were measured using NIH imageJ software calibrated to transform the number of pixels (viewed on a computer monitor) into micrometers from an image of the myosin ATPase stain.

The Differential Changes in Gene Expression in the Calf Gastrocnemius Muscle Following Ligation in Trained and Untrained HCR Rat vs. LCR Counterpart:

The patterns of expression of multiple genes are known to be essential for the understanding of the vascular adaptive response of the hind limb to arterial occlusion. These patterns were significantly different when the ligation followed 2 weeks of treadmill exercise in ischemic (Lt), nonischemic (Rt), and the ratio of ischemic to the non-ischemic hind limbs (Lt/Rt).

Vegfa is a strong indicator of angiogenesis in response to physiological and pathological hypoxia. Its mRNA expression in the control limb (Rt leg) of untrained LCR as compared to HCR was significantly higher, by approximately 47%, than HCR (Table 3.A; 1). Similarly, in the Lt ligated limb, Vegfa mRNA expression was found to be increased in the LCR, but the trend was not significant ($p=0.1$) (Table 3.A; 14). The ratio of Lt/Rt was increased slightly in the same level (3%) in these phenotypes (Table 3.A; 27).

The expression of Vegfa receptors, Flt1 and Kdr, were not altered significantly ($p>0.05$) in Rt (Table 3.A; 2-3), Lt (Table 3.A; 15-16), or even Lt/Rt ratio (Table 3.A; 28-29) between the phenotypes in untrained groups. However, the overall expressions were higher in LCR than HCR. Vegfa expression in the exercise pre-ligation group was distinct. The control limb (Rt leg) showed no significant difference ($P>0.05$) in Vegfa expression in gastrocnemius muscle between the phenotypes (Table 3.B; 1), while the Lt leg showed a significant 55% increase in Vegfa levels in LCR as compared with HCR (Table 3.B; 14). The ratio of Lt/Rt wassignificantly higher, by 48%, in LCR as compared

with HCR, but its trend showed a reduction in the Vegfa expression when compared with its Rt leg (Table 3.B; 27).

Flt-1 expression in the Rt leg was not significantly different between the trained phenotypes (Table 3.B; 2), but in the Lt leg of LCR it was 50% higher than HCR (Table 3.B; 15). The Lt/Rt ratio was ~50% higher in LCR than HCR, but was not statistically significant (P=0.09) (Table 3.B; 28). The expression of Kdr followed Flt-1 expression with no significant difference in Rt leg between the trained phenotypes (Table 3.B; 3). However, the Lt leg showed an approximate 58% increase, bordering statistical significance (p=0.05), in LCR as compared with HCR (Table 3.B; 16). The Lt/Rt ratio was significantly higher by about 64% in LCR than HCR. (Table 3.B; 29).

Hif1a is the sensitive mediator for hypoxia that mediates the activity of Vegf for angiogenesis. However, its mRNA expression in untrained, either in the Rt (Table 3.A; 4), Lt (Table 3.A; 17), or Lt/Rt ratio (Table 3.A; 30), and trained phenotypes, Rt (Table 3.B; 4), Lt (Table 3.B; 17), or Lt/Rt ratio (Table 3.B; 30) were not significantly elevated at the 14^{th} day after femoral artery ligation. Although, overall Hif1a mRNA expression was higher ($p>0.05$) in LCR than the HCR.

The expression of other hypoxia-related transcription factors includes Nfkb, Angpt1, and Angpt2. The gene expressions of Nfkb and Angpt1 were not significant ($p>0.05$) in either untrained Rt (Table 3.A; 6-7), Lt (Table 3.A; 19-20), and Lt/Rt ratio (Table 3.A; 32-33) or in trained Rt (Table 3.B; 5-6), Lt (Table 3.B; 18-19), and Lt/Rt ratio (Table 3.B; 31-32) between the phenotypes. However, they were generally higher in LCR than HCR.

Angpt2 expression in the untrained LCR group at the phenotypic baseline was significantly higher, by approximately 70%, than HCR (Table 3.A; 8). In the Lt leg, Angpt2 expression was 54% higher, but not statistically significant, in LCR than HCR (Table 3.A; 21). The Lt/Rt ratio was lower in LCR than HCR (P>0.05) (Table 3.A; 34) because Angpt2 expression decreased in the ligated leg of LCR while it increased in HCR. In exercise pre-ligation, Angpt2 expression in Rt (Table 3.B; 7) and Lt leg (Table 3.B; 20) were 50% and 54% higher, respectively, in LCR than HCR. However, the Lt/Rt ratio was increased in HCR to a level similar (~15%) to LCR (p>0.05) (Table 3.B; 33).

Other genes related to an inflammatory response such as Ccl2, Icam, Vcam, Il6, Pdgfrb, and Pdgfa showed distinct patterns in the LCR compared to HCR. Under the influence of ischemia alone without exercise, the phenotypes did not show any significant increase (p>0.05) for Ccl2 (Table 3.A; 9, 22, and 35), Vcam (Table 3.A; 10, 23, and 36), Icam (Table 3.A; 11, 24, and 37), Il6 (Table 3.A; 5, 18, and 31), Pdgfrb (Table 3.A; 12, 25, 38), and Pdgfa (Table 3.A; 13, 26, 39). However, the patterns of expression were different between the phenotypes under the influence of exercise pre-ligation.

Vcam gene expression in the Rt leg was nearly comparable in these phenotypes (p>0.05) (Table 3.B; 11). However, in the Lt leg, the Vcam expression was significantly higher, by 63%, in LCR than HCR (Table 3.B; 24) with a Lt/Rt ratio reaching a 65% increase in LCR compared with HCR (Table 3.B; 37).

Icam gene expression was the same between the phenotypes in Rt leg (Table 3.B; 12). In the Lt leg, the Icam gene expression in LCR was 35% higher than HCR, but not significant (P=0.1) (Table 3.B; 25). However, the level of significance increased when

the Lt/Rt ratio was calculated to show that the percentage change of Icam expression in LCR was about 35% higher ($p<0.05$) than HCR (Table 3.B; 38).

Pdgfa gene expression, after 2 weeks of treadmill exercise followed by 14 days of Lt femoral artery ligation, was higher in HCR than LCR in the Rt leg without reaching a significant difference ($p>0.05$) (Table 3.B; 10). However, in the Lt leg, the Pdgfa expression increased significantly, by 24%, as compared with LCR (Table 3.B; 23). The Lt/Rt ratio was also higher in HCR than LCR, but was not significant ($p=0.2$) (Table 3.B; 36). Pdgfrb gene expression in the Rt limb showed a 40% significant increase in HCR than LCR (Table 3.B; 9).

Unlike Pdgfa, Pdgfrb expression in the Lt leg was not significantly ($p>0.05$) higher in LCR than HCR, with HCR showing a reduction in its Pdgfrb gene expression (Table 3.B; 22). When we took a look at how much the percentage change differed between the phenotypes, we found a 62% ($P<0.05$) increased change in the Lt leg/Rt leg of LCR as compared with HCR (Table 3.B; 35). Ccl2 gene expression did not change much between the phenotypes, either in Rt (Table 3.B; 8), Lt (Table 3.B; 21), or in the ratio of Lt/Rt (Table 3.B; 34).

Il6 gene expression was 52% higher in the Rt leg of HCR than LCR, but was not statistically significant ($p=0.1$) (Table 3.B; 13). Additionally, the Lt legs between the phenotypes showed almost similar level of Il6 expression (Table 3.B; 26) and the Lt/Rt ratio was non-significantly ($P=0.2$) ~43% higher in LCR than HCR (Table 3.B; 39).

Table 3.A. Gene Expression in Gastrocnemius Muscle of Non-Exercised HCR and LCR Rats Following Two Weeks of Abrupt Left Femoral Artery Ligation

No.	Genes	LCR	HCR
Non-Ischemic Gastrocnemius Muscle (right leg (r))			
1	Vegfa	0.38± 0.02*	0.20 ± 0.04
2	Flt1	0.02 ± 0.003	0.03 ± 0.01
3	Kdr	0.04 ± 0.004	0.03 ± 0.01
4	Hif1a	0.02 ± 0.003	0.03 ± 0.01
5	Il6	$8.8x10^{-5} \pm 1.5x10^{-5}$	$8.6x10^{-5} \pm 1.6x10^{-5}$
6	Nfkb	0.02 ± 0.001	0.03 ± 0.002
7	Angpt1	0.01 ± 0.001	0.009 ± 0.001
8	Angpt2	0.01 ± 0.001*	0.002 ± 0.001
9	Ccl2	0.003 ± 0.0005	0.004 ± 0.004
10	Vcam	0.0033 ± 0.001	0.003 ± 0.001
11	Icam	0.01 ± 0.001	0.005 ± 0.001
12	Pdgfrb	0.038 ± 0.003	0.043 ± 0.004
13	Pdgfa	0.02 ± 0.001	0.023 ± 0.002
Ischemic Gastrocnemius Muscle (Left leg (L))			
14	Vegfa	0.383 ± 0.1	0.20 ± 0.1
15	Flt1	0.03 ± 0.01	0.02 ± 0.01
16	Kdr	0.043 ± 0.01	0.021 ± 0.01
17	Hif1a	0.03 ± 0.01	0.02 ± 0.01
18	Il6	$0.0001 \pm 3.7x10^{-5}$	$9.9x10^{-5} \pm 3x10^{-5}$
19	Nfkb	0.02 ± 0.006	0.02 ± 0.003
20	Angpt1	0.013 ± 0.005	0.004 ± 0.001
21	Angpt2	0.01 ± 0.002	0.004 ± 0.001
22	Ccl2	0.007 ± 0.004	0.009 ± 0.001
23	Vcam	0.05 ± 0.001	0.004 ± 0.001
24	Icam	0.02 ± 0.002	0.004 ± 0.001
25	Pdgfrb	0.032± 0.011	0.036 ± 0.006
26	Pdgfa	0.23 ± 0.003	0.025 ± 0.003
Ratio of Ischemic Over Non-Ischemic Muscle (L/r)			
27	Vegfa	1.03 ± 0.2	1.05 ± 0.3
28	Flt1	1.13 ± 0.2	0.8 ± 0.3
29	Kdr	1.2 ± 0.3	0.8 ± 0.2
30	Hif1a	1.13 ± 0.2	0.8 ± 0.3
31	Il6	1.5 ± 0.3	1.2 ± 0.2
32	Nfkb	1.2 ± 0.5	0.8 ± 0.13
33	Angpt1	1.9 ± 1.05	1.1 ± 0.2
34	Angpt2	1.2 ± 0.43	1.4 ± 0.5
35	Ccl2	2.45 ± 1.13	0.91 ± 0.11
36	Vcam	1.45 ± 0.4	1.1 ± 0.2
37	Icam	1.004 ± 0.3	0.84 ± 0.1
38	Pdgfrb	0.764 ± 0.233	0.774 ± 0.078
39	Pdgfa	0.97 ± 0.2	1.03 ± 0.1

*Significant differences between phenotypes at corresponding side.

Table 3.B Gene Expression in Gastrocnemius Muscle Following Exercise preconditioning For Left Femoral Artery Ligation in HCR and LCR Rats.

No.	Genes	LCR	HCR
Exercised-Non-Ischemic Gastrocnemius Muscle (Right Leg (r))			
1	Vegfa	0.29± 0.032	0.25 ± 0.035
2	Flt1	0.037 ± 0.008	0.034 ± 0.008
3	Kdr	0.038 ± 0.004	0.0503 ± 0.009
4	Hif1a	0.021 ± 0.002	0.023 ± 0.004
5	Nfkb	0.023 ± 0.001	0.028 ± 0.003
6	Angpt1	0.014 ± 0.002	0.013 ± 0.002
7	Angpt2	0.008 ± 0.001*	0.004 ± 0.0005
8	Ccl2	0.004 ± 0.001	0.004 ± 0.002
9	Pdgfrb	0.038 ± 0.007	0.063 ± 0.007*
10	Pdgfa	0.019 ± 0.001	0.021 ± 0.002
11	Vcam	$0.0001 \pm 2.4 \times 10^{-5}$	0.003 ± 0.001
12	Icam	0.003 ± 0.0003	0.002 ± 0.0004
13	Il6	$0.0001 \pm 2.4 \times 10^{-5}$	$0.0002 \pm 6.6 \times 10^{-5}$
Exercised-Ischemic Gastrocnemius Muscle (Left Leg (L))			
14	Vegfa	0.328 ± 0.046*	0.148 ± 0.024
15	Flt1	0.033 ± 0.006*	0.017 ± 0.003
16	Kdr	0.052 ± 0.013	0.023 ± 0.003
17	Hif1a	0.037 ± 0.008	0.0199 ± 0.004
18	Nfkb	0.026 ± 0.003	0.024 ± 0.001
19	Angpt1	0.017 ± 0.002	0.012 ± 0.002
20	Angpt2	0.009 ± 0.002	0.004 ± 0.001
21	Ccl2	0.002 ± 0.0004	0.001 ± 0.0004
22	Pdgfrb	0.051 ± 0.007	0.04 ± 0.009
23	Pdgfa	0.018 ± 0.001	0.024 ± 0.002
24	Vcam	0.006 ± 0.001*	0.002 ± 0.0004
25	Icam	0.003 ± 0.001	0.002 ± 0.0004
26	Il6	$0.0001 \pm 4.5 \times 10^{-5}$	$0.0001 \pm 4.7 \times 10^{-5}$
Ratio of Ex-Ischemic Over Ex-Non-Ischemic Muscle (L/r)			
27	Vegfa	1.149 ± 0.106*	0.594 ± 0.048
28	Flt1	1.319 ± 0.332	0.6481 ± 0.14
29	Kdr	1.396 ± 0.338*	0.507 ± 0.082
30	Hif1a	1.767 ± 0.367	1.001 ± 0.224
31	Nfkb	1.143 ± 0.106	0.888 ± 0.077
32	Angpt1	1.42 ± 0.279	1.01 ± 0.303
33	Angpt2	1.185 ± 0.222	1.174 ± 0.231
34	Ccl2	0.459 ± 0.37	0.478 ± 0.074
35	Pdgfrb	1.711 ± 0.31*	0.644 ± 0.095
36	Pdgfa	1.03 ± 0.104	1.237 ± 0.111
37	Vcam	1.923 ± 0.411*	0.657 ± 0.119
38	Icam	1.398 ± 0.196*	0.917 ± 0.09
39	Il6	1.484 ± 0.398	0.848 ± 0.335

*Significant differences between phenotypes at corresponding side.

DISCUSSION

Peripheral arterial disease (PAD) is an ischemic injury of the lower limbs which commonly causes intermittent claudication. It affects patient mobility and quality of life, leading to a sedentary status. This advances the deterioration of the patient's condition both locally, by developing critical limb ischemia and increasing the likelihood of amputation, and systemically, by increasing the risk of developing coronary artery and cerebrovascular atherosclerosis. Preventing PAD by a simple, safe, and cheap modality such as exercise will prolong a good quality of life by promoting systemic and local skeletal muscle health. It is well established that increased levels of physical activity, exercise, and fitness decrease cardiovascular mortality (21-22) by modifying the expression of genes that are linked to vascular health, function, and structure (167). Some of the health benefits and adaptations of aerobic exercise on skeletal muscle morphology and exercise performance include increased capillarization, increased mitochondrial density (proteins, oxidation, and function), increased aerobic and anaerobic capacities, and improved neural adaptations (48). The model of intrinsic aerobic capacity will help to better understand which phenotype will better adapt to lower limb ischemia following 2 weeks of gradually increased mild intensity treadmill exercise. This model infers there is genetic susceptibility to the effect of active exercise pre-ligation on vascular remodeling in response to femoral arterial ligation. This model also helps to understand the benefit of the exercise in preventing PAD in the elderly who have higher risk to develop PAD and may not be willing to perform exercise therapy after developing PAD due to the severity of the condition and the presence of comorbidities.

The goal of this study was to determine how the vascular adaptive response between rats bred for low intrinsic aerobic capacity and high intrinsic aerobic capacity are different through the use of arterial ligation following 2 weeks of mild intensity graded treadmill exercise. The results of this study showed that rats with low endurance running capacity phenotype (LCR) have a better vascular adaptive response to ligation following mild intensity treadmill exercise in a model of peripheral arterial obstructive disease. The results also provide a distinct corroboration that the high endurance running capacity phenotype has an unchanged vascular adaptive response to ligation following the type and intensity of this exercise. This can be explained due to the low exercise threshold received by the rats, as their system is usually stimulated and adapted to higher intensity exercise than what they received in this study.

Differential Changes After Two Weeks of Ligation Without Exercise

The muscle-capillary interaction following permanent arterial ligation was obviously distinct between the phenotypes. LCR rats' muscle-capillary interaction was altered by the arterial ligation. The number of capillaries attached per muscle fiber (NCAF) and share factor (SF) prominently decreased in the LCR phenotype, whereas in HCR rats these parameters were unchanged from the non-ischemic leg by acute arterial ligation (figure 3.1C and D). The mean muscle fiber cross-sectional area was quite low in LCR but was increased in HCR. The changes in mean muscle fiber area indicate the severity of ischemic muscle injury in LCR than HCR. The capillary density changes were negatively correlated to muscle fiber area. Therefore, CD was increased in LCR rats due to the reduction in their muscle fiber area while CD was

lower in HCR rats the increase in their muscle fiber area. These results indicate that the vascular response of LCR rats is less primed to adapt to the arterial occlusion, while HCR rats have an underlying intrinsic resistant mechanism against the arterial ligation. One possible mechanism behind the differential adaptation to ischemia is that HCR mean muscle fiber area is genetically and significantly lower than LCR rats (figure 3.2E). This inherent difference is crucial both in maintaining the close muscle-capillary relationship and in resisting the obstruction in blood flow. Our linear regression data, which is consistent with others (191, 205), shows that mean muscle cross-sectional area was negatively, and significantly, correlated with CD (figure 2.6). It suggests that having a smaller muscle cross-sectional area, prior to the development of acute arterial occlusion, might protect against mild injury of ischemic muscle through reducing diffusion distance or surface area between capillary and myofiber. These results are reliable with our previous data (unpublished) and others who also found that the cross-sectional area of HCR muscle fiber was 35% smaller than LCR (88), and corresponds with those who found that lower muscle cross-sectional area in HCR rats was due to higher intrinsic capillary density (87). In this study, only mean muscle cross-sectional area was significantly different in the non-ischemic leg between the phenotypes. The capillary density was higher, but did not reach statistical difference in HCR as compared with LCR, which is possibly due to the low sample size (figure 3.2A). However, our previous study (unpublished data) and others (89) found a significant greater CD in the control leg of HCR than LCR. The significant decrease in mean muscle cross-sectional area in LCR as compared to HCR after acute arterial ligation indicates that the injury was more severe in LCR, and leads us to infer

that their physical activity was significantly affected. This is reliable with a human study that found resistance training at a low intensity leads to an increase in muscle size and strength when combined with blood flow restriction, and that without muscle training hypertrophic promoting factors, muscle cannot be hypertrophied (139). Although, HCR rats in this trial did not receive exercise training except for their spontaneous cage activity, which may resemble mild aerobic exercise in its effect. In LCR rats, mean muscle fiber area significantly decreased after arterial ligation with a related increase in CD. This suggests there is a different vascular adaptive response to ischemia by increasing capillary surface area with an increase in muscle atrophy. The possible mechanism is as follows: through decreasing the physical activity of the ischemic limb and shifting dependence on the other limb, a state of intermittent claudication is presented to compensate for the low blood supply to the ischemic tissue, therefore controlling muscle necrosis.

LCR rats at this time point did not show a significant increase in any angiogenic or inflammatory genes expression except for an increase in Vegfa mRNA expression. This indicates that LCR rats respond to two weeks of arterial ligation through initial muscle fiber atrophy and expression of Vegfa. Additionally, another possible late response to arterial ligation is initiation of angiogenesis, since the number of capillaries did not increase in this period of time, confirming that either vascular sprouting will take place later on to present a closer muscle-capillary relationship or the process might be attenuated intrinsically.

However, HCR rats did not show a significant change in any angiogenic or inflammatory gene expression. This suggests that HCR rats resisted the arterial ligation

injury by possibly depending on intrinsic collateral circulation that already conducted blood flow under normal circumstances without exercise, and that the influence of the share stress stimulus was below the threshold or needed a stronger stimulus to propagate its response. The overall observation from these results clearly showed there are differential baseline changes in the muscle-capillary relationship that are responsible for the differential musculovascular adaptive responses between the phenotypes, at least under the influence of arterial ligation.

Differential Changes in Exercise Pre-Ligation

LCR and HCR intrinsically differ in their muscle-capillary interference, which is regulated by an intrinsic aerobic capacity phenotype that reversibly interacts with systemic and local metabolism. For example, skeletal muscle mass, CD, and fiber composition have been found to play roles in the pathogenesis of insulin resistance (97). Our study is a unique, in that we found the skeletal muscle-capillary relationship was strengthened by exercise pre-ligation in LCR rats. If LCR skeletal muscle is not inherently strong to face unilateral arterial ligation, exercise pre-ligation modality significantly improved it genetically and morphologically while this relationship in HCR rats remained steady.

Muscle-capillary relationship in the LCR Rt leg of exercise pre-ligation showed a deterioration in some parameters. Mean cross-sectional area, number of capillaries attached per fiber (NCAF), and share factor (SF) were deteriorated in LCR Rt leg as compared with HCR Rt leg. The possible reason why these parameters were negatively affected in exercise pre-ligation is likely due to its low intrinsic aerobic capacity

phenotype. When the muscle that intrinsically has low aerobic capacity begins to exercise for two weeks, this leads to physiological changes in the muscle, such as increased myofiber size or hypertrophy and increased capillary number as an adaptive response.

However, when that bout of exercise is followed by two weeks of a sedentary state, the hind limb muscle will atrophy at a rate more rapid than the process of hypertrophy, and capillaries will be degenerated (Guyton physiology). This might suggest that when a muscle remains unused for two weeks after bouts of exercise, the muscle will no longer be able to preserve the physiological adaptation. In other words, the effect of long-term exercise on the muscle-capillary interface is less than fourteen days. Lennon et al. reported that exercise-induced cardio-protection against myocardial stunning persisted for 9 days, but was absent 18 days after the cessation of exercise training (125). These parameter changes (decreased mean cross-sectional area, number of capillaries attached per fiber (NCAF), share factor (SF), and CFPE) were similar to what have been seen in the ischemic limb after ligation without exercise in the same LCR phenotype.

Although the causes are different, the results are the same. That is until we combined the two factors together (i.e. exercising the animals for two weeks and followed by ligation for another two weeks) and found a favorable effect on these parameters in the left leg: increased mean cross-sectional area, number of capillaries attached per fiber (NCAF), share factor (SF), and CFPE). This data is similar to the study that showed when exercise was followed by intermittent occlusion of the lower limb, muscle function and structure improved; with a difference between this study and ours in that we used a permanent occlusion instead of intermittent (172). This suggests that not only intermittent occlusion can be preconditioned against muscle damage through the use

of exercise, but that permanent occlusion can also strengthen the muscle structure if preceded by tread mill exercise in low intrinsic aerobic runner capacity phenotype.

In HCR Rt leg, exercise pre-ligation caused increases in the mean muscle cross-sectional area, NCAF, and SF with a slight increase in CFPE. It is not surprising that CD decreased, as it is negatively correlated with mean muscle cross-sectional area (191, 205). These same parameters were oppositely higher in HCR Rt leg than LCR Rt leg, suggesting that the high aerobic capacity phenotype is well-primed to adapt to exercise as compared with LCR phenotype. However, when we exercised HCR rats for two weeks before the unilateral ligation of the Lt femoral artery, we found no effect on the muscle-capillary interaction, as the Lt (ischemic) over the Rt (non-ischemic) ratio of almost all parameters have a ratio ~ 1. These results infer that exercise pre-ligation in HCR phenotype is not as significant as in the LCR phenotype; the possibility of this being due to the effect of mild ischemia is negligible in front of primed intrinsic collaterals that already preconditioned the muscle against ischemia or the sedentary effect during the period of ischemia. Our previous study showed that when HCR rats started treadmill exercise three days post Lt femoral artery ligation, the parameters were favorably increased, creating a close muscle-capillary relationship. However, these same parameters were deteriorated in LCR.

Angiogenic gene expression supports the relationship between muscle and its capillarity and can be observed histologically in the LCR. In the LCR Rt leg, Vegfa expression decreased by 25% as a result of exercise (was 0.38 in the non-ligated non-exercised limb and became 0.29 in exercised non-ligated limb). This change is consistent with the low mean muscle cross-sectional area and low NCAF previously shown. LCR

still had higher Vegfa expression than HCR in the Rt limb, although it slightly decreased in HCR Rt leg from the baseline control leg. The Lt leg of LCR had increased expression of Vegfa under the effect of exercise and ligation, while HCR Lt leg showed further reduction of Vegfa expression. Although the difference between HCR and LCR was not significant, the trend was near significant ($p=0.1$). The ratio of Lt/Rt was 50% higher in LCR than HCR. These results suggest that exercise pre-ligation has an influence on Vegfa expression; more so than the effect of ligation alone in these phenotypes. They also suggest that exercise pre-ligation acts as a synergistic single factor for increasing Vegfa expression in LCR rats only.

The Flt1 and Kdr expressions were 50% higher in the LCR Lt leg than HCR, which is consistent with the vegfa expression in each phenotype. This data shows that Vegfa and its receptors are phenotypically expressed during exercise preconditioning and ischemia, with higher levels present in LCR than HCR. This is consistent with histological data which indicates ongoing angiogenesis more in LCR than HCR.

Similar to Vegfa expression, Angpt2 levels were higher in LCR Rt leg than HCR under the influence of exercise preconditioning. A further increase was observed with a comparable percentage change in both phenotypes in Lt/Rt ratio under the influence of both exercise preconditioning and ischemia. Unlike Angpt2, Angpt1and Hif1a were only elevated in LCR Lt leg under the influence of exercise pre-ligation and there was no effect of exercise pre-ligation on Angpt1 and Hif1a in HCR. Additionally, there was no phenotypic differential response in its expression under exercise preconditioning alone. Presence of phenotypic differential responses in Angpt2 expression (higher in LCR than HCR), either under the influence of exercise preconditioning alone or under the influence

of exercise pre-ligation with a comparable percentage change between the phenotypes, suggests there is a different mechanism regulating baseline Angpt2 levels and therefore, different angiogenic mechanisms between HCR and LCR.

Nfkb was oppositely expressed between the phenotypes. Expression of Nfkb, under the influence of exercise preconditioning, was higher in HCR Rt leg than LCR, while after exercise pre-ligation (Lt leg), Nfkb expression was increased in the LCR phenotype and decreased in the HCR phenotype. However, this was not a significant change between the phenotypes, suggesting there is no phenotypic difference in Nfkb expression under the effect of exercise pre-ligation. The increase in Nfkb expression in each phenotype was not associated with muscle atrophy since the results from histology showed an increase in the mean muscle fiber area, indicating that the role of Nfkb in this model is toward regulating inflammatory, cell survival, and proliferation responses (158). Ccl2/Mcp1 is the downstream target of Nfkb signaling (43) and its expression mirrored that of Nfkb expression, in that there was an increased expression in the hypertrophic muscle of Rt HCR due to exercise and Lt LCR due to exercise pre-ligation.

