INFLUENCE OF EXERCISE TRAINING ON OXIDATIVE CAPACITY AND
UTRASTRUCTURAL DAMAGE IN SKELETAL MUSCLES OF AGED HORSES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School
of The Ohio State University

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ABSTRACT

During the aging process there is a progressive decrease in the ability of skeletal muscle to generate and sustain force and power, and these functional adaptations occur in concert with profound morphological and biochemical adaptations. Our overall aim was to investigate the utility of the horse as a model for studying age-related and exercise training-induced adaptations to skeletal muscle morphology and biochemistry that are typically observed in humans. Our first objective was to determine the age-related changes in myosin heavy chain (MHC) composition and muscle oxidative and glycolytic capacity in 18 horses ranging in age from 2 to 30 years. Our second objective was to determine the effect of 10 weeks of treadmill exercise training on muscle oxidative capacity and ultrastructural damage in 6 aged female Quarter horses (>23 years of age). There was no significant correlation between horse age and the proportions of type-IIA and type-IIX MHC isoforms ($r = 0.2$ and -0.03 , respectively, $P > 0.05$). The percent of type-I MHC isoforms decreased significantly with advancing age ($r = -0.56$, $P < 0.05$). Muscle citrate synthase (CS) activity decreased significantly with increasing age ($r = -0.41$, $P < 0.05$), whereas lactate dehydrogenase (LDH) activity increased with age ($r = 0.44$, $P < 0.05$). However, the activity of muscle 3-OH acyl COA dehydrogenase (HAD) did not change with aging ($r = 0.11$, $P >$

0.05). Maximal aerobic capacity ($\text{VO}_{2\text{max}}$) of 6 aged mares increased by 22% after 10 weeks of exercise training. After training, the percent of type-IIA MHC isoforms of the semimembranosus increased (11%, $P < 0.05$) while the percent of type-IIX MHC isoforms of triceps significantly decreased (3%, $P < 0.05$). Only triceps muscle showed significant increases ($P < 0.05$) in both CS and HAD activities by 41% (from 20.4 ± 1.8 to 28.7 ± 1.3 $\mu\text{mol/g/min}$) and 72% (from 9.9 ± 1.4 to 17.1 ± 1.2 $\mu\text{mol/g/min}$) after conditioning, respectively, in spite of no changes in LDH activity. Attenuation of the exercise-induced ultrastructural muscle damage at both the same absolute and the same relative workloads after exercise training occurred only in the semimembranosus muscle. The results of the present study suggest that age-related changes in equine skeletal muscle may differ from those in humans. However, similar to humans, the oxidative capacity of equine skeletal muscle appears to decrease with age, but intense aerobic exercise training can overcome this age-related reduction in functional capacity. This finding is consistent with a training-induced transition of MHC isoforms in aged skeletal muscle that shifts toward more oxidative isoforms after training. Electron microscopic analysis provides evidence that aerobic exercise training can attenuate the severity of the exercise-induced ultrastructural damage in aged skeletal muscle at given absolute and/or at the same relative workloads. However, the exercise training-induced adaptations in mammalian skeletal muscle may be muscle-group specific.

Dedicated to my Lord and my family

ACKNOWLEDGMENTS

I would like to thank my adviser, Dr. Steven T. Devor, for all of his intellectual support and guidance along with his continuous understanding and encouragement throughout this enormous process. Without him, my education here would not have been the same.

I would like to thank Dr. Kenneth W. Hinchcliff for all of his tremendous support, time, and enthusiasm, which made this dissertation possible.

I would like to thank Dr. Mamoru Yamaguchi for all of his unlimited technical and intellectual support, and for providing equipment along with his thoughtful encouragement.

I would like to thank Dr. Laurie A. Beard for all of her time and assistance in this project.

I would like to thank all of my colleagues, especially Chad D. Markert for his many hours of assistance in my second home, the biochemistry laboratory.

I would like to thank the College of Veterinary Medicine at The Ohio State University for the equine research fund, and thank all of the doctors and students of Veterinary Medicine for providing their time, technical support, and especially for their care of my lovely mares.

I would like to thank Wuthichai (Pok) Klomkleaw and Anne-E. Handley for their time and technical support, and especially for educating me in all the electron microscopic analyses.

I would like to thank William J. Fink at Ball State University for providing detailed enzymatic analysis protocols.

I would like to thank my former Master's advisers, Dr. Bruce W. Craig and Dr. Sung J. Park, for their continuous support and guidance throughout my education in the US.

I would like to thank Kay Yeager for her warm support as my American mom during my OSU life.

I would like to thank all of my family, especially both my parents and parents-in-law for their prayers and patience. Most of all, I would like to thank my best friend, my wife, for just being with me every moment. Without my lovely wife and kids, my life here might have been very different and much more difficult.

Most of all, I would like to sincerely thank my Lord for all.

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FIELDS OF STUDY

Major Field: Education

Special Area: Exercise Science

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CHAPTER 1

INTRODUCTION

During the aging process, the ability of mammalian skeletal muscle to produce and sustain power output decreases (96). A decrease in muscle mass and a loss of the number of motor units are partially responsible for this age-related reduction in the functional capacity of muscle (46, 54). As age increases, the oxidative capacity of human skeletal muscle also declines (20, 75), but this adaptation appears to be muscle group specific (41). Evidence of the decreased oxidative capacity is seen in both the rate of mitochondrial protein synthesis and activity of oxidative enzymes such as cytochrome c oxidase and citrate synthase (41, 75). The reduced oxidative capacity appears to be related to the age-related decrease in aerobic capacity and muscle performance (20, 41). There is also evidence of diminished glycolytic capacity in aged skeletal muscle (94).

In addition, skeletal muscle of humans appears to be more susceptible to contraction-induced injury with advancing age (59). Muscles of aged mammals also show a prolonged and/or incomplete recovery response during the degeneration and regeneration process after the injury (7). Muscle contractile properties tend to decline with aging, which is indicated by prolonged contraction and relaxation times during stimulated muscle contractions (61). Motor unit

remodeling is also partially responsible for the reduced functional properties of muscle (54), and this age-related phenomenon may contribute to a greater susceptibility to injury in aged muscle. These age-related adaptations are due to inevitable physiological changes and exacerbated by physical inactivity during the aging process (23).

Since physical inactivity is partly responsible for age-related changes in skeletal muscles, exercise training induces positive adaptations and attenuates the reduction in functional capacity with aging (28, 29, 32, 33, 39). Vigorous resistance exercise training enhances the mitotic activity of satellite cells, possibly by activating the secretion of growth factors via the autocrine system (1, 14, 45). Thus, exercise training accelerates the regenerative process of muscle protein synthesis (23, 44), thereby enhancing skeletal muscle hypertrophy and ultimately maintenance of lean body mass in the old (8, 15, 32, 33, 48, 96, 99).

During exercise-induced muscle hypertrophy, there is a shift in MHC isoforms toward type-IIA MHC isoforms (48, 82, 94). The rate of exercise-induced shifts in MHC isoforms decreases with increasing age in humans (48). Endurance exercise training partially attenuates the decline in maximal aerobic capacity associated with training-induced increases in activities of aerobic enzymes with aging (47, 49). Furthermore, compared with young individuals, healthy and relatively physically active older individuals exhibit very little decline in skeletal muscle oxidative capacity with aging (49).

Following severe damage, regenerated muscle fibers of older rats are markedly resistant to contraction-induced injury (22). The evidence indicates that

the damage-induced muscle fiber degeneration and regeneration cascade, which follows exercise training, may enhance resistance to contraction-induced cellular damage in skeletal muscle of older individuals. Thereby, exercise training may assist older individuals in preventing abrupt injury associated with age-related impairments such as a reduction in muscle strength, impaired mobility, and tendency to fall (28, 42). In addition, a progressive exercise-training program will induce the recruitment of type II fibers by increasing load, which may slow the loss of muscle contractile properties by diminishing a transformation to type I fiber via the age-related motor unit remodeling. Consequently, even though there may be altered skeletal muscle adaptations in the old, exercise training will aid the elderly in the prevention of the loss of functional capacity by maintaining their muscular structure.

Therefore, the overall aim of the experiments that comprise the present dissertation was to determine whether the aged horse was an appropriate model for age-related decrements in muscle function in human beings. The working **hypothesis** was that aerobic conditioning of aged horses would reduce the amount of skeletal muscle damage induced by acute exercise, and that this reduction in damage would be associated with conditioning-induced adaptations in the expression of MHC isoforms and marker enzymes of fuel utilization.

*Based upon our working hypothesis, **specific aims*** of the present investigation were to: 1) define the characteristics of muscle of aged horses and in particular to characterize the age-related changes in the expression of MHC isoforms and the activity of marker enzymes of energy metabolism. This aim

was consistent with our intention to investigate the utility of the horse as a model of age-related changes in muscle function in humans: 2) Evaluate the effects of 10 weeks of treadmill exercise training on; a) magnitude of ultrastructural skeletal muscle damage occurring in response to incremental exercise tests performed before and after conditioning; b) expression of skeletal muscle MHC isoforms; and, c) activity of skeletal muscle enzymes indicative of oxidative and glycolytic metabolism.

*To achieve the specific aims, the following **specific hypotheses** were tested:*

1) Skeletal muscle of aged horses will be associated with:

a) an increased expression of type-I MHC; b) a decreased expression of type-IIx MHC; c) a decreased activity of citrate synthase (CS, which reflects citric acid cycle activity) and 3-hydroxy-acyl CoA dehydrogenase (HAD or β -HAD, which reflects lipid oxidative capacity); and d) a decreased activity of lactate dehydrogenase (LDH, which reflects glycolytic capacity).

2) Compared with skeletal muscles from aged sedentary horses, skeletal muscles from aged horses conditioned by aerobic exercise for 10 weeks will:

a) exhibit reduced ultrastructural muscle damage in response to one bout of incremental exercise at the same absolute intensity as the first exercise test (VO_{2max} test); b) exhibit reduced ultrastructural muscle damage in response to one bout of incremental exercise at the same relative intensity as the first exercise test; c) demonstrate an increased expression of type-IIa MHC isoforms; d) demonstrate a decreased expression of type-IIx MHC isoforms; e) possess an increased activity of CS (citric acid cycle activity) and HAD (lipid oxidative

capacity); f) and possess a decreased or unchanged activity of lactate dehydrogenase (LDH, which reflects glycolytic capacity).

In the present investigation, the exhaustive incremental exercise tests (VO_{2max} test) played a role in creating exercise-induced ultrastructural muscle damage and were used for the determination of exercise training intensity for each horse.

CHAPTER 2

REVIEW OF LITERATURE

Overview of the Age-Related Adaptations in Mammalian Skeletal Muscle

The aging process typically is responsible for physical impairments such as a reduction in muscle strength and impaired mobility, which in humans lead to an increased tendency to suffer from falls. This phenomenon seems to be primarily due to a decrease in the ability of muscle to generate and sustain power output in association with changes in muscular structure and function during aging (46, 96). A decrease in muscle mass and a loss of motor unit number partially induces the age-related reduction in functional capacity of muscle.