This data is complementary with the possible role of Nfkb in skeletal muscle under physiological stress alone, or both physiological and pathological stress combined. Il-6 is also affected down-stream of Nfkb signaling. The level of Il-6 expression was increased in both HCR Rt leg, where there was an increase in mean muscle fiber area due to exercise, and in LCR Lt limb, due to the combination of exercise pre-ligation; although its level was lower than its HCR counterpart. Expression of Nfkb also lead to the expression of adhesion molecules. Our data

showed an increase in Vcam and Icam expressions in the Lt leg of LCR as compared with HCR under the influence of exercise pre-ligation. There was no differential response in these genes under the influence of exercise between the phenotypes. This data suggests that low intrinsic aerobic capacity phenotype has a higher risk of endothelial dysfunction, with a possibility of increased risk of atherosclerosis and atherothrombosis, and that exercise pre-ligation will not protect from endothelial dysfunction. Furthermore, this data is comparable to what has been reported regarding the concept that atherosclerosis is a diffuse disease that increases the likelihood of PAD patients developing stroke and acute myocardial infarction (56), although we looked only below the level of ligation (hind limb). Nfkb has also been reported to possibly participate in the expression of Pdgfa-inducible genes (170).

Pdgfa showed higher gene expression in HCR but showed no change in LCR, likely due to the effect of exercise pre-conditioning or ischemia following the exercise. On the other hand, Pdgfrb expression decreased in HCR and increased in LCR in exercise pre-ligation leg. This may indicate that HCR rats are more reliant on Pdgfa than Pdgfb, since Pdgfa only binds to Pdgfra, while LCR rats are more reliant on Pdgfb/pdgfd than Pdgfa, since Pdgfb/pdgfd have high affinities for Pdgfrb (23). Pdgfa plays an important role in myocyte formation and differentiation. Histologically, mean muscle fiber size was consistent with Pdgfa expression in HCR. In the HCR Rt leg (under the effect of exercise without ligation) and Lt leg (under the effect of exercise followed by ligation), the muscle fiber hypertrophied and Pdgfa expression was elevated. However, this mechanism seems phenotypically dependent. In LCR Rt leg, there was muscle atrophy with a decrease in Pdgfrb expression and no

change in Pdgfa expression. Similarly, when there was muscle hypertrophy in the Lt leg of LCR, following exercise pre-ligation, Pdgfrb expression increased while Pdgfa expression did not change, indicating different myogenesis mechanism between the phenotypes in response to exercise and ischemic stress. It is important to understand that each case in PAD needs to be treated separately from other cases. This data will add more to our current knowledge of PAD and help in sorting PAD patients into LCR and HCR phenotypes. Additionally, this data shows that rats of LCR and HCR phenotypes are convenient models to conduct further experiments and generate information that may be successfully translated to clinical applications.

Exercise preconditioning (EXP) activates cellular stress to protect against oxidative, mechanical, and metabolic stressors that might lead to pathological effects (119). EXP has been used clinically to protect against ischemia-reperfusion injury, type 2 diabetes, and muscle atrophy (42, 46, 54). EXP has been associated with an increase in capillary density (CD), leading to low stroke damage in ischemic rats (41). It has also been shown to prevent acute myocardial infarction and AMI-induced acute cardiac injury (216). EXP also has been found to decrease neonatal incision surgery-induced enhanced hyperalgesia (65). Moreover, EXP has been shown to prevent right ventricular diastolic dysfunction induced by monocrotaline-induced pulmonary arterial hypertension (168). In addition, EXP has been found to facilitate a decreased recovery time for patients, through improved functionality and a reduction in pain when performing activities of daily living, after joint surgery (182, 192, 214), and protects against acute doxorubicin cardiotoxicity for up to 10 days (94). Lastly, in a

rat model, EXP has been recorded to facilitate functional recovery from a localized injury to the spinal cord (27).

CONCLUSION

This study showed that two weeks of treadmill exercise can precondition hind limb occlusive disease in low intrinsic aerobic running capacity phenotype through remodeling the musculovascular adaptive response. This finding will help the elderly with high risks of developing PAD to be protective against the development of the disease and therefore, will decrease the clinical and economic burdens placed on PAD patients and the government, respectively.

CHAPTER 4: THE DIFFERENCIATIL IMPACT OF ANDROGEN AND DOPAMINE D3 RECEPTORS ON SKELETAL MUSCLE SATELLITE CELL PROLIFERATION

Abstract:

Peripheral arterial disease among the female is detrimental than in the male population. Estrogen, androgen and dopamine receptors have been reported as participating in the cardiovascular disease protection. High intrinsic aerobic capacity rats are protected against cardiovascular risk factors. We studied, *in vivo*, hind limb ischemia to elucidate the differential association and function of AR, ERα, and D3R between HCR and LCR in response to ischemia. We also explored *in vitro*, how the phenotypes differentially affect AR/D3R signaling interaction on satellite cell growth at early and late time points. HCR and LCR, Female rats, 40 weeks, n = 16 each, and dopamine treated with unilateral femoral artery ligation. Skeletal muscle was harvested from both hind limbs. We also used D_3KO female mice (B6.129S4-*DrD3*$_{tm1Dac}$/J) and their associated wild types (C57BL/6 mice), 22 weeks, n= 6. Samples were assessed in rats and mice by immunofluorescent for AR, ERα, and D3R protein expressions, and in rats for AR/ERα gene expression ratio. We found there is an increase AR/Eα gene expression ratio in HCR ischemic and non-ischemic limb as compared to LCR (P=0.005). However, protein expressions were not significant in the rats and mice. *In vitro*, we found that that LCR satellite cells have different AR/D3R signaling interaction between early growth and late growth time points while HCR cells have similar receptors interactions between early and late time points, which involved activation of both receptors signaling pathways.

INTRODUCTION

We mentioned in chapters 1, 2, and 3 the evidence regarding the tremendous effect of aerobic phenotype on angio-inflammatory gene expressions and skeletal muscle-capillary relationship. Here, we will provide additional evidence about the role of estrogen, androgen, and dopamine in vascular function and disease, as well as the biological characteristics of HCR and LCR that differentiate them from each other, suggesting a potential presence of neuroendocrine differences between these phenotypes.

HCR rats are characterized as having higher maximum oxygen uptake and resting metabolic rate, a leaner body mass, increased longevity, and are resistant to developing dyslipidemia, peripheral insulin resistance, diet induced-liver steatosis, depression, and metabolic syndrome in comparison with LCR. These differences are possibly due to a different underlying neuroendocrine profile between these phenotypes.

Several studies on testosterone prove the relationship between these biological characters and testosterone levels in human and animals. Studies in humans showed that men who had prostatic cancer and who were under androgen deprivation therapy for more than 3 months developed lower VO2max and lower resting metabolic rate compared to the group that received the therapy for less than three months (228).

Moreover, studies have shown that testosterone decreases visceral fat mass (143), improves insulin resistance (143), and increases lean body mass (144). Furthermore, studies have revealed that long-term testosterone replacement therapy in men with late onset hypogonadism have decreased obesity parameters and improved metabolic syndrome and health-related quality of life (143). Testosterone replacement therapy for hypogonadal and aging men caused decreases in total cholesterol and LDL

levels and no change in HDL (250). Testosterone has also been linked with depression in old men. A low free testosterone level in elderly men over 70 years of age was associated with a higher prevalence of depression (3). Hormonal replacement therapy has been found to increase life expectancy in both genders. Testosterone replacement therapy for men with late onset hypogonadism increased the survival rate by 9-10% in 5 years, which is similar to that of eugonadal men with normal endogenous testosterone level.

Likewise, menopausal women who have undergone estrogen replacement therapy have an increased survival rate of 2.6% within 5 years (32). Fatty liver is also associated with low testosterone concentration in both human and animal studies. In 495 men diagnosed with non-alcoholic fatty liver disease, 251 of them had low serum testosterone levels with the absence of an alternative cause (76). In animal studies, mice with a liver-specific androgen receptor deletion have higher liver steatosis than obese controls (134).

Clinical and basic experimental studies have provided evidence about the atheroprotective effect of estrogen, testosterone, and dopamine on blood vessels. While studies regarding the role of androgen on vasculature have been controversial for a long time, in recent years a great work has suggested that androgens exert protective effects against the development of atherosclerosis in both genders, and in both humans and animals. Although the effect of testosterone on cardiovascular events and mortality in women has not yet been evaluated in large cohort studies, a prospective cohort study that followed up on more than 2000 women after more than a 4-year period found that women with the lowest levels of testosterone had the

greatest risk of developing a cardiovascular event, independent of traditional risk factors (68). Studies on the hind limb occlusive model in animals found that androgen receptors are essential for the revascularization response in ischemia in both male and female mice (103). Moreover, the hormone testosterone has also been found to increase numerical density of capillaries in the left ventricle after injecting aging male rats with testosterone for 4 months (64). The mechanisms underlying the associations of low testosterone with CVD are complex and poorly understood.

However, estradiol and dihydrotestosterone administration in gonadectomized male mice have been reported to increase angiogenesis through increasing vascular branching and diameter, respectively (213). CVD incidence among women is low before menopause, but progressively elevates after the beginning of menopause. This increase is thought to result partially from the loss of endogenous estrogen (149). However, androgens also decrease with respect to increasing age (35). Epidemiological studies provided strong evidence that hormone replacement therapy provides cardioprotection in postmenopausal women (100), while other studies do not (91). It has been suggested that the inconsistency in these results is due to the diversity of responses to hormonal replacement therapy. LCR and HCR rats are generated from heterogeneous N:NIH founder population. Whether AR/ERα ratio in gastrocnemius muscle is differentially associated with acute hind limb ischemia between the female HCR and LCR rats, and whether AR differentially regulates skeletal muscle repair during ischemia has yet to be studied.

Sex steroid receptors commonly function as transcription initiators of genes, followed by the subsequent synthesis of several proteins, so as to regulate cell

function during physiological and pathological responses. In regard to the synthesis of dopamine, the rate limiting enzyme is tyrosine hydroxylase. A study has explored the androgen responsive element in the tyrosine hydroxylase region, base pairs 1562 to 1328, upstream of the promotor (98). Studies have shown that testosterone treatment increased or maintained tyrosine hydroxylase activity, which decreases after castration (1). HCR and LCR also differ in their dopamine systems in the brain. A study, using various brain areas involved in movement, motivation, and fatigue in HCR and LCR, found that HCR have prolonged dopamine availability compared to LCR. They found that D2R (and not D1R) in HCR was higher than in LCR. This data directs toward exploring additional determinants regarding the intrinsic differences in endurance capacity, and especially toward exploring the role of dopamine, peripherally, at the level of skeletal muscle.

In peripheral arterial ligation, dopamine was found to play a role in remodeling the vasculature. D2R was found to prolong the post-ischemia recovery. In a mouse model, D2 knockout mice showed a faster recovery rate and stimulation of angiogenesis in response to unilateral hind limb ischemia compared to wild-type (194). D3R was also found to play a role in ischemia. Its activation was found to improve the neurological function, learning, and memory of rats after global cerebral ischemia/reperfusion (229). This data is important in order to find whether the HCR phenotype also has a higher dopamine D3 receptor expression at the level of the skeletal muscle than LCR, and if that difference is responsible for skeletal muscle repair after ischemia.

MATERIALS AND METHODS

Animal Strains and Experimental Groups

HCR and LCR, female rats, 40 weeks, n= 16 each, generation 32. Eight HCR and eight LCR rats were received left femoral artery ligation while right limb served as internal control and at day 14 animals were sacrificed. These animals were obtained originally from Drs. Lauren Koch and Steven Britton at the University of Michigan. They have previously described the artificial selection used for the generation of the HCR and LCR strains (10, 111-112). Briefly, each sex was selected from the founder population (N: NIH stock). Two-way artificial selective breeding was used to create low capacity runner (LCR) and high capacity runner (HCR) strains that were divergent for treadmill running capacity (run time until exhaustion on a graded treadmill exercise test). The 13 lowest and 13 highest running capacity rats of each sex were randomly paired for mating. This pattern was repeated over subsequent generations to produce the divergent strains using a rotational breeding scheme. In the present study, animals from generation 32 were used. All the animals were exposed to treadmill exercise for 5 days at 11 weeks of age when the animals were phenotyped for treadmill running capacity. Upon verifying the phenotypes of the animals, animals were prepared for shipping at 14 weeks of age, or as soon after as weather conditions (airport tarmac temperatures < 85°F) permitted.

Once received by the Department of Comparative Medicine at ECU, the animals were maintained under mandatory quarantine for 10 weeks before they were released for study. Rats were provided standard rat chow and water *ad libitum*, and

were kept on a 12 h light/ 12 h dark time schedule until sacrifice. We also used D_3KO female mice (B6.129S4-*DrD3*$_{tm1Dac}$/J; kind gift from Dr. Clemens Stefan) and their associated wild types (C57BL/6 mice), 22 weeks, n= 3 each were anesthetized by inhalation of isoflurane in a sealed jar and decapitated. Animal procedures were conducted in accordance with American Physiological Society guidelines for the humane and safe use of animals, and all protocols involving animals used for these experiments were approved by the East Carolina University Animal Care and Use Committee.

Hind Limb Femoral Artery Occlusion. Femoral artery occlusion was produced as outlined by others (135, 136, 184). Briefly, rats were anesthetized with ketamine xylazine (0.1ml/100g body weight ip). The fur was shaved from the inner thigh and the surgical sight was cleaned with Betadine and 70% alcohol. Utilizing a small incision, the left femoral artery was isolated and three ligatures of 6-0 surgical silk was placed to cause an ischemic condition in the downstream tissue. One ligature was placed 5-6mm distal to the inguinal ligament, a second was placed on a collateral artery, and the third was placed 5-6 mm distal to the first ligature. When all three ligatures were in place, the incision was closed and the animal was given a Buprenex injection (0.1ml/100g body weight IP) and placed in its cage to recover. Animals were allowed to wake up and have unlimited access to food and water. Animals were sacrificed at day 14 following placement of the ligature. This procedure produces a uniform occlusion of the femoral arteries that reduces blood flow reserve capacity to 10–20% of normal but remains sufficient to meet resting blood flow requirements (243, 244, 245).

Quantitative Distribution Immunofluorescence. We used standard protocol for dual labeling immunofluorescence in skeletal muscle as described elsewhere (55), but we used TBS instead of PBS. Briefly, right (Rt) and left (Lt) gastrocnemius muscles for each animal were excised and flash-frozen in isopropanol, which then was cooled in liquid nitrogen. The samples were stored at -80°C until cut. The muscle in the OCT were cut into serial transverse muscle sections (thickness 8um) were slice at -20°C using cryostat and immediately post-fixed in 4%paraformaldehyde (sc-281692; Santa Cruz Biotechnology Inc.) on super-frosted plus microscopic slides (VWR International, LLC, Suwanee, GA) and then blocked for 2 hours with 10% normal goat serum (ab 7481, Abcam) in Tris buffered saline containing 0.2 % Tween-20(TBST). After overnight incubation with the dual labeled primary antibody (recommended dilution for D1R and D3R is 1:260) or unlabeled primary antibody, which were based on concentration titration (recommended dilution for AR antibody 2µg/ml, and for ER antibody is 10ug/ml), the sections were washed 3X with TBS, then incubated with the secondary antibody (1:200 recommended by the company) for 2 hours and washed 1X with TBS containing 0.1 Triton-X. In conjugated primary antibodies, the second wash was skipped and coverslips were applied with vectashield mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA). The following primary antibodies were used; rabbit anti-AR antibody (sc-816; Santa Cruz Biotechnology Inc.), mouse anti-ER-alpha (MA1-310, Invitrogen). The myocyte specific mouse anti-α-Actin (sarcomeric) antibody (Sigma Aldrich, St Louis, MO) was used to verify that the sections analyzed were skeletal muscle cells. The

secondary antibodies were: FITC-conjugated goat anti-rabbit IgG (ab97079, Abcam) and cy3-conjugated goat anti-mouse (ab97035, Abcam). The conjugated primary antibodies used were: D1R antibody [Alexa Fluor 488] (NB110-60017AF488, Novus) and D3R polyclonal antibody, Cy5 conjugated (bs-1743R-cy5, Bioss). In indirect staining, control sections were incubated with only secondary antibodies to determine non-specific binding. A random sample of images across the skeletal muscle section were acquired at wavelength 639 and 488 nm, analyzed by laser-based confocal microscopy using relative reading of mean intensity per field area, keeping parameters constant throughout the acquisition process. Data were presented as mean intensity/field area.

RNA Isolation, Reverse Tanscriptase-PCR, and Real-Time PCR. Total RNA was extracted from harvested rat gastrocnemius muscle using TRIzol reagents (Invitrogen) and cDNA was generated using the High-Capacity cDNA Reverse Transcription kit from Applied Biosystems (Thermofisher) following the manufacturer's protocol. qReal-Time PCR was performed using 5̀ fluorescent labeled primers for AR and ERα utilizing TaqMan Fast Advanced Master Mix (Life Technology) following the manufacturer's protocol. Actinb was used as the reference gene since CT values for this gene did not change with treatment. The qReal-Time PCR detection system used was the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). LCR and HCR internal control Rt legs samples were used for all data to be normalized with treated samples of other legs using the ratio of $2^{-\Delta}$CT of treated sample/$2^{-\Delta}$CT of controlled sample method.

Isolation and Culture of Primary Satellite Skeletal Muscle Cells (SSC).

Dopamine and androgen receptors exist in many tissues, including rat skeletal muscle tissue. However, the physiological importance of interaction of dopamine with androgen receptors in the regulation of skeletal muscle satellite cell function in these phenotypes is unclear. In this study, isolation and purification of satellite cells (SSC) were modified from the previous description (34). Gastrocnemius muscle tissue from both legs were obtained from the two experimental groups D3KO and WT mice (20 weeks old) or HCR and LCR rats (30 weeks old) rinsed in pre-warmed DMEM (Dulbeco's Modefied Eagle Medium; high glucose, with 4500 mg/L glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate) supplemented with 1% antibiotics (15240-062, ThermoFisher) and 1% Na-pyruvate (11360070, ThermoFisher). In a petri dish containing DMEM and using dissecting forceps, connective and fat tissue was removed from the muscle. Muscle was then transferred into a 60-mm petri dish with DMEM and cut into $3mm^3$ fragments. Muscle suspension was then transferred into a 15-ml conical tube and centrifuged at low speed (~200xg) for 4 minutes. Supernatant were aspirated and discarded. DMEM were used to re-suspend fragments up to 2ml for muscles from one animal and the whole bulk was transferred into 35-mm. The remaining sticky fragments were washed with 700µl of DMEM and added to the dish. Next, 300 µl of 1% pronase was added to the dish so as to achieve 3ml of 0.1% pronase solution in mice, or 6ml in rats. Using the pronase enzyme instead of collagenase may inhibit survival and proliferation of non-myogenic cells and increase the purity of isolated satellite cell populations (34). The dish with the enzymatic digestion was incubated in the cell culture incubator for an hour with gentle mixing every 15-20 min.

At the end of the digestion period, the enzyme and muscle fragment solution was transferred to a 15 ml conical tube and centrifuged at 400 x g for 5 min. The supernatant was removed and the pellet was re-suspended in 5 ml of 10% FBS in pre-warmed DMEM instead of 10% horse serum because we found changing the serum type from horse serum to fetal bovine serum has a similar effect (44,147). After enzymatic digestion, mechanical trituration was used to release the still attached satellite cells. The muscle trituration occurred by passing the fragments (20 times) through a 10-ml glass pipette until the tissue passed easily through the tip of the pipette. Then muscle was allowed to settle at room temperature to separate large fragments from the supernatant. Separated supernatant was transferred in to a 15 ml tube. The process of trituration was repeated by adding 5 ml of 10% FBS into the tube contained the remaining large fragments using 9" cotton-plugged glass pipette until all pieces easily passed through it. Supernatant was added to the rest of the previously collected supernatant in the 15 ml tube.

To eliminate residual debris from the suspension, a 40-µm cell strainer in 50 ml conical tube was used for the collected supernatant and additional 1 ml volume of DMED was added for maximum cell recovery. Next, cell suspension was centrifuged at ~1000 x g for 10 min, the supernatant was discarded and the pellet was suspended in 1ml of standard growth media. After this, cells were purified before plating by performing the pre-plating technique, where cell suspension is distributed onto uncoated tissue culture plastic for a period of 15-30 minutes to further enrich the isolated myogenic cell population (63,186). Fibroblasts or epithelial cell present in the

muscle suspension rapidly adhere to the uncoated tissue culture plastic, and the satellite cells remaining in suspension (15, 190) can be collected and plated on the gelatin-coated 24-well plate. Gelatin is commonly used with satellite cells culture for optimal cell proliferation (44). Cells remained in the incubator undisturbed for 3 days. After that, the media was removed and the plate was washed two times with 1x DPBS and replaced with fresh complete media contained DMEM with 1% antibiotics, 1% chicken embryo extract (100356-958, VWR), and 20% Fetal Bovine Serum (10091-148, ThermoFisher).

Cell Identification and Purification. Cells after isolation process and before plating on coated tissue culture plastic, were purified by a simple technique, which is called pre-plating technique. In this technique, cells that in suspension where distributed onto uncoated tissue culture plastic for a period of 15-30 minutes to further enrich the isolated myogenic cell population (186, 63). Fibroblasts or epithelial cells present in the muscle suspension rapidly adhere to the uncoated tissue culture plastic, and the satellite cells remaining in suspension (15, 190) can be collected and plated on the gelatin-coated 24-well plate.

For identification of satellite cell markers we used fluorescent immunocytochemistry staining protocol to detect $Pax7^{+}$ marker and MyoD for satellite cells progeny as previously described with slight modification (34, 201). Briefly, square cover slips (18x18-1, ThermoFisher) were first disinfected by 75% ethanol and coated with 2% gelatin by setting them in gelatin-filled 35-petri dish and then allowing the slides to dry by placing them onto an irregular surface so as to avoid adhesion to the surface. Coated cover slips were then placed in each well of a 6-well

plate and kept in the cell culture hood under ultraviolet rays for one hour before plating cells. Cells were plated at a density of 2000-4000 cells per well and sat undisturbed overnight to allow for cell attachment to the bottom of the wells. Cells then were rinsed with serum free media three times. An equal volume of media and pre-warmed 4% paraformaldehyde (sc-281692; Santa Cruz Biotechnology Inc.) was added on the culture for 10 minutes at room temperature. Cultures were then rinsed three times with Tris-buffered saline (TBS; 0.05 M Tris, 0.15 M NaCl, pH 7.4) and permeabilized with 0.5% Triton X-100 in TBS containing 1% goat serum (ab 7481, Abcam) for blocking and kept at room temperature for 2 hours. Cultures were then rinsed three times with tris-buffer saline containing 0.5% Tween-20 (TBST) followed by incubation with primary antibodies for 2 hours at room temperature. Cells were then incubated with the primary labeled Alexa Fluor 405 and 467 antibodies (NBP 2-34706AF647, Novus) at dilution of 1:50, and primary labeled MyoD Alexa Fluor 467 at dilution of 1:50 (sc-377460 AF647, Santa Cruz) for 2 hours at room temperature, and washed 3X with TBST. Vectashield mounting medium with or without DAPI (Vector Laboratories, Inc., Burlingame, CA) was used to avoid masking the nuclear marker. Images were captured by confocal microscope at 20x magnification.

Cell Viability Evaluation. By using automated counter (Cellometer Bright Field Automated Cell Counter 2000, Nexcelom), the cell viability reagent kit, and the trypan blue dye exclusion assay (0.4%, Sigma).

Cell Culture Experiments. Performed using previously published protocol and materials used in satellite cells isolation from mouse gastrocnemius muscle with slight adjustment for rat muscle (34, 124,129). Cells from passage 1-2 were grown to 80%

confluency in T75 flasks with normal culture media (NCM) containing 20% FBS and 1% chicken embryo extract in regular standard cell culture incubator. After cells were grown to sub-confluence, they were diluted to the required volume of phenol red-free and serum-free DMEM containing 0.2 % FBS, and plated in monolayer at a density of less than 60,000 cells per well via 300 µl aliquots into 96-well (Falcon, Corning, NY USA) (pre-coated with 2% gelatin) in triplicate. Experiment was done in two separate replicates and the average was calculated. Before treatment, each plate was exposed to serum-free media for 2 hr to synchronize cells, followed by incubation in normal culture medium for 36-48 hr depend on the experimental type. We conducted experiments on Sprague Dawley and HCR/LCR rats for studying cell growth at different time points.

Cell Growth Test. Growth properties of skeletal muscle cell (SSC) were detected using an automated cell counter (Cellometer Bright Field Automated Cell Counter 2000, Nexcelom). The isolated cells were seeded into 96-well plate at density of less than 60,000 cell per well with 300 µl CCM. Cells were then incubated at 37 $^{\circ}$C with 5% CO2 for 12 hours, followed by incubation in serum free media for 2 hours. After 2 hours, the media was changed to normal complete media with drugs added as follow: first triplicate were cells without treatment, second triplicate were cells treated with D3R antagonist, 10 µM (93) SB 27701A (kind gift from Dr. Clemens Stefan) the third triplicate were cells treated with androgen, 10 nM 5α-Androstan-17β-ol-3-one, dihydrotestosterone DHT (A8380, Sigma) (142), the 4th triplicate were cells treated with androgen antagonist, 50 nM Flutamide (F9397, Sigma) (206), Flutamide, the 5th triplicate were cells treated with D3R agonist, 10 µM pramipexole (kind gift from Dr.

Stefan Clemens) + androgen, the 6th triplicate were cells treated with D3R antagonist + androgen antagonist, the seventh triplicate were cells treated with D3R antagonist + D3R agonist + androgen, the last triplicate were cells treated with D3R antagonist + androgen. This test was repeated four times in the same plate for four trials with four different time points after replacing the media with addition of drugs as follow: 6 hour, 12 hour, 24 hour, 36 hour and 48 hours where cells were trypsinized with 1x Trypsin-EDTA phenol red-free solution (15400054, ThermoFisher) and incubated for 2-3 minutes at 37 °C. Cells were then neutralized using similar volume of Trypsin Neutralizer Solution (1x) (R002100, ThermoFisher). An equal volume of the cell suspension and Trypan blue (15250061, ThermoFisher) were mixed and a Cellometer Bright Field Automated Cell Counters for accurate cell counting, total cell concentration, and trypan blue viability. Data are presented as (treated group/control x 100) in each group at 6, 12, 24, 36, and/or 48 hours.

Statistics. Statistical analysis was performed using data analysis software in Microsoft Excel worksheet (Windows 10). Data are expressed as means ± SEM, and a p value <0.05 was considered statistically significant. Single factor ANOVA tests were used to compare between LCR and HCR to determine statistical significance among the groups (two groups at a time) comparing three different ways: internal control data of LCR with the counterpart of HCR group, ischemic limb data of LCR with its counterpart of HCR, and finally the ratio of Lt ischemic/non-ischemic Rt limb (Lt/Rt) of LCR with the HCR counterpart. Similarly, relative gene expression levels were calculated by comparing q-RT PCR data, after normalizing them to the internal standard housekeeping gene (Actinb), of non-ischemic Rt limb of LCR with its

counterpart of HCR and comparing ischemic Lt limb and the Lt/Rt ratio of LCR with HCR counterpart groups as explained above. For cell culture groups, the statistical analysis was based on the comparison among the groups at the same time point using single factor ANOFVA and paired t-test to find if there was a statistical difference between two groups at a time. Significant differences were accepted in all circumstances where $p < 0.05$.

RESULTS

The Differential Changes in AR, ERα, AR/ERα Genes and Proteins Expression in The Calf Gastrocnemius Muscle 14 Days Following Ligation Between LCR and HCR:

The AR and ERα genes expression in the non-ischemic limb, ischemic limb, and the ratio of ischemic over non-ischemic limbs (L/r) did not change significantly ($P \geq 0.05$) between the phenotypes (Figure 4.1 A and B). However, the AR/ERα gene expression ratio was significantly higher (32% and 42%) in the HCR non-ischemic and ischemic limbs respectively than LCR groups ($P < 0.05$), but when we calculated this ratio in the ischemic limb/non-ischemic limb (L/r) between the phenotypes, the trend did not reach statistical difference ($P=0.3$) (Figure 4.1 C).

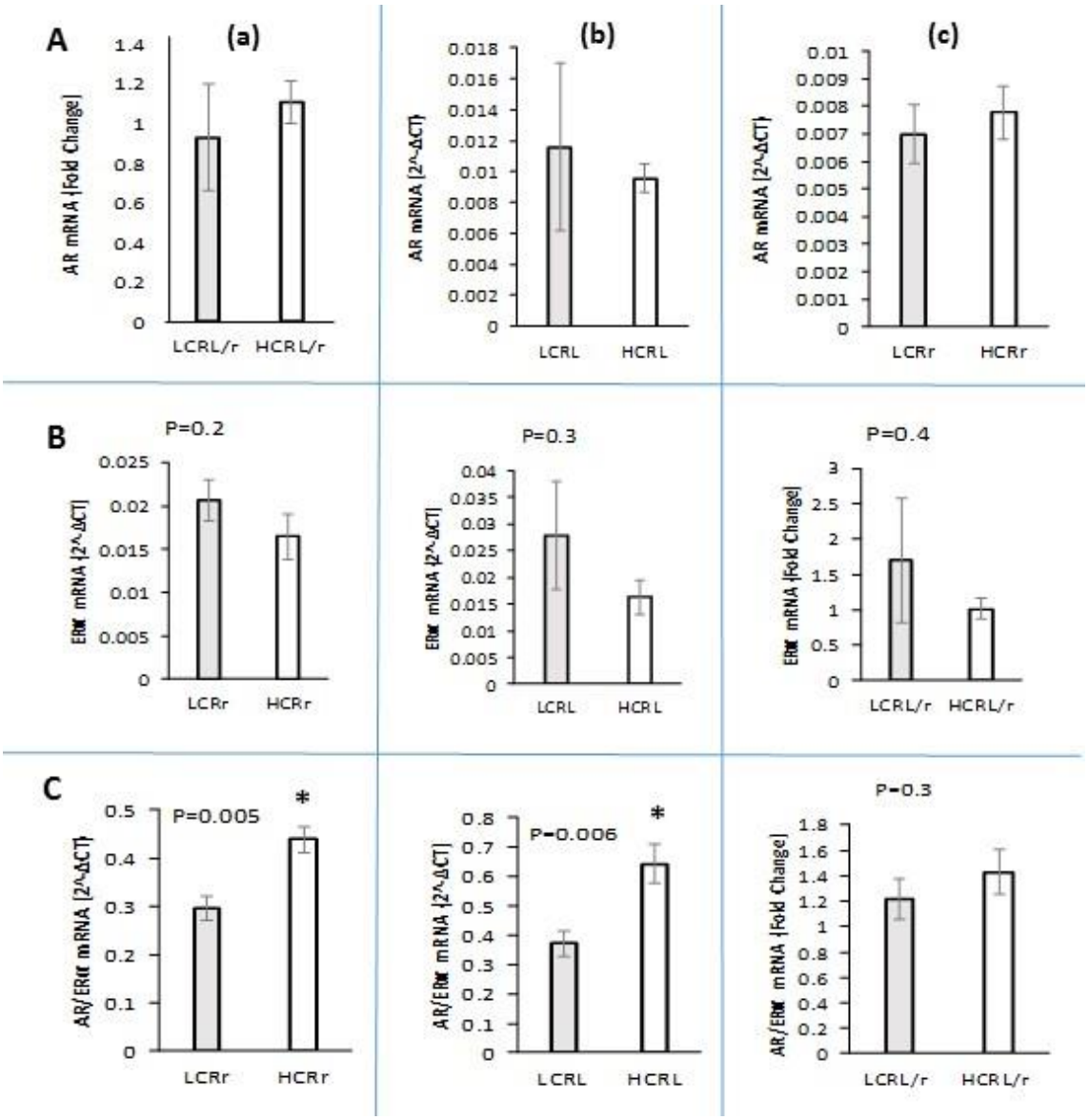

FIGURE 4.1. AR, ERα, and AR/ERα Gene expression ratio in Gastrocnemius muscle of non-ischemic, ischemic and the ratio of ischemic to non-ischemic limbs in HCR and LCR rats following two weeks of abrupt left femoral artery ligation. AR gene expression (A), ERα gene expression (B), and AR/ERα gene expression ratio (C) in non-ischemic (a), ischemic (b), and ischemic/non-ischemic (c) limbs between HCR and LCR after acute ligation. AR, androgen receptor; ERα, estrogen receptor alpha; AR/ ERα, the ratio of androgen receptor over estrogen receptor alpha. Left limb is the ischemic limb in all animals (HCRL and LCR L), and right limb is non-ischemic internal control limb (HCRr and LCRr). N = 10-11, * $p < 0.05$, mean ± SEM.

The protein expression of AR, ERα, and their ratio were not significantly changed between the phenotypes (P> 0.05) (Figure 4.2).

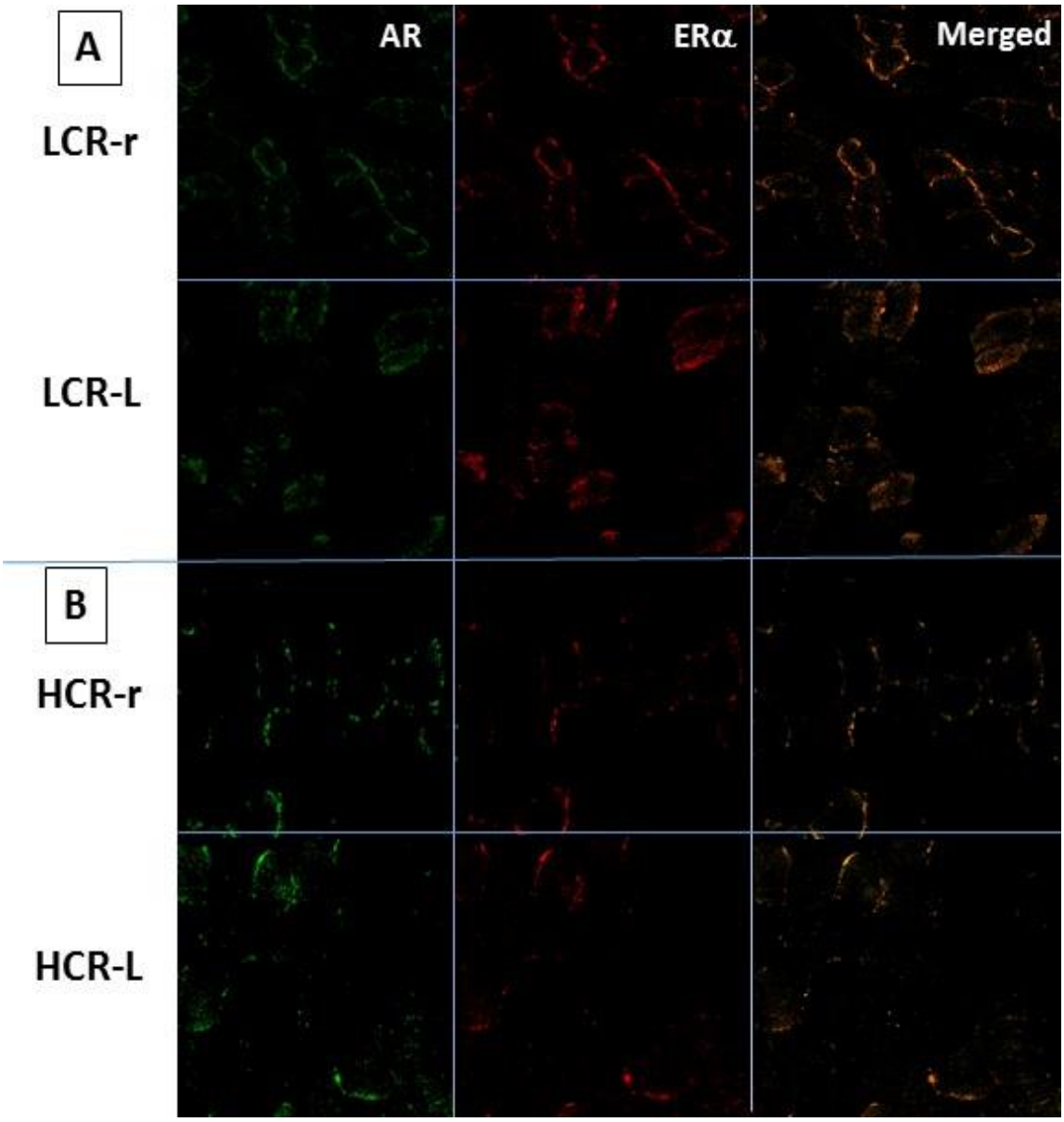

FIGURE 4.2. Androgen and estrogen receptor α protein expression in gastrocnemius muscle of high capacity and low capacity runner rats after 14 days of hind limb ischemia. Shown representative confocal microscopy photographs of AR (in green), ERα (in red) and the merged of both colors on gastrocnemius muscle frozen section in non-ischemic and ischemic limbs of LCR (A) and HCR (B) after acute left femoral arterial ligation.

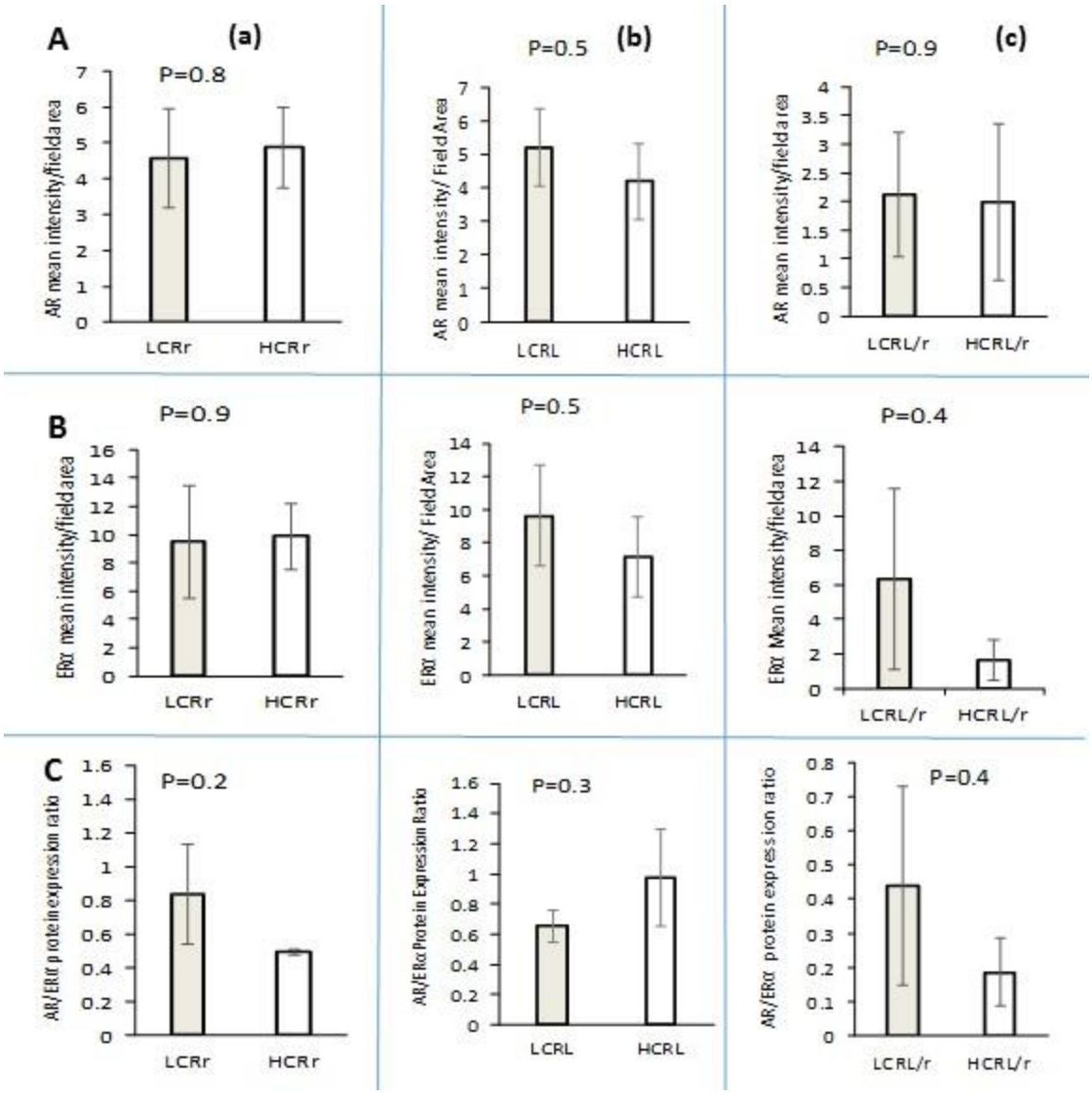

Figure 4.3 Quantification of confocal images for AR (A) ERα (B) and AR/ERα (C) protein expression in the non-ischemic limb (a), ischemic limb (b), and the ratio of ischemic to non-ischemic limb (c) between HCR and LCR after the ischemia; AR, androgen receptor; ERα, estrogen receptor alpha; LCRr, right limb of low capacity runner rat; HCRr, right limb of high capacity runner rat; LCRL, left limb of low capacity runner rat; HCRL, left limb of high capacity runner rat; n=10. The error bars represent standard error.

The Differential Changes in D1R and D3R Proteins Expression in The Gastrocnemius Muscle 14 Days Following Ligation Between LCR and HCR:

There were no differential changes in stimulatory 'D1R' and inhibitory 'D3R due to intrinsic differences in endurance capacity after hind limb arterial ligation either in non-ischemic or ischemic limbs or the ratio of ischemic to non-ischemic limbs (Figure 4.4).

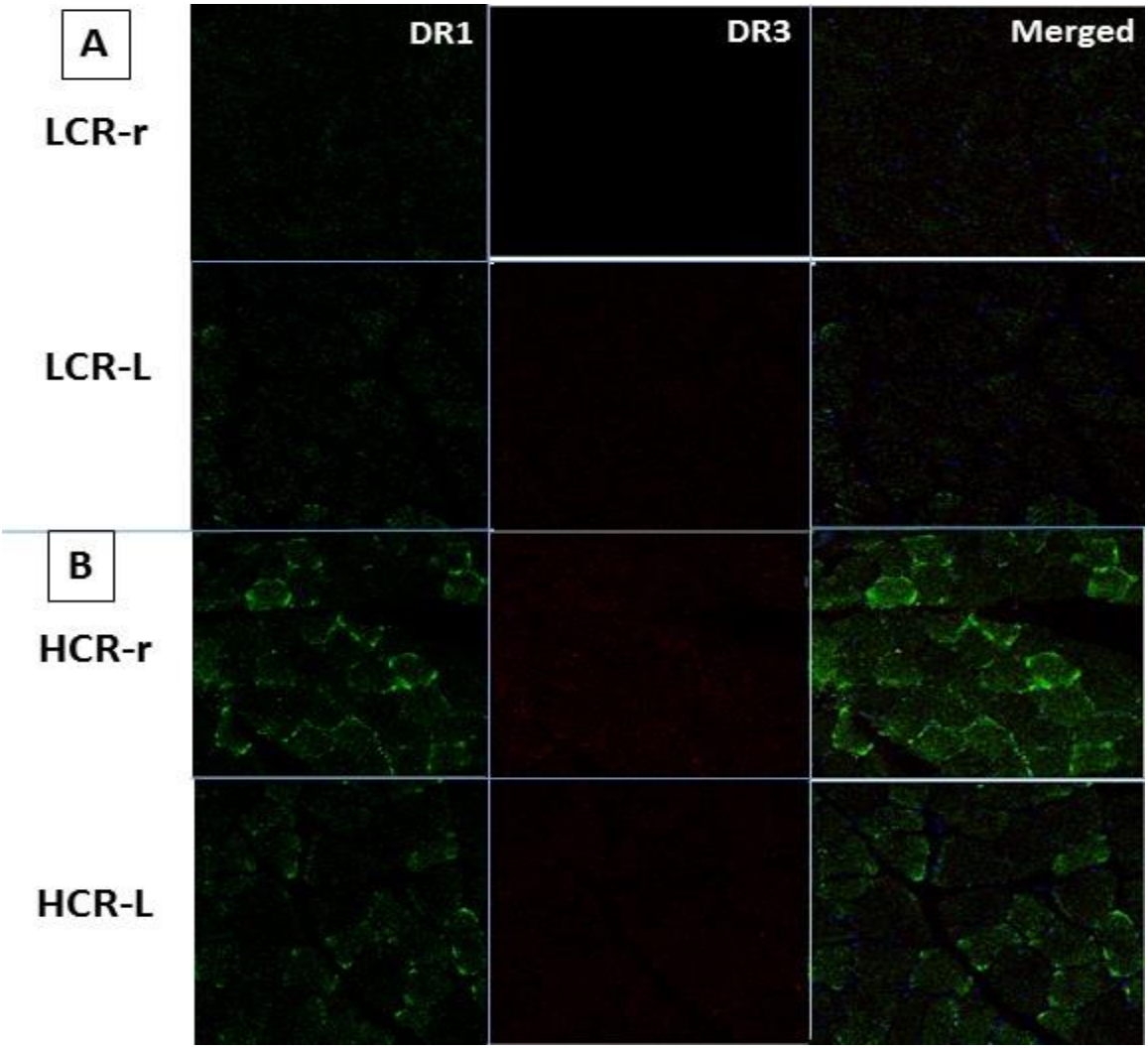

FIGURE 4.4. Representative confocal microscopy photographs of D1R (in green), D3R (in red) and the merged of both colors with neuclei stained with DAPI (in blue) on gastrocnemius muscle frozen cross-section in non-ischemic (LCRr & HCRr) and ischemic (LCRL & HCRL) limbs in LCR (A) and HCR (B) after acute left femoral arterial ligation; D1R, dopamine 1 receptor; D3R, dopamine 3 receptor.

Similarly, WT and D3KO mice showed no statistical differences in D1R and D3R protein expression in gastrocnemius frozen cross-section under baseline condition (Figure 4.5).

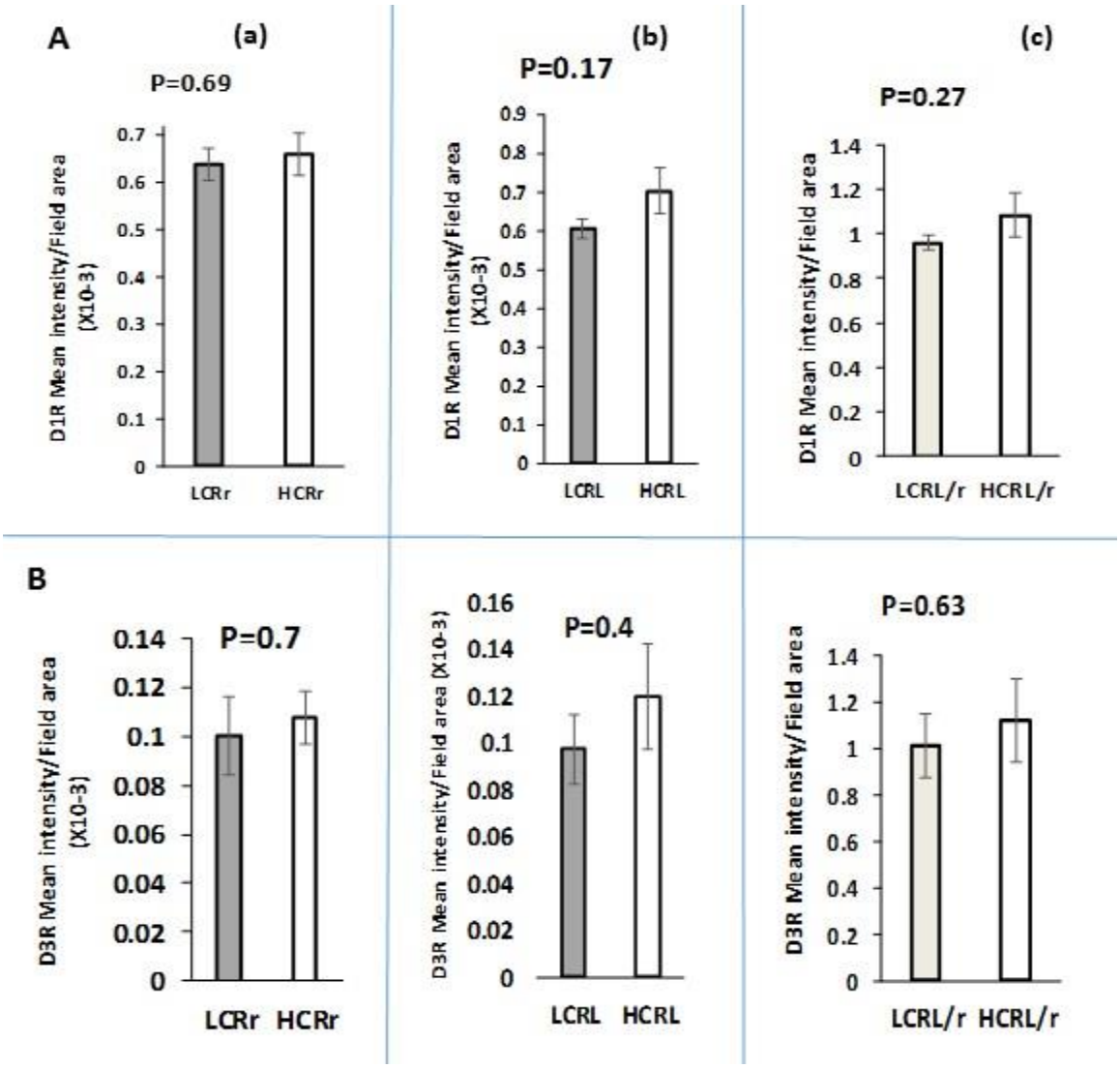

Figure 4.5. Quantification of confocal immunoflourescent images for Dopamine D1 and D3 receptors protein expression in gastrocnemius muscle of high and low capacity runner rats after 14 days of hind limb ischemia. D1R (A) and D3R (B) protein expression in the non-ischemic limb (a), ischemic limb (b), and the ratio of ischemic to non-ischemic limb (c) between HCR and LCR after the ischemia. N = 10-11; mean ± SEM.

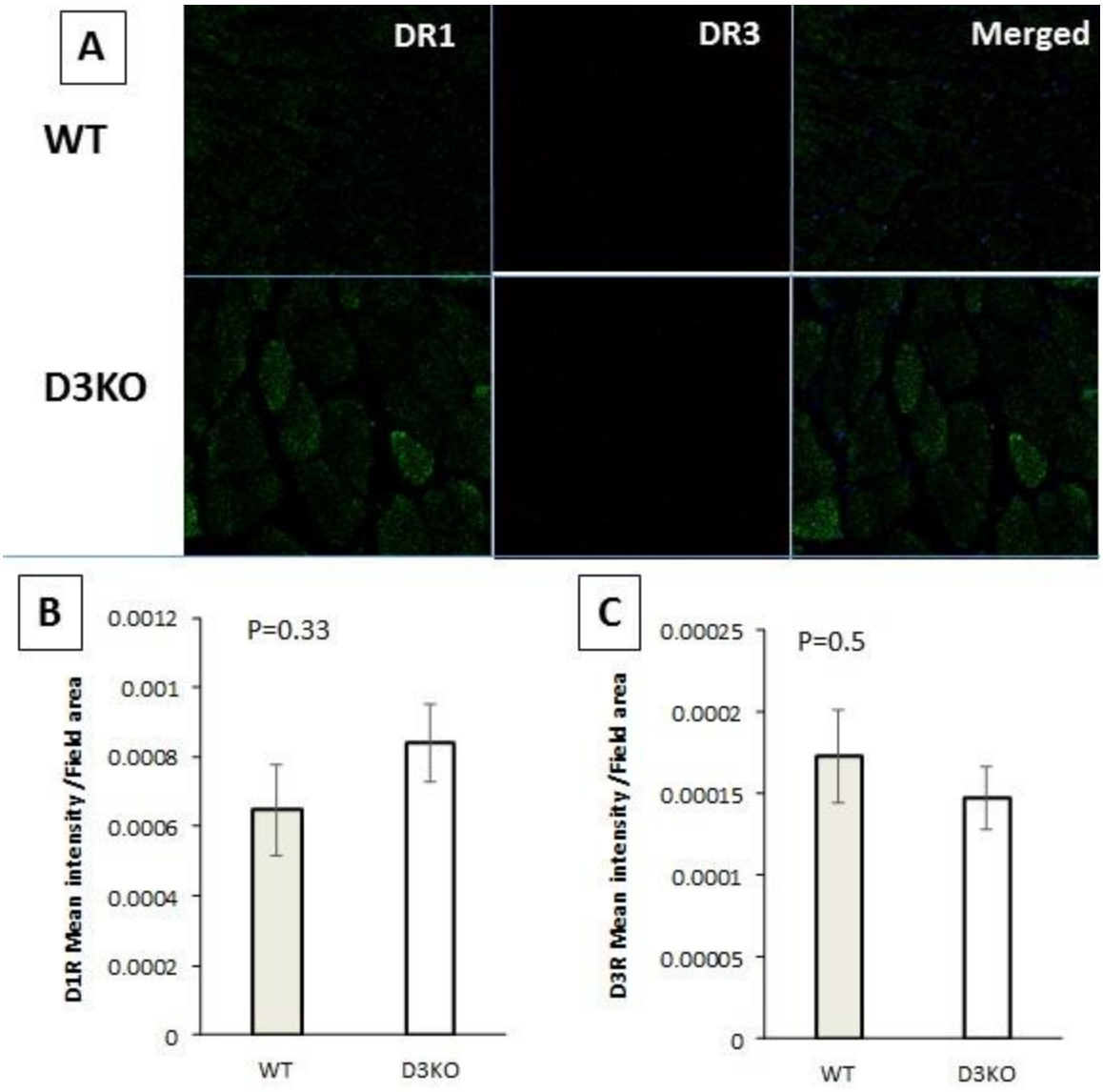

FIGURE 4.6. Representative confocal microscopy photographs of D1R (in green), D3R (in red) and the merged of both colors with neuclii stained with dapi (in blue) on gastrocnemius muscle frozen section in WT and D3KO mice at baseline condition (A). Quantification of relative immunoflourescence for Dopamine D1(B) and D3 (C) receptors protein expression in gastrocnemius muscle of WT and D3KO mice at baseline condition. D1R, dopamine 1 receptor; D3R, dopamine 3 receptor; WT, Wild-Type mice; D3KO, dopmine receptor 3 knockout mice; n=6, Mean ± SEM.

In Vitro Cell Growth Assessment of Satellite Cells Under The Influence of Androgen and Dopamine 3 Receptor Agonists/Antagonists.