The decline in muscle mass with aging is a response to a decrease in diameter and number of muscle fibers, especially type IIb fibers (79), and eventually results in a decrease in the total number of motor units while increasing the size of the remaining motor units (54). The slow (type I) to fast twitch (type II) fiber ratio appears to increase as age increases. This observation seems to be related to a transformation from type II to type I fibers in response to the motor unit remodeling, which is associated with altered functional demands due to a weight gain as a result of physical inactivity with aging (54). Further, as

age increases, the oxidative capacity of human skeletal muscle was shown to be reduced (20, 75) in association with the age-related decrease in aerobic capacity and muscle performance (20, 41). There also has been evidence of diminished glycolytic capacity in aged skeletal muscle (94). These age-related reductions in functional capacity of skeletal muscle are known to be related to both physical inactivity and the inevitable consequences of the aging process (28, 96).

Since physical inactivity is one of the major contributors to the age-related changes in skeletal muscles, exercise training appears to induce positive adaptations and attenuates the reduced functional capacity observed during the aging process (9, 15, 22, 29, 32, 51, 96). Vigorous exercise training, especially resistance training, is not only known to induce an increase in regional and total lean mass and decrease regional and total fat mass in older men (90), but also results in greater adaptations in type II fiber area (9), where the major atrophy occurs during the aging process. However, a number of studies suggest that in comparison with a young population, the quantity of exercise training-induced skeletal muscle adaptations tends to be less in older mammals (7, 8).

Exercise-Induced Muscle Damage

A number of studies have provided evidence that muscle pain and soreness may be experienced after certain intense exercises (particularly those with a bias toward lengthening contractions) and is thought to result from muscle fiber disruption or connective tissue damage. However, in order to stimulate muscle hypertrophy to counteract age-related atrophy, relatively heavy loads

must be applied during exercise training to induce muscle damage (29, 52, 58). In addition, it has also been demonstrated that applying the proper mode of exercise is even more crucial to elicit efficient skeletal muscle adaptations in response to exercise training (31, 35, 40, 58).

It is well documented that even heavy resistance exercise training will not induce optimal muscle hypertrophy unless eccentric (lengthening) muscle actions are involved (35, 40), as lengthening muscle actions appear to play a major role in inducing skeletal muscle damage (31, 34). Therefore, contraction-induced injury protocols consisting of lengthening contractions have been the most frequently utilized method for studies focused on skeletal muscle adaptations to resistance training in both human (31, 44, 76, 77) and animal models (7, 11, 23).

Lieber et al. (56) investigated three types of experimental treatments on the rabbit tibialis anterior to examine the mechanism of eccentric action induced muscle injury: 1) isometric action, 2) passive stretch, and 3) eccentric action. They demonstrated structural alterations in the rabbit muscle similar to those observed in humans. Using the electron microscope, they found tears in the myofibrils as well as cross striation band disturbances in the group that performed eccentric muscle action. These changes occur originally in the myofibrillar Z- and A-bands. The Z bands appeared to be particularly smeared, streamed, and even totally disrupted (56). Lieber et al. also reported potential mechanisms of muscle damage in fast glycolytic (FG) muscle fibers that fatigued in the first few minutes of the exercise period. The FG fibers became unable to regenerate ATP and entered a rigor state. As stiffness of the rigor fibers

increased, mechanical disruption occurred in these fibers due to subsequent stretch. However, isometric or concentric (shortening) contractions would not be expected to cause such damage, because even though the FG fibers enter a state of rigor, there is no force to elongate them.

Several studies have demonstrated various ways to evaluate the amount of structural damage of exercised muscle. The investigations are not only directly assessing ultrastructural damage at the level of the light or electron microscope (7, 23, 76, 77), but are also assessing damage indirectly by assessing the serum levels of muscular damage markers, such as creatine kinase (CK) (18, 19, 44, 59) and troponin I (16, 58, 84). Although the serum concentration of skeletal troponin I (sTnI) increases following severe physical work (84), it is not as sensitive as cardiac troponin I (cTnI), which undergoes degradation and circulates in blood even in response to minimal cellular necrosis. Thus, cTnI is used for patients with acute heart failure as the earliest marker of myocardial damage (16).

Creatine kinase (CK) in the blood is considered a marker of muscle tissue damage because it is a muscle enzyme that leaks out of the muscle once it is damaged (50). However, using serum CK activity as a muscular damage indicator has been criticized issue, and suggested by many researchers to be an inaccurate method for assessing the quantity of muscle cellular disruption (18, 19, 50, 53, 59). Clarkson et al. (18) assessed muscle soreness and serum CK activity in 28 college women and found no significant difference in a rise of blood CK levels following isometric, eccentric, and concentric exercise. The results of

their study suggest that even though CK levels increase following all three contraction protocols, the increase in blood CK activity may not be sensitive enough to differentiate the quantity of muscle damage.

Exercise-induced Ultrastructural Muscle Damage Response in the Old

One of the most important issues for older individuals who are willing to participate in exercise training programs is how a well-designed and appropriate exercise training program can diminish exercise-induced muscle injury and soreness. This issue is of major importance for exercise physiologists working with an aged population. However, it is a fact that a certain minimum level of cellular damage is required even for old individuals to achieve beneficial adaptations from exercise training. Consequently, the question is how much damage is enough to provide benefits?

As discussed, the quantity of skeletal muscle adaptations also tends to be less in older mammals (7, 59). The reduced adaptations in aged population are noted in greater ultrastructural muscle damage (59) and delayed rate of muscle recovery (7) in response to lengthening muscle stimulations. In both aging human (59) and animal studies (100), it has been suggested that older sedentary individuals exhibit higher levels of ultrastructural muscle damage in exercised skeletal muscle than their young sedentary counterparts in response to an acute exercise stimulus.

Manfredi et al. (59) compared ultrastructural muscle damage following high intensity lengthening exercise in 5 young (20-30 yrs old) and 5 older (59-63

yrs old) men at the light and electron microscopic levels. All subjects performed three bouts of 15-minute lengthening exercise at 90%, 80%, and 70% of their maximal shortening power output. The investigators also determined whether serum CK activity was a reliable damage indicator. There was no significant difference in elevated CK levels between age groups following exercise. However, biopsy samples from the vastus lateralis of older subjects studied with EM demonstrated focal damage to approximately 90% of the fibers, while muscle samples from young subjects yielded 5 to 50% focal damage. Observations at the light microscope level also indicated greater muscle damage in older than in young subjects. These data demonstrate no relationship between CK activity and actual structural damage shown in each subject. Therefore, serum CK activity appeared to be a poor indicator for evaluating quantitative of muscle damage, as previously proven in other investigations (18, 50, 53, 59).

Another line of supportive evidence showed that after lengthening contractions, the extensor digitorum longus (EDL) muscles of old mice appeared to be more susceptible to contraction-induced damage than EDL of young mice, and free radical treatments contribute to the secondary or delayed onset muscle damage (100). Possible explanations for the greater susceptibility to injury in aged muscles are age-related muscle atrophy, motor unit remodeling, and muscle weakness (28). These factors are also profoundly associated with the diminished regeneration process in skeletal muscles of aged animals.

Conversely, prolonged stimulatory contractions during longitudinal exercise training appear to result in similar consequences of skeletal muscle

damage between young and old humans (44, 76). Roth et al. (76) examined ultrastructural muscle damage at the electron microscopic level in young (25 ± 3 year old) and older (69 ± 3 year old) males after heavy-resistance strength training (HRST). The training program consisted of 9 weeks of HRST and 5 sets of high-volume (55 total repetitions) knee extensions of the dominant leg, and the non-dominant leg served as a control. The results of their study suggest that after 9 wk of HRST, increases in ultrastructural damage in the vastus lateralis muscle were similar in young and older individuals. This finding is supported by Hurley et al. (44), who showed that in older subjects, 16-week HRST resulted in substantial reduction in plasma CK activity and muscle soreness at the end of the training program. Furthermore, older individuals showed a 43% increase in muscle strength and a 7.2% increase in mid thigh muscle area response to the 16-week training protocol.

The conflicting results may potentially be due to differences in the exercise protocols in each study. Cross sectional acute exercise protocols seem to result in greater ultrastructural muscle damage in older groups than in young groups as a result of the sudden trauma to the impaired skeletal muscle structure seen in aging process. In contrast, longitudinal exercise training protocols appear to induce similar levels of muscular damage between young and older groups, possibly due to the chronic adaptive effects of long-term training.

Although **no investigation has been conducted using horses**, Devor and Faulkner (1999) recently provided evidence that in old rats, muscle fibers that have progressed through a regenerative process of protein synthesis after

severe damage are markedly resistant to contraction-induced injury, and that the level of resistance to injury was similar to that of young rats (23). This is further evidence of the beneficial effects of exercise for aged animals, suggesting that the process of degeneration and regeneration facilitated by certain amount of muscle damage during exercise training may help old horses avoid the injury associated with acute exercise and age-related impairments in muscle function.

However, it is not well documented that old horses exhibit similar age-related decrements in muscle function and responses to exercise training and muscle damage as do rats and humans. Horses may represent a novel and useful model for study of age-related changes in muscle function. Further, horses may be an ideal model for the age-related declines noted in humans because horses offer some advantages, such as their size, demeanor, and tractability.

The positive attributes of horses mean that repeated measurement of variables in muscle samples is viable, and that training-induced changes in oxygen consumption (a key indicator of exercise conditioning) can be readily measured. A horse model for the study of muscle damage may also be beneficial because of the ease of administering pharmacologic agents and measuring their effect on athletic performance and skeletal muscle morphology and biochemistry. Although studies in small mammalian species such as rats and mice have provided valuable information for humans to understand physiological adaptations to the aging process and conditioning, these species are not be a perfect model for age-related changes in humans because of

several limitations including their size and a lack of repeated sampling from the same individuals.

Effect of Exercise Training on the Alterations in the Expression of Myosin Heavy Chain Isoforms of Aged Skeletal Muscle in association with Exercise-induced Muscle Hypertrophy

Skeletal muscle is a major storage site of free amino acids and protein in the body. After complete maturation of the body, the maintenance of protein quantity is achieved by a repair process of protein breakdown and synthesis. Protein synthesis in the process of muscle fiber regeneration and hypertrophy occurs via the function of satellite cells and anabolic hormones such as human growth hormone (hGH) and insulin like growth factors (IGFs) (1). Fong et al. (30) proposed a possible mechanism of protein synthesis by demonstrating that the infusion of methionyl-hGH can induce intracellular amino acid accrual and increase levels of myofibrillar protein mRNA during hospitalized nutritional support. The results of this study suggest that hGH is a potential therapy to combat wasting of lean body mass.

Devol et al. (22) investigated whether there was local regulation of IGF gene expression during work-induced muscle hypertrophy in young rats. They suggest that the expression of IGF genes is enhanced by signals propagated during the muscle growth, and IGF-I mRNA levels could be modulated in the absence of hGH. Their data also indicate that the local production of IGF-I and IGF-II mRNAs may play a role in the regulation of skeletal muscle protein synthesis by enhancing the proliferative activity of satellite cells (14).

Since there is a decrease in the population and activity of satellite cells and anabolic hormones with aging (21, 81), vigorous physical activity may be required for older individuals to maintain the protein quantity in their atrophied skeletal muscles by inducing the degeneration/regeneration process of protein breakdown and synthesis. It has been well established that heavy exercise training (HET) appears to enhance muscle protein synthesis, thereby promoting skeletal muscle hypertrophy in both young (48, 87) and older individuals (8, 9, 15, 29, 32, 95, 99). HET may generate a stimulatory signal for the activation of growth factors and satellite cells. Since several types of proteins exist in skeletal muscle, it is important to measure the synthesis of specific proteins, such as myosin and actin (63).