We analysed the cell growth of skeletal muscle satellite cells isolated from gastrocnemius muscle of Sprague Dawley rats treated with different pharmacological AR and D3R agonist/antagonist in order to test their roles in cell physiology at different times points (6 hr, 24 hr, 36 hr, and 48 hr). As seen below (Figure 4.7) we found that the D3R antagonist (SB 277011A) significantly decreased cell grwoth (~32%) as compared to vehicle at 6 hr, but increases cell growth (72%) as compared to the vehicle at 36 hr ($P<0.05$). Howevere, there was no significant change in cellular growth at 24 hr and 48 hr.

Cells treated with DHT (10 nM) showed no statistical difference at 6 and 24 hr as compared to the vehicle. However, DHT at 36 hr increased cell growth (~60%) ($P<0.05$) as compared with cells treated with combination of DHT +D3R agonist and antagonist, and decreased cell growth (50%) ($P<0.05$) as compared with cells treated with D3R antagonist alone. At 48 hr, DHT decreased cell growth as compared to 36 hr, but still had higher impact on the cell growth at this time point around 50% in comparison with vehicle ($P<0.05$).

Cells that received Flutamide (50 nM) showed no significant changes in cellular growth in 24 hr and 36 hr while significant decrease (~25%) at 6 hr and (~30% increase) at 48 hr as compared to the vehicle. The group of cells that co-treated with combination of SB277011A and DHT drugs did not showed significant changes at 6, and 24 hr. Nevertheless, at 48 hr cells showed doubling in their total numbers as compared with the vehicle ($P<0.05$).

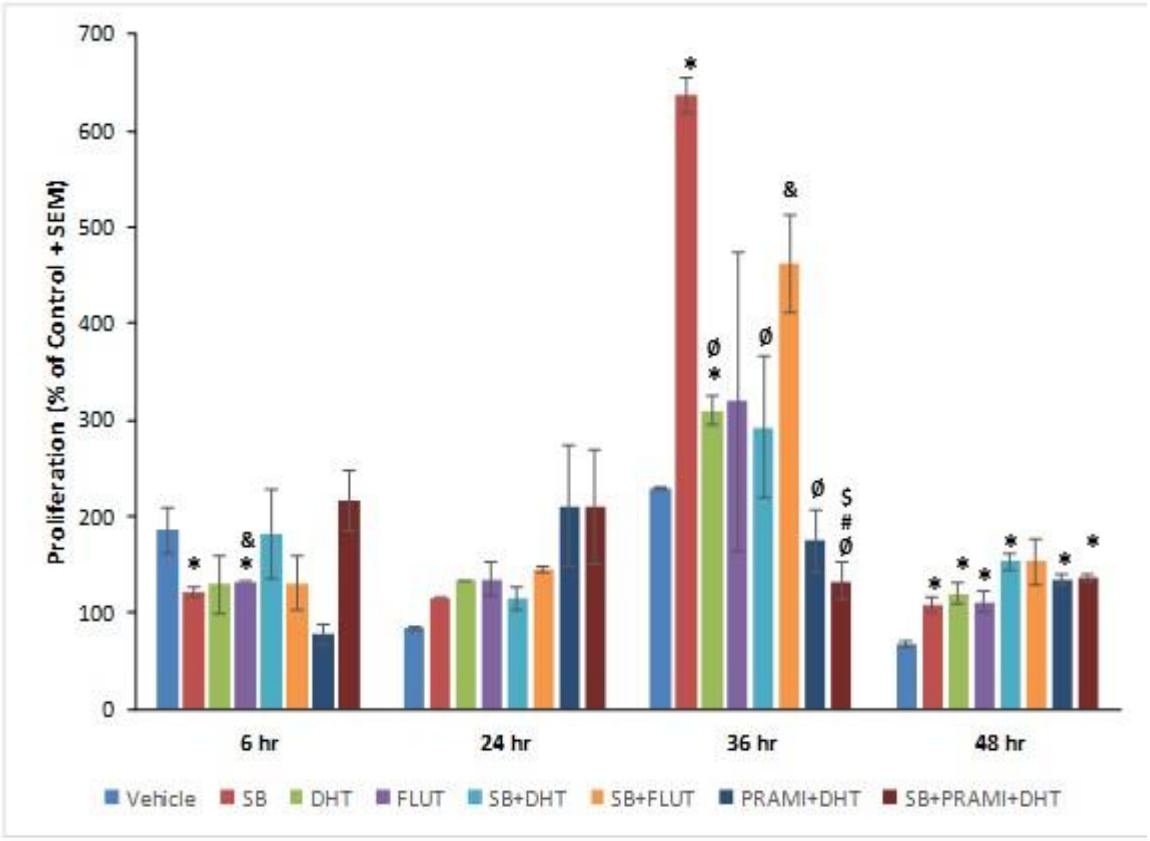

FIGURE 4.7. Effects of both androgen and D3R agonist, antagonist, and their combinations on Sprague Dawley skeletal muscle cell growth at four different time points (6 hr, 24 hr, 36 hr, and 48 hr). Nine groups where treated with different drugs or vehicles as shown by colors of the columns. Data represented by the percentage of control (treated group/control group x100) ± SEM. DHT, dihydrotestosterone; SB, SB277011A; Drugs concentrations as follows: SB 277011A and Pramipexole (10µM) each; DHT (10 nM); Flutamide (50 nM). To avoid confusion, we used several symbols to mark statistical trends between the groups, where *****: as compared to the vehicle; ***$***: as compared to SB277011A + Flutamide; ***&***: as compared to Pramipexole + DHT; ***#***: as compare to DHT; **ø**: as compared to SB277011A; n=4. Single factor ANOVA test was used to analyze the data between the groups; $P<0.05$.

Cells that received a combination of D3R and AR antagonists (SB277011A + Flutamide) increased growth at only 36 hr. At 36 hr, the growth increased ~61% as compared with group received (Pramipexole + DHT).

Treatment with (Pramipexole + DHT) caused a 75% decrease in growth (at 36 hr) then ~ 50% increase in cell growth (at 48 hr) ($P<0.05$). There was no significant difference between this group and the other groups at 12 hr and 24 hr.

The last group of cells that were treated with three drugs (SB + Pramipexole + DHT) showed effect at 36 hr with (80%, 55%, 65%) statistical difference in cell growth in comparison with the SB277011A, DHT, and SB+ Flutamide, respectively, and 50% increase in cell growth as compared to the vehicle.

Early cell growth (at 6 hr), in the presence of DHT:

The effect of DHT on LCR cell growth pretreated with dopamine antagonist was not changed significantly as compared to cells pretreated with dopamine agonist. In HCR, satellite cells that pretreated with dopamine agonist, there was (44%) significant decrease in cell growth as compared to cells pretreated with dopamine agonist. Between the phenotypes, there was (70%) higher growth in LCR cells that pretreated with dopamine antagonist as compared to HCR cells under similar condition (Figure 4.8).

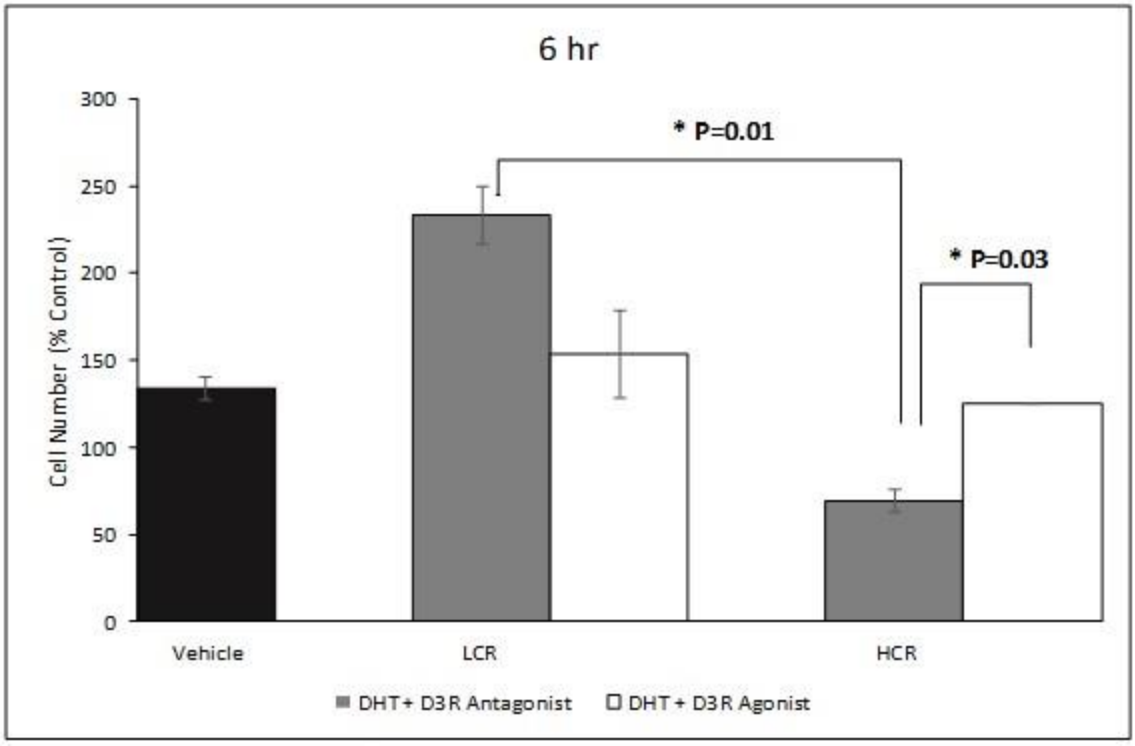

FIGURE 4.8. The effect of Dihydrotestosterone on LCR and HCR satellite cell number treated with D3R antagonist/agonist at early time point. Data represented cell growth by percentage of control (treated group/untreated control group x100) ± SEM. DHT, Dihydrotestosterone (10 nM); D3R antagonist, dopamine D3 receptor antagonist (SB, SB277011A 10μM); D3R agonist, dopamine D3 receptor agonist (Pramipexole 10μM); LCR, low capacity runner rat; HCR, high capacity runner rat; SEM, standard error of the mean; hr, hour. *: represent statistical significant. n=4. Paired t-test was used to compare within the groups of LCR or HCR and single factor ANOVA test was used to analyze the data between HCR and LCR groups; $P<0.05$.

Late cell growth (at 36 hr), in the presence of DHT:

In the presence of DHT, LCR cells that pretreated with dopamine antagonist had (85%) significant decrease in their growth as compared to cells pretreated with dopamine agonist ($P<0.05$). However, in HCR satellite cells, there was no significant changes between cells pretreated with dopamine antagonist and cells pretreated with dopamine agonist. Similarly, there were no significant changes found between the phenotypes (Figure 4.9). Both time points for both phenotypes are illustrated in (Figure 4.10) for convenience visualization.

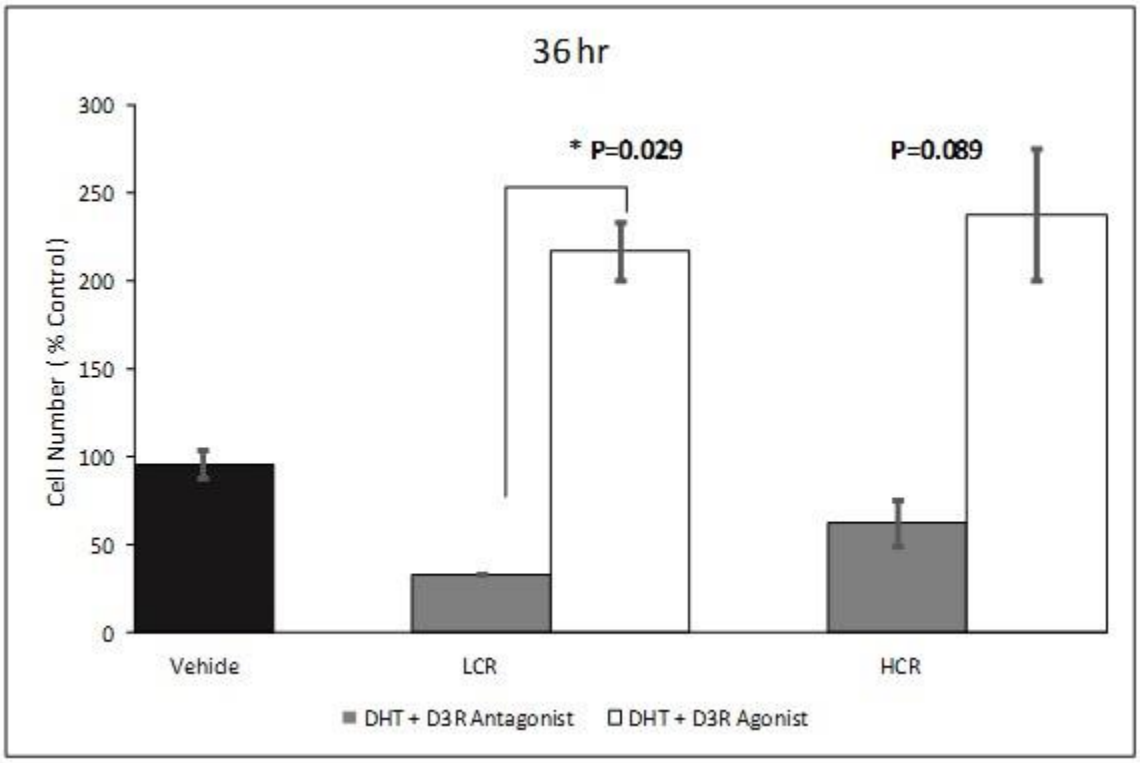

FIGURE 4. 9. The effect of Dihydrotestosterone on LCR and HCR satellite cell growth treated with D3R antagonist/agonist at late time point. Data represented cell number by percentage of control (treated group/untreated control group x100) ± SEM. DHT, Dihydrotestosterone (10 nM); SB, SB277011A (D3R antagonist 10µM); Pramipexole (D3R agonist 10µM); LCR, low capacity runner rat; HCR, high capacity runner rat; SEM, standard error of the mean; hr, hour. *: represent statistical significant. n=4. Paired t-test was used to compare within the groups of LCR or HCR and single factor ANOVA test was used to analyze the data between HCR and LCR groups; $P<0.05$.

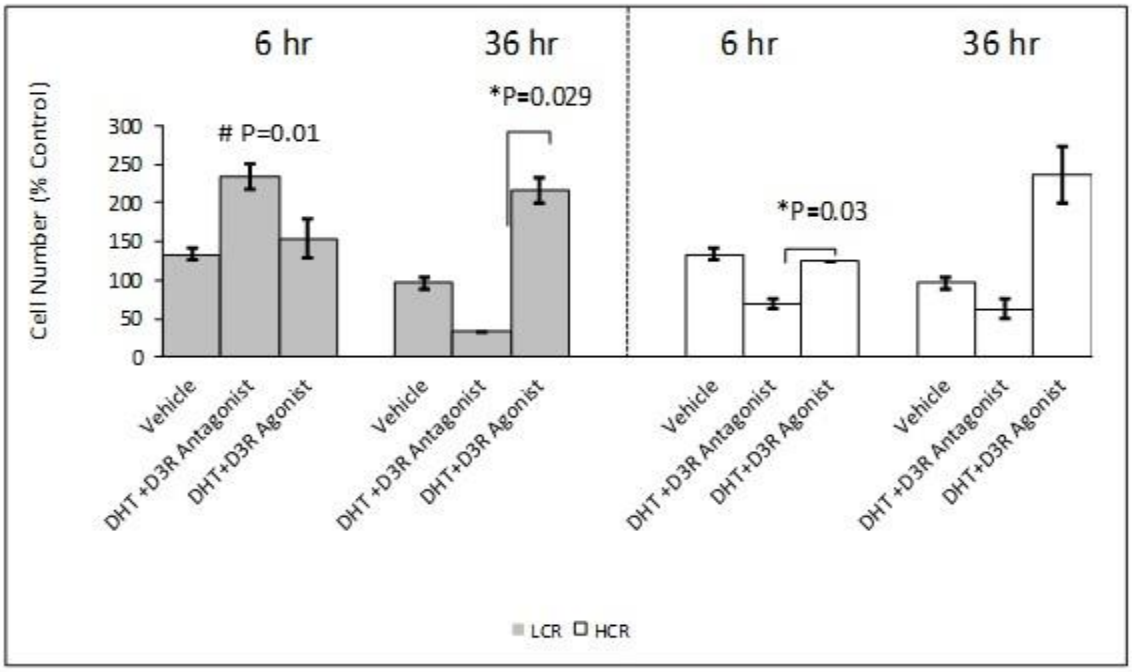

FIGURE 4.10. The effect of Dihydrotestosterone on LCR and HCR satellite cell number treated with D3R antagonist/agonist at both time points. Data represented cell number by percentage of control (treated group/untreated control group x100) ± SEM. DHT, Dihydrotestosterone (10 nM); SB, SB277011A (D3R antagonist 10µM); Pramipexole (D3R agonist 10µM); LCR, low capacity runner rat; HCR, high capacity runner rat; SEM, standard error of the mean; hr, hour. *: represent statistical significant within the phenotype. #: represent statistical significant with the corresponding HCR group. n=4. Paired t-test was used to compare within the groups of LCR or HCR and single factor ANOVA test was used to analyze the data between HCR and LCR groups; $P<0.05$.

Early cell growth (at 6 hr), in the presence of Pramipexole:

In LCR, cells that pretreated with AR agonist showed (13.5%) significant decrease in cell growth as compared with cells pretreated with AR antagonist ($P<0.05$). However, in HCR, there was no significant difference between cells pretreated with AR agonist and cells

pretreated with AR antagonist. There was also no significant difference between the phenotypes in both treatment (Figure 4.11).

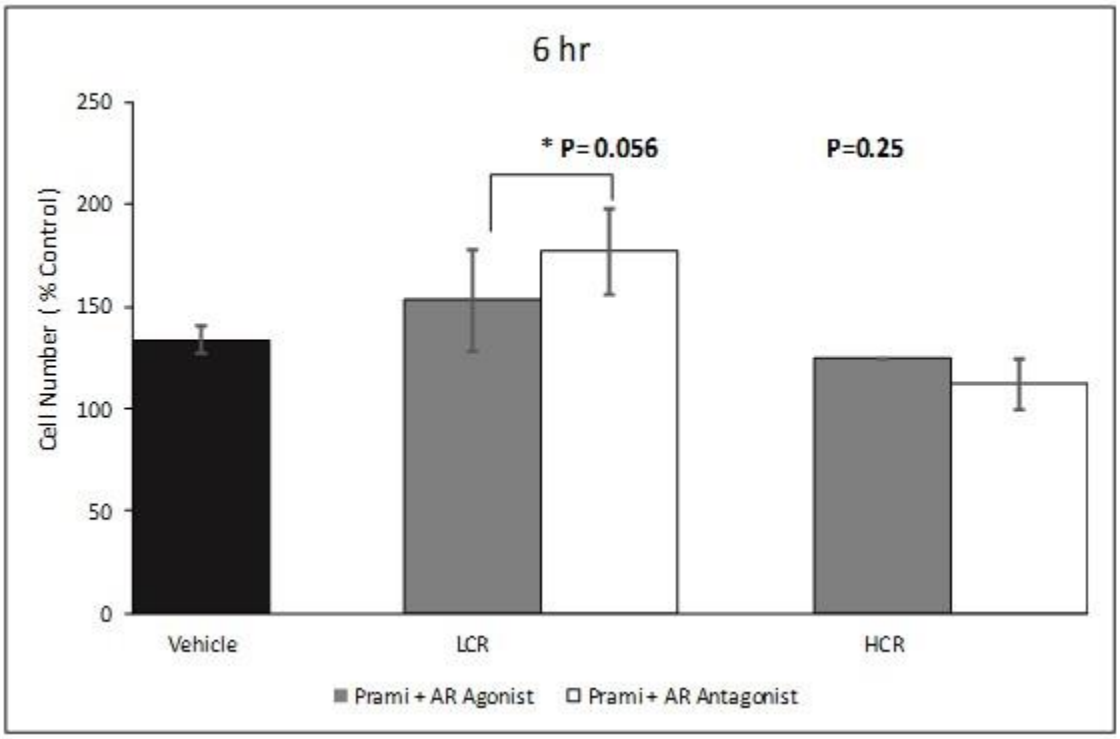

FIGURE 4.11. The effect of Pramipexole on LCR and HCR satellite cell number treated with AR antagonist/agonist at early time point. Data represented cell growth by percentage of control (treated group/untreated control group x100) ± SEM. Prami, Pramipexole (D3R agonist 10µM); AR agonist, androgen receptor agonist (Dihydrotestosterone 10 nM); AR antagonist, androgen receptor antagonist (Flutamide 50 nM); LCR, low capacity runner rat; HCR, high capacity runner rat; SEM, standard error of the mean; hr, hour. *: represent statistical significant. n=4. Paired t-test was used to compare within the groups of LCR or HCR and single factor ANOVA test was used to analyze the data between HCR and LCR groups; $P<0.05$.

Late cell growth (at 6 hr), in the presence of Pramipexole:

In LCR, cells that pretreated with androgen receptor agonist showed (54%) significant increase in cell growth as compared with cells pretreated with androgen receptor antagonist (P=0.05). However, there was no significant difference between cells pretreated with androgen receptor agonist, and cells pretreated with androgen receptor antagonist. Between the phenotypes, there was no significant difference in both treatments (Figure 4.12). For a complete visualization of the effect at both time points for both phenotypes, see (Figure 4.13).

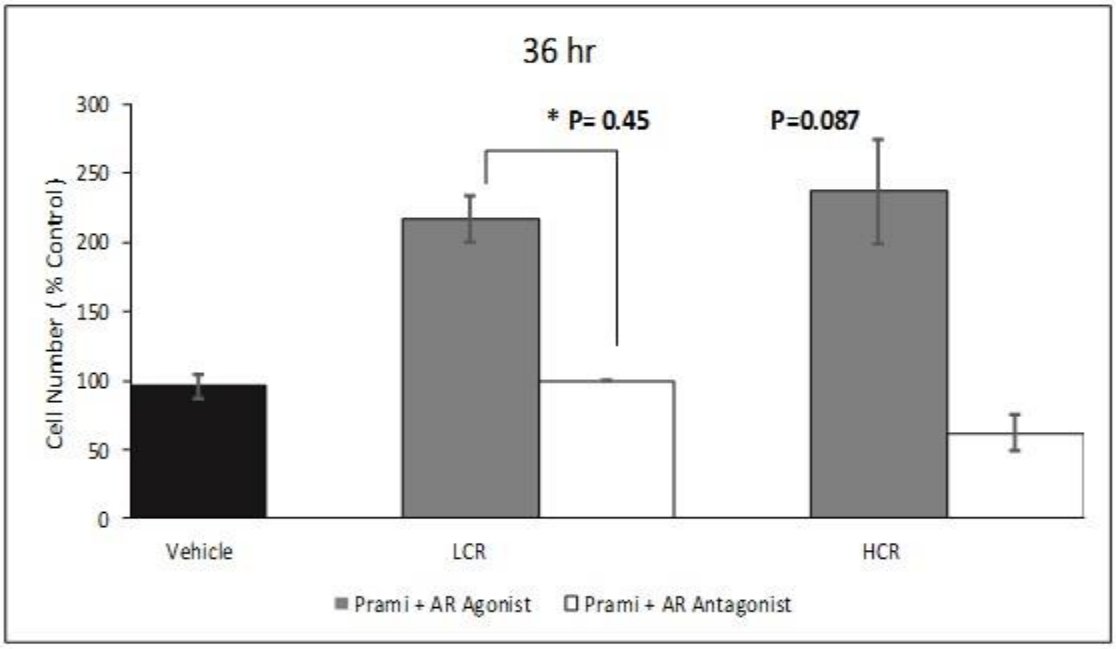

FIGURE 4.12. The effect of Pramipexole on LCR and HCR satellite cell number treated with AR antagonist/agonist at late time point. Data represented cell umber by percentage of control (treated group/untreated control group x100) ± SEM. Prami, Pramipexole (D3R agonist 10μM); AR agonist, androgen receptor agonist (Dihydrotestosterone 10 nM); AR antagonist, androgen receptor antagonist (Flutamide 50 nM); LCR, low capacity runner rat; HCR, high capacity runner rat; SEM, standard error of the mean; hr, hour. *: represent statistical significant. n=4. Paired t-test was used to compare within the groups of LCR or HCR and single factor ANOVA test was used to analyze the data between HCR and LCR groups; $P \leq 0.05$.

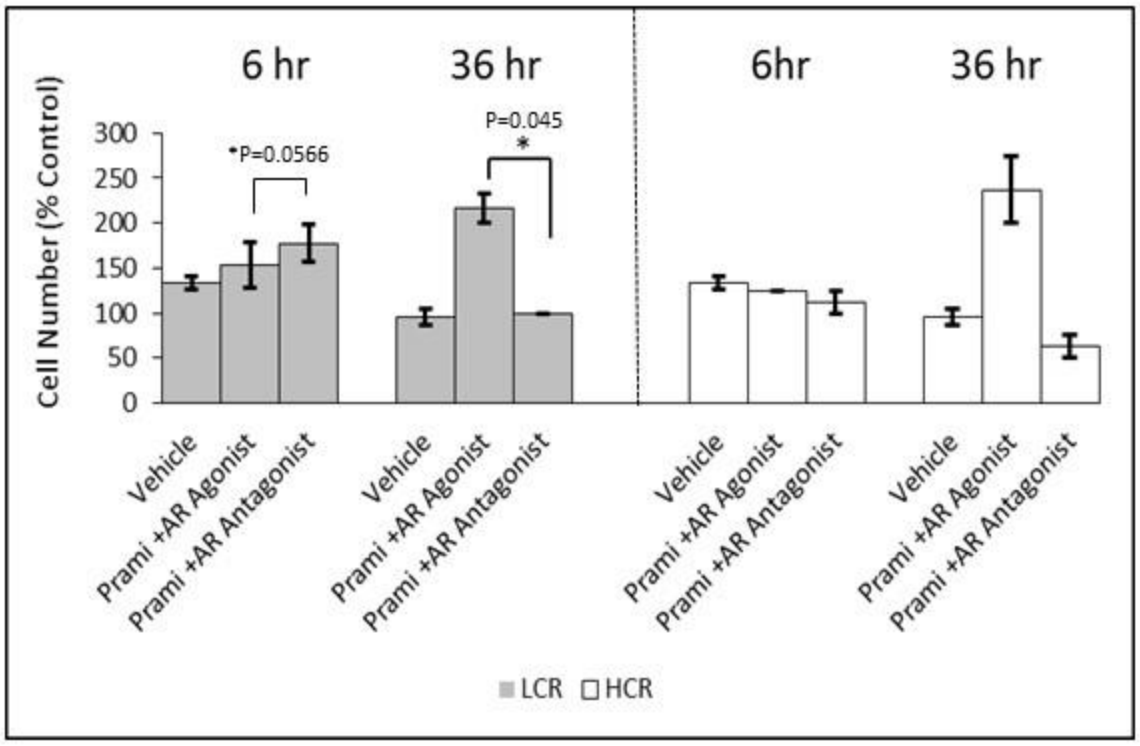

FIGURE 4.13. The effect of Pramipexole on LCR and HCR satellite cell number treated with AR antagonist/agonist at both time points. Data represented cell number by percentage of control (treated group/untreated control group x100) ± SEM. Prami, Pramipexole (D3R agonist 10µM); AR agonist, androgen receptor agonist (Dihydrotestosterone 10 nM); AR antagonist, androgen receptor antagonist (Flutamide 50 nM); LCR, low capacity runner rat; HCR, high capacity runner rat; SEM, standard error of the mean; hr, hour. *: represent statistical significant. n=4. Paired t-test was used to compare within the groups of LCR or HCR and single factor ANOVA test was used to analyze the data between HCR and LCR groups; $P \leq 0.05$.

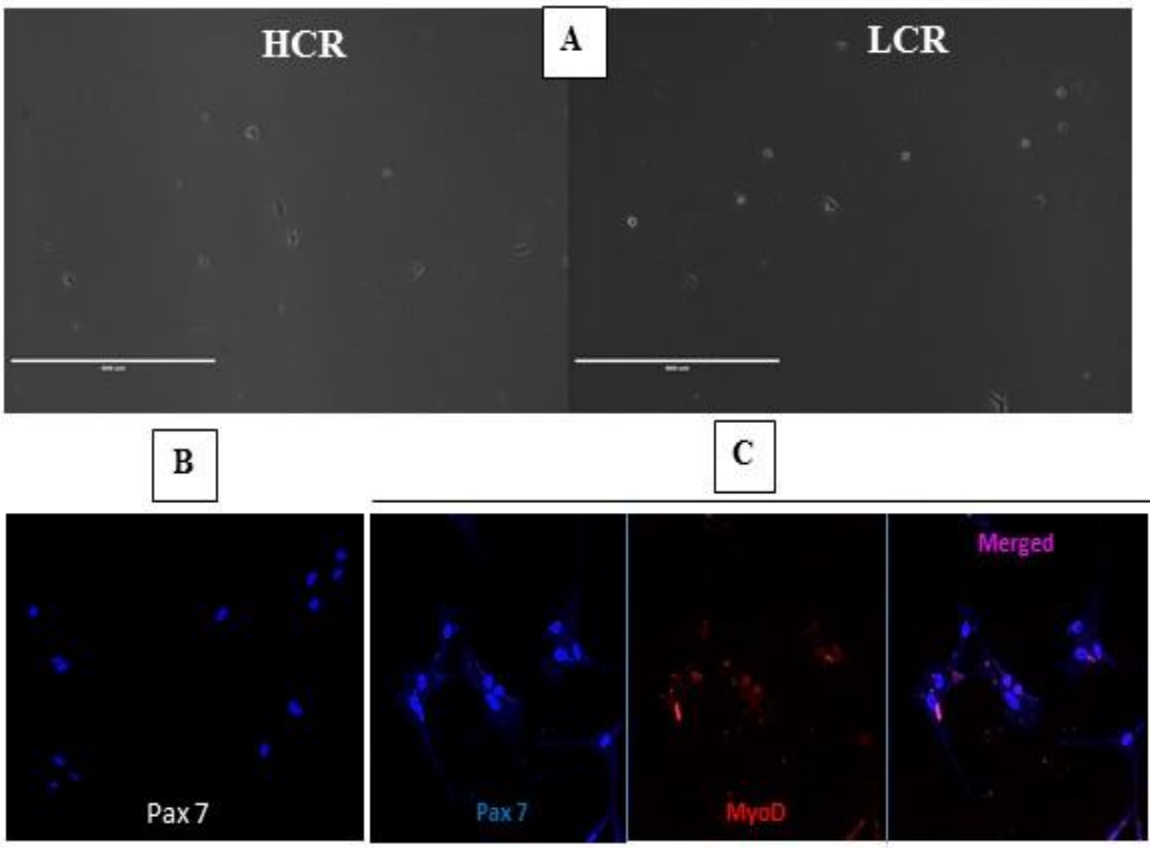

FIGURE 4.14. Identification of satellite cells from gastrocnemius muscle of both high capacity runner (HCR) and low capacity runner (LCR) rats. Shown representative phase contrast micrographs depicting the morphology of HCR and LCR myogenic cultures in rich growth media at day 3 according to protocols detailed in this chapter (A); confocal microscopy photographs of identified satellite cells by nuclear marker Pax7 at day3 (B) and by both Pax 7 (in blue), MyoD (in red), and the merged of both colors (in pink) for proliferating satellite cell progeny after Day 6 of isolation (C); Pax7, paired box protein-7; MyoD, myogenic differentiation antigen.

DISCUSSION

Peripheral arterial disease (PAD) among the female population has been known to be more detrimental than in the male population (9, 74, 240, 255). Sex hormones have been reported as participating in the cardiovascular disease protection. Not only can low levels of estrogen be risky in relation to cardiovascular disease (old concept), but lower levels of androgen has also been found to be associated with the onset of cardiovascular disease. Although females also express the androgen receptor in cardiovascular system, the study of association of androgen receptor expression with the severity of PAD in female individuals is not as extensively studied as in male individuals.

The model of intrinsic aerobic endurance capacity provides a better understanding of the differential association of sex steroid receptors with lower limb arterial ligation. This model helps answer questions such as *how the intrinsic factors (genes) play roles in differentially distributing sex steroid receptors (AR and ERα) during vascular remodeling in response to arterial ligation. It also helps in better understanding the benefit from increase sex steroid receptors in prevention and treatment of PAD through developing a novel molecular therapy for PAD patients who can't tolerate exercise therapy.*

The goal of this study was to determine whether there are associations of sex steroid receptors (AR and ERα) and dopamine receptors (D1R and D3R) in the differential vascular adaptive responses between female rats with low intrinsic running capacity and high intrinsic running capacity and if these receptors play role in the skeletal muscle cell repair. The results of this study showed that rats

selectively bred for high endurance running capacity (HCR) and low endurance running capacity (LCR) have a different AR/ERα gene expression ratio in non-ischemic and ischemic limbs after 14 days of left femoral artery ligation. The results suggest the importance of the AR/ERα genetic ratio in influencing vascular adaptive response to peripheral vascular occlusive disease in females with differences in intrinsic aerobic capacity.

AR/ERα Differential Changes Between HCR and LCR After Acute Ligation:

The AR/ ERα gene expression ratio was apparently different between the phenotypes. HCR rats' AR/ERα gene expression ratio was significantly higher than LCR groups (32% in non-ischemic limb, P=0.005 and 42% in ischemic limb, P=0.006). However, the proteins expression ratios of these receptors were not significantly changed between the phenotypes although the protein ratio was lower in HCR than LCR. The possible interpretation for non-significant change of the AR/ERα protein ratio between the phenotypes could be due to the time period between the initial ligation and tissue collection (14 days) very long to cause loss of the acute response of these receptors (minutes to hours). As we know sex steroid receptors are part of highly regulated system in our body (Endocrine). The mechanism by which these receptors are regulated is very complex. When the ligation occurs (stimulus), the receptors are activated (translocated to the nucleus), leading to the replacement of these receptors immediately from the stored receptors in the cytoplasm. While the active receptors act as transcription factors for several genes involved in vascular remodeling, they also transcribe for new sex steroid receptors synthesis from previously degraded receptors by ubiquitination process in

proteasome. Another possible interpretation for unchanged AR/ERα protein ratio is because we obtained the total distribution including the nuclear fraction. It is possible that the differences between the phenotypes is at the nuclear level (functional steroid receptors). At the level of nuclear AR/ERα gene ratio, higher ratio in the HCR than LCR indicates more activation and replenishment of the androgen receptor relative to estrogen receptor alpha (acute response within hours), causing low to unchanged protein levels, followed by more receptor degradation and synthesis (within days) which causes higher mRNA levels.

The importance of AR/ERα ratio has been reported in cardiovascular studies. In 1994, Mosnarova et al. studied the vascular reactivity of male rabbit after one month of sex hormone administration (estradiol or testosterone twice per week). They reported that hyperestrogenemia and change in the estrogen-androgen level ratio caused changes in vascular reactivity to several vasoreactive compounds. They suggest that a higher level of estrogen to androgen ratio in males is a risk factor to several vascular diseases (157). Another study was in 2010 conducted by Naessen et al. on prevalent cardiovascular disease in 70-year-old men and women. They found that prevalent cardiovascular disease was associated with indication of lower androgen precursors, an increase in aromatase activity, and higher estradiol levels in both sexes. This suggests an endogenous response to developing atherosclerosis, instead of a causation relationship (162), which supports our previous studies on these animals where we found higher markers of endothelial dysfunction in LCR as compared to HCR (Chapter 2).

In 2012, Naessen et al. studied the endogenous estrogen levels in 70-year-old women and men and assessed the common carotid arteries' intima, media and their ratio thickness. They found low androgen levels, high aromatase activity and high estrogen levels were associated with unhealthier artery wall (thick intima, thin media, and high intima to media ratio) in females as well as in males (161). Although we did not measure the androgen and estrogen hormones in these animals, we conducted these experiments because we strongly believe that HCR are considered androgen-oriented phenotype opposite to LCR. HCR rats characterized by having higher maximum oxygen uptake and resting metabolic rate, lean body mass, longevity, and are resistant to developing dyslipidemia, peripheral insulin resistance, diet induced-liver steatosis, depression, and metabolic syndrome in comparison with LCR. These differences are possibly due to at least different underlying endocrine hormonal profile between these phenotypes.

Several studies on testosterone prove the relationship between these biological characters and testosterone levels in human and animals. Studies in human showed that men who had prostatic cancer and who were under androgen deprivation therapy for more than 3 months developed lower VO2max and lower resting metabolic rate compared to the group that received the therapy for less than three months (228). Moreover, studies have showed that testosterone decreased visceral fat mass (143), improved insulin resistance (143), and increase lean body mass (144). Also, studies revealed that long-term testosterone

replacement therapy in men with late onset hypogonadism decrease obesity parameters and improved metabolic syndrome and health-related quality of life (143).

Testosterone replacement therapy for hypogonadal and aging men caused decrease in total cholesterol and LDL levels and no change in HDL (250). Hormonal replacement therapy has been found increases the longevity in both genders. Testosterone replacement therapy for men with late onset hypogonadism increased the survival rate by 9-10% in 5 years, which is similar to that of eugonadal men with normal endogenous testosterone level, and in menopausal women with estrogen replacement therapy, their survival rate increased by 2.6% in 5 years (32).

Fatty liver is also associated with low testosterone concentration in both human and animal studies. In 495 men diagnosed with non-alcoholic fatty liver disease, 251 of them have low serum testosterone level with absence of an alternative cause (76). In animal studies, mice with liver-specific androgen receptor deletion have higher liver steatosis than obese controls (134). On the other hand, estrogen signaling has been shown interrelated with the metabolic syndrome and its risk factors (153). It is also reported that LCR is known as a genetic model of metabolic syndrome (183). Epidemiological studies provided a strong evidence that hormone replacement therapy gives cardioprotection in postmenopausal women (100), while other studies do not (91). It has been suggested that inconsistency in these results is due to heterogeneity in response to

hormonal replacement therapy. LCR and HCR rats are generated from heterogeneous N: NIH founder population.

D1R/D3R Differential Changes Between HCR and LCR After Acute Ligation:

Dopamine receptors play an important role in regulating vascular pathophysiology. In this study we predict three possible causes that explain HCR benefits following hind limb ischemia, which summarized in (Figure 4.13). In our study we examined the differential changes in the protein expression of D1R and D3R, which are the stimulatory and inhibitory receptors respectively for dopamine release and synthesis after 14 days of abrupt ligation of unilateral femoral artery. The distribution of these receptors in non-ischemic and ischemic limbs were not statistically different between the HCR and LCR rats although they were higher in HCR than LCR. The possible interpretation of these results could be that these receptors are different functionally and not at the level of protein expression. Again, the level of these proteins that have been identified between the phenotypes represent the total distribution of these receptors in the cell whether that stored in vesicles (in active) or on the cell membrane (active). It is possible that in LCR rats the receptors are present mostly in the inactive form while HCR receptors are present in the active form. What we have shown in our study is the total distribution, indicating possible functional differences in these receptors between HCR and LCR. Studies on D3R are lacking in skeletal muscle. Most studies were conducted on kidney and central nervous system. In spinal cord neurons, it has been shown that in D3KO mice, the mRNA of D3R was higher

than that of WT mice. The study also showed that the distribution of the D3R was locally different between WT and D3KO where the receptor found bind with membrane in WT but not in D3KO as evidence for functional knockout in D3KO mice (251). This study also supported our findings in D3KO mice as compared to WT mice that the immunofluorescence staining for D3R and D1R were unchanged between the groups indicating functional knockout instead of protein expression levels differences although D3R was overall lower in D3KO mice than WT mice.

D1R has been linked to muscle mass preservation and muscle force production (188). We have reported early in this study (Figure 2.1 E) that the area of muscle fiber of HCR ischemic limb was significantly higher than LCR counterpart. However, it has been shown that D1R centrally in different brain areas was not different between HCR and LCR (53). A study conducted by Teresa E. Foley et al. they used high capacity runners (HCR) and low capacity runners (LCR) and studied various brain areas involved in movements, motivation and fatigue and found D2R (and not D1R) in HCR was higher than LCR (53). It is possible that the D2R and not D1R or D3R is responsible for regulating skeletal muscle cell repair during injury or even physiologically. Another way of interpretation is that peripheral dopamine system regulating skeletal muscle cell repair during injury, or even physiologically, is different than central dopaminergic signaling or by other dopamine receptors. D1R stimulation in model for hind limb arterial ligation was associated with enhanced bone marrow-derived cell mobilization & post ischemic angiogenesis (187). In our study, we did not investigate the infiltration of immune cells. However, we found

increased gene expression of angiogenic markers Vegfa and Angiop2 in LCR but not in HCR (Table 2.A) suggesting different mechanism that LCR own or different dopamine receptor type involvement for vascular remodeling during ischemia such as D2R. It has been shown that in unilateral hind limb ischemia of D2KO mice there was increased recovery and angiogenesis stimulation compared to WT mice (194) which is most likely that LCR differs from HCR in the lack or dysfunction of D2R. However, in vitro studies can answer the functional query about D1R and D3R in HCR and LCR.

The Effect of the Interaction Between Androgen Receptor and Dopamine D3 Receptor During Cell Growth of Sprague Dawley Rat Satellite Cells *In Vitro:*

Culturing satellite skeletal muscle cells isolated from adults female Sprague Dawley rats treated with D3R antagonist were helpful in determining the functional status of dopamine receptor. They were also helpful in building a relationship between the protein levels and function during SSC growth in normal culture medium for 36-48 hr. D3R antagonist (SB277011A) caused significant decrease in the cell growth at early time point (6 hr) then it increased the growth to very high level at 36 hr. This means its dysfunctional negative effect was shown after 6 hr of incubation for 2 hr in serum free media and that effect was time dependent specifically at 36 hr when the growth peak was the highest. It has been shown that the expression of dopamine receptors depends on the state of cellular activation and the concentration and time of exposure to dopamine (6). Dihydrotestosterone (DHT) treated cells showed an increase in growth only at 48 hr indicating that DHT works through

genomic receptors since Flutamide had no effect on cell growth at all and it did not abolish the effect of DHT on cell growth. This suggests that DHT worked on non-androgenic receptors such as estrogen receptors beta through its metabolite 3β-androstanediol which is a potent and selective agonist ERβ (113). DHT abolished the effect of SB 277011A on cell growth at 36 hr. This means DHT/R act as transcription factor and upregulate D3R. This led to two opposite reactions: D3R inhibition by SB and D3R upregulated (activated) by DHT. Thus, the end result of these two reactions is zero because they subtracted each other. The final response was for DHT effect at 48 hr through non-AR response. Cells received D3R agonist (Pramipexole) and DHT did not show increase cell growth before 48 hr while at 48 hr the cell growth effect not different from DHT effect. However, in the first 6 hr cell growth decreased as in SB 277011A and Pramipexole treated groups. This could be due to the 48 hr length of the study, as others used up to 24 hr (248) although we replaced the drugs and media first at 6hr then after 12 hr until the experiment was complete. It is also possible due to rebound effect of the drugs as these drugs are sensitive to the air and have short bioactivity at 36 hr. When the drug activity reduced, the effect was rebound as in SB 277011A treated group. In Pramipexole treated cells, we did not see any effect after 6 hr so it could be the drug not associated with rebound effect because it is activating to the receptors or due to drug toxicity since these cells are satellite skeletal muscle cells and their dopamine receptors expressions were not high (Fig. 4.4). One would say why the toxicity in the first 6 hr only, it is possible that cells tolerated the drugs in the late time points. Clinically Pramipexole was associated with augmentation and tolerance with long-term treatment of restless leg syndrome (238). Another study

reported that long-term and not acute stimulation of D3R was found to promote neuronal proliferation in rat hippocampus (82, 85). This controversy effect strongly suggests a time-dependent effect. The treatment by SB277011A, Pramipexole, and DHT together interestingly decreased cell growth at 36 hr. The cell growth due to the effect of SB277011A was attenuated by the activation of D3R (Pramipexole) although D3R agonist by itself at 10 μM concentration had no effect on skeletal muscle satellite cell growth. Simultaneous inhibition and activation of D3R had an inhibitory effect at 36 hr.

Studies reported contradictory effects of D2R function on cell proliferation. It has been reported that D2R antagonist increased cell proliferation and decreased cell death of pancreatic β-cells in human (193) and in animals (58). On the other hand, D2-like receptor activation increased cell proliferation in the embryonic mouse Telencephalon by dopamine (180).

D3R agonist (PD-128907) has been shown to have no effect on vascular smooth muscle cell proliferation (130), which is similar to what we found in our study. However, we used pramipexole as D3R agonist instead of PD-128907 while others studied the activation of D4R by (PD 168077) on vascular smooth muscle cells proliferation and migration in vivo and found inhibitory effect on neointemal formation (rat model of hyperinsulinemia) (249). However, D3R antagonist (U-99194A) in neonatal sub-ventricular neurons showed decreased cell proliferation (106). This study is opposite to what we reported here. The differences in the D3R antagonists and the cell type that they used in their studies were different than our study, which could suggest that the effect of D3R inhibition on cell growth is drug

and cell type dependent. Other studies showed that stimulation of D3R attenuates renal ischemia/reperfusion injury via increased linkage with Gα12 (230) while other studies reported inhibition of D3R by SB277011A it increase survival rate in mice model of Anorexia nervosa (110).

The Effect of the Interaction Between Androgen Receptor and Dopamine D3 Receptor on LCR and HCR Satellite Cells Growth *In Vitro:*

Previous work has confirmed that HCR intrinsically differs in prolonged dopamine availability in the brain when compared to LCR (53). Clinical and basic experimental studies provided huge evidences about the atheroprotective effect of testosterone on blood vessels (68, 104, 152, 200). *In vivo*, we studied the distribution of AR and D3R in ischemic and non-ischemic gastrocnemius of HCR and LCR. Although we did not find significant differences between these phenotypes in term of availability of these receptors, however, not always availability of protein is an indicator of functioning cells. The important determinant of cell function is based on how is the condition of signaling pathway of the receptor. Signaling pathway can be activated or inhibited in order to initiate cellular response, whether through direct action of the receptor on the cell or indirectly through interaction with other receptor which also is as important as the direct effect. Therefore, we conducted an *in vitro* study to determine the signaling pathway resulted from the interaction of AR and D3R in isolated satellite cells from each phenotype. This study helped better understand the mechanism by which HCR differs from LCR, which may explain the differential response between the phenotype to various stimuli in vivo and in vitro.

Here we conducted in vitro experiment to study satellite cell growth at two different time points to investigate the early effects of signaling pathway interaction at 6 hr and the late effect at 36 hr. We chose two time points because of the limitation in using primary stem cells isolated from adult animal, where cell yield is low. These time points were based on the several conducted experiments that we have done on satellite cells of Sprague Dawley rats and fibroblasts that isolated from mice. This preliminary data showed that androgen has a late effect after 24 hr and dopamine effect occurred at early ours.

In these phenotypes we found interesting differential effects resulting from the interaction of AR and D3R. Early time point, when cells treated with DHT and D3R antagonist, we found that there is an opposite effect on cell growth between the phenotypes. In LCR, cell number was increased while in HCR, cell number was decreased. At late time point, LCR showed an effect that was opposite to what we see at 6 hr while the effect in HCR at late time point was similar to what we see at early time point. These data indicate that the growth of satellite cells progeny in both phenotypes are enhanced with the exposure to DHT and Pramipexole, and suppressed when cells exposed to DHT and D3R antagonist, and these effects occur only at 36 hr. However, at 6 hr, DHT and Pramipexole only increased the number of HCR cells and decreased it in LCR cells. These results are in line with several studies that have reported the effect of Pramipexole on promoting neuronal cell proliferation (221, 222), and the role of D3R stimulation in improving neurological function, learning, and memory in a model of global cerebral ischemia/reperfusion (71, 229). Stimulation of D3R has also been suggested as playing a protective role toward the attenuation of renal

ischemia/reperfusion injury (230). However, D3R antagonist (U-99194A) in neonatal sub-ventricular neurons showed decreased cell proliferation (153). This study is opposite to what we reported here in LCR. The differences in the agents that they used for D3R antagonists and the cell type in their studies were different from our study, which could suggest that the effect of D3R inhibition on cell growth is drug, cell type dependent, and or phenotype dependent.

Cells that were stimulated with Pramipexole (D3R agonist) and treated with androgen receptor agonist, at the early time point, androgen receptor agonist in LCR decreased cell number and androgen antagonist increased cell number in LCR, but not in HCR. At late time point, the effect of androgen antagonist on cell number was no longer protective as at 6 hr, it decreased cell number in LCR while treatment with androgen receptor agonist improved the LCR cell number. On the other hand, treatment with androgen or its receptor blocker not significantly changed cell number at both time points in HCR.

These interesting data showed that intrinsic aerobic capacity is an important factor in regulating myocyte cellular response to growth through the interaction of neuroendocrine signaling pathway. In high capacity runner phenotype, AR and D3R need to interact together to enhance the cell growth at early and late time points while in LCR, early time point requires only one of these receptor to be blocked in order to enhance the growth as early as 6 hr, while at 36 hr (late time point), both receptors need to be activated in order to enhance the cell growth.

These data indicate that both phenotypes have differential mechanisms through the interactions between the two receptors in response to cell growth at early time

point. One of these mechanisms in LCR satellite cells is that suppression of cell growth occurs through activation of both AR and D3R simultaneously while activation of one of any of them with blocking the other one has an enhancement effect on cell growth. However, the mechanism for HCR satellite cell growth is different from LCR. HCR requires an activation of both receptors to enhance the growth at early time point.

These data also may explain the controversial effect of androgen and dopamine in cardiovascular diseases. The time points that are required to initiate the effect of the interaction between the two signaling pathways of AR and D3R are very important in low intrinsic aerobic capacity phenotype, which is the phenotype that is more prone to developing metabolic disease. It also may explain the detrimental effect of cardiovascular diseases in female population as compare to the male population (9,173, 240, 252). Although the effect of testosterone on cardiovascular events and mortality in women has not yet been evaluated in large cohort studies, a prospective cohort study in more than 2000 women, that were followed up after more than four years from the start of the study, found that women with the lowest levels of testosterone had the greatest risk of a cardiovascular event independent of traditional risk factors (68). In LCR satellite cells, at late time point, treatment with androgen blocker caused significant reduction in cell number as compared with treatment with androgen although this is not the case at early time point when androgen blocker worked better than androgen.

As clearly shown at early time point, HCR results infer that there is an androgen effect dependent on activation of dopamine receptor. This effect is through

the rapid non-genomic signaling pathway. This is supported by our results when we treated with androgen blocker, it did not abrogate the effect on cell growth. This results also in line with a study that showed androgen alters brain catecholamine content and blood pressure in the testicular feminized male rats. They found testosterone manipulation increases blood pressure and correlates with dopamine content in the different brain regions through non-genomic or non-classic androgen receptor signaling (49). This suggests that androgen receptor pathway requires activating dopamine D3 receptor in order to initiate cell growth at early time point possibly through non-genomic pathway.

On the other hand, LCR story at early time point is much more complicated than in HCR, because the results infer uncommon effect of DHT and D3R agonist on cell growth (opposite to HCR). LCR showed increased the growth when treated with (DHT + D3R antagonist) and with (Pramipexole + AR antagonist). In the first group, (DHT + D3R antagonist), there was no significant difference when we compared it to (DHT + D3R agonist) although cell number was increased, indicating that androgen signaling pathway may not depend on dopamine signaling pathway. In the second group (Pramipexole + AR antagonist), the cell number significantly increased as compared to cells treated with androgen instead of the blocker. This indicates that dopamine signaling pathway depends on androgen signaling, and that effect has negative response, which may be point to that dopamine D3 receptor activation interrupts androgen signaling pathway causing negative effect on cell growth. However, interestingly, this mechanism of action is not activated when androgen receptor is blocked. This result was supported by the idea that activation of inhibitory

dopamine receptor (D2R), reduced androgen receptor level through mediating protein degradation pathway in prostate cancer cell line, and induced cell-cycle arrest (247).

In HCR at late time point, receptors signaling in cell treated with DHT was similar as in the early time point although the effect between treated groups with dopamine agonist/or antagonist was not different, which may indicate that signaling pathway is mainly due to the effect of D3R activation. These results are supported by our finding that showed blocking of androgen receptor caused cell decreased cell number, and D3R activation is not enough to initiate similar effect of androgen on cell growth. This data indicates that AR signaling requires an active D3R to initiate cell growth and vice versa. The results are in line with several studies that reported androgen modulates dopamine system (98). A study showed that androgen treatment increased the activity of tyrosine hydroxylase, rate limiting enzyme in dopamine synthesis (1), and its gene expression (1, 73, 207, 217). On the other hand, dopamine also regulated androgen signaling (116). A study recorded that the ability of nandrolone (anabolic androgen steroid) to modulate motivation and reward-related effects of psychostimulant drugs, such as amphetamine, is dependent on activation of androgen receptor (116).

In LCR, late time point cell number was not different than HCR. This indicates that there is no differential effect on satellite cells growth between the phenotypes in respect to AR/D3R signaling interaction. However, at late time point, it was interesting because the effect of the signaling interaction was opposite to what we see at early time point. This indicates that LCR phenotype uses different mechanism at two different time points. These differences may be contribute to the

controversial effects of androgen and dopamine in cardiovascular disease that found in the literatures. At early time point, LCR cell number was depended on one of the receptor activation and inactivation of the other receptor. However, in the late time point, cell growth required both receptors being active in order to enhance the cell growth.

CONCLUSION

This study showed that low intrinsic aerobic capacity phenotype satellite cells have different AR/D3R signaling interaction between early and late growth time points while high intrinsic aerobic capacity phenotype have similar interaction between early and late time points, which involved activation of both receptors signaling pathways.