In a last few decades, a number of investigators have developed a technique to measure myosin heavy chain (MHC) and frequently utilize this technique to assess muscle protein synthesis (48, 86). While the age-associated changes in skeletal muscle have shown to be muscle specific (41), in humans, the type-II to type-I MHC isoform ratio was less in older muscles (66-77 yr) than in young muscles (21-24 yr), due to a shift from type II to type-I MHC isoforms during the aging process (94). During the muscle hypertrophic process, however, there appears to be a shift in MHC isoforms from type-IIIX toward type-IIA (48, 85). This shift in myosin isoforms shows an agreement with fiber type shifts from type-IIIX (fast glycolytic) to type-IIA (fast oxidative) following heavy exercise training in humans (86). Although aged skeletal muscle shows a increase in type II fiber area (15) (mainly due to increase in type IIa) following

exercise training as well as an identical MHC phenotype profile observed in a young group in rats (85), the rate of exercise-induced shifts in MHC isoforms seems to decrease with increasing age in humans (48).

Horse skeletal muscle possesses 3 MHC isoforms, MHC-I (slow oxidative), -IIA (fast oxidative), and -IIX isoforms (fast glycolytic), and has 3 fiber types containing a single MHC (I, IIA, IIX) and 2 hybrid types co-expressing 2 MHC isoforms (I+IIA, IIA+IIX) (70). Untrained horses also demonstrate significant increases in the percent of type I fibers (100%) and decreases in the percent of type IIb fibers (-50%) with aging (69). In contrast to humans, it has been demonstrated that among 5 age groups of trained horses (1–24 years), the oldest group had a greater percentage of fast MHC (73.3%) than young horses (67%), and there was a significant correlation between age and performance index (2). However, the effect of age on MHC isoform distribution and activity of marker enzymes of fuel metabolism has not been investigated in untrained horses, nor has the effect of training in aged horses been determined.

Similarities in age-related changes in skeletal muscles have been documented in various mammalian species including humans, rats, mice, and dogs (15), and the adaptive responses of aged human skeletal muscle to exercise training are well documented as previously discussed. However, few studies document similar detailed information related to horses. Prolonged endurance exercise training appears to result in a conversion of MHC isoforms from IIX (non-oxidative) to IIA, and toward type-I MHC isoforms (highly oxidative) corresponding with the volume/duration of exercise training in horses (67, 82).

This exercise training-induced fast-to-slow shift in MHC is identical to findings in other mammalian species and relates to the improvement in muscle oxidative capacity (88). In addition, fiber type proportions and fiber size in equine skeletal muscle appear to be directly related to the athletic ability of the horse for endurance events (67). These are similar to findings in trained humans. However, an age-related decrease in the type-II to type-I MHC isoform ratio and muscle atrophy appear to be associated with a reduction in the maximal aerobic capacity with aging regardless of training status (66).

Since horses are one of a few mammalian species that undergo exercise-training, and even participate in athletic events, the horse may be a useful model to provide further evidence and support findings in humans regarding conditioning-induced skeletal muscle adaptations associated with muscle performance.

Effect of Exercise Training on the Metabolic Characteristics of Aged Skeletal Muscle in association with Maximal Oxygen Consumption (VO_{2max})

With increasing age, the oxidative capacity of human skeletal muscle has been shown to decline (20, 75), although it appears to be muscle-group specific (41). This age-related reduction in the oxidative capacity has been shown by a reduction in activity of oxidative enzymes such as citrate synthase, 3-OH acyl CoA dehydrogenase, and cytochrome c oxidase in association with a decreased rate of mitochondrial protein synthesis (41, 75). Among the number of oxidative enzymes, citrate synthase (CS) is known to be one of the key enzymes in the

citric acid cycle because of its role in an irreversible reaction from oxaloacetate to citrate in mitochondria, while 3-OH acyl CoA dehydrogenase (HAD) is a key mitochondrial enzyme for assessing lipid oxidative capacity (36).

This age-related reduction in muscle oxidative enzyme activities also appears to correlate with the age-related decrease in whole body aerobic capacity and muscle performance (20, 41), which has been indicated by a significant correlation between CS activity in the gastrocnemius muscle and maximum oxygen consumption (VO_{2max}) data. In addition, the age-related decline in VO_{2max} is seen with fit individuals as well, and the rate of decline is approximately 0.5% per year (5, 41).

Reduced skeletal muscle mass may also contribute to the age-associated decline in VO_{2max} (66). Further, Welle et al. (94) recently demonstrated that the mRNA encoding several enzymes involved in glycolysis and glycogenolysis appear to be less abundant in the vastus lateralis muscle of aged humans, which may be evidence of diminished glycolytic capacity in aged skeletal muscle. However, endurance exercise training seems to partially attenuate an age-related decrease in the maximal aerobic capacity in association with training-induced increase in the activities of aerobic enzymes (47). Furthermore, in healthy and relatively physically active old individuals compared with young individuals, there was no decline in skeletal muscle oxidative capacity with aging (49).

Although no muscle enzymatic changes with aging have been reported in untrained horses, older untrained mares have substantially (-24%) lower maximal

aerobic capacity ($VO_{2\max}$) in comparison with young mares (60). It has been demonstrated that a 12-wk endurance training program induced a significant increase in the activities of CS and HAD in young horses (38). Moreover, exercise training seems to overcome the age-related alterations in the oxidative capacity in equine skeletal muscles. Essen et al. (26) provide evidence that, in skeletal muscle of race (trained) horses, the aerobic (CS) and lipid oxidative (HAD) capacities appear to increase with aging. However, endurance exercise training seems to have minimal effect on glycolytic capacity (lactate dehydrogenase (LDH) activity) regardless of age in both humans and horses (26, 57). Although the training-induced adaptations in muscle oxidative and glycolytic capacity have been well documented (37, 73, 74, 93), a training study needs to be conducted on older sedentary horses in order to confirm the findings in humans and horses regarding aging.

Effect of Exercise Training on the Blood Lactate Concentration during the Incremental Exercise Tests

The lactate shuttle hypothesis suggests that lactate plays a crucial role in distributing carbohydrate potential energy, as a gluconeogenic precursor, among various tissues and organs, (i.e., between: cytosol and mitochondria, muscle and blood, active and inactive muscles, oxidative (red) and glycolytic (white) muscle beds, blood and heart, blood and liver, and liver and other tissues) (3, 6). The contributions of both lactate shuttle systems vary, dependent upon the types (i.e., intensity and duration) of exercise in both humans and animals.

The results of isotope tracer studies suggest that both the rate of appearance (R_a) and disappearance (R_d) of blood lactate increase with an increase in exercise intensity because lactate clearance rate (R_d) depends on production rate (R_a) via a concentration gradient; the higher the level of lactate, the greater the force required to remove it (3, 6). Bergman and associates (3) also suggest that in humans, the arterial-venous (a-v) lactate difference indicates that there was a positive linear relationship between %lactate R_d from leg lactate uptake and exercise intensity ($r = 0.98$), and active limbs accounted for the most of whole body lactate oxidation (70%).

Blood lactate appears to be proportionally elevated with increasing exercise intensity due to the rate of lactate production (R_a) by active muscle groups exceeding the rate of lactate clearance (R_d) by various tissues and organs including exercising muscles. It has been demonstrated that both high-intensity and endurance exercise training reduce the response of blood lactate concentrations at given absolute workloads (3). Although neither the potential mechanism nor the age-related adaptations are well established in both humans and horses, the reduced blood lactate concentration is known to be due to the increased lactate clearance rate (R_d) at given absolute workloads after endurance training in association with the increased activity of lactate transporters (4, 24, 27, 65).

CHAPTER 3

METHODOLOGY

I. Summary of Experimental Plan and Design

Experiment 1: Determination of Age-related Changes in Muscle Structure and Enzyme Activity of Horses

Specific Aim:

To define the characteristics of skeletal muscle of aging horses. Specifically, to characterize the age-related alterations in myosin heavy chain (MHC), fiber type distribution, and the activity of marker enzymes of energy metabolism. This aim is consistent with our intention to investigate the utility of the horse as a model of age-related changes in muscle function in humans.

Objectives:

To measure MHC isoform distribution and the activity of marker enzymes in skeletal muscle of horses ranging in age from 2 to 30 years old.

Rationale:

The aging process is associated with marked changes in MHC distribution and activity of enzymes indicative of both carbohydrate and fat oxidation in humans and laboratory animals. Such changes have only been superficially examined in horses (69) and it is not well established whether the changes occur in horses, a naturally athletic species.

In the overall context of our aim to determine the suitability of horses as a model for investigation of age-related changes in muscle function in humans, it is crucial that we determine whether in fact changes similar to those observed in humans occur in aging horses. It is highly likely, given the conservation of skeletal muscle structural and function characteristics across a wide range of mammalian species (15), that age-related changes in horses will mimic those of humans. However, it is essential that we demonstrate this relationship in conjunction with our investigation of the similarity of exercise-conditioning associated changes in muscle characteristics in aging horses.

Experiment 2: Determination of the Effect of Aerobic Conditioning on Muscle Composition and Damage Response to Exhaustive Incremental Exercise***Rationale:***

Based on studies with humans and rats, we expect that: 1) The magnitude of ultrastructural muscle damage in response to an acute bout of exhaustive incremental exercise in old sedentary horses will be reduced by aerobic

conditioning; 2) There will be a shift in expression of MHC isoforms from type-IIX (non-oxidative) to type-IIA or type-I (oxidative) isoforms in response to 10 weeks of aerobic conditioning; 3) The oxidative, but not glycolytic, capacity of exercising muscles will be enhanced by endurance exercise training; and 4) Aerobic conditioning will enhance whole body oxidative capacity, as indicated by an increase in the maximal rate of oxygen consumption.

Experimental Design:

Six old healthy unconditioned mare Quarter horses were initially deconditioned by 12 weeks of enforced box stall confinement. Following deconditioning, the first incremental exercise test (test 1) was performed by each horse. Subsequently the horses underwent 10 weeks of aerobic conditioning by running 3 times each week on a treadmill. Horses completed another two incremental exercise tests, one after 8 weeks of training (test 2) and the final test after 10 weeks of training (test 3). Test 2 (same absolute workload) was of identical speed and duration as test 1, thereby yielding information regarding the influence of 8 wks of conditioning on muscle damage incurred while performing the same workload. Exercise test 3 (same relative workload) was an incremental exercise test to fatigue (as was test 1) and provided information about the influence of conditioning on muscle damage following maximal work.

During the first and third incremental exercise tests the maximal rate of oxygen consumption (VO_{2max}), blood lactate and creatine kinase (via jugular venous blood samples), highest speed attained, and duration of exercise were

measured. Before and immediately after (<5 min) each exercise test muscle samples were collected from the triceps, semimembranosus, and masseter muscles. Samples from masseter muscle served as a non-weight bearing control as it underwent no increase in neural recruitment or mechanical loading during exercise. Muscle samples were analyzed for the determination of the magnitude of ultrastructural damage responses, the expression of MHC isoforms, and the activity of oxidative (citrate synthase (CS) and 3-OH-acyl-CoA dehydrogenase (HAD or β -HAD)) and glycolytic (lactate dehydrogenase (LDH)) enzymes in muscles from exercised trained horses.