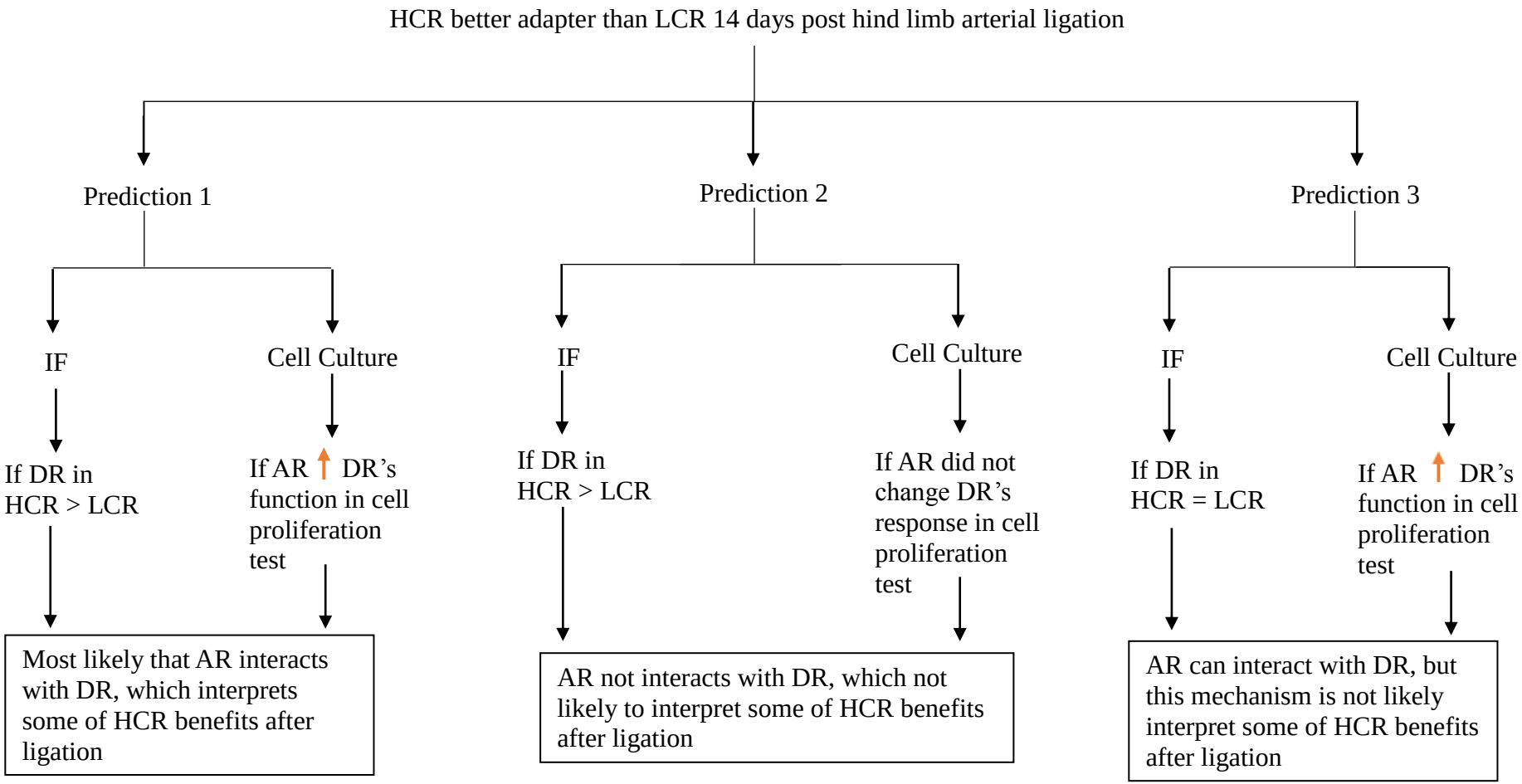

Figure 4.15. Approach to Prediction of mechanism for differences in vascular adaptive response between HCR and LCR 14 days post hind limb arterial ligation. HCR, high capacity runner rat; LCR, low capacity runner rat; IF, immunofluorescent; DR, dopamine receptor; AR, androgen receptor; Black arrow indicates the flow of the chart; Red arrow indicates increase in the parameter.

CHAPTER 5: INTRINSIC EXERCISE CAPACITY ON HIND LIMB ARTERIAL LIGATION: DIFFERENTIAL IMPACT OF ACTIVE EXERCISE AND ANDROGEN SIGNALING ON POST-ISCHEMIC RECOVERY

Peripheral arterial disease (PAD) is a common disease that affects 8.5 million people in the United States and around 202 million people worldwide (174). Exercise response in humans is determined mainly by 70% of intrinsic aerobic capacity (70%) and by active exercise (10-40%) (16). This suggests that genetic predisposition or intrinsic aerobic capacity are likely to contribute in vascular injury pathogenesis and remodeling. Studying how genetic factors interact with active exercise and determining the mechanisms that drive peripheral arterial disease in susceptible phenotypes is crucial for two reasons: first, it helps in optimizing exercise to receive beneficial outcomes; and second, it may provide an understanding of novel mechanisms in the development of PAD in intrinsic aerobic phenotypes that can contribute to the pathophysiology of the disease process.

This thesis evaluated the interaction of active exercise capacity with intrinsic aerobic capacity and their impact on musculovascular adaptive response to hind limb ischemia injury. The data presented in this thesis is built on a strong body of knowledge that high intrinsic aerobic capacity running phenotype is a disease resistant to several models and risk factors of metabolic and cardiovascular diseases. This includes factors such excess weight, insulin resistance, elevated blood pressure (239), dyslipidemia (13, 156, 218), and previous unpublished work on peripheral occlusive disease. We hypothesized that high capacity running phenotype depends on androgen/dopamine signaling as one of the adaptive mechanisms that protects against peripheral arterial ischemia, making exercise and hind limb ischemia interact in favorable responsive outcomes, opposite to the low

capacity running phenotype. Herein we discovered that rats selectively bred for high endurance running capacity (HCR) and low endurance running capacity (LCR) have different responses to exercise post-ligation in a model of peripheral arterial obstructive disease. The results also provide distinct elements supporting notion that the low endurance running capacity phenotype has a higher risk for developing exercise post-ligation injury due to insufficient priming in the anatomical structure for adapting to ischemic stress.

Additionally, in this same phenotype, treadmill exercise can precondition hind limb arterial occlusive disease through remodeling the musculovascular adaptive response. Thus, the underlying differences in HCR and LCR phenotypes might suggest there are differences in factors regulating musculovascular adaptive response, such as angiogenic and inflammatory gene expressions during ischemia. The importance of exercise capacity, exercise post–ligation as a therapeutic modality in PAD (72), exercise pre-ligation as a preventive method against developing PAD (199), and the importance of sex steroid receptors, and dopamine receptor in modulating cardiovascular stresses lead us to hypothesize: *in a model of femoral arterial occlusion, there are differences in outcomes resulting from the interaction of exercise capacity and the underlying intrinsic aerobic capacity phenotypes that modifies the AR/D3R signaling pathway during skeletal muscle injury.*

To test this hypothesis, we used a model of PAD, through ligation of the left femoral artery, that our lab previously used and evaluated in these phenotypic rats, where muscle vascular adaptations were different between female LCR & HCR phenotypes after acute ligation and without exercise. We used this model for several

reasons. It is a commonly employed hind limb ischemia model, simple, cheap, provides well-developed outcomes, develops milder ischemia, has normal rest blood flow, and has a low level of necrosis due to collateral involvement, similar to what is seen clinically in PAD patients (115). For the second part of the hypothesis, we used cell culture for an *in vitro* study of satellite cell growth to investigate the differential interaction of AR/D3R signaling pathway between the phenotypes, because we believe that knowing the pathophysiology at baseline may help understanding how these phenotypes respond differentially to ischemia. The reason for our selection to cell culture study, because it provides excellent model systems for studying the normal physiology and cell growth response to drugs with consistency and reproducibility.

To investigate the interaction between active exercise and intrinsic exercise capacities on muscle-capillary relationship and gene expressions 14d post-ligation, we divided each phenotype into exercise pre-ligation and exercise post-ligation. Exercise pre-ligation animals were exposed to exercise preconditioning before ligation (-14 days) while exercise post-ligation were exposed to exercise after ligation (beginning on day 3 post-surgery) and continuing until day 14, when the animals were sacrificed. Each exercised animal engaged in motorized treadmill running as a type of mild intensity supervised exercise training. Animals were subjected to 5 days on the treadmill at a very low speed (10 m/min) to condition them to the treadmill environment. Next, animals began exercising for sessions of 5 minutes at 14-16 m/min for 5 days per week for two weeks; the duration of exercise was gradually increased by 5 min per day, until they were trained for 60 minutes. Another group of

each phenotype was not subject to exercise sessions so as to compare the changes in the muscle-capillary relationship, angiogenic and inflammatory gene expressions, and protein expressions between the two phenotypes.

The effect of acute ligation on the phenotypes was distinct. There were not observed changes in the muscle-capillary relationship of HCR rats except for muscle hypertrophy, which may be due to the presence of body weight (resistant exercise) and blood flow restrictions (139). In LCR rats there was an observed decrease in both NCAF and SF, in addition to muscle hypertrophy. Changes in the muscle fiber area in addition to the low number of capillaries indicates the severity of ischemic muscle injury (191, 205). The differences between the phenotypes in response to arterial ligation suggest that HCR rats have an underlying intrinsic resistant mechanism, as is seen by the low muscle fiber area (88) and higher capillary density as compared to LCR (87).

Consistent with the histology results, HCR rats have no changes in angiogenic and inflammatory genes expression at day 14 post-ligation while LCR showed an increase in the gene and protein levels of Vegfa, indicating effective angiogenic drive (18). These results indicate that LCR rats respond to acute arterial ligation by initial muscle fiber atrophy and necrosis, then by functional gene expression of angiogenic markers, and lastly by angiogenesis. Since the number of capillaries did not increase, this confirms that active angiogenesis will take place later on to influence a closer muscle-capillary relationship. On the other hand, HCR rats resist the arterial ligation injury. A possible underlying mechanism may be due to the presence of functional collateral circulation.

Some of the most interesting findings in this overall project resulted from our study of exercise post- (discussed in chapter 2) and exercise pre-ligation (discussed in chapter 3). Exercise post-ligation was observed to improve the muscle-capillary relationship in HCR while it deteriorated the same relationship in LCR. Additionally, although exercise pre-ligation improved the ischemia in LCR, there was almost no observed effect on ischemia in HCR! In exercise post-ligation, muscle capillary parameters in HCR rats, including CD, CFPE, and NCAF were increased while they were decreased in LCR rats. In HCR, muscle cross-sectional area was lower than LCR with higher CD and CFPE, indicating surviving muscle tissue underlying the muscle atrophy mechanism, despite lower Vegfa gene expression. In LCR, CD decreased with association of an 8-fold increase in muscle cross-sectional area, suggesting the presence of muscle tissue necrosis due to blood flow restriction (139) and compensatory hypertrophy. In LCR, lower CFPE is due to decreases in both CD and number of fibers, which in turn may be due to necrosis and increased mean muscle fiber cross-sectional area.

Taken together, these results show that HCR responds to exercise post-ligation by increasing CD, muscle atrophy, and collateral artery involvement, or faster angiogenic response. Therefore, it is expected to have accelerated muscle regeneration and normotrophy. On the other hand, LCR may have responded to exercise post-ligation via early muscle necrosis with compensatory hypertrophy, increased expression of angiogenic markers, late capillary formation, and insufficient early collateral artery involvement, as is explained by lower share factor-related inflammatory gene expressions in comparison with HCR.

In exercise pre-ligation, the muscle-capillary relationship caused improved outcomes in LCR while there was no effect in HCR. Increased mean cross-sectional area, NCAF, SF, and CFPE are all favorable effects of muscle-capillary parameters in LCR, with increased angiogenic gene expression (Vegfa, Flt1, Kdr, and Angiopoietin 2).

Although our study was on permanent occlusion, this data is similar to a study that showed when exercise was followed by intermittent occlusion of the lower limb, muscle structure and function was improved (172). Interestingly, the adhesion molecules Icam and Vcam were elevated in LCR compared to HCR. Icam and Vcam have been reported to be associated with endothelial dysfunction (56). This may suggest that low intrinsic aerobic capacity phenotype has a higher risk to develop endothelial dysfunction with the possibility of an increased risk of atherosclerosis and atherothrombosis; furthermore, exercise pre-ligation may not protect from endothelial dysfunction. In HCR rats subjected to exercise before ligation, we did not find changes in muscle-capillary interaction. However, there was only elevated gene expression in Pdgfa, one of the share stress-related inflammatory genes, indicating that Pdgfa alone is not enough to improve the muscle-capillary relationship in exercise pre-ligation.

Interestingly, LCR rats showed elevation in Pdgfrb and not Pdgfa, indicating different myogenesis signaling pathways between the phenotypes, since HCR may rely more on Pdgfa than Pdgfb. Accordingly, Pdgfa ligand only binds to the Pdgfa receptor, while LCR may rely more on Pdgfb/Pdgfd than Pdgfa since Pdgfb/Pdgfd have high affinities to Pdgfrb. These results are important to understand the

pathophysiology of the interaction between exercise capacity and intrinsic aerobic capacity with respect to peripheral arterial disease. It is inferred that each PAD patient needs to be treated differently in respect to preventing the onset of the disease. Although this project will add additional knowledge to this field of study, further work is needed to determine specific targets that successfully translate clinically toward sorting PAD patients into "high and low capacity runners" for improved disease outcomes.

As can be seen in the beginning of this dissertation, clinical and basic experimental studies have provided evidence about the atheroprotective effect of estrogen, testosterone, and dopamine on blood vessels. Herein, we were wondering whether the background of these phenotypes differentially modulate these receptors, specifically AR and Dopamine receptor, as these receptors commonly associated with controversial effect on cardiovascular disease. The heterogeneity of these phenotypes makes studying these receptors interesting. To determine whether there are differential associations of sex steroid receptors (AR and ERα) and dopamine receptors (D1R and D3R) between HCR and LCR after acute femoral arterial ligation, protein and gene expression were investigated from gastrocnemius muscle of ischemic and non-ischemic HCR and LCR rats. The results of this study showed that AR/ERα gene ratio was differentially expressed between the phenotypes, in that HCR rats have a higher ratio than LCR rats (P=0.005). However, the protein expression ratio of these receptors was not significantly changed between the phenotypes. D1R and D3R protein expressions were also not significantly different between the phenotypes. We also evaluated the protein expression of D1R and D3R in WT and

D3KO mouse to determine whether there are differential changes at baseline. Nonetheless, we found there are no differences between WT and D3KO in expression of D1R and D3R. Therefore, we concluded that there is possible functional knockout (251) of these receptors in HCR and LCR.

Here we conducted in vitro experiment to study satellite cell growth at two different time points to investigate the early effects of signaling pathway interaction at 6 hr and the late effect at 36 hr. To evaluate the role of these receptors and their interactions with each other in satellite cell growth, we performed a satellite cell growth study at two different time points, early time point that was tested at 6 hr, and late time point that was tested at 36 hr. We chose two time points because of the limitation in using primary stem cells isolated from adult animal, where cell yield is low. These time points were based on the several conducted experiments that we have done on satellite cells of Sprague Dawley rats and fibroblasts that isolated from mice. This preliminary data showed that androgen has a late effect after 24 hr and dopamine effect occurred at early hours. Cells from each phenotype were incubated with various combinations of the drugs: DHT (10 nM), Pramipexole (10μM), Flutamide (50 nM), and SB277011A (10μM).

In vitro study showed that intrinsic aerobic capacity is an important factor in regulating cellular response to growth through the interaction of neuroendocrine signaling pathway. In high capacity runner phenotype, AR and D3R need to interact together to enhance the cell growth at early and late time points while in LCR, at early time point, cells requires only one of these receptor to be blocked in order to replicate as early as 6 hr, while at 36 hr (late time point), both receptors need to be activated in order

to enhance the cell growth. These results suggesting that both phenotypes have differential molecular mechanisms and interactions between the two receptors in response to cell growth at early time point.

This data also may explain the controversial effect of androgen and dopamine in cardiovascular diseases. The time points of the effect of the interaction between the two signaling pathways of AR and D3R are very important in low intrinsic aerobic capacity phenotype, which is the phenotype that is more prone to developing metabolic disease. It also may explain the detrimental effect of cardiovascular diseases in female population as compare to the male population (9,173, 240, 252).

In response to D3R agonist, early time point LCR cells showed better response to cell growth with Androgen blocker. However, this effect is no longer present at late time point. Oppositely, in HCR, androgen has no detrimental effect neither at early time point nor at late time point. This is interesting finding! This finding may explain some of the differential changes in vascular adaptive response to ischemia alone or to ischemia with exercise between the phenotypes. In both situations, HCR phenotype was protected from the ischemic effect while LCR was severely deteriorated (without exercise and with exercise post-ligation). It is possible that the negative outcomes that LCR faced 14 days after ligation could be due to the early negative effect of the interaction of androgen receptor with dopamine signaling pathway. This effect might cause delay in muscle repair as it was shown, *in vitro,* suppressing the satellite cell growth. Satellite cells play crucial role in skeletal muscle regeneration which also depend on androgen receptor signaling pathway. In LCR, this

signaling pathway for somehow interacts negatively while in HCR this signaling interaction is more favorable causing them to grow.

This data also might explain why LCR responded to exercise pre-ligation better than exercise post-ligation. At early time point, LCR cells showed decreased in the cell number when treated with D3R agonist in response to androgen. It has been reported that androgen decreased the gene expression of D3R in the brain (185). Our data suggests that this signaling occurs through genomic pathway. When cells treated with androgen blocker, it caused an increase in cell number. Since this mechanism takes hours to days to occur, exercise pre-ligation may helpful in the LCR phenotype to precondition this negative effect resulting from the interaction between AR and D3R. This preconditioning masked the early effect of this interaction and allow for better late response to face the insult, causing better outcome, as when exposed to ligation after the exercise. In exercise post-ligation, LCR cannot stop the reaction and the muscle will go through the early phase of this signaling which faces the time when ischemic injury occurs, making it difficult to rehab rapidly.

References

1. **Adler A, Vescovo P, Robinson JK and Kritzer MF.** Gonadectomy in adult life increases tyrosine hydroxylase immunoreactivity in the prefrontal cortex and decreases open field activity in male rats. *Neuroscience* 89: 3: 939-954, 1999.

2. **Ahimastos AA, Pappas EP, Buttner PG, Walker PJ, Kingwell BA and Golledge J.** A meta-analysis of the outcome of endovascular and noninvasive therapies in the treatment of intermittent claudication. *J. Vasc. Surg.* 54: 5: 1511-1521, 2011.

3. **Almeida OP, Yeap BB, Hankey GJ, Jamrozik K and Flicker L.** Low free testosterone concentration as a potentially treatable cause of depressive symptoms in older men. *Arch. Gen. Psychiatry* 65: 3: 283-289, 2008.

4. **Araki T, Kato H, Shuto K, Fujiwara T and Itoyama Y.** Effect of cerebral ischemia on dopamine receptors and uptake sites in the gerbil hippocampus. *Eur. Neuropsychopharmacol.* 7: 4: 275-282, 1997.

5. **Arras M, Ito WD, Scholz D, Winkler B, Schaper J and Schaper W.** Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. *J. Clin. Invest.* 101: 1: 40-50, 1998.

6. **Arreola R, Alvarez-Herrera S, Perez-Sanchez G, Becerril-Villanueva E, Cruz-Fuentes C, Flores-Gutierrez EO, Garces-Alvarez ME, de la Cruz-Aguilera DL, Medina-Rivero E, Hurtado-Alvarado G, Quintero-Fabian S and Pavon L.** Immunomodulatory Effects Mediated by Dopamine. *J. Immunol. Res.* 2016: 3160486, 2016.

7. **Baffour R, Garb JL, Kaufman J, Berman J, Rhee SW, Norris MA and Friedmann P.** Angiogenic therapy for the chronically ischemic lower limb in a rabbit model. *J. Surg. Res.* 93: 2: 219-229, 2000.

8. **Ballermann BJ, Dardik A, Eng E and Liu A.** Shear stress and the endothelium. *Kidney Int. Suppl.* 67: S100-8, 1998.

9. **Bauer T, Boeder N, Nef HM, Mollmann H, Hochadel M, Marco J, Weidinger F, Zeymer U, Gitt AK and Hamm CW.** Fate of Patients With Coronary Perforation Complicating Percutaneous Coronary Intervention (from the Euro Heart Survey Percutaneous Coronary Intervention Registry). *Am. J. Cardiol.* 116: 9: 1363-1367, 2015.

10. **Belch JJ, Topol EJ, Agnelli G, Bertrand M, Califf RM, Clement DL, Creager MA, Easton JD, Gavin JR,3rd, Greenland P, Hankey G, Hanrath P, Hirsch AT, Meyer J, Smith SC, Sullivan F, Weber MA and Prevention of Atherothrombotic Disease Network.** Critical issues in peripheral arterial disease detection and management: a call to action. *Arch. Intern. Med.* 163: 8: 884-892, 2003.

11. **Beunen G and Thomis M.** Genetic determinants of sports participation and daily physical activity. *Int. J. Obes.* 23: S55-S63, 1999.

12. **Bhagat B and Wheeler N.** Effect of amphetamine on the swimming endurance of rats. *Neuropharmacology* 12: 7: 711-713, 1973.

13. **Bikman BT, Woodlief TL, Noland RC, Britton SL, Koch LG, Lust RM, Dohm GL and Cortright RN.** High-fat diet induces Ikkbeta and reduces insulin sensitivity in rats with low running capacity. *Int. J. Sports Med.* 30: 9: 631-635, 2009.

14. **Blair SN.** Physical Fitness and All-Cause Mortality. *JAMA* 262: 17: 2395, 1989.

15. **Blau HM and Webster C.** Isolation and characterization of human muscle cells. *Proc. Natl. Acad. Sci. U.S.A.* 78: 9: 5623-5627, 1981.

16. **BOUCHARD C, LESAGE R, LORTIE G, SIMONEAU J, HAMEL P, BOULAY MR, PERUSSE L, THERIAULT G and LEBLANC C.** Aerobic performance in brothers, dizygotic and monozygotic twins. *Med. Sci. Sports Exer.* 18: 6: 639-646, 1986.

17. **Braddock M, Schwachtgen JL, Houston P, Dickson MC, Lee MJ and Campbell CJ.** Fluid Shear Stress Modulation of Gene Expression in Endothelial Cells. *News Physiol. Sci.* 13: 241-246, 1998.

18. **Brandao D, Costa C, Canedo A, Vaz G and Pignatelli D.** Endogenous vascular endothelial growth factor and angiopoietin-2 expression in critical limb ischemia. *Int. Angiol.* 30: 1: 25-34, 2011.

19. **BRAY MS, HAGBERG JM, PÃRUSSE L, RANKINEN T, ROTH SM, WOLFARTH B and BOUCHARD C.** The Human Gene Map for Performance and Health-Related Fitness Phenotypes. *Med. Sci. Sports Exer.* 41: 1: 35-73, 2009.

20. **Britton SL and Koch LG.** Animal models of complex diseases: an initial strategy. *IUBMB Life* 57: 9: 631-638, 2005.

21. **Buckwalter JB, Curtis VC, Valic Z, Ruble SB and Clifford PS.** Endogenous vascular remodeling in ischemic skeletal muscle: a role for nitric oxide. *J. Appl. Physiol.(1985)* 94: 3: 935-940, 2003.

22. **Buschmann IR, Hoefer IE, van Royen N, Katzer E, Braun-Dulleaus R, Heil M, Kostin S, Bode C and Schaper W.** GM-CSF: a strong arteriogenic factor acting by amplification of monocyte function. *Atherosclerosis* 159: 2: 343-356, 2001.

23. **Cao Y, Cao R and Hedlund EM.** R Regulation of tumor angiogenesis and metastasis by FGF and PDGF signaling pathways. *J. Mol. Med. (Berl)* 86: 7: 785-789, 2008.

24. **Castro-Hernández, J., Afonso-Oramas, D., Cruz-Muros, I., Salas-Hernández, J., Barroso-Chinea, P., Moratalla, R., Millan, M.J. & González-Hernández, T.** Prolonged treatment with pramipexole promotes physical interaction of striatal dopamine D3 autoreceptors with dopamine transporters to reduce dopamine uptake. *Neurobiology of Disease*, vol. 74, pp. 325-335, 2014.

25. **Cavallotti C, Mancone M, Bruzzone P, Sabbatini M and Mignini F.** Dopamine receptor subtypes in the native human heart. *Heart Vessels* 25: 5: 432-437, 2010.

26. **Chalothorn D, Clayton JA, Zhang H, Pomp D and Faber JE.** Collateral density, remodeling, and VEGF-A expression differ widely between mouse strains. *Physiol. Genomics* 30: 2: 179-191, 2007.

27. **Chang CK, Chou W, Lin HJ, Huang YC, Tang LY, Lin MT and Chang CP.** Exercise preconditioning protects against spinal cord injury in rats by upregulating neuronal and astroglial heat shock protein 72. *Int. J. Mol. Sci.* 15: 10: 19018-19036, 2014.

28. **Charge SB and Rudnicki MA.** Cellular and molecular regulation of muscle regeneration. *Physiol. Rev.* 84: 1: 209-238, 2004.

29. **Chen D, Zhao M and Mundy GR.** Bone morphogenetic proteins. *Growth Factors* 22: 4: 233-241, 2004.

30. **Chiu JJ, Wung BS, Hsieh HJ, Lo LW and Wang DL.** Nitric oxide regulates shear stress-induced early growth response-1. Expression via the extracellular signal-regulated kinase pathway in endothelial cells. *Circ. Res.* 85: 3: 238-246, 1999.

31. **Coffey VG, Shield A, Canny BJ, Carey KA, Cameron-Smith D and Hawley JA.** Interaction of contractile activity and training history on mRNA abundance in skeletal muscle from trained athletes. *Am. J. Physiol. Endocrinol. Metab.* 290: 5: E849-55, 2006.

32. **Comhaire F.** Hormone replacement therapy and longevity. *Andrologia* 48: 1: 65-68, 2016.

33. **Cooter GR and Stull GA.** The effect of amphetamine on endurance in rats. *J.Sports Med. Phys. Fitness* 14: 2: 120-126, 1974.

34. **Danoviz ME and Yablonka-Reuveni Z.** Skeletal muscle satellite cells: background and methods for isolation and analysis in a primary culture system. *Methods Mol. Biol.* 798: 21-52, 2012.

35. **Davey DA.** Androgens in women before and after the menopause and post bilateral oophorectomy: clinical effects and indications for testosterone therapy. *Womens Health. (Lond)* 8: 4: 437-446, 2012.

36. **Davis JM and Bailey SP.** Possible mechanisms of central nervous system fatigue during exercise. *Med. Sci. Sports Exer.* 29: 1: 45-57, 1997.

37. **Davis JM, Zhao Z, Stock HS, Mehl KA, Buggy J and Hand GA.** Central nervous system effects of caffeine and adenosine on fatigue. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284: 2: R399-404, 2003.

38. **De Lautour H, Taylor WJ, Adebajo A, Alten R, Burgos-Vargas R, Chapman P, Cimmino MA, da Rocha CP, Day R, Harrold LR, Helliwell P, Janssen M, Kerr G, Kavanaugh A, Khanna D, Khanna PP, Lin C, Louthrenoo W, McCarthy G, Vazquez-Mellado J, Mikuls TR, Neogi T, Ogdie A, Perez-Ruiz F, Schlesinger N, Ralph Schumacher H, ScirÃ¨ CA, Singh JA, Sivera F, Slot O, Stamp LK, Tausche A, Terkeltaub R, Uhlig T, van dL, White D, Yamanaka H, Zeng X and Dalbeth N.** Development of Preliminary Remission Criteria for Gout Using Delphi and 1000Minds Consensus Exercises. *Arthritis Care Res*. 68: 5: 667-672, 2016.

39. **De Moor, M. H. M., Stubbe JH, Boomsma DI and De Geus, E. J. C.** Exercise participation and self-rated health: Do common genes explain the association? *Eur. J. Epidemiol.* 22: 1: 27-32, 2006.

40. **Derevenco P, Stoica N, Sovrea I and Imreh S.** Central and peripheral effects of 6-hydroxydopamine on exercise performance in rats. *Psychoneuroendocrinology* 11: 2: 141-153, 1986.

41. **Ding Y, Li J, Luan X, Ding YH, Lai Q, Rafols JA, Phillis JW, Clark JC and Diaz FG.** Exercise pre-conditioning reduces brain damage in ischemic rats that may be associated with regional angiogenesis and cellular overexpression of neurotrophin. *Neuroscience* 124: 3: 583-591, 2004.

42. **Ding YH, Young CN, Luan X, Li J, Rafols JA, Clark JC, McAllister JP,2nd and Ding Y.** Exercise preconditioning ameliorates inflammatory injury in ischemic rats during reperfusion. *Acta Neuropathol.* 109: 3: 237-246, 2005.

43. **Donadelli R, Abbate M, Zanchi C, Corna D, Tomasoni S, Benigni A, Remuzzi G and Zoja C.** Protein traffic activates NF-kB gene signaling and promotes MCP-1-dependent interstitial inflammation. *Am. J. Kidney Dis.* 36: 6: 1226-1241, 2000.

44. **Doumit ME and Merkel RA.** Conditions for isolation and culture of porcine myogenic satellite cells. *Tissue Cell* 24: 2: 253-262, 1992.

45. **Dufour SP, Ponsot E, Zoll J, Doutreleau S, Lonsdorfer-Wolf E, Geny B, Lampert E, FlÃ¼ck M, Hoppeler H, Billat V, Mettauer B, Richard R and Lonsdorfer J.** Exercise training in normobaric hypoxia in endurance runners. I. Improvement in aerobic performance capacity. *J. Appl. Physiol.* 100: 4: 1238-1248, 2006**.**

46. **Dupont-Versteegden EE, Fluckey JD, Knox M, Gaddy D and Peterson CA.** Effect of flywheel-based resistance exercise on processes contributing to muscle atrophy during unloading in adult rats. *J. Appl. Physiol.(1985)* 101: 1: 202-212, 2006.

47. **Duscha BD, Robbins JL, Jones WS, Kraus WE, Lye RJ, Sanders JM, Allen JD, Regensteiner JG, Hiatt WR and Annex BH.** Angiogenesis in skeletal muscle precede improvements in peak oxygen uptake in peripheral artery disease patients. *Arterioscler. Thromb. Vasc. Biol.* 31: 11: 2742-2748, 2011.

48. **Egan B and Zierath JR.** Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell. Metab.* 17: 2: 162-184, 2013.

49. **Ely D, Toot J, Salisbury R and Ramirez R.** Androgens alter brain catecholamine content and blood pressure in the testicular feminized male rat. *Clin. Exp. Hypertens.* 33: 2: 124-132, 2011.

50. **Eriksson M, Rasmussen F and Tynelius P.** Genetic Factors in Physical Activity and the Equal Environment Assumption â€“ the Swedish Young Male Twins Study. *Behav. Genet.* 36: 2: 238-247, 2006.

51. **Fagiani E and Christofori G.** Angiopoietins in angiogenesis. *Cancer Lett.* 328: 1: 18-26, 2013.

52. **Feng X, Degos V, Koch LG, Britton SL, Zhu Y, Vacas S, Terrando N, Nelson J, Su X and Maze M.** Surgery Results in Exaggerated and Persistent Cognitive Decline in a Rat Model of the Metabolic Syndrome. *Anesthesiology* 118: 5: 1098-1105, 2013.

53. **Foley TE, Greenwood BN, Day HE, Koch LG, Britton SL and Fleshner M.** Elevated central monoamine receptor mRNA in rats bred for high endurance capacity: implications for central fatigue. *Behav. Brain Res.* 174: 1: 132-142, 2006.

54. **Fontana L, Klein S and Holloszy JO.** Effects of long-term calorie restriction and endurance exercise on glucose tolerance, insulin action, and adipokine production. *Age (Dordr)* 32: 1: 97-108, 2010.

55. **Fu, R., Liu, J., Fan, J., Li, R., Li, D., Yin, J. & Cui, S.** Novel evidence that testosterone promotes cell proliferation and differentiation via G protein-coupled receptors in the rat L6 skeletal muscle myoblast cell line. *Journal of Cellular Physiology* 227: 1: 98-107, 2012.

56. **Fuster V, Moreno PR, Fayad ZA, Corti R and Badimon JJ.** Atherothrombosis and high-risk plaque: part I: evolving concepts. *J. Am. Coll. Cardiol.* 46: 6: 937-954, 2005.

57. **Gale NW and Yancopoulos GD.** Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev.* 13: 9: 1055-1066, 1999.

58. **Garcia Barrado MJ, Iglesias Osma MC, Blanco EJ, Carretero Hernandez M, Sanchez Robledo V, Catalano Iniesta L, Carrero S and Carretero J.** Dopamine modulates insulin release and is involved in the survival of rat pancreatic beta cells. *PLoS One* 10: 4: e0123197, 2015.

59. **Garcia-Cardena G, Comander J, Anderson KR, Blackman BR and Gimbrone MA,Jr.** Biomechanical activation of vascular endothelium as a determinant of its functional phenotype. *Proc. Natl. Acad. Sci. U.S.A.* 98: 8: 4478-4485, 2001.

60. **Gardner AW, Montgomery PS and Parker DE.** Physical activity is a predictor of all-cause mortality in patients with intermittent claudication. *J. Vasc. Surg.* 47: 1: 117-122, 2008.

61. **Gerald MC.** Effects of (+)-amphetamine on the treadmill endurance performance of rats. *Neuropharmacology* 17: 9: 703-704, 1978.

62. **Gesta S, Tseng YH and Kahn CR.** Developmental origin of fat: tracking obesity to its source. *Cell* 131: 2: 242-256, 2007.

63. **Gharaibeh B, Lu A, Tebbets J, Zheng B, Feduska J, Crisan M, Peault B, Cummins J and Huard J.** Isolation of a slowly adhering cell fraction containing stem cells from murine skeletal muscle by the preplate technique. *Nat. Protoc.* 3: 9: 1501-1509, 2008.

64. **Goncalves L, de Souza RR, Maifrino LB, Caperuto EC, Carbone PO, Rodrigues B and Gama EF.** Resistance exercise and testosterone treatment alters the proportion of numerical density of capillaries of the left ventricle of aging Wistar rats. *Aging Male* 17: 4: 243-247, 2014.

65. **Gong X, Jiang J and Zhang M.** Exercise preconditioning reduces neonatal incision surgery-induced enhanced hyperalgesia via inhibition of P38 mitogen-activated protein kinase and IL-1beta, TNF-alpha release. *Int. J. Dev. Neurosci.* 52: 46-54, 2016.

66. **Gordon CM.** Run, Jump, and Be Merry: How Much Exercise Is Needed for Building Young Bones? *J. Bone Miner. Res.* 29: 6: 1322-1324, 2014.

67. **Gorgens SW, Eckardt K, Jensen J, Drevon CA and Eckel J.** Exercise and Regulation of Adipokine and Myokine Production. *Prog. Mol. Biol. Transl. Sci.* 135: 313-336, 2015.

68. **Greenhill C.** Reproductive endocrinology: Low testosterone increases risk of cardiovascular events in women. *Nat. Rev. Endocrinol.* 6: 11: 597, 2010.

69. **Gustafsson T, Rundqvist H, Norrbom J, Rullman E, Jansson E and Sundberg CJ.** The influence of physical training on the angiopoietin and VEGF-A systems in human skeletal muscle. *J. Appl. Physiol.(1985)* 103: 3: 1012-1020, 2007.

70. **Guzman JA, Rosado AE and Kruse JA.** Dopamine-1 receptor stimulation attenuates the vasoconstrictive response to gut ischemia. *J. Appl. Physiol.(1985)* 91: 2: 596-602, 2001.

71. **Hall ED, Andrus PK, Oostveen JA, Althaus JS and VonVoigtlander PF.** Neuroprotective effects of the dopamine D2/D3 agonist pramipexole against postischemic or methamphetamine-induced degeneration of nigrostriatal neurons. *Brain Res.* 742: 1-2: 80-88, 1996.

72. **Hamburg NM and Balady GJ.** Exercise rehabilitation in peripheral artery disease: functional impact and mechanisms of benefits. *Circulation* 123: 1: 87-97, 2011.

73. **Hammond DN, Wainer BH, Tonsgard JH and Heller A.** Neuronal properties of clonal hybrid cell lines derived from central cholinergic neurons. *Science* 234: 4781: 1237-1240, 1986.

74. **Han DK, Faries PL, Chung C, Weaver MV, Tadros RO, Ting W and Vouyouka AG.** αutcomes of Femoropopliteal Stenting in Women: 3-Year Results of the DURABILITY II Trial. *Ann. Vasc. Surg.* 30: 110-117, 2016.

75. **Hart, C.A., Tsui, J., Khanna, A., Abraham, D.J. & Baker, D.M.** Stem cells of the lower limb: Their role and potential in management of critical limb ischemia, *Experimental Biology and Medicine,* vol. 238, no. 10, pp. 1118-1126, 2013

76. **Hazlehurst JM and Tomlinson JW.** Non-alcoholic fatty liver disease in common endocrine disorders. *Eur. J. Endocrinol.* 169: 2: R27-37, 2013.

77. **Heil M, Ziegelhoeffer T, Pipp F, Kostin S, Martin S, Clauss M and Schaper W.** Blood monocyte concentration is critical for enhancement of collateral artery growth. *Am. J. Physiol. Heart Circ. Physiol.* 283: 6: H2411-9, 2002.

78. **Helisch A, Wagner S, Khan N, Drinane M, Wolfram S, Heil M, Ziegelhoeffer T, Brandt U, Pearlman JD, Swartz HM and Schaper W.** Impact of mouse strain differences in innate hind limb collateral vasculature. *Arterioscler. Thromb. Vasc. Biol.* 26: 3: 520-526, 2006.

79. **Herring MP, Sailors MH and Bray MS.** Genetic factors in exercise adoption, adherence and obesity. *Obes. Rev.* 15: 1: 29-39, 2013.

80. **Heyes MP, Garnett ES and Coates G.** Central dopaminergic activity influences rats ability to exercise. *Life Sci.* 36: 7: 671-677, 1985.

81. **Hiatt WR, Armstrong EJ, Larson CJ and Brass EP.** Pathogenesis of the limb manifestations and exercise limitations in peripheral artery disease. *Circ. Res.* 116: 9: 1527-1539, 2015.

82. **Hiramoto T, Kanda Y, Satoh Y, Takishima K and Watanabe Y.** Dopamine D2 receptor stimulation promotes the proliferation of neural progenitor cells in adult mouse hippocampus. *Neuroreport* 18: 7: 659-664, 2007.

83. **Hirsch AT, Criqui MH, Treat-Jacobson D, Regensteiner JG, Creager MA, Olin JW, Krook SH, Hunninghake DB, Comerota AJ, Walsh ME, McDermott MM and Hiatt WR.** Peripheral arterial disease detection, awareness, and treatment in primary care. *JAMA* 286: 11: 1317-1324, 2001.

84. **Hoch RV and Soriano P.** Roles of PDGF in animal development. *Development* 130: 20: 4769-4784, 2003.

85. **Hoglinger GU, Rizk P, Muriel MP, Duyckaerts C, Oertel WH, Caille I and Hirsch EC.** Dopamine depletion impairs precursor cell proliferation in Parkinson disease. *Nat. Neurosci.* 7: 7: 726-735, 2004.

86. "How Much Exercise is Optimal for Heart Health?" *Harvard Health Publications. Harvard Heart Letter*, 2016.

87. **Howlett RA, Gonzalez NC, Wagner HE, Fu Z, Britton SL, Koch LG and Wagner PD.** Selected contribution: skeletal muscle capillarity and enzyme activity in rats selectively bred for running endurance. *J. Appl .Physiol.* 94: 4: 1682-1688, 2003.

88. **Howlett RA, Kirkton SD, Gonzalez NC, Wagner HE, Britton SL, Koch LG and Wagner PD.** Peripheral oxygen transport and utilization in rats following continued selective breeding for endurance running capacity. *J. Appl. Physiol.* 106: 6: 1819-1825, 2009.

89. **Hoydal MA, Wisloff U, Kemi OJ, Britton SL, Koch LG, Smith GL and Ellingsen O.** Nitric oxide synthase type-1 modulates cardiomyocyte contractility and calcium handling: association with low intrinsic aerobic capacity. *Eur. J. Cardiovasc. Prev. Rehabil.* 14: 2: 319-325, 2007.

90. **Hu FB, Willett WC, Li T, Stampfer MJ, Colditz GA and Manson JE.** Adiposity as Compared with Physical Activity in Predicting Mortality among Women. *N. Engl. J. Med.* 351: 26: 2694-2703, 2004.

91. **Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B and Vittinghoff E.** Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *JAMA* 280: 7: 605-613, 1998.

92. **Hussain T and Lokhandwala MF.** Renal dopamine receptors and hypertension. *Exp. Biol. Med. (Maywood)* 228: 2: 134-142, 2003.

93. **Hussein N, Amawi H, Karthikeyan C, Hall FS, Mittal R, Trivedi P, Ashby CR,Jr and Tiwari AK.** The dopamine D3 receptor antagonists PG01037, NGB2904, SB277011A, and U99194 reverse ABCG2 transporter-mediated drug resistance in cancer cell lines. *Cancer Lett.* 396: 167-180, 2017.

94. **Hydock DS, Lien CY, Schneider CM and Hayward R.** Exercise preconditioning protects against doxorubicin-induced cardiac dysfunction. *Med. Sci. Sports Exer.* 40: 5: 808-817, 2008.

95. **J. S, P. K, P. C and T. G.** Food consumption and body composition in mice selected for high wheel-running activity. 171: 8: 651-659, 2001.

96. **Jacobi J, Tam BY, Wu G, Hoffman J, Cooke JP and Kuo CJ.** Adenoviral gene transfer with soluble vascular endothelial growth factor receptors impairs angiogenesis and perfusion in a murine model of hind limb ischemia. *Circulation* 110: 16: 2424-2429, 2004.

97. **Jensen CB, Storgaard H, Madsbad S, Richter EA and Vaag AA.** Altered skeletal muscle fiber composition and size precede whole-body insulin resistance in young men with low birth weight. *J. Clin. Endocrinol. Metab.* 92: 4: 1530-1534, 2007.

98. **Jeong H, Kim MS, Kwon J, Kim KS and Seol W.** Regulation of the transcriptional activity of the tyrosine hydroxylase gene by androgen receptor. *Neurosci. Lett.* 396: 1: 57-61, 2006.

99. **Jose PA, Eisner GM and Felder RA.** Regulation of blood pressure by dopamine receptors. *Nephron Physiol.* 95: 2: p19-27, 2003.

100. **Kalin MF and Zumoff B.** Sex hormones and coronary disease: a review of the clinical studies. *Steroids* 55: 8: 330-352, 1990.

101. **Kalinski MI, Dluzen DE and Stadulis R.** Methamphetamine produces subsequent reductions in running time to exhaustion in mice. *Brain Res.* 921: 1-2: 160-164, 2001.

102. **Kellam S, Liu J, Leung P, Smith D, Bush JA and Olvera N.** Reducing Adiposity Indicators: How Much Exercise Dosage Is Needed? *Med. Sci. Sports Exer.* 43: 350, 2011.

103. **Kelly DM and Jones TH.** Testosterone: a vascular hormone in health and disease. *J. Endocrinol.* 217: 3: R47-R71, 2013.

104. **Khaw KT, Dowsett M, Folkerd E, Bingham S, Wareham N, Luben R, Welch A and Day N.** Endogenous testosterone and mortality due to all causes, cardiovascular disease, and cancer in men: European prospective investigation into cancer in Norfolk (EPIC-Norfolk) Prospective Population Study. *Circulation* 116: 23: 2694-2701, 2007.

105. **Khong TK, Selvanayagam VS, Sidhu SK and Yusof A.** Role of carbohydrate in central fatigue: a systematic review. *Scand. J. Med. Sci. Sports* 27: 4: 376-384, 2017.

106. **Kim Y, Wang WZ, Comte I, Pastrana E, Tran PB, Brown J, Miller RJ, Doetsch F, Molnar Z and Szele FG.** Dopamine stimulation of postnatal murine subventricular zone neurogenesis via the D3 receptor. *J. Neurochem.* 114: 3: 750-760, 2010.

107. **Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S and Epstein SE.** Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ. Res.* 94: 5: 678-685, 2004.

108. **Kinnaird T, Stabile E, Zbinden S, Burnett MS and Epstein SE.** Cardiovascular risk factors impair native collateral development and may impair efficacy of therapeutic interventions. *Cardiovasc. Res.* 78: 2: 257-264, 2008.

109. **Kishigami S and Mishina Y.** BMP signaling and early embryonic patterning. *Cytokine Growth Factor Rev.* 16: 3: 265-278, 2005.

110. **Klenotich SJ, Ho EV, McMurray MS, Server CH and Dulawa SC.** Dopamine D2/3 receptor antagonism reduces activity-based anorexia. *Transl. Psychiatry.* 5: e613, 2015.

111. **Koch LG and Britton SL**. Aerobic metabolism underlies complexity and capacity. *J. Physiol* 2007.

112. **Koch LG and Britton SL**. Artificial selection for intrinsic aerobic endurance running capacity in rats. *Physiol Genomics* 5: 45-52, 2001.

113. **Kohtz AS and Frye CA.** Dissociating behavioral, autonomic, and neuroendocrine effects of androgen steroids in animal models. *Methods Mol. Biol.* 829: 397-431, 2012.

114. **Koteja P, Garland T, Sax JK, Swallow JG and Carter PA.** Behaviour of house mice artificially selected for high levels of voluntary wheel running. *Anim. Behav.* 58: 6: 1307-1318, 1999.

115. **Krishna SM, Omer SM and Golledge J.** Evaluation of the clinical relevance and limitations of current pre-clinical models of peripheral artery disease. *Clin. Sci.(Lond)* 130: 3: 127-150, 2016.

116. **Kurling-Kailanto S, Kankaanpaa A, Hautaniemi J and Seppala T.** Blockade of androgen or estrogen receptors reduces nandrolone's ability to modulate acute reward-related neurochemical effects of amphetamine in rat brain. *Pharmacol. Biochem. Behav.* 95: 4: 422-427, 2010.

117. **Kutlu B, Darville MI, Cardozo AK and Eizirik DL.** Molecular regulation of monocyte chemoattractant protein-1 expression in pancreatic beta-cells. *Diabetes* 52: 2: 348-355, 2003.

118. **Lam SY, Liu Y, Ng KM, Lau CF, Liong EC, Tipoe GL and Fung ML.** Chronic intermittent hypoxia induces local inflammation of the rat carotid body via functional upregulation of proinflammatory cytokine pathways. *Histochem. Cell Biol.* 137: 3: 303-317, 2012.

119. **Lawler JM, Rodriguez DA and Hord JM.** Mitochondria in the middle: exercise preconditioning protection of striated muscle. *J. Physiol.* 594: 18: 5161-5183, 2016.

120. **Leamy LJ, Pomp D and Lightfoot J.** A search for quantitative trait loci controlling within-individual variation of physical activity traits in mice. 11: 1: 83, 2010.

121. **LEE I and SKERRETT PJ.** Physical activity and all-cause mortality: what is the dose-response relation? *Med. Sci. Sports Exer.* 33: S459-S471, 2001.

122. **Lee I.** Dose-Response Relation Between Physical Activity and Fitness. *JAMA* 297: 19: 2137, 2007.

123. **Legaz Arrese A, MunguÃa Izquierdo D and Serveto Galindo JR.** Physiological Measures Associated with Marathon Running Performance in High-Level Male and Female Homogeneous Groups. *Int. J. Sports Med.* 27: 4: 289-295, 2006.

124. **Lemini C, Estela Avila M, Medina M, Sanchez C, Figueroa A, Garcia-Mondragon MJ, Vargas-Vazquez A and Jimenez-Orozco A.** Proliferative Properties of 17beta-aminoestrogens in MCF-7 Human Breast Cancer Cells. *Basic Clin. Pharmacol. Toxicol.* 120: 3: 235-242, 2017.

125. **Lennon SL, Quindry J, Hamilton KL, French J, Staib J, Mehta JL and Powers SK.** Loss of exercise-induced cardioprotection after cessation of exercise. *J. Appl. Physiol.(1985)* 96: 4: 1299-1305, 2004.

126. **Lerman I, Harrison BC, Freeman K, Hewett TE, Allen DL, Robbins J and Leinwand LA.** Genetic variability in forced and voluntary endurance exercise performance in seven inbred mouse strains. *J. Appl. Physiol.* 92: 6: 2245-2255, 2002.

127. **Ley K, Gaehtgens P, Fennie C, Singer MS, Lasky LA and Rosen SD.** Lectin-like cell adhesion molecule 1 mediates leukocyte rolling in mesenteric venules in vivo. *Blood* 77: 12: 2553-2555, 1991.

128. **Li HZ, Guo J, Gao J, Han LP, Jiang CM, Li HX, Bai SZ, Zhang WH, Li GW, Wang LN, Li H, Zhao YJ, Lin Y, Tian Y, Yang GD, Wang R, Wu LY, Yang BF and Xu CQ.** Role of dopamine D2 receptors in ischemia/reperfusion induced apoptosis of cultured neonatal rat cardiomyocytes. *J. Biomed. Sci.* 18: 18-0127-18-18, 2011.

129. **Li HZ, Han LP, Jiang CM, Li H, Zhao YJ, Gao J, Lin Y, Ma SX, Tian Y, Yang BF and Xu CQ.** Effect of dopamine receptor 1 on apoptosis of cultured neonatal rat cardiomyocytes in simulated ischaemia/reperfusion. *Basic Clin. Pharmacol. Toxicol.* 102: 3: 329-336, 2008.

130. **Li Z, Yu C, Han Y, Ren H, Shi W, Fu C, He D, Huang L, Yang C, Wang X, Zhou L, Asico LD, Zeng C and Jose PA.** Inhibitory effect of D1-like and D3 dopamine receptors on norepinephrine-induced proliferation in vascular smooth muscle cells. *Am. J. Physiol. Heart Circ. Physiol.* 294: 6: H2761-8, 2008.

131. **Lightfoot JT, Turner MJ, Daves M, Vordermark A and Kleeberger SR.** Genetic influence on daily wheel running activity level. *Physiol. Genomics* 19: 3: 270-276, 2004.

132. **Lightfoot JT, Turner MJ, Pomp D, Kleeberger SR and Leamy LJ.** Quantitative trait loci for physical activity traits in mice. *Physiol. Genomics* 32: 3: 401-408, 2008.

133. **Lillioja S, Young AA, Culter CL, Ivy JL, Abbott WG, Zawadzki JK, Yki-Jarvinen H, Christin L, Secomb TW and Bogardus C.** Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. *J. Clin. Invest.* 80: 2: 415-424, 1987.

134. **Lin HY, Yu IC, Wang RS, Chen YT, Liu NC, Altuwaijri S, Hsu CL, Ma WL, Jokinen J, Sparks JD, Yeh S and Chang C.** Increased hepatic steatosis and insulin resistance in mice lacking hepatic androgen receptor. *Hepatology* 47: 6: 1924-1935, 2008.

135. **Lloyd PG, Prior BM, Li H, Yang HT and Terjung RL.** VEGF receptor antagonism blocks arteriogenesis, but only partially inhibits angiogenesis, in skeletal muscle of exercise-trained rats. *Am. J. Physiol. Heart Circ. Physiol.* 288: 2: H759-68, 2005.

136. **Lloyd PG, Prior BM, Yang HT and Terjung RL.** Angiogenic growth factor expression in rat skeletal muscle in response to exercise training. *Am. J. Physiol. Heart Circ. Physiol.* 284: 5: H1668-78, 2003.

137. **Lyu X, Li S, Peng S, Cai H, Liu G and Ran X.** Intensive walking exercise for lower extremity peripheral arterial disease: A systematic review and meta-analysis. *J. Diabetes* 8: 3: 363-377, 2016.

138. **Macera CA, Hootman JM and Sniezek JE.** Major public health benefits of physical activity. 49: 1: 122-128, 2003.

139. **Madarame H, Neya M, Ochi E, Nakazato K, Sato Y and Ishii N.** Cross-transfer effects of resistance training with blood flow restriction. *Med. Sci. Sports Exer.* 40: 2: 258-263, 2008.

140. **Mahoney EM, Wang K, Keo HH, Duval S, Smolderen KG, Cohen DJ, Steg G, Bhatt DL, Hirsch AT and Reduction of Atherothrombosis for Continued Health (REACH) Registry Investigators.** Vascular hospitalization rates and costs in patients with

peripheral artery disease in the United States. *Circ. Cardiovasc. Qual. Outcomes* 3: 6: 642-651, 2010.

141. **Maia JAR, Thomis M and Beunen G.** Genetic factors in physical activity levels. *Am. J. Prev. Med.* 23: 2: 87-91, 2002.

142. **Malena A, Pennuto M, Tezze C, Querin G, D'Ascenzo C, Silani V, Cenacchi G, Scaramozza A, Romito S, Morandi L, Pegoraro E, Russell AP, Soraru G and Vergani L.** Androgen-dependent impairment of myogenesis in spinal and bulbar muscular atrophy. *Acta Neuropathol.* 126: 1: 109-121, 2013.

143. **Marin P, Holmang S, Jonsson L, Sjostrom L, Kvist H, Holm G, Lindstedt G and Bjorntorp P.** The effects of testosterone treatment on body composition and metabolism in middle-aged obese men. *Int .J. Obes. Relat. Metab. Disord.* 16: 12: 991-997, 1992.

144. **Marin P, Oden B and Bjorntorp P.** Assimilation and mobilization of triglycerides in subcutaneous abdominal and femoral adipose tissue in vivo in men: effects of androgens. *J. Clin. Endocrinol. Metab.* 80: 1: 239-243, 1995.

145. **Mazari FA, Shahin Y, Khan JA, Samuel N, Carradice D, McCollum PT and Chetter IC.** Comparison of Use of Short Form-36 Domain Scores and Patient Responses for Derivation of Preference-Based SF6D Index to Calculate Quality-Adjusted Life Years in Patients with Intermittent Claudication. *Ann. Vasc. Surg.* 34: 164-170, 2016.

146. **McDermott MM.** Lower extremity manifestations of peripheral artery disease: the pathophysiologic and functional implications of leg ischemia. *Circ. Res.* 116: 9: 1540-1550, 2015.

147. **McFarland DC, Doumit ME and Minshall RD.** The turkey myogenic satellite cell: optimization of in vitro proliferation and differentiation. *Tissue Cell* 20: 6: 899-908, 1988.

148. **Meier P, Gloekler S, Zbinden R, Beckh S, de Marchi SF, Zbinden S, Wustmann K, Billinger M, Vogel R, Cook S, Wenaweser P, Togni M, Windecker S, Meier B and Seiler C.** Beneficial effect of recruitable collaterals: a 10-year follow-up study in patients with stable coronary artery disease undergoing quantitative collateral measurements. *Circulation* 116: 9: 975-983, 2007.

149. **Mendelsohn ME and Karas RH.** The protective effects of estrogen on the cardiovascular system. *N. Engl. J. Med.* 340: 23: 1801-1811, 1999.