II. Methods and Procedures

Experiment 1: Cross Sectional Study

Animals:

All experiments were conducted in the laboratories of the Section of Sport and Exercise Sciences and the Department of Veterinary Clinical Sciences at The Ohio State University in Columbus, Ohio. In order to define the characteristics of skeletal muscle of aging horses, 18 horses of various ages from 2 to 30 years were obtained from either donation to the Veterinary Teaching Hospital, horses from the teaching herd, or in response to a request made to clients of the Equine Ambulatory Service. Upon the approval by the Veterinary Teaching Hospital Executive Committee and the Institutional Laboratory Animal

Care and Use Committee (ILACUC) at The Ohio State University, an informed consent was provided to all clients and obtained prior to their participation.

Horses were selected to represent light breeds (Thoroughbred, Standardbred, Quarterhorse, and cross breeds and derivatives of these breeds) because of their genetic and ancestral similarity. Heavy horse breeds (Percherons, Clydesdales, etc) and ponies or miniature horses were not sampled. Horses selected were not in active athletic training. All horses had no history of lower extremity joint, bone, or muscle trauma or disease.

Experimental Protocol:

Muscle samples were collected by excisional biopsy of the semimebranosus muscle or immediately (<2 minutes) post mortem. The biopsy was performed after induction of local anesthesia by injection of 10 ml of mepivacaine hydrochloride and surgical preparation of the area. The 3 cm skin incision was closed with interrupted skin sutures and dressed with antimicrobial ointment. Horses were administered tetanus prophylaxis and sutures were removed in 7-10 days.

Analytical techniques:

Muscle samples were processed as described for the accompanying exercise study (see Experiment 2).

Experiment 2: Longitudinal Study

Animals:

Six old healthy untrained Quarter horse mares, from 23 to 30 years of age, were used for the longitudinal study. The study was approved by the Veterinary Teaching Hospital Executive Committee and ILACUC at the Ohio State University. Overnight dexamethasone suppression tests were normal in all mares. Body weight was 487 ± 24 kg. Prior to initiation of the study, horses underwent a 12-week period of detraining. During the detraining period, horses were housed in box stalls and fed grass hay and mixed grain sufficient to meet the National Research Council recommendations for the nutrient requirements of horses and to maintain an ideal body condition score (5-6 on a scale of 9).

Experimental Protocol:

During the first month of the 12-week detraining period, horses underwent at least 1 or 2 familiarization sessions per week on the treadmill and associated respiratory gas collection system in order to avoid any incidents and invalid results due to unaccustomed testing environments. The first muscle samples (Pre-1) were taken from triceps, semimembranosus, and masseter muscles using an open biopsy technique 3 days before the horses underwent the first incremental exercise test. The second biopsy sampling (Post-1) was performed immediately (<5 minutes) after the first incremental exercise test. Horses then

underwent an exercise training protocol consisting of treadmill exercise 3 days per week for 10 weeks (25).

Exercise training was an interval treadmill exercise training (see appendix C), which involved a total of 4 different exercise protocols throughout the 10 weeks of training. The exercise training intensity for the first training protocol was determined based upon the results of the first incremental exercise (VO_{2max}) test for each horse, and the training protocol was updated every other week based on performance and response to given workloads. For example (see appendix C), exercise consisted of walking (2 meters per second, m/s) for 2 minutes on a treadmill inclined at a 4° slope. Treadmill speed was increased to a speed calculated to require 40% VO_{2max} and maintained for 2 minutes. Then, treadmill speed was increased rapidly and maintained at a speed calculated to require 60% VO_{2max} for 5 minutes, at which point treadmill speed was increased to a speed calculated to require 80% VO_{2max} for 5 minutes. The horses then ran at 60% VO_{2max} for 5 minutes before walking at 2 m/s for 1 minute. Total exercise time was 20 to 22 minutes, 15 minutes of which was at a relatively high intensity. At all other times when not training, the horses were in box stalls.

As previously described, after 8 weeks of aerobic conditioning, horses performed an incremental exercise test that mimicked the initial test in speed attained and time on the treadmill. This test required horses to perform the same absolute workload as the first exercise test that was conducted before the initiation of conditioning, regarding the speed, grade, and duration of the incremental test. The same absolute-test at wk-8 allowed the evaluation of

changes in the magnitude of ultrastructural muscle cell damage response to the same absolute stress in comparison to that at pre-conditioning. Muscle samples were also taken in the same manner (i.e., pre- and post-exercise samples) from the same muscle groups (see muscle sampling procedure for details). Samples collected following 8 weeks of conditioning were labeled Pre-2 (collected before the same absolute-test) and Post-2 (collected after the same absolute-test).

Following two more weeks of aerobic conditioning (at the end of 10-week aerobic conditioning), collection of muscle samples and the incremental exercise tests were repeated. The additional 2 weeks of training period was to allow horses to recover from exercise-induced cellular damage by the same absolute-test at wk-8. This incremental test after 10-week conditioning (same relative workload) was another exhaustive exercise test, which was a measurement of training-induced adaptation to maximal aerobic capacity (VO_{2max}). The muscle samples were collected before and after the exercise test and labeled Pre-3 and Post-3, respectively, which permitted the determination of training-induced skeletal muscle adaptation in ultrastructural damage response to the same relative stress following the aerobic conditioning.

For all incremental tests, horses were positioned on the treadmill for measurement of VO_2 , VCO_2 , and calculation of respiratory exchange ratio (RER) using an open circuit calorimeter. Respiratory gas exchange was measured throughout exercise.

Experimental Protocol

As illustrated above, Pre-1 specimens were analyzed to determine the characteristics of skeletal muscle of old sedentary horses and served as baseline data for the training adaptation. Using light and electron microscopy, Post-1 muscle samples were compared with Pre-1 to evaluate the magnitude of ultrastructural damage following one bout of maximal exercise (incremental exercise test) on a treadmill, and the same comparisons were made between Pre-2 and Post-2, and between Pre-3 and Post-3 muscle samples. Ultimately, as previously described, the differences in the degree of ultrastructural damage between Pre-1 and Post-1 were not only compared with differences between Pre-2 and Post-2 in order to determine the training effect on ultrastructural damage responses to the same absolute external stress on aged equine skeletal muscles, but also compared with differences between Pre-3 and Post-3 muscle

samples to evaluate the extent of ultrastructural damage response to the same relative external stress on exercising muscles after the conditioning.

Additionally, Pre-1 and Pre-3 samples were analyzed and compared to evaluate the effects of exercise training on MHC isoform expression as well as oxidative and glycolytic capacities of exercise trained muscles in association with a change in maximal aerobic capacity (VO_{2max}) after training.

Muscle Sampling Procedure:

Muscle samples were taken from the triceps, semimembranosus, and masseter muscles by using the open biopsy technique. All pre- and post-exercise biopsy samples were collected from the right and left sides of each muscle group, respectively. First, the horses were sedated by intravenous injection of detomidine (40 (g/kg). The area for biopsy was then desensitized by injection of mepivacaine (2% solution, 10ml) and aseptically prepared.

Immediately after muscle sample collection, the samples were trimmed and dissected longitudinally in approximate sizes of 2mm length and 1mm thickness. The isolated tissue samples were placed in 5% phosphate-buffered glutaraldehyde fixative solution (5% glutaraldehyde, 0.05 M sodium phosphate), pH 7.4, overnight at 4°C for EM analyses. The remaining muscle sections were frozen in liquid nitrogen immediately after the isolation and stored at -80°C for bio- and histo-chemical analyses.

Blood Sampling Procedure:

Blood sampling catheters were inserted into the right jugular vein after treatment with local anesthesia. For lactate analysis, blood samples were obtained immediately before (pre) and after (post) each incremental test and also taken at the end of every 90-second stage throughout the duration of each incremental exercise test. Blood samples for creatine kinase (CK) analysis were taken before, immediately after, and 4 & 24 hours after each incremental test. Plasma and serum were harvested within 30 min of blood collections for determination of plasma lactate concentration and serum CK activity, respectively.

All blood samples were centrifuged for 15 minutes at the speed of 1500xg, and supernatants were aliquoted either into tubes containing YSI (The Yellow Springs Instruments Life Sciences) blood lactate preservative for plasma lactate or into normal storage tubes for serum CK analysis. All samples were then kept at -20 °C and stored at -80°C for later blood analyses.

Analytical techniques:***A. Measurement of VO₂max (Indirect calorimetry):***

The incremental exercise test consisted of each horse running on a treadmill (Sato, BIAB Industrial, Uppsala, Sweden) inclined at 4° for 90 seconds at 3 m/s, after which the treadmill speed was increased by 1 m/s every 90 seconds until the horse was unable to maintain its position on the treadmill. VO₂ was measured at 10-second intervals during the exercise test with an open

circuit calorimeter (Oxymax-XL, Columbus Instruments, Columbus, OH) as previously described (25). $\text{VO}_{2\text{max}}$ was defined as the value at which VO_2 reached a plateau despite further increases in speed. A plateau was defined as a change in VO_2 of $< 4 \text{ ml/kg/min}$ with increase in speed.

B. Histological analyses:

(1) Muscle fixation and analysis: Damage to muscle fibers was evaluated by light and electron microscope (LM and EM) analysis of cross sections and longitudinal sections of muscle fibers, as described by Yamaguchi et al. (98). After the first fixation in 5% phosphate-buffered glutaraldehyde fixative solution overnight, muscle sections were washed in 0.05 M sodium phosphate buffer at least 3 times and post-fixed in a buffered solution of 1% osmium tetroxide for 24 hours. After the second fixation, muscle samples were rewashed in phosphate buffer (3X) and dehydrated through a graded ethanol series and subsequently in acetone. All muscle preparation procedures were conducted using cold liquids.

After post-fixation, each specimen was embedded in epoxy resin and polymerized for 5 days at 60°C . Thick sections ($3 \mu\text{m}$) were cut on an ultramicrotome (LKB BROMMA 2088 Ultratome® V, Sweden) with a pre-made glass knife and stained on a glass slide for light microscope analysis. Thick sections were also observed under LM (Leitz Wetzlar, Dolan Scientific Instruments, Houston, Dallas, Texas) in order to determine the region of specimen for thin sectioning. Ultrathin sections (60 to 90 nm) were cut with a diamond knife on the same ultramicrotome and stained with uranyl acetate and

lead citrate. The sections were examined with a Philips EM-300 transmission electron microscope (Philips Electronic Instruments, Holland) operating at 60 kV. Approximately 6 to 10 pictures were taken under EM for each muscle at magnifications of 2800X, 5000X, 18000X, and 35000X.

(2) Semi-Quantification of muscle damage: The semi-quantitative assessments of morphological damage to fibers were made at the light and electron microscopic (LM and EM) levels on pictures of longitudinal sections of each muscle sample. The characteristics of aerobic exercise-induced ultrastructural cell damage were defined as follows: a) An oblique appearance due to individual myofibrils not returning to resting length; b) Splitting of sarcomere/myofibrils or Z-disc; c) Z-disc streaming; and d) Z-disc disruption. All EM pictures were grouped for each horse and muscle group. Investigators were unaware of horse identification and sampling time when evaluating EM.

The EM pictures, taken at five thousand times (5000X) magnification, were used for the semi-quantification of ultrastructural damage on each muscle sample. The longitudinal sections were evaluated based upon those characteristics as described above. The number of damage signs and damaged areas on 5,000X magnification pictures of each muscle group were carefully analyzed and scaled from 0 as minimum to 10 as maximum damage. Differences between scaled pre- and post-muscle damage levels were compared for all 3 incremental exercise tests in order to determine the magnitude of ultrastructural muscle damage.

C. Histochemical and biochemical analyses:

(1) Myosin heavy chain (MHC) electrophoresis: MHC electrophoresis was performed following the protocol of Talmadge and Roy (89). A standard sample of adult rat costal diaphragm muscle (Sigma, St. Louis, MO) was included in gels, and used as a control, which has shown to contain four different MHC isoforms identified as type I, IIA, IIX, and IIB (83). Approximately 10 mg of isolated muscle sample was homogenized in a 1:100 dilution in Tris buffer (62.5 mM Tris, pH 6.8). Electrophoresis was performed in 8% separating gel with 30% (w/v) glycerol and 14% stacking gel with 30% glycerol. Based on pre-determined total protein concentrations by protein assay, 10 microliter aliquots of diluted myosin, which contained 5 micrograms of protein, were subjected to electrophoresis for 26 hours at 285 V and 4°C with 20cm long vertical gels. Separating gels were stained with Coomassie blue and scanned with the Mode GS-700 Imaging Densitometer (BIO-RAD, Hercules, CA) to determine the optimal density and percentage of each MHC isoform.

(2) Enzyme analyses: Muscle tissue stored at -80°C was weighted at -20°C. The activity of citrate synthase (CS) as a measure of citric acid cycle activity was determined at 30°C using spectrophotometric techniques (17). The activities of 3-OH-acyl-CoA dehydrogenase (HAD) as a measure of lipid oxidation and lactate dehydrogenase (LDH) as a measure of glycolytic capacity were analyzed at 25°C using fluorimetric techniques as described by Chi et al. (17). For the CS assay, tissue was homogenized in a solution of Tris-buffer 100mM and triton X-100 0.1% in distilled H₂O to give a 5g/100ml solution. For

HAD and LDH assay, tissue was homogenized in a solution of glycerol 50%, K₂HPO₄ 20mM, 2-mercaptoethanol 5mM, EDTA 0.5mM, and BSA 0.05% in distilled H₂O to give a 100 dilution with muscle tissue.

Assay mixture. 1) *Citrate synthase*: TRIS-buffer 0.1 M pH 8.35, acetoacetyl-CoA 22.5 mM, oxaloacetate 25 mM, and 5,5'-dithiobis-(2 nitrobenzoic acid) 5mM. 2) *3-OH-acyl-CoA dehydrogenase*: Imidazole buffer 150mM, pH 6.1, (15mM imidazole base, 135 mM imidazole HCl), NADH 0.1 mM, acetoacetyl-CoA 0.2 mM, EDTA 1 mM, and bovine plasma albumin (BSA) 0.05%. 3) *Lactate dehydrogenase*: imidazole-HCl buffer, pH 7.0 (12mM imidazole base, 8mM imidazole HCl), pyruvate 0.1mM, NADH 0.05mM, and bovine serum albumin 0.02% as described by Passonneau and Lowry (64).

(3) Blood analyses: Plasma blood lactate concentrations were measured using commercial kits (Sigma Chemical Co. Kits, St. Louis, MO) on a microplate reader (Power Wave X, BIO-TEK® Instruments INC., Winooski, Vermont). The activity of creatine kinase in serum was analyzed by The Ohio University Veterinary Hospital Clinical Laboratory using an automated analyzer.

D. *Statistical analysis*:

Descriptive statistics were calculated, and the data summarized in graphical form where appropriate. MHC isoform expression, enzymatic activities, and VO_{2max} test data were analyzed using a dependent t-test to determine whether significant effects of exercise training existed. The effect of training was considered significant at $P < 0.05$.

Expression of MHC isoforms and enzyme activity were examined graphically, and the normality of distribution was tested by the Wilkes-Shapiro test. Non-normal variables were normalized by transformation (logarithmic or arcsine), and the hypothesis of age-related changes were tested using a Pearson Product Correlation Coefficient (r) analysis between age and changes in MHC isoform distribution and the activity of marker enzymes with a Tukey post-hoc test where appropriate.

Blood lactate concentrations were compared at the same speeds on each exercise trial by using a one-way repeated measures analysis of variance (ANOVA) with a Turkey post-hoc test. Although this comparison provided the training-induced adaptation on the metabolic rate of blood lactate at a given workload, it was limited to analyzing only those speeds that all horses achieved. Therefore, in order to examine the effect of exercise training on blood lactate concentrations in association with the improved exercise duration of aged horses during the same relative incremental exercise tests, the area under the lactate versus time curve was calculated for each horse on each occasion by using the trapezoidal rule, and then a one-way repeated measures ANOVA was used to compare these values on each occasion.

CHAPTER 4

RESULTS

Experiment 1: Determination of Age-related Changes in Muscle Structure and Enzyme Activity of Horses

Myosin Heavy Chain (MHC) Composition and Total Protein Concentrations with Aging

Myosin heavy chain (MHC) composition was not significantly altered with increasing age from 2 to 30 yrs in 18 horses (Figure 2, 3, and 4). There was no statistically significant correlation between horse age and the percentages (%) of all 3 MHC isoforms including type-I, type-IIA, and type-IIX ($P > 0.05$; $r = -0.36$, 0.2 , and -0.03 , respectively, $n = 18$). However, excluding an outlier, the data of a 9-year-old horse, the percent of type-I MHC isoforms decreased significantly with advancing age ($P < 0.05$; $r = -0.557$, $n = 17$).

There was no significant change ($r = -0.123$, $P > 0.05$) in muscle total protein concentration with aging in 18 horses (Figure 1).

Muscle Oxidative and Glycolytic Capacity with Aging

Activities of muscle oxidative (CS and HAD) and glycolytic enzymes (LDH): The activity of muscle citrate synthase (CS) decreased significantly with increasing age ($r = -0.406$, $P < 0.05$, $n = 18$). However, the activity of muscle 3-OH acyl CoA dehydrogenase (HAD) did not change with age (Figure 6; $r = 0.113$, $P > 0.05$). On the other hand, the activity of muscle lactate dehydrogenase (LDH) increased with advancing horse age (Figure 7; $r = 0.44$, $P < 0.05$).

Experiment 2: Determination of the Effect of Aerobic Conditioning on Muscle Composition and Damage Response to Exhaustive Incremental Exercise

Effect of Exercise Training on the Maximum Oxygen Consumption (VO_{2max}) and Horse Body Weights (kg)

Both absolute ($L \cdot min^{-1}$) and relative ($ml \cdot kg^{-1} \cdot min^{-1}$) values of the maximum oxygen consumption (VO_{2max}) of 6 aged mares were increased by 16% and 22%, respectively, after the 10 weeks of treadmill exercise training, and the improvement was statistically significant ($P < 0.05$). There was a significant decrease (4.2%, $P < 0.05$) in horse body weights (kg) after the 10 weeks of treadmill exercise training (Table 1).

Effect of Exercise Training on Plasma Lactate Concentrations during the Incremental Exercise Tests

There was a statistically significant decrease ($P < 0.05$) in plasma lactate concentration at both 5 and 6 meters per second (m/s) of the treadmill speeds during the incremental exercise tests following both 8 and 10 weeks of treadmill exercise training (Figure 8). The peak blood lactate concentrations were obtained at 6 m/s (7.7 ± 0.6 mmol/l) and 7 m/s (6.5 ± 1.8 mmol/l) during the first and the same absolute incremental exercise trials, respectively. During the incremental exercise trial performed after 10 weeks of training, the peak lactate value was achieved at 9 m/s (11.4 ± 0.6 mmol/l).

There was a significant improvement in treadmill run time to exhaustion of aged horses after 10 weeks of exercise training (24%, $P < 0.05$, from 9.1 ± 0.4 minutes to 11.3 ± 0.5 minutes). The maximum treadmill speeds attained during the incremental exercise trials were increased from 6.5 ± 0.3 m/s to 8.2 ± 0.4 m/s after conditioning (25.6%, $P < 0.05$). This training-induced improvement in performance has been confirmed by a statistically significant increase ($P < 0.05$) in total lactate response (sec•mmol/l) during the same relative incremental exercise test compared with either the values during the first (pre-training) incremental exercise test or the values during the second (same absolute) incremental exercise test.

Effect of Exercise Training on MHC Composition and Total Protein Concentration

There was no alteration in MHC composition in the masseter muscle of all 6 aged horses after the 10 weeks of exercise conditioning ($P > 0.05$) (Table 3). In contrast, there was a significant increase (10.9%, $P < 0.05$) in % type-IIA MHC isoform expression of the semimembranosus muscle after conditioning (Table 3). Furthermore, % type-IIIX MHC isoform expression of triceps decreased significantly (3.1%, $P < 0.05$) after conditioning (Table 3). Although it was not statistically significant ($P > 0.05$), after training, % type-I and % type-IIIX MHC isoforms of the semimembranosus muscle were decreased by 11.8% and 3.1%, respectively, while % type-I and % type-IIA MHC isoforms of triceps increased by 4.2% and 3.8%, respectively (Table 3).

None of the 3 muscle groups showed a significant increase ($P > 0.05$) in total protein concentrations after the 10 weeks of conditioning (Table 2). In addition, there was no significant difference in total protein concentration between all 3 muscle groups ($P > 0.05$).

Effect of Exercise Training on Muscle Oxidative and Glycolytic Capacity

Muscle Citrate Synthase (CS): The activity of CS (Table 4) in triceps muscle increased significantly by 41% ($P < 0.05$) after 10 weeks of conditioning, while CS activities in semimembranosus and masseter muscles were not changed significantly after the conditioning ($P > 0.05$). In addition, masseter

muscle showed significantly (approximately 200%) greater CS activity in comparison with triceps and semimembranosus muscle groups ($P < 0.05$).

Muscle 3-OH Acyl CoA Dehydrogenase (HAD): Similar to CS activity, triceps muscle showed a significant increase ($P < 0.05$) in the activity of muscle HAD (Table 4) by 72% after 10 weeks of exercise conditioning. There were no significant changes in HAD activity in either the semimembranosus or masseter muscles. In addition, HAD activities in masseter muscle were significantly (approximately 3X) greater in comparison to those in semimembranosus and triceps muscles ($P < 0.05$).

Muscle Lactate Dehydrogenase (LDH): There were no significant changes in the activities of LDH (Table 4) after the 10 weeks of exercise conditioning in all muscle groups ($P > 0.05$). However, LDH activity in masseter muscles was significantly less ($P < 0.05$) by approximately 50% in comparison to that in semimembranosus and triceps muscles.

Effect of Exercise Training on Muscle Fiber Damage and Creatine Kinase Response

As shown in Pictures 2, 5, and 8, there was only minor damage signs observed in all 3 muscle groups (Pre-1 semimembranosus, triceps, and masseter) prior to the first incremental exercise test. After each incremental exercise test at week 0, 8, and 10 weeks of conditioning, longitudinal muscle sections showed certain levels of increase in muscle structural damage on both

LM and EM examination (Pictures 2, 3, 4, 5, 6, and 7). The only exception was the control (masseter) muscle group (Picture 8).

The characteristics of ultrastructural muscle cell damage (Picture 1) in the present study were different from those previously reported characteristics of the contraction-induced damage in other studies (23, 76, 77). Therefore, the present study is the first to introduce these unique observations, which were defined by the present investigators as “aerobic exercise-induced ultrastructural muscle damage.” The characteristics of aerobic exercise-induced ultrastructural cell damage (see Picture 1) were defined as follows: a) Oblique appearance due to individual myofibrils not returning to resting length; b) Splitting of sarcomere/myofibrils or Z-disc; c) Z-disc streaming; d) Z-disc disruption. All pre- and post-muscle samples of each incremental exercise test were compared, and differences in scaled ultrastructural damage levels were determined as described earlier.

Only in the semimembranosus muscle (Pictures 2, 3, and 4), was there a tendency for decrease in severity of ultrastructural fiber damage at the same absolute and the same relative workloads. In response to the first incremental exercise trial prior to exercise conditioning, the difference in damage scale scores between Post-1 and Pre-1 muscle sections was 9 (pre-training damage response, $\text{Post-1} - \text{Pre-1} = 9$). In response to the same absolute incremental exercise trial after 8 weeks of conditioning, the difference in damage scale scores between Post-2 and Pre-2 muscle sections was 7 ($\text{Post-2} - \text{Pre-2} = 7$). Further, in response to the same relative incremental exercise trial after 10 weeks of

conditioning, the difference in damage scale scores between Post-3 and Pre-3 muscle sections was also 7 ($\text{Post-3} - \text{Pre-3} = 7$). Therefore, damage responses were diminished from scale score 9 to scale score 7 after exercise conditioning at both same absolute and the same relative workloads. In triceps muscles (Pictures 5, 6, and 7), however, there were similar responses in pre- and post-training ultrastructural cell damage levels after 8 weeks of training (damage scale score difference between Pre-2 and Post-2 muscle sections = 4), while less damage was observed after 10 weeks of training (damage scale score difference between Pre-3 and Post-3 muscle sections = 3). Therefore, the damage response at the same absolute workloads was not different from pre-training damage response (damage scale score difference between Pre-1 and Post-1 muscle sections = 4), although damage level was reduced at the same relative workloads. The control (masseter) muscle group did not show any difference in damage responses before and after training (Picture 8).

Exercise conditioning did not significantly affect the activity of serum creatine kinase (CK) at any sampling time points after the incremental exercise tests ($P > 0.05$) (Figure 9). In comparison with each pre-exercise CK value, however, there was a significant increase in CK activity ($P < 0.05$) after all 3 incremental exercise tests; at post-0 and post-4hrs of the first incremental exercise test (wk-0); at post-0 and post-24hrs of the second incremental exercise test (the same absolute, wk-8); and at post-24hrs of the last incremental exercise test (the same relative, wk-10).

Aging Effect on Total Protein Concentration

Figure 1: Age-related changes in muscle total protein concentration. $r = -0.12$ ($P > 0.05$).

Figure 2: Age-related changes in %type-I myosin heavy chain (MHC) isoform expression in semimembranosus muscle. $r = -0.36$ ($P > 0.05$, $n = 18$); excluding the data of a 9-year-old horse ($n = 17$), $r = -0.557$, $P < 0.05$.

Figure 3: Age-related changes in %type-IIA myosin heavy chain (MHC) isoform expression in semimembranosus muscle. $r = 0.2$ ($P > 0.05$).

Figure 4: Age-related changes in %type-IIx myosin heavy chain (MHC) isoform expression in semimembranosus muscle. $r = -0.03$ ($P > 0.05$).

Figure 5: Age-related changes in muscle citrate synthase activity. $r = -0.41$, $P < 0.05$.

Figure 6: Age-related changes in muscle 3-OH acyl COA dehydrogenase activity. $r = 0.11$ ($P > 0.05$).

Figure 7: Age-related changes in muscle lactate dehydrogenase activity. $r = 0.44$, $P < 0.05$.

Figure 8: Effect of exercise training on plasma lactate concentration. Mean $\pm$ SEM, Week-0 = an incremental exercise test before exercise training; Week-8 = an incremental exercise test after 8 weeks of exercise training; Week-10 = an incremental exercise test after 10 weeks of exercise training; * indicates statistically significant difference between pre- and post-exercise training lactate values at $P < 0.05$, $n = 6$.

Figure 9: Effect of exercise training on serum creatine kinase (CK) activity.

Mean $\pm$ SEM, Week-0 = an incremental exercise test before exercise training; Week-8 = an incremental exercise test after 8 weeks of exercise training; Week-10 = an incremental exercise test after 10 weeks of exercise training; Pre-0 = immediately before an incremental exercise test; Post-0 = immediately after an incremental exercise test; Post-4hrs = 4 hours after an incremental exercise test; Post-24hrs = 24 hours after incremental exercise test; no statistically significant difference between pre- and post-exercise training CK values ($P > 0.05$); * indicates statistically significant difference in post-incremental exercise test CK values compared with pre-exercise test CK values at $P < 0.05$, $n = 6$.

	Before Training	After Training
Body weight (kg)	487 ± 24	466 ± 22*
VO _{2max} (relative) (ml•kg ⁻¹ •min ⁻¹)	80.4 ± 3.0	98.3 ± 2.7*
VO _{2max} (absolute) (l•min ⁻¹)	39.3 ± 3.0	45.8 ± 2.2*

Table 1: Effect of exercise training on maximum oxygen consumption (VO_{2max}) and body weight. Means ± SEM, * indicates statistically significant difference between pre- and post-exercise training values at $P < 0.05$, $n = 6$.

	Before Training	After Training
Semimembranosus (%)	11.6 ± 1.1	11.5 ± 0.2
Triceps (%)	10.2 ± 0.6	11.9 ± 0.4
Masseter (%)	10.3 ± 0.4	11.6 ± 0.6

Table 2: Effect of exercise training on muscle total protein concentration. Means ± SEM, no statistically significant difference between pre- and post-exercise training values at $P < 0.05$, $n = 6$.

Muscle Group	MHC isoforms (%)	Before Training	After Training
Semimembranosus	MHC type-I	12.1 ± 1.9	10.7 ± 1.8
	MHC type-IIA	29.7 ± 2.4	32.9 ± 1.5*
	MHC type-IIX	58.2 ± 1.7	56.4 ± 1.1
Triceps	MHC type-I	8.8 ± 2.6	9.2 ± 2.3
	MHC type-IIA	33.0 ± 1.1	34.4 ± 1.5
	MHC type-IIX	58.2 ± 1.7	56.4 ± 1.6*
Masseter	MHC type-I	64.2 ± 5.7	57.9 ± 2.3
	MHC type-IIA	13.4 ± 3.4	16.0 ± 2.1
	MHC type-IIX	22.4 ± 2.4	26.1 ± 0.6

Table 3: Effect of exercise training on percent myosin heavy chain (MHC) isoform expression. Means ± SEM, * indicates statistically significant difference between pre- and post-exercise training values at $P < 0.05$, $n = 6$.

Muscle Enzyme	Muscle Group	Before Training	After Training
Citrate synthase ($\mu\text{mol/g/min}$)	Semimembranosus	19.6 ± 1.8	21.3 ± 3.0
	Triceps	20.4 ± 1.8	$28.7 \pm 1.3^*$
	Masseter	39.1 ± 4.9	35.1 ± 2.5
3-OH acyl COA dehydrogenase ($\mu\text{mol/g/min}$)	Semimembranosus	10.3 ± 0.7	10.9 ± 1.1
	Triceps	9.9 ± 1.4	$17.1 \pm 1.2^*$
	Masseter	32.0 ± 5.2	26.5 ± 1.6
Lactate dehydrogenase ($\mu\text{mol/g/min}$)	Semimembranosus	169.1 ± 4.5	173.4 ± 4.8
	Triceps	170.0 ± 5.1	170.1 ± 4.1
	Masseter	84.7 ± 5.2	84.2 ± 4.4

Table 4: Effect of exercise training on muscle enzyme activity. Means $\pm$ SEM, * indicates statistically significant difference between pre- and post-exercise training values at $P < 0.05$, $n = 6$.

Picture 1: “Aerobic Exercise-induced Ultrastructural Muscle Damage” at the Electron Microscopic (EM) Level. The characteristics of aerobic exercise-induced ultrastructural cell damage were defined as follows: a) Oblique appearance due to individual myofibrils not returning to resting length; b) Splitting of sarcomere/myofibrils or Z-disc; c) Z-disc streaming; and d) Z-disc disruption, Post = post-incremental exercise test; x = magnification.

Picture 2: Ultrastructural muscle damage in Semimembranosus at light and electron microscopic (LM & EM) levels after the first incremental exercise (VO_{2max}) test before exercise training. Pre = pre-1st incremental exercise test; Post = post-1st incremental exercise test; x = magnification.

Picture 3: Ultrastructural muscle damage in Semimembranosus at LM and EM levels after the second (same absolute) incremental exercise test after 8 wks of exercise training. Pre = pre-2nd incremental exercise test; Post = post-2nd incremental exercise test; x = magnification.

Picture 4: Ultrastructural muscle damage in Semimembranosus at LM and EM levels after the third (same relative) incremental exercise test after 10 wks of exercise training. Pre = pre-3rd incremental exercise test; Post = post-3rd incremental exercise test; x = magnification. A longitudinal section of Semimembranosus (Pre) shows hypercontracted sarcomeres.

Picture 5: Ultrastructural muscle damage in Triceps at LM and EM levels after the first incremental exercise (VO_{2max}) test before exercise training. Pre = pre-1st incremental exercise test; Post = post-1st incremental exercise test; x = magnification.

Picture 6: Ultrastructural muscle damage in Triceps at LM and EM levels after the second (same absolute) incremental exercise test after 8 wks of exercise training. Pre = pre-2nd incremental exercise test; Post = post-2nd incremental exercise test; x = magnification. A longitudinal section of Triceps (Pre) shows hypercontracted sarcomeres.

Picture 7: Ultrastructural muscle damage in Triceps at LM and EM levels after the third (same relative) incremental exercise test after 10 wks of exercise training. Pre = pre-3rd incremental exercise test; Post = post-3rd incremental exercise test; x = magnification. Longitudinal sections of both Triceps (Pre) and (Post) show hypercontracted sarcomeres.

Picture 8: Ultrastructural muscle damage in Masseter at LM and EM levels after the first incremental exercise (VO_{2max}) test before exercise training. Pre = pre-1st incremental exercise test; Post = post-1st incremental exercise test; x = magnification.

Picture 9: Potential evidence of the *Exercise-induced Muscle Regeneration Process?* - Potential Formation of *Myotube* or *Capillaries*?

CHAPTER 5

DISCUSSION

Experiment 1: Determination of Age-related Changes in Muscle Structure and Enzyme Activity of Horses

Age-related Changes in MHC Composition and Total Protein Concentration

In the present study, neither myosin heavy chain (MHC) composition nor total protein concentration in the semimembranosus muscle of horses was significantly altered with advancing age. Therefore, our hypothesis, which stated that skeletal muscle of aged horses will be associated with an increased expression of type-I MHC isoform and a decreased expression of type-II MHC isoform, was not supported. However, excluding an outlier, the data of a 9-year-old horse, the percent of type-I MHC isoforms appeared to decrease with advancing age ($P < 0.05$; $r = -0.557$, $n = 17$). Further, the Pearson Product Correlation Coefficient (r) illustrates a tendency for the percent of type-II MHC to increase with advancing age ($r = 0.36$, $P > 0.05$).

As previously discussed, in untrained humans, the type-II to type-I MHC isoform ratio increases with increasing age possibly due to a shift from type-II to type-I MHC isoforms during the aging process (94), but the age-associated

skeletal muscle adaptations appear to be muscle-group specific (41). The age-related transformation from type-II to type-I fibers is thought to be due to motor unit remodeling, which is associated with altered functional demands due to a weight gain as a result of physical inactivity with aging (54).

There are very few investigations performed with sedentary horses related to age-associated physiological adaptations (69). A study by Rivero and his associates in 1993 is the only equine research study that examined age-related adaptations in *untrained* male and female horses (n = 143) with various age groups and breeds (69). Similar to humans, they reported an increase in the percent of type-I fibers as well as a decrease in the percent of type-II fibers in the gluteus medius muscle of untrained horses with advancing age.

A study utilizing race horses also reported that compared with the triceps, vastus, and semitendinosus, the gluteus medius was the only muscle that possessed a higher type-I/II ratio in the oldest age group (from 10 to 28 years) than in the younger groups (26). This finding suggests that the age-related adaptations in equine skeletal muscle may be also muscle-group specific. Additionally, using horses from 1 to 6 years old, Roneus et al. (72, 73) also demonstrated an increase in percent type-I fibers and a decrease in percent type-IIb fibers with aging in trained horses.

Therefore, the present MHC results agree with previously reported data for aging horses, especially for trained horses (2). However, the present MHC data conflict with previous findings for aging humans (94).

Age-related Changes in Muscle Oxidative and Glycolytic Capacity

As we hypothesized, the oxidative capacity of the semimembranosus muscle declines with advancing horse age, although the adaptation is enzyme specific. The age-associated reduction in mitochondrial citrate synthase (CS) activity indicates a decrease in activity of the citric acid cycle with advancing age. However, no age-related alteration in muscle 3-OH acyl CoA dehydrogenase (HAD) activity was observed, which suggests that the lipid oxidative capacity is not altered during the aging process. Further, lactate dehydrogenase (LDH) data indicate that the muscle glycolytic capacity increases with advancing age. Therefore, the present results of the age-related changes in CS activity supported our oxidative capacity hypothesis, but the activities of HAD and LDH did not.

No enzyme activity profile is available in untrained horses. However, the age-related declines in muscle oxidative capacity have been well documented in sedentary humans (20, 41, 43, 75). A decline in the activity of mitochondrial enzymes such as CS and cytochrome c oxidase have been reported in the vastus lateralis (VL) of aging sedentary humans, and these declines appear to be associated with the changes in the rate of mitochondrial protein synthesis with aging (75). Similarly, Houmard et al. (41), demonstrated a decline in CS activity in the gastrocnemius, but not in the VL of aging humans, and the age-related changes in CS activity were related to the maximal oxygen consumption (VO_{2max}).

Although potential mechanisms are not clear, a tendency for alterations in the MHC composition seen in the present study may account for our findings in muscle enzymatic changes [$\downarrow$ in percent of type-I MHC ($P < 0.05$ without outlier) $\Rightarrow \downarrow$ in CS and concomitant $\uparrow$ in percent of type-II MHC ($P > 0.05$, $r = 0.36$) $\Rightarrow \uparrow$ in LDH] due to the known characteristics of type-I and II MHC isoforms (82). Another potential explanation for our findings in the age-related skeletal muscle adaptations may be associated with either a variation in training history of the 18 horses or a low number of younger horses ($n = 3$ from 1 to 10 years old) among the 18 horses. Therefore, further investigations may be necessary with a larger sample size of horses in various age ranges in order to verify the age-associated findings of the present study.

Experiment 2: Determination of the Effect of Aerobic Conditioning on Muscle Composition and Damage Response to Exhaustive Incremental Exercise

Effect of Exercise Training on the Maximum Oxygen Consumption (VO_{2max}) in association with Changes in Horse Body Weights (kg)

Aged mares lost approximately 20 kilograms (kg) of body mass following 10 weeks of treadmill exercise training (Table 1). Based on the body weights, the relative values of the maximum oxygen consumption (relative VO_{2max}) of the 6 aged mares were significantly increased after 10 weeks of treadmill exercise training. As the decreased horse body weights resulted in an increase in the mean values of relative VO_{2max} (ml/kg/min) regardless of the training effect, the

absolute $\text{VO}_{2\text{max}}$ values (L/min) play a role in confirming the training-induced improvement in both the maximum aerobic capacity and the treadmill run time to exhaustion of 6 aged horses.

There is no data available from horses regarding to the age-related changes in the whole body maximum aerobic capacity ($\text{VO}_{2\text{max}}$). In humans, however, it is well established that maximum aerobic capacity declines with increasing age (20, 43), and the rate of decline in $\text{VO}_{2\text{max}}$ with age does not appear to relate with individual differences in training status (43, 97). Although no training-induced physiological adaptations have been previously evaluated in aged mares, the findings of the present study suggest that relatively intense aerobic exercise training can markedly improve the maximum aerobic capacity of older individuals.

Effect of Exercise Training on Plasma Lactate Concentrations during the Incremental Exercise Tests

The results of the present study support previous findings that aerobic exercise training reduces plasma lactate concentrations at given absolute workloads (3), and the adaptation is known to be due to the improved lactate clearance rate (R_d) after endurance training in association with the increased activity of lactate transporters (24, 27).

The mean of treadmill run times to exhaustion in the present study was improved from 6.5 m/s to 8.2 m/s following 10 weeks of exercise training in aged mares. The improved endurance exercise capacity has been confirmed by

greater total lactate responses (sec•mmol/l), which were assessed by comparisons of the calculated area under the lactate-time curve based on the trapezoidal rule. The area under the curve method also allows us to demonstrate a reduced lactate response at given absolute workloads during the same absolute incremental exercise trial after training.

Effect of Exercise Training on MHC Composition and Total Protein Concentration

The results of the present study support our hypothesis related to MHC isoform composition. We observed an increase in the percent of type-IIA MHC as well as a decrease in the percent of type-IIIX in the muscle groups of exercise-trained aged mares, and no alteration in MHC composition of the unloaded control (masseter) muscle after the aerobic exercise training. Additionally, total protein concentration was not altered by with exercise training, and there were also no differences between muscle groups in aged horses.

Again, although no training study has been conducted with aged mares, Essen et al. (26) demonstrated in race horses that there was no difference in the ratio of type-I/II fibers in all age groups. They also suggest that training, rather than age, is regarded as the factor responsible for inducing the changes in muscle composition (26). The hierarchical response of training predominating over age in horses is also supported by studies in healthy trained horses with age ranging from 1 to 6 years (72, 73). In another recent aging study with trained horses (age from 1 to 24 years), the older group possessed the greatest

proportion of type-II MHC isoforms, and significant correlations have been found between performance and the percent of type-II MHC isoform expression of the gluteus medius and biceps femoris muscles (2).

The findings in the present study have been recently supported by Serrano et al. (2000) in young horses, which demonstrated that 3 months of training induced similar changes in MHC composition (82). Prolonged aerobic exercise training (8 months) induces a further decline in the percent of type-IIIX MHC isoform expression as well as an increase in the percent of type-I MHC isoform expression (67, 82). Therefore, although our findings suggest that the training-induced skeletal muscle adaptations may be also muscle-group specific (41), the results indicate that the conversion of MHC isoforms is in the order of type-IIIX $\rightarrow$ IIA $\rightarrow$ I with increased duration of exercise training in horses (82). This shift in myosin isoforms shows an agreement with fiber type shifts from type-IIb to type-IIa following vigorous exercise training (86). Aged skeletal muscle also shows an increase in type-II fiber area (82) following relatively intense exercise training, and this is mainly due to increase in type-IIa fibers. However, the rate of exercise-induced myosin shifts seems to decrease with increasing age (48).

Further, even though the potential mechanism is not clear, a mechanical load applied to exercising skeletal muscle during treadmill running, may diminish the age-related motor unit remodeling (54) by enhancing the recruitment of type-II fibers (39). Consequently, vigorous exercise training may prevent the elderly from losing their functional capacity, which is related to the age-related fiber type transformation in association with motor unit remodeling.

Effect of Exercise Training on Muscle Oxidative and Glycolytic Capacity

The activity of mitochondrial CS and HAD in the triceps muscle of aged mares was markedly altered by 10 weeks of treadmill exercise training, whereas there was no alteration observed in muscle LDH activity after training. The results of the present study suggest that relatively intense aerobic exercise training can markedly improve the muscle oxidative capacity of aged horses (82), and the training-induced improvements in mitochondrial enzyme activity may be muscle-group specific. One of the potential mechanisms for the muscle-group specific adaptations is the greater amount of mechanical load applied on the relatively small triceps muscle compared with the semimembranosus muscle during the treadmill running as approximately 60% of body weight occupies the front portion of a horse body.

When skeletal muscle has adequate blood flow and O₂ delivery, muscle O₂ consumption in both older and young adults is dependent on intracellular processes within the exercising muscle (80). Further, in skeletal muscle of aged animals, aerobic pathways are particularly sensitive to both endurance and resistance exercise conditioning (47). Despite the fact that the whole body maximum aerobic capacity (VO_{2max}) is a function of the cardiovascular capacity, as seen in humans (57), the exercise training-induced increase in muscle oxidative capacity observed in the present study also appears to be associated with the training-induced improvement in the maximum oxygen consumption (VO_{2max}) in aged horses.

Regardless of mammalian age and species, endurance exercise training is known to induce an increase in the activity of the key aerobic enzymes that are markers of muscle oxidative capacity (17, 26, 36, 37, 38, 71, 82, 88). Although the adaptations appear to be glycolytic enzyme specific (73, 93), the majority of previous findings suggest no training-induced alterations in muscle LDH activity, which supports the data of the present study (26, 38). The adaptations in metabolic properties of muscle from exercise-trained animals are typically well coordinated with the training-induced transition in MHC composition (82, 88).

In addition, the proportion of type-I or -IIA fibers also appeared to be positively correlated to oxidative enzyme activities in horses (93). Stone et al. (88) demonstrated adaptive responses of hypertrophying skeletal muscle to 6-weeks of endurance running by measuring CS activity and MHC expression in Sprague-Dawley rats. They suggest that endurance training-induced increases in muscle oxidative enzyme content are compromised when the process of muscle hypertrophy occurs simultaneously (88). It has been also suggested that the pattern of MHC plasticity is regulated by the relative loading state imposed on the muscle during repetitive usage (88).

In the present study, ultrastructural analysis, via electron microscope, also provided supportive evidence of training-induced elevation in the activity of mitochondrial enzymes. Numerous swollen mitochondria were observed following the exercise conditioning and likely provide a greater space for CS activity (55). A larger population of swollen mitochondria were also observed in EM pictures of the masseter muscle (see Picture 8), which showed markedly

greater activities of both mitochondrial enzymes, CS and HAD, in comparison with the semimembranosus and triceps muscles.

Effect of Exercise Training on Muscle Fiber Damage and Creatine Kinase Response

The results of the present investigation related to the determination of the magnitude of ultrastructural muscle cell damage following exercise training are presented based on our new semi-quantitative analytical methods of EM pictures (5,000X) of longitudinal muscle sections that were taken from each experimental condition. Our results suggest that 10 weeks of relatively intense treadmill exercise training can diminish the magnitude of ultrastructural muscle cell damage of mechanically loaded muscles in response to the incremental exercise trials at the same absolute as well as at the same relative workloads. However, it also indicates that the training-induced attenuating effect on the severity of exercise-induced muscle damage may occur on a specific muscle group.

As discussed, no comparison has been made in any mammalian species between ultrastructural damage responses following pre- and post-exercise training at given absolute or at the same relative workloads. In addition, none of previous equine research has been conducted in the area of exercise-induced muscle damage, particularly with EM technology. However, it has been suggested that skeletal muscle of aged humans and animals appears to be more susceptible to contraction-induced damage than that of young individuals following an acute exercise stimulus (59, 100).

Roth et al. recently attempted to examine the effects of heavy resistance exercise training on ultrastructural muscle damage in young and aged individuals (76, 77) by using EM analysis. They demonstrated that 9 weeks of high-volume, heavy-resistance training can result in similar levels of ultrastructural muscle damage in the vastus lateralis of young and older men (76). Further, a follow-up report from the same research group suggests that the muscle damage levels appear to be greater in older women than young women, while a similar improvement in muscle strength was observed in both young and older groups following the same training regimen (77). However, the age and gender difference in training adaptations claimed by this group are questionable due to a lack of measurement of an acute exercise-induced damage response before training, as they only compared pre- and post-training muscle samples (76, 77).

In the present study, the training-induced attenuating effect was only observed in the semimembranosus muscle, but not in the triceps muscle. This muscle-group specific morphological consequence is in good agreement with our reported training adaptations on mitochondrial enzymatic activity (55). As previously discussed, the same theory can be applied on the potential mechanisms responsible for the muscle-group specific adaptations. There is greater mechanical stress applied to relatively small muscle, the triceps, compared with the semimembranosus muscle during the treadmill running due to the greater proportion of body weight at the front portion of the horse's body. This theory holds that the degree of damage to the triceps muscle of aged mares would be greater than that to the semimembranosus throughout the entire 10

weeks of treadmill exercise training. In addition, it is likely that after the same degree of contraction-induced injury, the extent of recovery is less in muscles from aged animals (7).

Satellite cells are known to be critically involved in the muscle fiber regeneration process, and their population and proliferative activity appears to be reduced during the aging process (81). The age-related changes in satellite cells may partially explain the age-related delay or incomplete recovery response following injury (7, 78). Consequently, it is possible that the failure in finding differences in the level of damage in the triceps muscle following each occasion may be due to the age-related diminished recovery. This may be especially true in the continuously damaged triceps due to the nature of the treadmill running in association with the nature of the horse's body.

Since no data is available regarding the characteristics of "aerobic exercise-induced ultrastructural cell damage" in skeletal muscles of any species, the unique observations from the present study provide new evidence of the early damage consequences in mammalian skeletal muscle following exhaustive treadmill running. One of the unique observations compared with those from resistance exercise was "an oblique appearance" in a certain portion of the longitudinal section area (see Picture 1). This phenomenon seems to be due to a number of individual myofibrils not returning to normal resting length after exhaustive treadmill exercise, as this activity requires tremendous numbers of relatively high-speed muscle contractions in comparison with resistance exercise.

The theory of “oblique appearance” in the present study based on our observation (see Picture 1) corresponds with previously reported potential explanations by Morgan and Allen (62). One of their explanations for an early decline in submaximal tension of damaged muscle at a given muscle length is a shift of optimum length on length-tension curves due to localized regions of overstretched sarcomeres in early events of muscle damage (62).

Another unique symptom of the myofibrils observed via EM was splitting of sarcomere/myofibrils or Z-discs (see Picture 1). Although no clear explanation is available at this point, one of potential mechanisms is an early consequence of the process of exercise-induced muscle hypertrophy. In other words, it may be an initiative stage for the process of adding myofibrils inside muscle fibers during the hypertrophic process after numerous lengthening muscle contractions.

Exponential blood CK elevations have been observed following all incremental exercise trials. However, the CK data of the present study also suggest that the measurement of blood CK activity as an indirect cell damage marker is not sensitive enough to detect our direct morphological evidence under LM and EM (18, 50, 59). As suggested by Faulkner et al. (28), in spite of the importance of direct morphological evidence of contraction-induced cellular damage, the magnitude of muscle damage is almost impossible to be quantified by direct methods. It is possibly due to the number of limitations or the nature of the EM analytical process, such as handling of small muscle specimens, qualities of sample sections and pictures, and subjectivity of investigators.

In the present study, a vigorous and methodical approach to perform a semi-quantitative analysis of EM pictures was employed. The investigators were blinded to all EM pictures, and ranking scales were created for all possible manipulators including picture quality, oblique section levels, and number and size of damaged areas. Therefore, all pre- and post-muscle samples for all experimental possibilities were compared for the final determination of the magnitude of morphological muscle damage. We believe that our new analytical approach in the present study may open another gate for future morphological studies in this area.

In conclusion, the results of the present study suggest that age-related changes in morphology and biochemistry of equine skeletal muscle may differ from those observed in humans. However, similar to humans, the oxidative capacity of equine skeletal muscle appears to decrease with advancing age, but relatively intense aerobic exercise training can overcome the age-related reduction in muscle oxidative capacity. This finding is consistent with an exercise training-induced transition of MHC isoforms in aged skeletal muscle that appears to become more oxidative after training.

A new EM analysis herein provides evidence that aerobic exercise training can attenuate the severity of the exercise-induced ultrastructural cell damage in aged skeletal muscle at given absolute and/or at the same relative workloads. However, these age-related and exercise training-induced adaptations in mammalian skeletal muscle may be muscle-group specific.

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APPENDIX A

INSTITUTIONAL LABORATORY ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

THE OHIO STATE UNIVERSITY
INSTITUTIONAL LABORATORY ANIMAL CARE AND USE COMMITTEE

ACTION OF THE COMMITTEE

 X Original Review

 Periodic Review

ANIMAL USE PROTOCOL:

**OIAO082 Ultrastructural Muscle Damage and Muscle Oxidative Capacity in Old Horses,
Steven T. Devor, Kenneth W. Hinchcliff and Jeong-su Kim, Dept. of Physical
Activity and Educational Services**

The Institutional Laboratory Animal Care and Use Committee took the following action on the protocol cited above:

 X Approved

 Disapproved

 Approved with Modifications*

* Modifications requested by the Committee have been accepted by the Investigator(s) and, therefore, the protocol is **APPROVED**.

Approval of the protocol is for the period 07/01/01 through 06/30/04. The Committee will periodically contact investigators to seek information about approved protocols. Periodic contact for this protocol is next scheduled for 06/02.

The Investigator(s) shall immediately bring to the attention of the Institutional Laboratory Animal Care and Use Committee any changes proposed for the approved protocol as they relate to the care or use of laboratory animals. The Committee will decide whether the extent or type of changes proposed warrant formal Committee review. If such a review is deemed necessary, the chairperson shall schedule the review for the earliest feasible time.

Prior to the initiation of any projects you should contact the attending veterinarian or person in charge of animal care within the college in which your animals are to be housed. Additional information is generally required to comply with the specific college needs before the initiation of any projects. Failure to make such contact may delay the initiating of this project.

Certification of review and approval will be transmitted to external sponsors, as required, by the Research Foundation. **INVESTIGATORS ARE RESPONSIBLE FOR CONVEYING A COPY OF THIS DOCUMENT TO THEIR SPONSORED PROGRAM DEVELOPMENT OFFICERS.**

Date 22 June 2001

Signed

(Chairperson)

ILACUC A - 05 (6/91)

APPENDIX B

A HORSE INFORMATION RECORD FORM WITH MUSCLE SAMPLING PROCEDURE FOR THE CROSS-SECTIONAL STUDY

EFFECTS OF EXERCISE TRAINING ON ULTRASTRUCTURAL MUSCLE DAMAGE AND MUSCLE OXIDATIVE CAPACITY IN OLD HORSES

Muscle Sampling Procedure for Cross-Sectional Study

- ✓ **Requirements**: must meet following requirements in order to participate in the present investigation
- ☐ **Sedentary** – must have not been regularly trained in the last 6 months
 - ☐ **No skeletal muscle diseases** (i.e. skeletal muscle disorder) in subject's history

Muscle Sample Information: Label – _____ (see labeling procedure below)

Sampling Date: _____ (m)/ _____ (d)/ _____ (y) & Time: _____ : _____ (am. or pm).

➤ Performed by: Dr. _____

1. ***Any other disease in history?*** – Yes or No. If your answer is Yes,
 - ☐ What kind? & How serious?:
 - ☐ When? & How long?:
2. ***Age*** – _____ yrs old
 - ☐ Group 1: 2 – 5 yrs
 - ☐ Group 2: 6 – 15 yrs
 - ☐ Group 3: 16 – 25 yrs
 - ☐ Group 4: 26 – 30 yrs
 - ☐ Group 5: 30+
3. ***Gender*** – _____ (M or F)
4. ***Breed*** – _____ (Thoroughbred, Standardbred, Quarterhorse, or etc.)
5. ***Sampling Protocol***
 - ☐ Muscle samples will be collected by excisional biopsy of the semimembranous muscle performed by Drs. Beard or Hinchcliff.
 - ☐ Biopsy will be performed after induction of local analgesia by injection of 10 ml of mepivacaine hydrochloride and surgical preparation of the area.
 - ☐ 3 cm skin incision will be closed with interrupted skin sutures and dressed with antimicrobial ointment.
 - ☐ Muscle samples will be lapped with prepared aluminum foil (cut & labeled)
 - ☐ Muscle samples will be frozen in liquid nitrogen within 15 seconds of collection and stored at – 80°C until analysis.
 - ☐ Horses will be administered tetanus prophylaxis and sutures will be removed in 7 – 10 days.
6. ***Labeling Procedure***
 - ☐ Age/Sex/Breed & Sampling Month/Date/Year/Time
 - ☐ i.e. – **20/M/T & 9/20/01/15** for 20 yrs/male/Thoroughbred sampled at 3:00 pm. on Sep/20th/2001

APPENDIX C

A SAMPLE EXERCISE TRAINING PROTOCOL

Old Horse Exercise Training Protocol (#4) (1/7/02~1/17)

Minute	Duration (minutes)	Intensity (%VO _{2max})	Speed for Each Horse (m/s)					
			275	325	328	330	346	69
0 - 1	1	Warm-up	1.9	1.9	1.9	1.9	1.9	1.9
1 - 2	1	~40%	3.0	3.0	3.0	3.0	3.0	3.0
2 - 7	5	55%	3.9	3.5	4.4	3.9	3.9	3.9
7 - 10	3	80%	5.6	4.8	6.4	5.6	5.6	5.6
10 - 13	3	55%	3.9	3.5	4.4	3.9	3.9	3.9
13 - 15	2	80%	5.6	4.8	6.4	5.6	5.6	5.6
15 - 20	5	55%	3.9	3.5	4.4	3.9	3.9	3.9
20 - 21	1	Cool-down	1.9	1.9	1.9	1.9	1.9	1.9

Please do following

- 1) TPR before exercise
- 2) Clean feet out before exercise
- 3) Turn fans on during exercise
- 4) Wash them after exercise

APPENDIX D

SYSTEMATIC SCALING FORMAT FOR THE SEMI-QUANTITATIVE UTRASTRUCTURAL MUSCLE DAMAGE ANALYSIS OF ELECTRON MICROSCOPE PICTURES

Electron Microscope Picture Analysis for Ultrastructural Muscle Damage
(Analysist must be blinded from all pictures prior to anaysis)

1. Characteristics of Aerobic Exercise-induced Ultrastructural cell damage on 5,000X EM pictures

- Oblique appearance due to individual myofibrils not returning to resting length
- Splitting of Sarcomere/Myofibrils or Z-disc
- Z-disc Streaming
- Z-disc Disruption

2. Damage Scaling System

<u>0</u>	1	2	3	4	<u>5</u>	6	7	8	9	<u>10</u>	
No symptoms of damage					Moderate	Most sever damage symptoms					

[illegible]

**Note: 'Oblique appearance' must be differentiated from the characteristics of oblique sections*