150. **Milani RV and Lavie CJ.** The role of exercise training in peripheral arterial disease. *Vasc. Med.* 12: 4: 351-358, 2007.

151. **Mitchell BD, Rainwater DL, Hsueh W, Kennedy AJ, Stern MP and Maccluer JW.** Familial Aggregation of Nutrient Intake and Physical Activity. *Ann. Epidemiol.* 13: 2: 128-135, 2003.

152. **Mohamad MJ, Mohammad MA, Karayyem M, Hairi A and Hader AA.** Serum levels of sex hormones in men with acute myocardial infarction. *Neuro Endocrinol. Lett.* 28: 2: 182-186, 2007.

153. **Monteiro R, Teixeira D and Calhau C.** Estrogen signaling in metabolic inflammation. *Mediators Inflamm.* 2014: 615917, 2014.

154. **Moore LL, Lombardi DA, White MJ, Campbell JL, Oliveria SA and Ellison RC.** Influence of parents' physical activity levels on activity levels of young children. *J. Pediatr.* 118: 2: 215-219, 1991.

155. **Morrell NW.** Pulmonary hypertension due to BMPR2 mutation: a new paradigm for tissue remodeling? *Proc. Am. Thorac. Soc.* 3: 8: 680-686, 2006.

156. **Morris EM, Whaley-Connell AT, Thyfault JP, Britton SL, Koch LG, Wei Y, Ibdah JA and Sowers JR.** Low aerobic capacity and high-fat diet contribute to oxidative stress and IRS-1 degradation in the kidney. *Am. J. Nephrol.* 30: 2: 112-119, 2009.

157. **Mosnarova A, Huzulakova I, Stecova A, Slamova J and Motesicka M.** The influence of 1-month sex hormones administration on the isolated rabbit vessels reactivity. *Acta Physiol. Hung.* 82: 3: 251-256, 1994.

158. **Mourkioti F and Rosenthal N.** NF-kappaB signaling in skeletal muscle: prospects for intervention in muscle diseases. *J. Mol. Med.(Berl)* 86: 7: 747-759, 2008.

159. **Munoz-Canoves P, Scheele C, Pedersen BK and Serrano AL.** Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword? *FEBS J.* 280: 17: 4131-4148, 2013.

160. **Myers J, Prakash M, Froelicher V, Do D, Partington S and Atwood JE.** Exercise Capacity and Mortality among Men Referred for Exercise Testing. *N. Engl. J. Med.* 346: 11: 793-801, 2002.

161. **Naessen T, Bergquist J, Lind L and Kushnir MM.** Higher endogenous estrogen levels in 70-year-old women and men: an endogenous response to counteract developing atherosclerosis? *Menopause* 19: 12: 1322-1328, 2012.

162. **Naessen T, Sjogren U, Bergquist J, Larsson M, Lind L and Kushnir MM.** Endogenous steroids measured by high-specificity liquid chromatography-tandem mass spectrometry and prevalent cardiovascular disease in 70-year-old men and women. *J. Clin. Endocrinol. Metab.* 95: 4: 1889-1897, 2010.

163. **Nagel T, Resnick N, Atkinson WJ, Dewey CF,Jr and Gimbrone MA,Jr.** Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells. *J. Clin. Invest.* 94: 2: 885-891, 1994.

164. **Nagel T, Resnick N, Dewey CF,Jr and Gimbrone MA,Jr.** Vascular endothelial cells respond to spatial gradients in fluid shear stress by enhanced activation of transcription factors. *Arterioscler. Thromb. Vasc. Biol.* 19: 8: 1825-1834, 1999.

165. **Neve KA, Seamans JK and Trantham-Davidson H.** Dopamine receptor signaling. *J. Recept. Signal Transduct. Res.* 24: 3: 165-205, 2004.

166. **Neves SR, Ram PT and Iyengar R.** G protein pathways. *Science* 296: 5573: 1636-1639, 2002.

167. **Newcomer SC, Thijssen DH and Green DJ.** Effects of exercise on endothelium and endothelium/smooth muscle cross talk: role of exercise-induced hemodynamics. *J. Appl. Physiol.(1985)* 111: 1: 311-320, 2011.

168. **Nogueira-Ferreira R, Moreira-Goncalves D, Silva AF, Duarte JA, Leite-Moreira A, Ferreira R and Henriques-Coelho T.** Exercise preconditioning prevents MCT-induced right ventricle remodeling through the regulation of TNF superfamily cytokines. *Int. J. Cardiol.* 203: 858-866, 2016.

169. **O'Donovan G, Blazevich AJ, Boreham C, Cooper AR, Crank H, Ekelund U, Fox KR, Gately P, Giles-Corti B, Gill JMR, Hamer M, McDermott I, Murphy M, Mutrie N, Reilly JJ, Saxton JM and Stamatakis E.** The ABC of Physical Activity for Health: A consensus statement from the British Association of Sport and Exercise Sciences. *J. Sports Sci.* 28: 6: 573-591, 2010.

170. **Olashaw NE, Kowalik TF, Huang ES and Pledger WJ.** Induction of NF-kappa B-like activity by platelet-derived growth factor in mouse fibroblasts. *Mol. Biol. Cell* 3: 10: 1131-1139, 1992.

171. **Onoue Y, Kuwatsuka K, Miyazaki I, Asanuma M, Kitamura Y and Sendo T.** Effects of bupropion and pramipexole on cell proliferation in the hippocampus of adrenocorticotropic hormone-treated rats. *Biol. Pharm. Bull.* 37: 2: 327-330, 2014.

172. **Page W, Swan R and Patterson SD.** The effect of intermittent lower limb occlusion on recovery following exercise-induced muscle damage: A randomized controlled trial. *J. Sci. Med. Sport* 20: 8: 729-733, 2017.

173. **Panettieri RA,Jr, Lazaar AL, Pure E and Albelda SM.** Activation of cAMP-dependent pathways in human airway smooth muscle cells inhibits TNF-alpha-induced ICAM-1 and VCAM-1 expression and T lymphocyte adhesion. *J. Immunol.* 154: 5: 2358-2365, 1995.

174. **Parizkova J.** Impact of daily work-load during pregnancy on the microstructure of the rat heart in male offspring. *Eur .J. Appl. Physiol. Occup. Physiol.* 34: 4: 323-326, 1975.

175. **Pedersen BK and Saltin B.** Evidence for prescribing exercise as therapy in chronic disease. 16: 3-63, 2006.

176. **Pedersen BK.** The diseasome of physical inactivity--and the role of myokines in muscle--fat cross talk. *J. Physiol.* 587: Pt 23: 5559-5568, 2009.

177. **Peterson JC, Charlson ME, Wells MT and Altemus M.** Depression, Coronary Artery Disease, and Physical Activity: How Much Exercise Is Enough? *Clin. Ther.* 36: 11: 1518-1530, 2014.

178. **Pierce GF, Mustoe TA, Altrock BW, Deuel TF and Thomason A.** Role of platelet-derived growth factor in wound healing. *J. Cell. Biochem.* 45: 4: 319-326, 1991.

179. **Pittaluga M, Casini B and Parisi P.** Physical Activity and Genetic Influences in Risk Factors and Aging: A Study on Twins. *Int. J. Sports Med.* 25: 5: 345-350, 2004.

180. **Popolo M, McCarthy DM and Bhide PG.** Influence of dopamine on precursor cell proliferation and differentiation in the embryonic mouse telencephalon. *Dev. Neurosci.* 26: 2-4: 229-244, 2004.

181. **Porcari, John P., 1955, Cedric X. Bryant 1960, and Fabio Comana.** *Exercise Physiology.* F.A. Davis Company, Philadelphia, PA, 2015.

182. **Pour AE, Parvizi J, Sharkey PF, Hozack WJ and Rothman RH.** Minimally invasive hip arthroplasty: what role does patient preconditioning play? *J. Bone Joint Surg. Am.* 89: 9: 1920-1927, 2007.

183. **Povel CM, Boer JMA, Reiling E and Feskens EJM.** Genetic variants and the metabolic syndrome: a systematic review. *Obes. Rev*. 12: 11: 952-967, 2011.

184. **Prior BM, Lloyd PG, Ren J, Li H, Yang HT, Laughlin MH and Terjung RL.** Time course of changes in collateral blood flow and isolated vessel size and gene expression after femoral artery occlusion in rats. *Am. J. Physiol. Heart Circ. Physiol.* 287: 6: H2434-47, 2004.

185. **Purves-Tyson TD, Owens SJ, Double KL, Desai R, Handelsman DJ, Weickert CS.** Testosterone induces molecular changes in dopamine signaling pathway molecules in the adolescent male rat nigrostriatal pathway. *PLoS One*. 2014;9(3):e91151. Published 2014 Mar 11. doi:10.1371/journal.pone.0091151

186. **Rando TA and Blau HM.** Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J. Cell Biol.* 125: 6: 1275-1287, 1994.

187. **Recalde A, Richart A, Guerin C, Cochain C, Zouggari Y, Yin KH, Vilar J, Drouet I, Levy B, Varoquaux O and Silvestre JS.** Sympathetic nervous system regulates bone marrow-derived cell egress through endothelial nitric oxide synthase activation: role in postischemic tissue remodeling. *Arterioscler. Thromb. Vasc. Biol.* 32: 3: 643-653, 2012.

188. **Reichart DL, Hinkle RT, Lefever FR, Dolan ET, Dietrich JA, Sibley DR and Isfort RJ.** Activation of the dopamine 1 and dopamine 5 receptors increase skeletal muscle mass

and force production under non-atrophying and atrophying conditions. *BMC Musculoskelet. Disord.* 12: 27-2474-12-27, 2011.

189. **Ricci A, Mignini F, Tomassoni D and Amenta F.** Dopamine receptor subtypes in the human pulmonary arterial tree. *Auton. Autacoid Pharmacol.* 26: 4: 361-369, 2006.

190. **Richler C and Yaffe D.** The in vitro cultivation and differentiation capacities of myogenic cell lines. *Dev. Biol.* 23: 1: 1-22, 1970.

191. **Ripoll E, Sillau AH and Banchero N.** Changes in the capillarity of skeletal muscle in the growing rat. *Pflugers Arch.* 380: 2: 153-158, 1979.

192. **Rooks DS, Huang J, Bierbaum BE, Bolus SA, Rubano J, Connolly CE, Alpert S, Iversen MD and Katz JN.** Effect of preoperative exercise on measures of functional status in men and women undergoing total hip and knee arthroplasty. *Arthritis Rheum.* 55: 5: 700-708, 2006.

193. **Sakano D, Choi S, Kataoka M, Shiraki N, Uesugi M, Kume K and Kume S.** Dopamine D2 Receptor-Mediated Regulation of Pancreatic beta Cell Mass. *Stem Cell Reports* 7: 1: 95-109, 2016.

194. **Sarkar C, Ganju RK, Pompili VJ and Chakroborty D.** Enhanced peripheral dopamine impairs post-ischemic healing by suppressing angiotensin receptor type 1 expression in endothelial cells and inhibiting angiogenesis. *Angiogenesis* 20: 1: 97-107, 2017.

195**. Sawada H, Saito T, Nickel NP, Alastalo TP, Glotzbach JP, Chan R, Haghighat L, Fuchs G, Januszyk M, Cao A, Lai YJ, Perez Vde J, Kim YM, Wang L, Chen PI,**

Spiekerkoetter E, Mitani Y, Gurtner GC, Sarnow P and Rabinovitch M. Reduced BMPR2 expression induces GM-CSF translation and macrophage recruitment in humans and mice to exacerbate pulmonary hypertension. *J. Exp. Med.* 211: 2: 263-280, 2014.

196. **Schainfeld RM and Isner JM.** Critical limb ischemia: nothing to give at the office? *Ann. Intern. Med.* 130: 5: 442-444, 1999.

197. **Schaper W and Scholz D.** Factors regulating arteriogenesis. *Arterioscler. Thromb. Vasc. Biol.* 23: 7: 1143-1151, 2003.

198. **Schaper W.** Collateral circulation: past and present. *Basic Res. Cardiol.* 104: 1: 5-21, 2009.

199. **Schiattarella GG, Perrino C, Magliulo F, Carbone A, Bruno AG, De Paulis M, Sorropago A, Corrado RV, Bottino R, Menafra G, Abete R, Toscano E, Giugliano G, Trimarco B and Esposito G.** Physical activity in the prevention of peripheral artery disease in the elderly. *Front. Physiol.* 5: 12, 2014.

200. **Sewdarsen M, Vythilingum S, Jialal I, Desai RK and Becker P.** Abnormalities in sex hormones are a risk factor for premature manifestation of coronary artery disease in South African Indian men. *Atherosclerosis* 83: 2-3: 111-117, 1990.

201. **Shefer G, Rauner G, Yablonka-Reuveni Z and Benayahu D.** Reduced satellite cell numbers and myogenic capacity in aging can be alleviated by endurance exercise. *PLoS One* 5: 10: e13307, 2010.

202. **SHEPHARD RJ.** Absolute versus relative intensity of physical activity in a dose-response context. *Med. Sci. Sports Exerc.* 33: S400-S418, 2001.

203. **Sherman JA, Hall A, Malenka DJ, De Muinck ED and Simons M.** Humoral and cellular factors responsible for coronary collateral formation. *Am. J. Cardiol.* 98: 9: 1194-1197, 2006.

204. **Shorten GD.** Fenoldopam: potential clinical applications in heart surgery. *Rev. Esp. Anestesiol. Reanim.* 48: 10: 487-491, 2001.

205. **Sillau AH and Banchero N.** Effect of maturation on capillary density, fiber size and composition in rat skeletal muscle. *Proc. Soc. Exp. Biol. Med*. 154: 3: 461-466, 1977.

206. **Simard J, Luthy I, Guay J, Belanger A and Labrie F.** Characteristics of interaction of the antiandrogen flutamide with the androgen receptor in various target tissues. *Mol. Cell. Endocrinol.* 44: 3: 261-270, 1986.

207. **Simerly RB, Young BJ, Capozza MA and Swanson LW.** Estrogen differentially regulates neuropeptide gene expression in a sexually dimorphic olfactory pathway. *Proc. Natl. Acad. Sci. U.S.A.* 86: 12: 4766-4770, 1989.

208. **Simon, Harvey**. "How Much Exercise do You Need?" *Harvard Health Publications*. Harvard Commentaries on Health, 2017.

209. **Sonenberg N and Hinnebusch AG.** Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* 136: 4: 731-745, 2009.

210. **Song Y, Coleman L, Shi J, Beppu H, Sato K, Walsh K, Loscalzo J and Zhang YY.** Inflammation, endothelial injury, and persistent pulmonary hypertension in heterozygous BMPR2-mutant mice. *Am. J. Physiol. Heart Circ. Physiol.* 295: 2: H677-90, 2008.

211. **Stewart AH and Lamont PM.** Exercise training for claudication. *Surgeon* 5: 5: 291-299, 2007.

212. **Suzuki H and Yoshitaka I.** Exercise Therapy for Intermittent Claudication in Peripheral Artery Disease. *E-J of Cardiology Practice.* 13: 1-13, 2015.

213. **Svensson J, Moverare-Skrtic S, Windahl S, Swanson C and Sjogren K.** Stimulation of both estrogen and androgen receptors maintains skeletal muscle mass in gonadectomized male mice but mainly via different pathways. *J. Mol. Endocrinol.* 45: 1: 45-57, 2010.

214. **Swank AM, Kachelman JB, Bibeau W, Quesada PM, Nyland J, Malkani A and Topp RV.** Prehabilitation before total knee arthroplasty increases strength and function in older adults with severe osteoarthritis. *J. Strength Cond Res.* 25: 2: 318-325, 2011.

215. **Tang GL, Chang DS, Sarkar R, Wang R and Messina LM.** The effect of gradual or acute arterial occlusion on skeletal muscle blood flow, arteriogenesis, and inflammation in rat hindlimb ischemia. *J. Vasc. Surg.* 41: 2: 312-320, 2005.

216. **Tao L, Bei Y, Lin S, Zhang H, Zhou Y, Jiang J, Chen P, Shen S, Xiao J and Li X.** Exercise Training Protects Against Acute Myocardial Infarction via Improving Myocardial Energy Metabolism and Mitochondrial Biogenesis. *Cell. Physiol. Biochem.* 37: 1: 162-175, 2015.

217. **Thanky NR, Son JH and Herbison AE.** Sex differences in the regulation of tyrosine hydroxylase gene transcription by estrogen in the locus coeruleus of TH9-LacZ transgenic mice. *Brain Res. Mol. Brain Res.* 104: 2: 220-226, 2002.

218. **Thyfault JP, Rector RS, Uptergrove GM, Borengasser SJ, Morris EM, Wei Y, Laye MJ, Burant CF, Qi NR, Ridenhour SE, Koch LG, Britton SL and Ibdah JA.** Rats selectively bred for low aerobic capacity have reduced hepatic mitochondrial oxidative capacity and susceptibility to hepatic steatosis and injury. *J. Physiol.* 587: Pt 8: 1805-1816, 2009.

219. **Topper JN and Gimbrone MA,Jr.** Blood flow and vascular gene expression: fluid shear stress as a modulator of endothelial phenotype. *Mol. Med. Today* 5: 1: 40-46, 1999.

220. **Tousoulis D, Davies GJ, Asimakopoulos G, Homaei H, Zouridakis E, Ahmed N and Kaski JC.** Vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 serum level in patients with chest pain and normal coronary arteries (syndrome X). *Clin. Cardiol.* 24: 4: 301-304, 2001.

221. **Van Kampen JM and Robertson HA.** A possible role for dopamine D3 receptor stimulation in the induction of neurogenesis in the adult rat substantia nigra. *Neuroscience* 136: 2: 381-386, 2005.

222. **Van Kampen JM, Hagg T and Robertson HA.** Induction of neurogenesis in the adult rat subventricular zone and neostriatum following dopamine D3 receptor stimulation. *Eur. J. Neurosci.* 19: 9: 2377-2387, 2004.

223. **Van Royen N, Hoefer I, Bottinger M, Hua J, Grundmann S, Voskuil M, Bode C, Schaper W, Buschmann I and Piek JJ.** Local monocyte chemoattractant protein-1 therapy increases collateral artery formation in apolipoprotein E-deficient mice but induces systemic monocytic CD11b expression, neointimal formation, and plaque progression. *Circ. Res.* 92: 2: 218-225, 2003.

224. **Van Royen N, Hoefer I, Buschmann I, Kostin S, Voskuil M, Bode C, Schaper W and Piek JJ.** Effects of local MCP-1 protein therapy on the development of the collateral circulation and atherosclerosis in Watanabe hyperlipidemic rabbits. *Cardiovasc. Res.* 57: 1: 178-185, 2003.

225. **Van Royen N, Piek JJ, Buschmann I, Hoefer I, Voskuil M and Schaper W.** Stimulation of arteriogenesis; a new concept for the treatment of arterial occlusive disease. *Cardiovasc. Res.* 49: 3: 543-553, 2001.

226. **Vegh A, Papp JG, Semeraro C, Fatehi-Hasanabad Z and Parratt JR.** The dopamine receptor agonist Z1046 reduces ischaemia severity in a canine model of coronary artery occlusion. *Eur. J. Pharmacol.* 344: 2-3: 203-213, 1998.

227. **Vina, J., Sanchis-Gomar, F., Martinez-Bello, V. & Gomez-Cabrera, M.** Exercise acts as a drug; the pharmacological benefits of exercise, *British Journal of Pharmacology*, vol. 167, no. 1, pp. 1-12, 2012.

228. **Wall BA, Galvao DA, Fatehee N, Taaffe DR, Spry N, Joseph D and Newton RU.** Reduced Cardiovascular Capacity and Resting Metabolic Rate in Men with Prostate Cancer Undergoing Androgen Deprivation: A Comprehensive Cross-Sectional Investigation. *Adv. Urol.* 2015: 976235, 2015.

229. **Wang W, Liu L, Chen C, Jiang P and Zhang T.** Protective effects of dopamine D2/D3 receptor agonist piribedil on learning and memory of rats exposed to global cerebral ischemia-reperfusion. *Neurosci. Lett.* 684: 181-186, 2018.

230. **Wang Z, Guan W, Han Y, Ren H, Tang X, Zhang H, Liu Y, Fu J, He D, Asico LD, Jose PA, Zhou L, Chen L and Zeng C.** Stimulation of Dopamine D3 Receptor Attenuates Renal Ischemia-Reperfusion Injury via Increased Linkage With Galpha12. *Transplantation* 99: 11: 2274-2284, 2015.

231. **Warburton DER.** Health benefits of physical activity: the evidence. *Can. Med. Assoc. J.* 174: 6: 801-809, 2006.

232. **Warburton DER.** Prescribing exercise as preventive therapy. *Can. Med. Assoc. J.* 174: 7: 961-974, 2006.

233. **Waters RE, Terjung RL, Peters KG and Annex BH.** Preclinical models of human peripheral arterial occlusive disease: implications for investigation of therapeutic agents. *J. Appl. Physiol.(1985)* 97: 2: 773-780, 2004.

234. **Weitz JI, Byrne J, Clagett GP, Farkouh ME, Porter JM, Sackett DL, Strandness DE and Taylor LM.** Diagnosis and Treatment of Chronic Arterial Insufficiency of the Lower Extremities: A Critical Review. *Circulation* 94: 11: 3026-3049, 1996.

235. **Wen CP, Wai JPM, Tsai MK, Yang YC, Cheng TYD, Lee M, Chan HT, Tsao CK, Tsai SP and Wu X.** Minimum amount of physical activity for reduced mortality and extended life expectancy: a prospective cohort study. *Lancet* 378: 9798: 1244-1253, 2011.

236. **Williams MH and Thompson J.** Effect of varient dosages of amphetamine upon endurance. *Res. Q.* 44: 4: 417-422, 1973.

237. **Wilmore JH, Green JS, Stanforth PR, Gagnon J, Rankinen T, Leon AS, Rao DC, Skinner JS and Bouchard C.** Relationship of changes in maximal and submaximal aerobic

fitness to changes in cardiovascular disease and nonndash]insulin-dependent diabetes mellitus risk factors with endurance training: The HERITAGE Family Study. *Metab. Clin. Exp.* 50: 11: 1255-1263, 2001.

238**. Winkelman JW and Johnston L.** Augmentation and tolerance with long-term pramipexole treatment of restless legs syndrome (RLS). *Sleep Med.* 5: 1: 9-14, 2004.

239. **Wisloff U, Najjar SM, Ellingsen O, Haram PM, Swoap S, Al-Share Q, Fernstrom M, Rezaei K, Lee SJ, Koch LG and Britton SL**. Cardiovascular risk factors emerge after artificial selection for low aerobic capacity 14. *Science* 307: 418-420, 2005.

240. **Wisman PP, Tangelder MJ, van Hattum ES, de Borst GJ and Moll FL.** Young women with PAD are at high risk of cardiovascular complications. *Eur. J. Vasc. Endovasc. Surg.* 43: 4: 441-445, 2012.

241. **Witt KA, Mark KS, Huber J and Davis TP.** Hypoxia-inducible factor and nuclear factor kappa-B activation in blood-brain barrier endothelium under hypoxic/reoxygenation stress. *J. Neurochem.* 92: 1: 203-214, 2005.

242. **Yamamoto Y and Oelgeschlager M.** Regulation of bone morphogenetic proteins in early embryonic development. *Naturwissenschaften* 91: 11: 519-534, 2004.

243. **Yang HT and Feng Y.** bFGF increases collateral blood flow in aged rats with femoral artery ligation. *Am. J. Physiol. Heart Circ. Physiol.* 278: 1: H85-93, 2000.

244. **Yang HT and Terjung RL.** Angiotensin-converting enzyme inhibition increases collateral-dependent muscle blood flow. *J. Appl. Physiol.(1985)* 75: 1: 452-457, 1993.

245. **Yang HT, Feng Y, Allen LA, Protter A and Terjung RL.** Efficacy and specificity of bFGF increased collateral flow in experimental peripheral arterial insufficiency. *Am. J. Physiol. Heart Circ. Physiol.* 278: 6: H1966-73, 2000.

246. **Yang Y, Tang G, Yan J, Park B, Hoffman A, Tie G, Wang R and Messina LM.** Cellular and molecular mechanism regulating blood flow recovery in acute versus gradual femoral artery occlusion are distinct in the mouse. *J. Vasc. Surg.* 48: 6: 1546-1558, 2008.

247. **Yang, Y., Mamouni, K., Li, X., Chen, Y., Kavuri, S., Du, Y., Fu, H., Kucuk, O. & Wu, D.** Repositioning Dopamine D2 Receptor Agonist Bromocriptine to Enhance Docetaxel Chemotherapy and Treat Bone Metastatic Prostate Cancer, *Molecular cancer therapeutics*, vol. 17, no. 9, pp. 1859-1870, 2018.

248. **Yin T, He S, Shen G, Ye T, Guo F and Wang Y.** Dopamine receptor antagonist thioridazine inhibits tumor growth in a murine breast cancer model. *Mol. Med. Rep.* 12: 3: 4103-4108, 2015.

249. **Yu C, Wang Z, Han Y, Liu Y, Wang WE, Chen C, Wang H, Jose PA and Zeng C.** Dopamine D(4) receptors inhibit proliferation and migration of vascular smooth muscle cells induced by insulin via down-regulation of insulin receptor expression. *Cardiovasc. Diabetol.* 13: 97-2840-13-97, 2014.

250. **Zgliczynski S, Ossowski M, Slowinska-Srzednicka J, Brzezinska A, Zgliczynski W, Soszynski P, Chotkowska E, Srzednicki M and Sadowski Z.** Effect of testosterone replacement therapy on lipids and lipoproteins in hypogonadal and elderly men. *Atherosclerosis* 121: 1: 35-43, 1996.

251. **Zhu H, Clemens S, Sawchuk M and Hochman S.** Unaltered D1, D2, D4, and D5 dopamine receptor mRNA expression and distribution in the spinal cord of the D3 receptor knockout mouse. *J. Comp. Physiol. A. Neuroethol Sens. Neural Behav. Physiol.* 194: 11: 957-962, 2008.

252**. Zylla MM, Brachmann J, Lewalter T, Hoffmann E, Kuck KH, Andresen D, Willems S, Eckardt L, Tebbenjohanns J, Spitzer SG, Schumacher B, Hochadel M, Senges J, Katus HA and Thomas D.** Sex-related outcome of atrial fibrillation ablation: Insights from the German Ablation Registry. *Heart Rhythm* 13: 9: 1837-1844, 2016.

APPENDEX: ANIMAL CARE AND USE COMMITTEE PROTOCOL APPROVAL

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

June 9, 2015

Robert Lust, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Lust:

Your Animal Use Protocol entitled, "Cardiovascular Consequences of Artificial Selection Based on Aerobic Running Capacity" (AUP #Q330) was reviewed by this institution's Animal Care and Use Committee on 6/9/15. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

East Carolina University
Tomorrow starts here.

Animal Care and
Use Committee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834-4354

252-744-2436 office
252-744-2355 fax

May 1, 2018

Robert Lust, Ph.D.
Department of Physiology
Brody 6N-98
East Carolina University

Dear Dr. Lust:

Your Animal Use Protocol entitled, "Cardiovascular Consequences of Artificial Selection Based on Aerobic Running Capacity" (AUP #Q330a) was reviewed by this institution's Animal Care and Use Committee on May 1, 2018. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure