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Postmenopausal women on hormone replacement therapy (HRT) have been reported to be stronger when compared to women who are not using HRT. The first goal of this study was to investigate whether muscle morphology was altered in women who use HRT when compared to women who do not use HRT. In addition, this study examined the combined effects of a 6-month moderate-intensity strength training (ST) routine and HRT on the neuromuscular system of early postmenopausal women. Because not all the women completed the ST, this study was separated into two separate analyses, baseline (n=17; 7 HRT, 10 non-HRT) and training (n=14; 6 HRT, 8 non-HRT). ST consisted of two exercises (squat and dead lift), two days a week, for 6-months. *Vastus lateralis* muscle biopsies were taken at baseline and 6-months after exercise training. Biopsy samples were sectioned and analyzed histochemically for muscle fiber type and fiber cross-sectional area (CSA). In addition, voluntary knee extension strength was assessed at 30°/sec using an isokinetic dynamometer at these two time points. At baseline there were no significant differences in knee extensor strength between groups (HRT: 443 ± 121 N, non-HRT: 490 ± 106 N). Regardless of hormone status, Type I fibers were significantly larger (p=.005) in CSA (Type I = 3705 ± 877μm^2; Type II = 2790 ± 756μm^2). However, there were significantly more

Type II fibers ($p<.0001$) (61.5 ± 7.9% of total) and consequently, Type II fibers occupied significantly more total fiber area $p=.0012$) (Type I = 45.3 ± 7.4%; Type II = 54.7 ± 7.4%). No significant differences were found in the fiber type distributions of the HRT (37.9 ± 2.5% Type I, 62.1 ± 2.5% Type II) and non-HRT (38.9 ± 2.9% Type I, 61.1 ± 2.9% Type II) groups. There were no significant differences in fiber CSA of Type I fibers (HRT: 3615 ± 886 μm^2, non-HRT: 3769 ± 912 μm^2) or Type II fibers (HRT: 2770 ± 722 μm^2, non-HRT: 2849 ± 804 μm^2) obtained from the two groups. Six months of ST had no effect on the strength, fiber CSA, and fiber type distribution for HRT and non-HRT subjects. These results suggest that HRT does not alter muscle strength, fiber type distribution, and fiber CSA in early postmenopausal women.

The Effects of Hormone Replacement Therapy on Muscle Strength and Morphology in Early Postmenopausal Women

by

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INTRODUCTION

Declines in muscle strength and muscle mass with age has received much attention in recent years because of its contribution to falls, fractures, and an overall reduction in quality of life [17, 19, 22]. Studies have shown that strength training (ST) is an effective way to counter these adverse effects of aging by increasing muscle strength [5-7, 12, 23, 32, 33] and by improving muscle fiber morphology [5, 6, 32] in older individuals, particularly in older women.

In addition to the normal aging process, women go through a number of hormonal and physiological changes involved with menopause. According to Palacios [30], the gradual decrease of ovarian function leads to a significant reduction in the amount of estrogen produced and in circulation. These low levels of estrogen may cause several negative consequences which can be divided into three categories: short-term, medium-term, and long-term. The short-term symptoms are vasomotor symptoms (hot flushes and night sweats), sleep disturbances, depression, anxiety, fatigue, nervousness, and irritability. Medium-term symptoms include vaginal dryness, breast atrophy, skin and hair dryness, and urinary difficulties. Finally, long-term consequences may include increased risk for osteoporosis, cardiovascular disease, and Alzheimer's Disease [30].

Management of these symptoms has become increasingly important in the areas of health care and medicine. The majority of the above-mentioned symptoms are due to an estrogen deficiency, therefore, the aim of hormone replacement therapy (HRT) is to increase circulation levels of estrogen, and to mimic the levels that are seen in the woman's body before menopause. HRT is prescribed to relieve some of these symptoms, such as reduced hot flashes and night sweats, to reduce problems with depression and mood swings, and to improve on the conditions in the genital area [30]. More importantly, research has shown a decreased risk of osteoporotic fractures, improved blood-lipid profile, and a positive effect on arterial walls and blood vessels for women that are using HRT [30]. Current investigation is underway to determine potential risks that may be part of this therapy as well [18, 30].

To a large extent the effects of the menopause-related changes on the body have been studied. However, one area that still remains in question is the effect of HRT on skeletal muscle function of postmenopausal women [28]. Some studies have documented positive effects of HRT on preserving [13] and increasing [31, 37] muscle strength but others have not [27]. Skelton et al. [37] have shown that giving HRT to women 5-15 years after menopause results in approximately a 15% increase in the isometric maximal voluntary force (MVF) of the *adductor pollicis* (AP). These investigators believe that the mechanism behind the improvements in muscle function from HRT is due to a reversal of the process causing weakness at the menopause. Overall, they believe that HRT acts at the cellular level to change muscle strength [37].

Even fewer studies have looked at exercise and HRT, and their combined effects on muscle strength. Brown et al. [4] examined the effects of exercise training alone, and in combination with HRT, on muscle strength and fat free mass (FFM). For this study, weight-bearing exercises consisted of walking, jogging, and stepping, rather than strength training (ST) exercises. Strength tests were used to evaluate isometric, concentric, and isokinetic strength of the women before and after training. Both groups of women significantly increased their strength, yet significant differences in strength gains between the HRT women and the non-HRT women were not observed.

To date, studies employing moderate to high intensity ST in older subjects have been successful and confirm the positive impact of ST on healthy older women and men [5, 7, 12, 29, 32]. However, little is known about the interaction effect between HRT and resistance training. This project was part of a larger and more comprehensive study investigating the combined effects of a free-weight training program and HRT on early post-menopausal women (0-36 months post-menopause). The reason for selecting this target population is because it is thought that early menopause may be the best stage for administrating an intervention to prevent osteoporotic fractures in later years. The two hypotheses that were tested are as follows:

1) Women who use HRT will have increased strength and altered muscle morphology when compared to women who do not use HRT
2) HRT and ST will interact to produce greater changes in skeletal muscle than either will separately

We have tested the first hypothesis by addressing the following specific aims:

a) Women who use HRT will have increased voluntary neuromuscular strength versus those who do not use HRT
b) Women who use HRT will have increased fiber cross-sectional areas when compared to women who do not use HRT

We have tested the second hypothesis by addressing the following specific aims:

c) HRT and ST will produce greater gains in voluntary neuromuscular strength than ST only

d) HRT and ST will produce greater muscle fiber hypertrophy than ST only

The other areas of the larger project concentrate on changes in bone mineral density, neural function, single muscle fiber physiology, and balance with the same women. Once complete, the components of this study will provide us with information regarding the effects of HRT and ST for improvement of the neural, muscular, and skeletal systems in early post-menopausal women.

LITERATURE REVIEW

CHANGES IN THE MUSCLE WITH AGE

Physical frailty has been observed as the combined effects of muscle atrophy, declining muscle strength and power, fatigue, and injury [3]. It has also been attributed to declining muscle mass [8, 22], alterations on muscle fiber type [10], and neural changes [15, 20]. Although increased physical frailty is accepted as a part of aging, the exact mechanism behind the age-related loss in muscle strength is still unclear [3, 19, 27]. Because of this unknown, the degree to which we can prevent these age-related changes is also unclear [3].

In taking a closer look at this topic, age-related declines in strength and ability to perform functional activities has been linked with a loss of muscle mass and function [8, 22]. Hyatt et al. [17] have shown that these changes may predispose older individuals (69-75 years) to osteoporosis, atherosclerosis, and diabetes as well as to limitations in performing activities of daily living. The aim of this study was to examine the association of muscle strength and functional status in elderly individuals, with a range of disabilities. Functional status and activities of daily living were assessed by questionnaires and muscle strength was measured with isometric tests in the biceps and quadriceps muscles. Maximum voluntary contraction (MVC) of handgrip strength was measured with a hand-held dynamometer. The results of this study show that muscle weakness is associated with aging, yet it cannot be determined whether it is a cause or an effect. It is believed that declines in muscle strength in older people are multi-factorial, and may be due to disorders of the nervous and musculoskeletal systems [17].

Kallman et al. [19] take a closer look at the role of muscle loss in the age-related decline of grip strength. In order to investigate this, 847 volunteers (20-100 years old) were tested in grip strength, creatinine excretion, and forearm circumference. Both cross-sectional and longitudinal results show that grip strength increases well into the individuals' thirties, and then declines at an accelerated rate after age 40 [19]. This study demonstrated that grip strength and muscle mass are strongly correlated when using creatinine excretion and forearm circumference as the measures of muscle mass. Even though a significant correlation exists, other undetermined factors may play a role in the loss of strength with aging [19].

Similarly, Larsson et al. [22] investigated changes in muscle strength and speed of movement in men between the ages of 11-70 years old. Maximum values for isometric and dynamic strength, and speed of movement were measured in the quadriceps muscle. In addition, *vastus lateralis* muscle biopsies were taken from 51 of these subjects (22-65 years old). It was found that both isometric and dynamic strength increased up to the third decade, similar to Kallman et al. [19]. Strength remained constant to the fifth decade, and then decreased with increasing age. However, unlike Kallman, et al. [19], there was no correlation between the muscle circumference and decline in strength in the older individuals. When looking at the histochemistry results from the muscle biopsies, changes observed in muscle tissue were associated with the aging process. Here the major findings were a decreased proportion and a selective atrophy of Type II fibers.

In general, most investigations report a decrease in muscle strength with age, for both men and women. This decline begins in the forties and fifties, and decreases at a constant rate in the decades to follow.

STRENGTH TRAINING AS A COUNTERMEASURE

It has been well documented that high-intensity strength training (ST) results in significant increases in muscle strength and mass. In order to take a closer look at the changes accompanying individuals in ST programs, Frontera et al. [12] looked at the effects of strength and conditioning on older men. Twelve men (age 60-72 years old) participated in a 12-week ST program of the quadriceps muscle, at 80% of 1-repetition maximum (1-RM), (8 repetitions, 3 days/week). 1-RM is equal to the maximum amount of weight that can be lifted one time with proper technique through a full range of motion. Weekly measurements of the 1-RM strength showed that there was a progressive increase in quadriceps muscle strength throughout the training. This weekly strength gain, which averaged 5% per training session, was similar to the 4.4-5.6% increase in dynamic strength seen in young men (average age 28) who performed a similar training protocol (Rutherford et al. [35]). Muscle biopsies showed increases in Type I (34%) and Type II (28%) fiber area, indicating muscle hypertrophy for these older men [12].

In general these ST studies have been performed on men, and few studies have examined the effect of ST on women's strength and skeletal muscle adaptations. An exception has been the research conducted by Staron and colleagues [40, 41]. In their first study [41] they recruited twenty-nine women (mean age = 22 years), who were inactive and did not take part in any type of cardiovascular or resistance training. The high intensity ST program was 20 weeks long, and included 4 lower body strength exercises (squat, leg press, leg extension, and leg curls). Strength was measured and *vastus lateralis* muscle biopsies were taken prior to the start and at the conclusion of the training. After 20 weeks of training, a significant increase in isotonic strength for all of the exercises was found. In support of this, significant changes in muscle fiber sizes, where Type IIa increased by 45%, Type IIb/IIab increased 57%, and Type I increased 15%, after the 20 weeks of heavy resistance training, were also observed.

In 1991, Staron et al. [40] performed a follow-up to their study done in 1989, by using a group of 15 college-aged women. Eight of the women had taken part in the previous study, and were therefore considered previously trained (PT). Seven women who were not previously trained (NP) also volunteered for this study. The purpose of this investigation was to look at the effects of 30-32 weeks of detraining and then retraining in previously strength-trained, and untrained women. At the conclusion of the previous study, the PT women who were training returned to their normal daily activities, which did not involve regular exercise. Following this detraining phase (30-32 weeks), the women participated in a high-intensity ST program two times a week, consisting of three exercises (squat, leg press, and leg extension). *Vastus lateralis* muscle biopsies were taken from these women at the beginning of the training and at the conclusion of the 6-weeks of training. For the PT women, the results show that following the detraining period, there was a significant decrease of 14% in the Type IIa + IIb fiber areas. For these women there were decreases in the other fiber types, yet they were not significant. After retraining, the PT women showed significant increases in both Type IIa (18%) and

Type IIa + IIb fibers (30%) when compared to the detrained values. For the NP women, there were significant increases in all three major fiber types, where Type I increased 16%, Type IIa by 17%, and Type IIab + IIb by 28% after 6 weeks of training. The authors concluded that young women are capable of considerable improvements in strength and specific muscular adaptations after high intensity training. The results of the second study also show that rapid changes occur in women that were previously trained and not previously trained [40].

The positive results found in ST research with young women have provoked many researchers to look at these adaptations with older women. In fact, ST is thought to be an effective intervention against the age-related decline in muscle mass and strength. There are many studies that have looked at ST because it has the ability to positively affect these factors. Fielding [11] completed a review on the role of progressive resistance training in preservation of lean body mass (LBM) of the elderly. He shows that in general, studies have reported positive effects of both low-intensity and high-intensity resistance training on muscle function and size in both middle-aged (50-75 years old) and older (80-100 years old) individuals. More specifically, high intensity training studies show substantial increases in 1-RM muscle strength of the muscle being trained, in response to 8-12 weeks of ST.

Recently, Rhodes et al. [33] examined the effects of progressive resistance exercise (PRE) on dynamic muscular strength and bone mineral density. Sedentary and healthy women, ages 65-75, volunteered to take part in this 1-year study, and were assigned to either exercise or control groups. The exercise group took part in a training session 3 times a week, focusing on the major muscle groups (chest press, leg press, bicep curl, triceps extension, quadriceps extension, and hamstring curl). Muscle strength was measured by a hand-held dynamometer and by using 1-RM measures for several muscle groups. Through the course of the study, there were significant increases in strength varying from 19 to 53% in the women who were training. Overall, these investigators state that this increase in muscle strength may contribute to a better quality of life in older women.

Charette et al. [5] conducted a 12-week resistance training study with women between 64 and 86 years old. The training program focused on the lower extremities, and consisted of a 3-day/week routine. Strength tests and muscle biopsies were performed at baseline, week 2, week 7, and week 12. The findings show an increase in muscle strength ranging from 28 to 115% of their baseline values. Similar to what Frontera, et al. [12] found in older men, there was a 20% increase in the cross-sectional area of type II fibers, and slight (7%) increase in type I fibers for all of the women after 12-weeks of resistance training.

Along the same lines, Cress et al. [7], Morganti et al. [29], and Pyka et al. [32] reported increases in muscle strength for older women, who trained for 1-year. The main purpose of the study done by Cress et al. [7] was to determine the effects of long-term combined resistance-aerobic training on older women. The women (average age 72 years old) performed three 1-hour sessions per week, including both aerobic and resistance training exercises. In order to look at the effects of this program, both strength tests (using a dynamometer) and *vastus lateralis* muscle biopsies were performed. In conclusion, the investigators reported that the exercising women were able to increase their strength (9.4%), as well as their type II fiber size (29%), with this combined aerobic-resistance training program.

Next, Morganti et al. [29] observed women that were at least 5-years post-menopause, and showed increases in 1-RM muscular strength that ranged from 18 to 77%, after 1-year of training. Actually, the greatest gains for these women were seen within the first three months of training. For this experiment the training included five exercises that covered the large muscle groups of the body (leg press, knee extension, lateral pull down, trunk extension, and trunk flexion). The training intensity for the first three exercises was high and was set at 80% of their determined 1-RM, and the score of 16 based on the Borg scale of perceived exertion. This research indicates that there are continual increases in muscular strength, when post-menopausal women take part in high-intensity ST.

Similarly, Pyka et al. [32] found that after the initial increase in strength at three months, the changes in strength plateaued for the duration of the experiment. This study also looked at the fiber adaptations that occur with a 1-year resistance-training program, for older women (N=17) and men (N=8) (average age 68). In order to investigate this, muscle biopsies were taken from the *vastus lateralis* at baseline, and 15 and 30 weeks post-training. Cross-sectional area (CSA) of the Type I fibers increased in both 15 & 30 weeks of training, whereas the Type II fibers only increased at 30 weeks of training. Therefore it is thought that the increases in muscle strength that are rapidly achieved at the start of ST, are accompanied by hypertrophy of both the Type I and Type II fibers.

Most recently, Bemben et al. [1] looks specifically at early postmenopausal women (1-7 years post-menopause) and the musculoskeletal responses to high and low intensity resistance training programs. Both of these 6-month training programs produced similar training volumes (sets x reps x load). Results show that both high-intensity and low-intensity resistance training both improved muscular strength, especially for the lower body exercises, in early postmenopausal women. An indication of this study was that a low-intensity training program could be beneficial for the muscular fitness in women for whom high-intensity training may be contraindicated.

These studies demonstrate that ST is a safe and effective activity that counters the effects of aging on muscle. Although the results vary with training programs, in general these studies have shown an increase in muscle strength and in Type II fiber CSA, for older men and women.

GENDER DIFFERENCES IN MUSCLE FUNCTION WITH AGE

Another large area of research has been focused on the differences in muscle function between older men and women. Lindle et al. [23] performed a research study on 654 women and men between the ages of 20-93 years old. These investigators assess the differences in both age and gender in isometric, concentric, and eccentric peak torque of the knee extensors. The results show that both men and women exhibit similar age-related declines in knee extensor isometric and concentric strength. These decreases begin in the fourth decade, and continue to decrease at a rate of 8-10% per decade. The age-related decline in eccentric strength was about the same for men and women, however, women's reductions begin 10 years later than men. This difference shows that women are thought to better preserve their muscle quality (MQ = strength per unit muscle) with age, compared to men. The investigators believe that this difference is seen because older women have a greater capacity to store and use elastic energy. The results of this study also suggest a decline in the overall MQ with age, in both men and women.

Tracy et al. [44] compared MQ in older men and women, before and after a 9-week training program. Men showed greater increases in muscle volume and 1-RM strength, when compared to the women in this study. However, there was no difference between genders in the MQ response to ST. This suggests that there were similar neuromuscular adaptations in both groups from the ST program.

Recently, Roth et al. [34] examined how both gender and age influence muscle volume responses to a 6-month whole body strength training program. These authors found that there were neither age nor gender differences in the muscle volume responses to ST. In addition, across-the-board they found similar relative increases in thigh and quadriceps muscle volumes. As a result of this investigation, muscle volume, rather than muscle CSA was recommended for studying muscle mass responses to training.

HORMONE REPLACEMENT THERAPY AND AGING

Currently there is a tremendous amount of research being done in the area of hormone replacement therapy (HRT) for postmenopausal women. According to Palacios [30], estrogen has many important functions in the woman's body, such as to promote, regulate, and maintain the female reproductive structures, regulate bone remodeling, act on vasculature to protect from coronary heart disease, control fluid and electrolyte balance, and increase protein anabolism. The onset of menopause results in a gradual decrease in ovarian function, which ultimately causes a reduction of estrogen production and circulation. Unfortunately, low estrogen levels can have a number of negative consequences, both short- and long-term. HRT functions to increase circulating estrogen levels. By using this therapy, it is thought women can prevent the consequences of long-term estrogen deficiency and treat the common symptoms associated with menopause.

Even though there is a large amount of research examining HRT, controversy remains in several areas causing uneasiness for many women. Some of the areas of question include the effects of HRT on certain types of cancer, cardiovascular disease (CVD), and osteoporosis. There have been several review papers examining HRT, and the possible risks and benefits involved in the administration of this type of therapy for postmenopausal women.

Jacobs and Loeffler [18] review some of these controversial areas in the topic of HRT for postmenopausal women. One area of concern for women is the effect of HRT on breast cancer. There is evidence of an association between breast cancer and early menarche and late menopause, but there has not been enough conclusive research to prove that HRT is a cause of breast cancer.

According to Jacobs and Loeffler, when considering HRT, another questionable area is its effects on CVD. Two of the known risk factors for CVD are high low-density lipoproteins (LDL), and low high-density lipoproteins (HDL). In fact, within six months of menopause, it has been shown that there are significant increases in women's total cholesterol (~6%), LDL (~11%), and triglycerides (~9%). In this review it is stated that HRT can reverse these postmenopausal lipid profile changes, which is a protective mechanism against atherosclerosis and CVD. Palacios [30] also reports that HRT has a beneficial effect on the lipid profiles of postmenopausal women. More specifically, it is stated that HRT significantly increased HDL levels, as well as significantly decreased LDL levels. In addition to the favorable effects on lipid levels, HRT has been shown to positively modify both blood vessel dynamics, and affect the formation of atheroma on the arterial walls. In combination, these changes are beneficial to women who are using HRT, and they have a 35-45% lower risk of CVD than those women who are not using HRT.

Palacios [30] points out that another major consequence of estrogen deficiency is osteoporosis. Osteoporosis is characterized by a large reduction in bone mass, and with this reduction the bones are more prone to fracture and breaking. This condition is due to a combination of a low level of circulating estrogen and to an age-related bone loss. HRT is currently prescribed for both prevention of postmenopausal osteoporosis, and as a treatment to slow the progression of the disease. It is shown in numerous studies that HRT can conserve and increase bone mass. In fact, HRT is thought to restore calcium balance to premenopausal levels. Yet, in order to gain the maximum benefits against fractures, HRT must be used long-term, because bone loss seems to accelerate as soon as it has been discontinued.

Studies have also looked at the consequences of weight training, and how it alters bone density in both men and women. Maddalozzo and Snow [25] compared the effects of a moderate-intensity seated resistance-training program and a high-intensity free weight program on bone mass of older men and women. There were twenty-eight men (average age 55) and twenty-six women (average age 53) that met the criteria for this study. All of the women were postmenopausal and not taking HRT. The first 12-weeks was a control period, where the subjects continued their normal daily activities and dietary intake. After the 12-weeks, the subjects were randomly assigned to either a moderate- or high-intensity ST program. Both programs consisted of a 24-week routine, three days/week, and targeted all the major muscle groups of the body. Bone mass, body composition, muscle strength and power were all assessed at baseline, after 12, 24, and 36-weeks of training. These investigators saw changes on bone mineral density (BMD) after 6-months in the high-intensity training group, for both men and women. In addition, there were significant increases in muscle mass and strength in all groups. Consequently, ST may be beneficial to both men and women for preventing age-associated decline related to bones and muscles.

CHANGES IN MUSCLE STRENGTH WITH HORMONE REPLACEMENT THERAPY

Very little research has been done in the area of skeletal muscle changes due to the administration of HRT in women. In 1993, one study by Phillips et al. [31] looked to determine whether the loss of specific muscle force with age is hormone-dependent. Both HRT and non-HRT women took part in this research, and were between the ages of 42 and 72. Some of the women had been on HRT for 1-25 years. None of the subjects had any other treatment for menopause symptoms or osteoporosis. The maximal voluntary force (MVF) and the cross-sectional area (CSA) of the *adductor pollicis* (AP) of the right hand were measured for each subject. In general, the MVF was found by using a force transducer and the CSA was determined by measuring the thickness of the hand. The findings of this study show that women who were receiving HRT were protected against the decrease of specific force of the muscle that occurred following menopause. The authors speculated that the mechanism involved was located at the level of the cross-bridge. They claim that there is a possibility that hormonal influences could alter the sensitivity of the cross-bridge to metabolites (H^+ or P_i) or to some other factor. In the case of this study, the HRT was shown to prevent the action of the unknown factor.

In 1999, Skelton et al. [37], in the same laboratory looked at the changes in the AP muscle strength following a HRT intervention. In this investigation, the women recruited were generally healthy, 5-15 years post menopause, and had a body mass index (BMI) of 20-29 kg/m^2. Out of 122 women, a random group was selected to be administered HRT (Prempak C ® 0.625), for 6-12 months, while the others remained a control. The subjects were seen eight times, in addition to the initial visit, within a 12-month period. MVF and CSA were measured in the same way as mentioned in the previous study [31]. The results of this study show that giving HRT to women 5-15 years after menopause results in a significant increase in the MVF of the AP, when compared to the control group. In fact, the women who were the weakest at the start benefited the most from the HRT administration.

That same year, Greeves et al. [13] looked at the effects of hormone deficiency on quadriceps muscle strength in early postmenopausal women, over a period of 39 weeks. The HRT group was comprised of women within the first 1-3 year post-menopause, and began taking various preparations of therapy and the start of the study. The previously mentioned studies [31, 37] were both looking at changes in the *adductor pollicis*, while this study is looking at a weakness in a functional muscle group, the quadriceps, and the effects of menopause and HRT on muscle strength. It was demonstrated that there were significant decreases in isometric strength (-10%) and dynamic leg strength (-9%) in the control group, while there were no significant changes in the HRT group. Although these authors do not show a significant increase in strength, they present a possibility that HRT may offer protection against the muscle weakness that occurs within the first three years post-menopause. In addition, these authors have suggested that since estrogen and progesterone are both deficient at menopause, it is possible that low progesterone levels may be responsible for the loss of strength during early menopause.

Even though there is little research in this area, the amount that exists is very controversial. For example, both Seeley et al. [36] and Taaffe et al. [42] show that there was no evidence that estrogen is beneficial to muscle function of postmenopausal women. Seeley et al. [36], examined the association between estrogen and muscle strength, neuromuscular function, and the risk of falling, in women aged 65 and older. These researchers assessed six performance-based measures, including hip abduction, triceps extension, and handgrip strength, standing balance, gait speed, and timed chair stand. All of the strength measures were done by hand held dynamometers. Overall, these authors claim to find no evidence that HRT preserves or increases muscle strength, improves neuromuscular function, or prevents falling. In addition, they say that women currently using HRT were similar to women who had used HRT in previous years.

Taaffe et al. [42] looked to determine whether the beneficial effects of HRT, as reported by Phillips et al. [31], were also evident in larger muscle groups that are involved in movement, balance, and activities of daily living. Of the eighty-five women (ages 65-82) that they examined, thirty-seven were on stable doses of

HRT ranging from 2 to 43 years. The 1-RM method was used to determine dynamic muscle strength for these women, in five lower body exercises (leg press, knee extension, knee flexion, hip abduction, and hip adduction.) Bone mineral density (BMD) of the axial and appendicular skeleton was also assessed in this study, via dual x-ray absorptiometry. The results of this study show that there was no effect of HRT on lower body muscle strength, even though there was a significant increase in BMD for the HRT women.

Recent investigations by Meeuwsen et al. [27] explores the effects of tibolone, a tissue specific compound with a mixed (estrogenic, progestogenic, and androgenic) hormonal profile on skeletal muscle strength in postmenopausal women. Both upper body (handgrip) and lower body (quadriceps) maximal strength were measured at baseline and every three months for 1 year. The results of this study show that tibolone significantly increased handgrip strength when compared to placebo, however, there was no treatment effect observed in the isometric knee extension.

Although there are several reports of HRT and its possible effects on muscle, there has only been one study that involves the effects of muscle with HRT in combination with an exercise-training program. Brown et al. [4] organized an 11-month weight-bearing exercise program for postmenopausal women. Brown and her colleagues recruited fifty-eight women between the ages of 60-72 years old. There were three groups of women in this study, exercise and HRT (n=22), exercise only (n=20), and control (n=16). The HRT was administered to the 22 women at the start of the training, and was stopped at the conclusion of the training, at 11-months.

The training protocol began with a 2-month pre-training period that focused on enhancing flexibility, balance, reaction time, and to a modest degree strength. These exercises were intended to counteract some of the age-related declines in muscle strength and to prepare the subjects for the upcoming training program. During this pre-training, the exercises were performed using the subject's body weight, resistance bands, or gravity resistance.

In the 9-months to follow, the women participated in weight-bearing exercises, including walking, jogging, and stair climbing. Based on previous research [21], this type of training was used to enhance the women's bone mass, by using relatively high ground reaction forces. The initial recommendation was to walk for thirty minutes at an intensity of 60-70% of their maximum heart rate. The women were asked to attend a minimum of three sessions per week and encouraged to attend five sessions a week. After several weeks of this program, the intensity and duration were altered according to the women's abilities, and at the third month of the training, stair climbing and/or jogging were also incorporated into their program.

Several different strength tests were performed on these women at both pre- and post-training. First, for the hip extensor and abductor muscles, strength was assessed by using a hand-held dynamometer. Next, knee extension and flexion strength were measured by a Cybex II dynamometer. Finally, a maximal leg press, on a Nautilus machine, assessed simultaneous hip and knee extension. Similar to other research, this exercise program resulted in significant gains in strength. When the investigators averaged the percent improvement of strength in each area (isometric, concentric, and isokinetic) there was in increase of 16 $\pm$ 11%

in response to exercise only group, and 17 ± 13% in the exercise and HRT group. As we can see, the women who were receiving HRT did not increase the gains in strength beyond the women who just participated in exercise alone. However, this program did not include any strenuous ST exercises.

In summary, it is controversial whether HRT improves strength in postmenopausal women. In addition, no one to our knowledge has determined whether HRT has a positive interaction with ST in postmenopausal women.

METHODS

SUBJECTS

One hundred and fifty early postmenopausal women volunteered to take part in the overall exercise intervention study examining bone density, balance, and neural function. These women were recruited both by newspaper advertisements and by referrals from their physicians. The term "early postmenopausal" refers to women who were within their first 36 months of menopause. Post-menopausal status was confirmed by follicle-stimulating hormone (FSH) levels $\geq$ 20 mIUI/ml as assessed by each subject's personal physician. Their body mass index (BMI) was measured at the screening and needed to fall between 19-30 kg/m^2 in order to be part of the study. In addition, subjects could not be participating in any other type of ST outside of this study. Other reasons for excluding potential subjects included hypertension (140/90 mmHg), metabolic diseases, or any other physical contraindication that may have prevented participation in this study. For the HRT women, it was assumed that there was a relative consistency in the dosage of their HRT, since most women start with a standard 0.625 mg conjugated estrogens (Premarin ®), or .05 -.10 mg estradiol (Estraderm patch ®).

The subjects participating in this particular study were a subgroup of the larger study participants. Eighteen women volunteered to undergo pre-training and 6-month training muscle biopsies in addition to testing conducted in the larger study. Of these women, ten were not on HRT, while eight women had been on

HRT, for an average of 19 months (6-36 months). HRT consisted of 0.625 mg conjugated estrogens (n=6) or an 0.10 mg estrogen patch (n=1).

Both the larger study and this sub-study were approved by the Institutional Review Board (Appendix A). In addition, all subjects signed an informed consent prior to their participation (Appendix B).

STUDY DESIGN

A time-line of the study is presented in Figure 1. All baseline measurements were made prior to any type of training. In order to assure proper exercise technique, the initial 2-3 weeks of training was considered a learning phase, preceding the formal training intervention. At this time the women became comfortable with their trainer, the stretching and lifting techniques, and the routine that they were participating in. Training resistance during this period was gradually increased until they were training at the intensities and durations listed in Table 1. Once the subjects reached this level, they were considered to be in the training phase. Following 6-months of the training phase, all baseline measurements were repeated (6-month post-training data.)

0-months Baseline measurements	2-3 weeks Familiarization period	6-months Training period (see Table 1 for specific protocol)	End of 6- months Post-training measurements

Figure 1: Study design timeline

TRAINING PROTOCOL

All eighteen women participated in the ST program. All training sessions took place at Oregon State University, and were closely monitored by a personal trainer. Training sessions began with a total-body warm-up for 10-15 minutes, which entailed a variety of stretching activities. The women were also encouraged to practice these warm-up exercises at home.

The ST session consisted of 2 free-weight exercises, the squat and the dead lift. These exercises were performed at a moderate intensity (40-65% of 1-RM), 2 days a week, for 26 weeks. A moderate intensity was chosen in order to increase adherence. In addition, it was previously shown that these two exercises were safe and effective for increasing muscle mass and strength in older men and women [25]. The following periodization-training program was used:

Table 1: Training program [24]

Phase	Warm-up set	Working sets
Phase 1 (4-6 weeks)	1 set 10-12 repetitions	3 sets 15 repetitions 40-50% 1-RM
Phase 2 (4-6 weeks)	1 set 10-12 repetitions	3 sets 12 repetitions 50-60% 1-RM
Phase 3 (4-6 weeks)	1 set 10-12 repetitions	3 sets 8 repetitions 60-65% 1-RM
Phase 4 (1-week - active rest)	1 set 10-12 repetitions	3 sets 15 repetitions 30% of 1-RM

In all of the phases, there was a 1-minute rest period allowed between each set. In total, these four phases took 13 to 19 weeks to complete, and were then repeated until the 6-months of training was completed.

MEASUREMENT OF PHYSICAL ACTIVITY

At the beginning and the conclusion of the training, each subject was administered an Exercise and Physical Activity Questionnaire (Appendix C). The purpose of this questionnaire was to estimate the women's daily physical activity levels. This questionnaire has been validated for measuring physical activity levels of older women [9, 16].

BODY COMPOSITION

A whole body dual-energy x-ray absorptiometry scan (DXA, Hologic QDR-4500A Elite, Waltham, MA) was performed to quantify percent body fat, whole body lean mass, and left leg lean mass. The percentage of water in lean tissue was assumed to be 73.2%. Validation for this procedure for body composition has been conducted previously in this laboratory [24, 25].

MUSCULAR STRENGTH

Voluntary neuromuscular strength was measured at baseline and 6-months after training. Maximum voluntary strength of the left knee extensors was measured using an isokinetic dynamometer (KinCom 500H, Chattex Corp.). Each trial was corrected for gravity and performed at an angular velocity of 30 degrees per second. The speed of 30 degrees per second was chosen because previous experience indicated that values obtained at this slow velocity were more reproducible than values obtained during isometric contractions [24, 25]. For each test, the participants were instructed to perform 10-12 trials of the exercise at an intensity well below the maximum. This served as the warm-up prior to the maximum strength assessment. After the warm-up, subjects performed 3-5 maximal efforts in order to determine peak torque. Each maximal strength assessment was followed by a 1-minute rest period. This testing protocol has been used previously in this laboratory and is reliable for subjects within this population (CV = 6-8%) [24, 25].

MUSCLE BIOPSY

Muscle biopsies were obtained from all subjects at baseline and after 6-months of training. These biopsies were obtained 1 week after the muscular strength testing, but a few days prior to the familiarization phase of the training programs. The biopsy was performed by a physician (Dr. Jeff Mull, Oregon State University Health Services), and was taken from the left *vastus lateralis* under local anesthesia. Immediately after the biopsy was taken, it was divided into several pieces. One part of the biopsy was mounted onto a cork with TBS Mounting Medium®. Once the muscle was arranged vertically on the cork, it was quickly immersed upside-down into isopentane which was cooled to it's freezing point by liquid nitrogen. The muscle was kept in the isopentane until it was thoroughly frozen. The frozen cork-mounted muscle was placed in an airtight cryotube and stored at -80 degrees Celsius until further analysis.

MUSCLE HISTOCHEMISTRY

The muscle biopsy samples were thawed to -20 degrees Celsius and sectioned for histochemical and histological analyses. Sectioning of the muscle samples was done in a cryostat (Leica Co.) at -20 degrees Celsius. The cork-mounted muscle was fixed to a specimen mount with TBS Mounting Medium®. The blade of the cryostat was set-up to cut 8-10 μm sections of the muscle sample. The sections were picked up by #2 glass cover slips and were set aside to air dry at room temperature. Once dry, the cover slips were stored in a freezer (at 4 degrees Celsius) until all sectioning was complete.

The cover slips were then placed in Columbia jars where they were stained for myofibillar ATPase (mATPase). In order to distinguish between the three different fiber types, sections were stained at pH 9.4 after both alkaline (pH 10.0) and two different acid (pH 4.35 & 4.65) pre-incubations. Following the incubation in the pH 9.4 mATPase solution, sections were transferred to a cover-slip carrier and run through a series of reactions and washes (1% $CaCl_2$, 2% $CoCl_2$, 0.01M sodium barbital, and double-distilled water). The sections were then placed in 1% $(NH_4)_2S$ solution, followed by another wash in double-distilled water. The sections were then dehydrated rapidly in ascending alcohols and cleared in xylene, prior to being mounted on glass slides [2, 14]. A more complete description of the solutions and procedures used for the staining can be found in Appendix D and E.

HISTOLOGOCAL ANALYSIS

Following the staining, color photographs of the sections were taken at 100x. Photos were then developed and converted into digital files using a scanner. These images were used to determine the total fiber number and the fiber type percentage of each biopsy sample. The prevalence of Type I and Type II fibers were determined for all specimens; all countable fibers were included in order to calculate the prevalence of the different fiber types. Image analysis software (Scion Image ®) was used to determine the cross-sectional areas of the fibers. The muscle fibers selected for measurement were free of artifacts, had distinct cell borders, and had no tendency toward a longitudinal cut [5, 6]. Data were transferred into a Microsoft Excel document, and then into SPSS [38] for statistical analysis.

STATISTICAL ANALYSIS

One-way analysis of variances (ANOVA) were performed to detect differences in subject characteristics between the groups (HRT, non-HRT) at baseline. Two-way ANOVA's with main effects of group (HRT, non-HRT) and fiber types (Type I and II), were used to evaluate fiber type distributions, cross-sectional areas of fibers, and percent of total area at baseline. In addition, a two-way repeated measures analysis of variance (RM-ANOVA) was performed on those subjects who completed 6 months of training. The model had main effects of group (HRT, non-HRT) and training status (baseline, 6-months post training). When significant values were observed, a Tukey's HSD post-hoc test was performed to determine significant differences between means. Analysis was carried out using the SPSS statistical analysis software package [38].

RESULTS

The results of this study have been divided into two different sections: baseline analysis and training analysis. The baseline analysis compares the two groups of women (HRT and non-HRT) prior to the start of the strength-training program. The training analysis consists of the observations made between the two groups (HRT and non-HRT) over time, from baseline to immediately following the 6-month strength-training program. Two analyses were performed because not all of the women who began the study (baseline analysis) completed the 6-month training program.

BASELINE ANALYSIS

Subject characteristics

Of the eighteen women recruited for this study, seventeen (HRT = 7, non-HRT = 10) were included in the baseline analysis. One subject was excluded in the baseline analysis because of a muscle biopsy sample that was too small for valid interpretation.

Descriptive characteristics of the subjects are presented in Table 2. The HRT group was not significantly different from the non-HRT group in any of the baseline measurements. The HRT and non-HRT subjects were the same age, months post-menopause, weight, and height at baseline. In addition, the groups had similar body mass index (BMI), total lean body mass, and body fat percentages. The physical activity levels of these women were not significantly different at baseline, where the daily expenditure in METS (metabolic equivalents) was 70.8 ± 62.1 for the HRT group and 57.2 ± 34.0 for the non-HRT group.

Table 2: Subjects characteristics

	HRT (n=7)	non-HRT (n=10)
Age (y)	52.6 ± 2.5 (50.1-55.1)	53.0 ± 2.3 (50.7-55.3)
Post menopause (m)	19 ± 7 (12-26)	22 ± 16 (6-38)
Weight (kg)	64.3 ± 11.9 (52.4-76.2)	67.5 ± 11.5 (56-79)
Height (in)	64.3 ± 3.3 (61-67.6)	65.9 ± 3.5 (62.4-69.4)
BMI (kg/m^2)	24.0 ± 4.0 (20-28)	24.0 ± 2.8 (21.2-26.8)
Total Body Lean Mass (kg)	41.8 ± 7.5 (34.3-49.3)	44.5 ± 5.6 (38.9-50.1)
Body Fat (%)	31.9 ± 2.7 (29.2-34.6)	30.1 ± 7.9 (22.2-38)

Values are presented as means ± SD, with range in parentheses. HRT=hormone replacement therapy group; non-HRT=non-hormone replacement therapy group; n=number of subjects; y=years; kg=kilograms; in=inches; BMI=body mass index; m^2= meters squared.

Muscular strength

As shown in Table 3, the two groups were similar in left knee extensor strength and left leg lean mass at baseline. In addition, there was no significant difference between groups when looking at the relationship between leg strength and leg lean mass (N/kg).

Table 3: Muscular strength & lean mass measurements at baseline

Baseline	HRT (n=7)	non-HRT (n=10)
Left leg strength (knee extension) (N)	443 ± 121	490 ± 106
Left leg lean mass (kg)	6.59 ± 1.44	7.51 ± 1.06
Leg strength/leg lean mass (N/kg)	68.9 ± 17.6	66.0 ± 13.9

Values are presented as means ± SD. HRT=hormone replacement therapy group; non-HRT=non-hormone replacement therapy group; n=number of subjects; N=newtons; kg=kilograms

Muscle fiber type percentages

Figure 2 shows a representative muscle section stained for myofibrillar ATPase, at the pre-incubation of pH 10.0. In determining the fiber type percentages, due to the inconsistency of finding three distinct fiber types, we chose to assess the samples for Type I and II fibers only. At pre-incubation of pH of 10.0, Type I fibers appear light in color and Type II fibers are dark in color. The total number of fibers used for analysis was different for each sample but averaged 106 ± 40 fibers.

Figure 3 shows the fiber type percentages for each subject at baseline. Fiber type distribution varied between subjects in the HRT group ranging from

28.6% to 48.8% for Type I and between 51.2% and 71.4% for Type II. The non-HRT group fiber type distributions varied between 27.0% and 50.6% for Type I and 49.4% to 73.0% for Type II. The distribution of fiber types was not significantly different between groups at baseline. The HRT group had 37.9 ± 2.5% Type I and 62.1 ± 2.5 % Type II fibers, while the non-HRT group 38.9 ± 2.9% Type I and 61.1 ± 2.9% Type II fibers at baseline. However, when looking at the overall mean among subjects (n=17) the percent of Type I (38.5 ± 7.9%) and Type II (61.5 ± 7.9%) fibers, there was significant main effect ($p<.0001$), where regardless of the group, there was a higher percentage of Type II fibers across subjects.

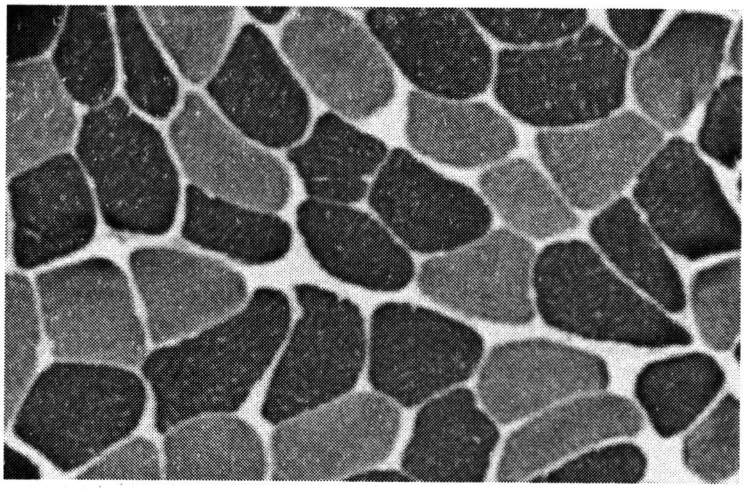

Figure 2: Photograph of muscle section, stained with myofibrillar ATPase at pH 10.0. Type I fibers are light in color; Type II fibers are dark in color.

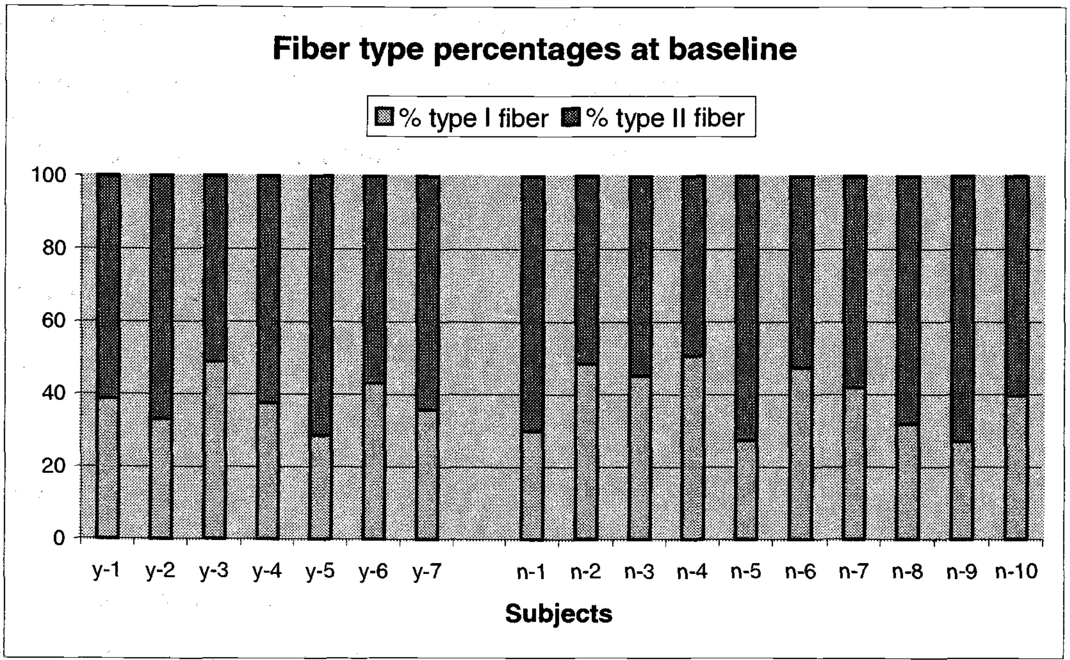

Figure 3: Muscle fiber type percentages for individuals at baseline. y=subject in HRT group (n=7); n=subject in non-HRT group (n=10).

Muscle fiber areas

Table 4 shows the mean fiber cross-sectional areas (CSA) for both Type I and II fibers at baseline. As stated previously, the muscle fibers that were used in the analysis were free of artifact, had distinct cell borders, and had no tendency towards a longitudinal cut. There were no significant differences between groups in fiber cross-sectional areas of Type I and II fibers. However, when looking at the overall mean among subjects (n=17), there was a significant main effect of type (p=.005) regardless of group, where Type II fibers were significantly smaller than Type I fibers.

The area occupied by each fiber type was determined by calculating the total area of the sample for both fibers, dividing this number by the number of fibers for each fiber type, then multiplying by 100, in order to get a percentage of the total area observed. There were no significant differences in percent fiber area between the two groups (Table 5). However, when looking at the overall mean among subjects (n=17), there was a significant main effect of fiber type (p=.0012) regardless of group, where the relative area occupied by Type I fibers was smaller than the area occupied by Type II fibers. Therefore, although the Type II fibers were smaller in CSA, because there were relatively more Type II fibers, the area occupied by Type II fibers was greater.

Table 4: Muscle fiber cross-sectional areas at baseline

Baseline	HRT (n=7)	non-HRT (n=10)	Total mean (n=17)
Type I fiber area (μm^2)	3615 ± 335	3769 ± 289	3705 ± 877
Type II fiber area (μm^2)	2770 ± 273	2849 ± 254	2790 ± 756*

Values are presented as mean ± SD. * Denotes significantly different (p=.005) from Type I fiber cross-sectional areas. HRT=hormone replacement therapy group; non-HRT=non-hormone replacement therapy group: n=number of subjects; μm^2=micrometers squared.

Table 5: Percent of total area for Type I and II fibers at baseline

Baseline	HRT (n=7)	non-HRT (n=10)	Total mean (n=17)
% area of Type I fibers	44.4 ± 6.7	45.6 ± 8.7	45.3 ± 7.4
% area of Type II fibers	55.6 ± 6.7	54.4 ± 8.7	54.7 ± 7.4*

Values are presented as mean ± SD. * Denotes significantly different (p=.0012) from Type I percent fiber area. HRT=hormone replacement therapy group; non-HRT=non-hormone replacement therapy group: n=number of subjects.

TRANING ANALYSIS

Subjects

Of the seventeen women (7 HRT, 10 non-HRT) considered in the baseline analysis, sixteen completed the 6-month study (6 HRT, 10 non-HRT) representing an attrition rate of 5.9%. The one woman (HRT group) who dropped out of the study did so for medical reasons unrelated to the study or the training program. In addition, there were two muscle biopsy samples (non-HRT group) that were excluded from the analysis because of artifact within the muscle biopsy samples. The remaining fourteen subjects' biopsies (6 HRT, 8 non-HRT) were used in the analysis of the training program.

Muscular strength

As presented in Table 6, there were no significant differences in voluntary knee extensor strength at 6-months between groups. In addition, there were no significant differences in strength between baseline and 6-months post-training in either group. At baseline the HRT group mean was 435 ± 131 N while at 6-months it was 425 ± 136 N. Similarly, at baseline the non-HRT group mean was 528 ± 65 N, while at 6-months it was 513 ± 98 N. Figures 4 and 5 show the changes in individual strength from baseline to 6-months after training.

When looking at the strength/lean mass ratio, there were no significant differences between groups at 6-months, or over time. At baseline, the HRT ratio was 69.4 ± 19.2 N/kg while at 6 months it was 64.6 ± 19.1 N/kg. The non-HRT ratio was 69.0 ± 7.9 N/kg at baseline and 64.1 ± 9.2 N/kg at 6-months. The individual changes in strength/lean mass from baseline to 6-months after training are shown in Figures 6 and 7.

Table 6: Muscular strength and lean mass measurements at 6-months

6-months	HRT (n=6)	non-HRT (n=8)
Left leg strength (knee extension) (N)	425 ± 136	513 ± 98
Left leg lean mass (kg)	6.72 ± 1.60	7.98 ± .80
Leg strength/leg lean mass (N/kg)	64.6 ± 19.1	64.1 ± 9.2

Values are presented as means ± SD. HRT=hormone replacement therapy group; non-HRT=non-hormone replacement therapy group; n=number of subjects; N=newtons; kg=kilograms

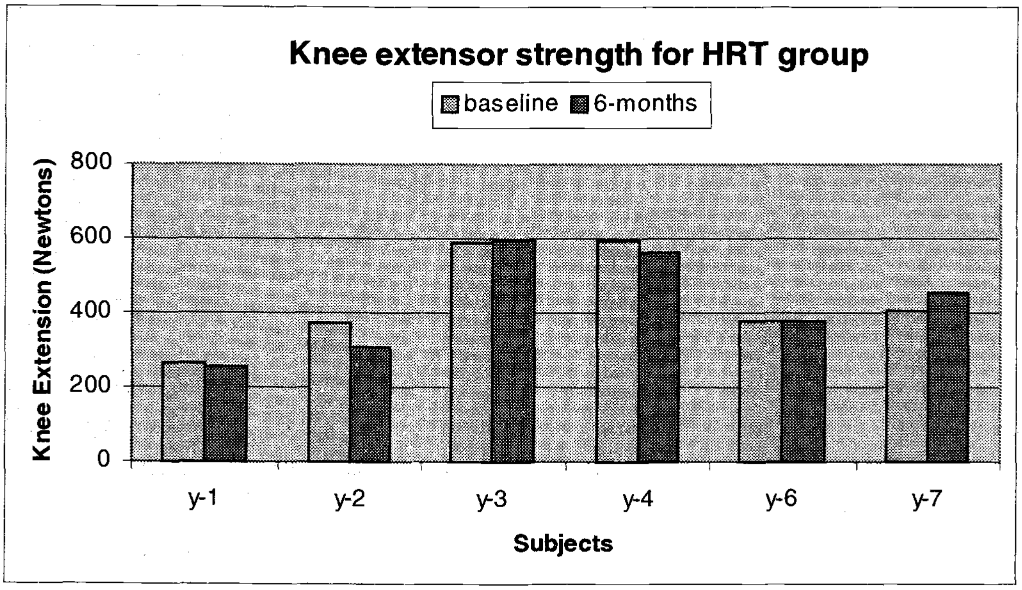

Figure 4: Changes in muscle strength for individuals in HRT group

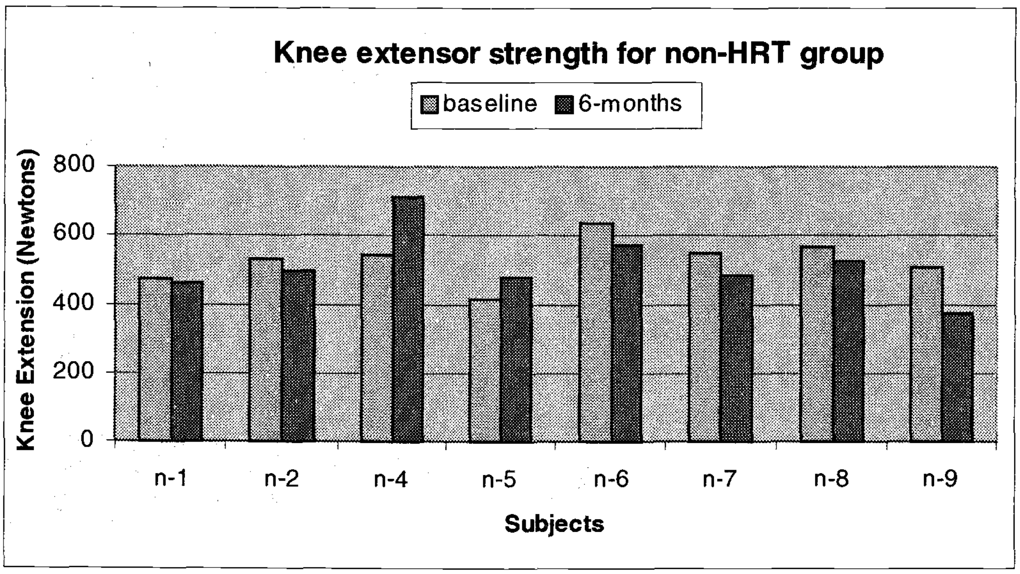

Figure 5: Changes in muscle strength for individuals in non-HRT group

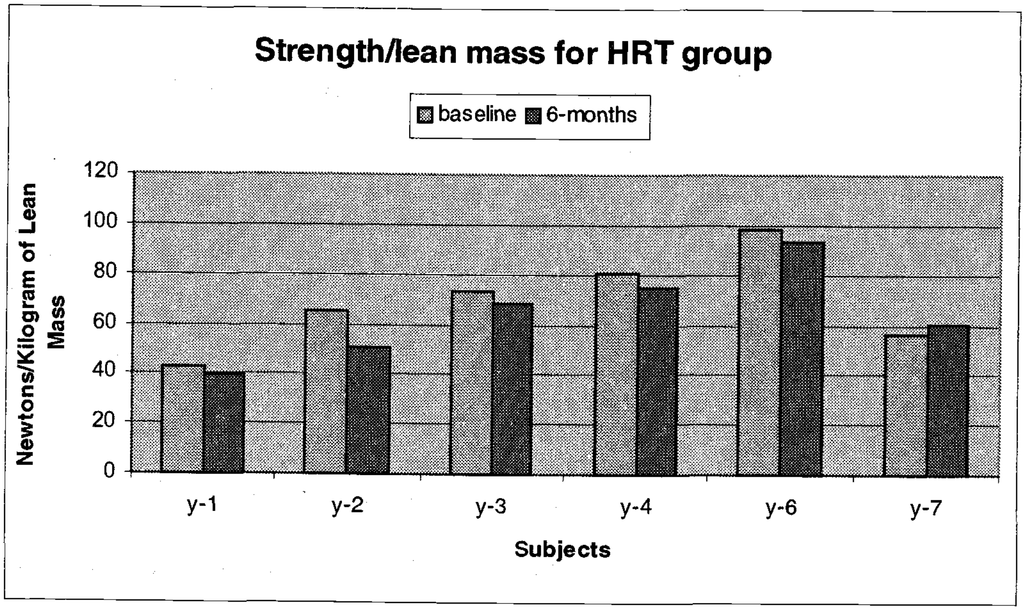

Figure 6: Changes in strength/lean mass for individuals in HRT group

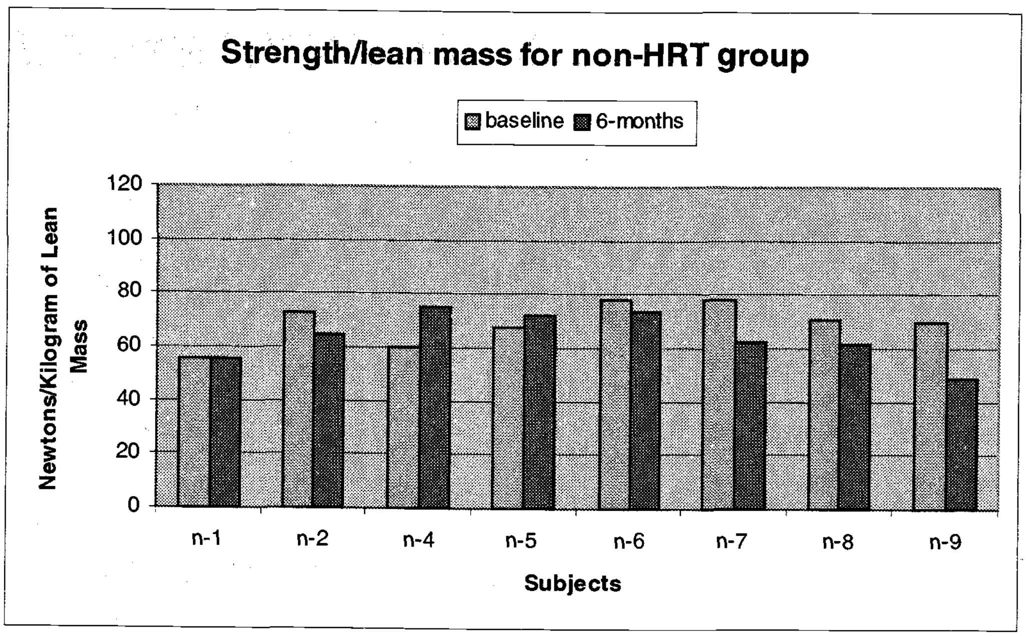

Figure 7: Changes in strength/lean mass for individuals in non-HRT group

Muscle fiber type percentages

The distribution of fiber number, calculated as a percentage of the total number of each fiber type, was not altered significantly after 6-months strength training in either group. The number of fibers for each sample used for the training analysis was on average 120 ± 37 fibers, which was not significantly different from the baseline analysis (106 ± 40). Over time there were no significant differences when looking at the muscle fiber type distributions, in either group (Table 7). Individual differences in Type II fiber percentages from baseline to 6-months can be seen in Figures 8, and it is assumed that the remainder of 100% consists of Type I fibers.

Table 7: Muscle fiber type distribution at baseline & 6-months

Type I (%)	HRT (n=6)	non-HRT (n=8)
Baseline	39.5 ± 5.7	38.0 ± 10.0
6-months	33.7 ± 4.4	35.7 ± 9.0
Type II (%)		
Baseline	60.6 ± 5.7	62.0 ± 10.0
6-months	66.3 ± 4.4	64.3 ± 9.0

Values are presented as mean ± SD. HRT=hormone replacement therapy group; non-HRT=non-hormone replacement therapy group: n=number of subjects.

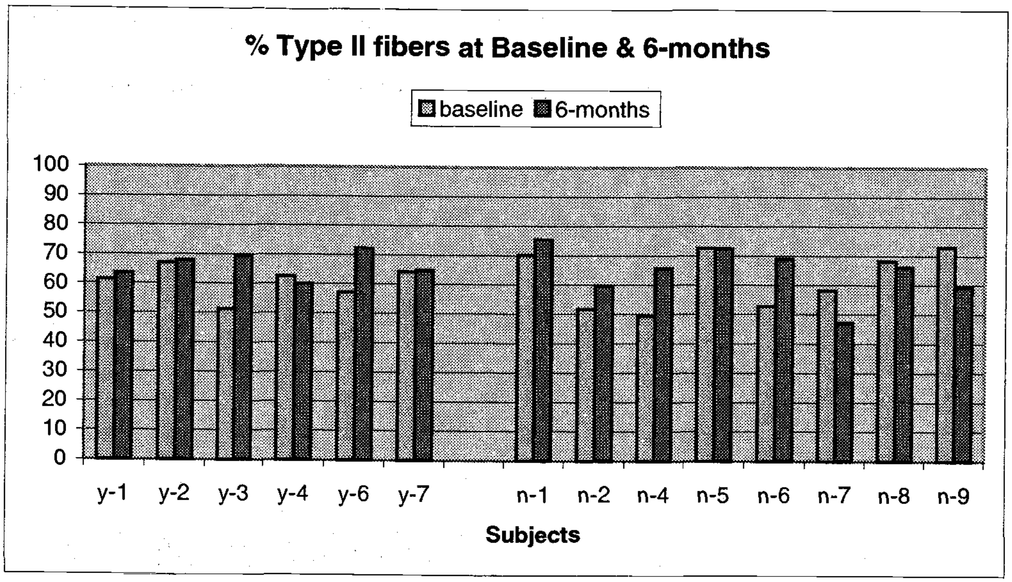

Figure 8: Type II muscle fiber percentages for individuals at baseline and 6-months. y=subject in HRT group (n=6); n=subject in non-HRT group (n=8).

Muscle fiber areas

The mean muscle fiber areas for Type I and Type II muscle fibers are listed in Table 8. There were no significant differences in muscle fiber CSA in either group after the 6-months of training. The HRT mean fiber CSA for Type I was 3545 μm^2 at baseline and 3530 μm^2 after 6-months of training. For the same group, the mean fiber CSA for Type II fibers was 2752 μm^2 at baseline and 2822 μm^2 after 6-months of training. The non-HRT mean fiber CSA for Type I was 3499 μm^2 at baseline and 3433 μm^2 after 6-months of training. For the same group, the mean fiber CSA for Type II fibers was 2822 μm^2 at baseline and 2774 μm^2 after 6-months of training.

Table 9 displays the average percent of total area of the muscle biopsy sample for the two different fiber types. There were no significant differences between groups or over time in both Type I and II percent of total area. Although there were no training effects on the percent areas, a similar trend was observed where there was an increased percent area for Type II fibers in both groups over time.

Table 8: Muscle fiber cross-sectional areas at baseline & 6-months

Type I fiber area (μm^2)	**HRT (n=6)**	**non-HRT (n=8)**
Baseline	3545 ± 527	3499 ± 482
6-months	3530 ± 698	3433 ± 709
Type II fiber area (μm^2)		
Baseline	2752 ± 544	2749 ± 547
6-months	2822 ± 398	2774 ± 417

Values are presented as mean ± SD. HRT=hormone replacement therapy group; non-HRT=non-hormone replacement therapy group; n=number of subjects; μm^2=micrometers squared.

Table 9: Percent of total area for Type I and II fibers at baseline and 6-months

% area of Type I fibers	**HRT (n=6)**	**non-HRT (n=8)**
Baseline	45.5 ± 6.7	45.5 ± 9.2
6-months	36.7 ± 5.5	41.8 ± 9.3
% area of Type II fibers		
Baseline	54.5 ± 6.7	54.5 ± 9.2
6-months	63.2 ± 5.5	58.6 ± 9.0

Values are presented as mean ± SD. HRT=hormone replacement therapy group; non-HRT=non-hormone replacement therapy group: n=number of subjects.

DISCUSSION

The current investigation addresses two hypotheses. The first hypothesis was that women who use hormone replacement therapy (HRT) have increased strength and muscle fiber cross-sectional area (CSA) compared to women who do not use HRT. The second hypothesis was that HRT and strength training (ST) will interact to produce greater gains in neuromuscular strength and skeletal muscle morphology in the HRT group compared to the non-HRT group. To our knowledge, this is the first time muscle histochemistry has been used to assess the effects of HRT and the interaction between HRT and ST on muscular adaptations in early postmenopausal women.

We found no difference between groups in strength, muscle fiber CSA, or fiber type distribution at baseline and after 6-months training. In addition, HRT did not interact with ST to enhance muscular strength. However, it is difficult to reject the 2nd hypothesis because the ST program did not improve strength.

EFFECTS OF HOMRMONE REPLACEMENT THERAPY ON MUSCULAR STRENGTH

The rationale for this study was that HRT has been found to improve voluntary neuromuscular strength of postmenopausal women. For example, Phillips et al. [31] found that those women on HRT were stronger when compared to the non-HRT controls. Further, Skelton et al. [37] found that when HRT was started in a group of non-HRT women, strength increased 5% and 15% after 26 and 52 weeks of treatment, respectively. Greeves et al. [13] found that HRT protected against a 10% loss of quadriceps muscle strength seen in the non-HRT group of early postmenopausal women. These results suggest that HRT plays an important role in maintaining strength in postmenopausal women.

Our data do not support the results of these previous studies since we found no difference in voluntary knee extensor strength between HRT and non-HRT women. This held for absolute strength and after normalizing for leg muscle mass. Our data is therefore consistent with both Seeley et al. [36] and Taaffe et al. [42] who found no evidence that estrogen was beneficial to muscle function of postmenopausal women. Seeley et al. [36] examined the association between estrogen and muscle strength, neuromuscular function, and the risk of falling, in women aged 65 and older. There was no evidence that HRT increased hip adductor, triceps extension, and handgrip strength. Likewise, Taaffe et al. [42] investigated whether the beneficial effects of HRT were evident in larger muscle groups that are involved in movement, balance, and activities of daily living. The results of this study show that there was no effect of HRT on dynamic muscle strength in lower body exercises.

Voluntary strength is dependent on neural and muscular factors. Thus, benefits of HRT at the muscle could be obscured by neural mechanisms affecting strength. Along these lines, Phillips et al. [31] and Skelton et al. [37] have concluded that HRT acts at the level of the muscle cells. Therefore we examined muscle fiber CSA and fiber type distribution, in biopsy samples from HRT and non-HRT women. The force produced by a fiber is proportional to its CSA. Also, fast fibers produce greater force per CSA than the slow fibers [43]. Therefore, differences in fiber CSA or fiber type might exist between HRT and non-HRT groups. However, there were no differences in voluntary knee extensor strength.

In summary, there were no significant differences in the CSA between HRT (Type I, 3615 ± 335 μm^2; Type II, 2770 ± 273 μm^2) non-HRT (Type I, 3769 ± 289 μm^2; Type II 2849 ± 254 μm^2) fibers, at baseline. There were also no significant differences in fiber type distribution between HRT (37.9 ± 2.5% Type I, 62.1 ± 2.5% Type II) and non-HRT (38.9 ± 2.9% Type I, 61.1 ± 2.9% Type II), at baseline. In addition, there were no significant differences in percent of fiber areas between HRT (Type I, 44.4 ± 2.5%; Type II, 55.6 ± 2.5%) and non-HRT (Type I, 45.6 ± 2.8%; Type II 54.4 ± 2.8%), at baseline.

Our results support the studies showing no effects of HRT on muscle strength. One strong point of the present study was that there were no significant differences in subject characteristics between the two groups at baseline. The main distinction between these two groups was that the HRT group was taking HRT prior to this study for the average of 19 months (6-36 months). Based on the intervention study of Skelton et al. [37], HRT improved strength significantly after only 26 weeks of treatment, in the women who were on average 10 years post-menopause. Therefore, it is assumed that a minimum of 6 months and an average of 19 months of HRT is sufficient to show effects.

Another strength was the morphological analysis of the muscle biopsy samples. There are several benefits of this measurement. First of all, it eliminates the effects of motivational factors that may cause limitations with measuring and interpreting voluntary muscle strength. In addition, it can be used as an additional measurement of changes within the musculoskeletal system, by looking at muscle fiber CSA and fiber type distribution.

The baseline CSA measurements of the present subjects are comparable to those in previous investigations (Table 10). Additionally, the variability in the measurements in this study is similar to those reported by these other investigations. In addition, Widrick et al. studied skinned fibers from a small portion of the same biopsy samples used in this study. After adjusting for the swelling that occurs during the skinning, there is very good agreement between the present CSA's determined by histochemistry and the CSA of the single fibers.

Table 10: Muscle fiber cross-sectional areas in recent studies

	Baseline	Type I (μm^2)	Type II (μm^2)
Present study - early PM women (40-57 yr) - muscle histochemistry	HRT Non-HRT	3614 ± 334 3769 ± 289	2770 ± 273 2849 ± 254
Widrick, et al. (abstract) - early PM women (40-57 yr) - single skinned fibers (values adjusted for swelling that occurs with skinning)	HRT Non-HRT	3383 ± 149 3678 ± 134	2901 ± 145† 2881 ± 160†
Staron, et al. [41] - young women (20-25 yr) - muscle histochemistry	Pre-Training	4253 ± 949	3370 ± 1048†
Cress, et al. [7] - older women (65-83 yr) - muscle histochemistry	Control Exercise	3979 ± 748 3305 ± 760	2733 ± 737† 2468 ± 878†
Pyka, et al. [32] - older women (61-78 yr) - muscle histochemistry	Control Exercise	3525 ± 472 3872 ± 259	3076 ± 273 3245 ± 183
Essen-Gustavsson & Borges [10] - men & women of all ages - muscle histochemistry	women 40 yr women 50 yr women 60 yr	3700 ± 700 3600 ± 700 3900 ± 700	3300 ± 800† 3500 ± 700† 3300 ± 500†

PM=postmenopausal, yr-years old, HRT=hormone replacement therapy; non-HRT=non-hormone replacement therapy; μm^2=micrometers squared; †=indicates Type IIa muscle fibers were differentiated.

One interesting finding is that women, regardless of hormone treatment, have a consistently larger Type I fiber CSA versus Type II fibers. A similar result has been observed by all of the investigators summarized in Table 10. This appears to be the opposite of what is observed in male subjects. When looking at college-aged men in McCall et al. [26] showed that prior to strength training young men typically have larger Type II fiber areas (6378 ± 1552 μm^2) when compared to Type I fiber areas (4196 ± 859 μm^2). In addition, Essen-Gustavsson & Borges [10], found that 50 year old men have the average of 4100 ± 700 μm^2 for Type I fibers and 4700 ± 700 μm^2 for Type II fibers.

Since fast fibers (Type II) produce greater force per unit CSA than slow fibers (Type I) [43] one possibility is women on HRT have a greater percent of Type II fibers. Thus we examined fiber type distributions at baseline. At this time, there were no significant differences between the two groups fiber type distributions. On average, the women had 38% Type I and 62% Type II fibers, regardless of group. The results are similar to what the distribution found by Staron et al. [39], who reported that younger women (average age 20.6 years) had 39% Type I fibers, and 55% Type IIa and IIb fibers combined.

There are several possible reasons why there have been a variety of outcomes for studies looking at the effects of HRT on skeletal muscle of early postmenopausal women. First of all, previous studies by both Phillips et al. [31] and Skelton et al. [37] have examined changes in the *adductor pollicis* muscle, whereas both Greeves et al. [13], and the current investigation looked at a larger weight-bearing muscle, the quadriceps. Greeves et al. [13] showed that HRT maintained strength, yet similar to the current investigation, no increases in strength were found. This brings about the question, are all muscles affected equally by HRT?

Moreover, Greeves et al. [13] suggest that low concentrations of progesterone may be the cause of decline in muscle strength following menopause. One possible reason why we may not have seen differences in the current investigation was because only two out of eight women were taking progesterone as part of their therapy. Lastly, in Skelton et al. [37] the most significant results have been found in women with the lowest initial force. According to this finding it is thought that extremely fit and strong women should be excluded from participation. In the present investigation, women with a wide variety of activity levels were included, from sedentary to extremely active. Therefore, variability in fitness level of the participants may be another reason for varying results found on the effects of HRT on muscle strength.

INTERACTION OF HORMONE REPLACEMENT THERAPY AND STRENGTH TRAINING

The second hypothesis tested was that HRT and ST will interact to produce greater gains in neuromuscular strength and skeletal muscle cross-sectional area in the HRT group compared to the non-HRT group. In order to interpret the training results of this study, it is important to first look at the effects of ST on muscular strength and morphology of postmenopausal women. Rhodes et al. [33] found significant increases in strength varying from 19-53% in older women (65-75 yr) who performed a 3 day/week total body resistance training routine for 1 year. Similarly, Cress et al. [6] saw a 9.4% increases in strength after a 3 days/week strength training program carried out for 1-year. Morganti et al. [29] used only a 2 days/week training protocol, yet still had significant findings in relation to strength increases (18-77%) after a year of training. Furthermore, Charette et al. [5] conducted a 12-week resistance training study with women between 64 and 86 years old and reported increases in strength ranging from 28 to 115% of their baseline values. Finally, Pyka et al. [32] found initial increases in strength (30-97%)

during the first three months, yet their strength plateaued for the remaining 9 months of the study. Each of these studies were similar, in that they investigated the effects of a 1-year strength training routine on the muscular strength in older, postmenopausal women. These studies demonstrate that older women have the capability of increasing strength by participating in various strength-training programs. None of the studies considered menopausal status and HRT as part of the investigation.

In the present study, there was no significant effect of the training program on the isometric strength of the knee extensors. In addition, when strength was adjusted for changes in lean muscle mass of the leg, there were no significant differences following the training protocol, in either group of women. Consistent with those findings, when looking at the changes in the muscle fiber CSA and fiber type distribution, there were also no significant differences. Since the training program had not effected the muscle strength or morphology, it is inconclusive as to whether HRT interacts with ST. Therefore, we can neither accept nor reject our hypothesis.

Although there have been several reports of HRT and its possible effects on muscle, there has only been one longitudinal study looking at a combination of HRT and exercise. Brown et al. [4] looked at the effects of an 11-months weight bearing exercise program on lower body strength (hip extensors and abductors, and knee extensors and flexors). In support of our findings, Brown et al. [4] found that women on HRT did not have increased gains in strength when compared to non-HRT women. However, when comparing this to the current investigation, the exercise program for their study consisted of weight bearing activities, such as running, jogging, and stair climbing, not intense strength training.

LIMITATIONS

There were several limitations to this study that occurred in both the baseline and training analyses. The most prominent limitation was the non-randomization of the women into two groups. In this case, the women had previously chosen to partake in HRT or not. Therefore, subjects were not randomized into two groups prior to their HRT treatment. According to Jacobs and Loeffler [18] women who are taking HRT are more likely to be active and fit, therefore, we may have selection bias for the HRT group. Another limitation was the small sample size. Due to the invasiveness of the muscle biopsy procedure, few women volunteered to be a part of this study sub-group. An additional limitation to the current study was the lack of measurement of hormone concentration at the baseline, and at the conclusion of 6-months of training. Although the women were all postmenopausal, there is a wide range of hormone concentration, which may have confounded the results. Lastly, due to the complexity of the histochemical procedures, one final limitation to this investigation was that we did not classify the fast fibers into IIa or IIb subgroups. When analyzing the samples, we were able to distinguish between the different fast fibers on some of the samples, but not all. Therefore, because of this limitation, we were only able to classify the fibers into two general categories, Type I and [41] II fibers. However, IIb fibers make up a very small percent of total fibers and they have a very similar CSA when compared to IIa fibers [41]. Thus, the inability to distinguish subgroups of fast fibers is unlikely to alter the main conclusions of this study.

Although there are several limitations to this investigation, there were several delimitations that were present. Our baseline measurement of fiber CSA and fiber type distribution are in very good agreement with previous studies [7, 10, 32, 41]. This suggests that our data are valid measurements of these variables. Also the histochemical and performance results are consistent. For instance, when there was no significance in muscle strength or strength/lean mass ratio, there was also no matching alterations in fiber CSA or fiber distributions.

Another strength of this study was the target population. Early postmenopausal women is a group that has received limited attention, yet there has been growing interest in this population in recent years because of the importance of this time period for prevention of osteoporosis and other chronic conditions. Lastly, to our knowledge, this is the first time muscle histochemistry has been used to assess the effects of HRT and the interaction between HRT and ST on muscular adaptations in early postmenopausal women.

RECOMMENDATIONS & CONCLUSIONS

There are several recommendations suggested for future research. One would be, to use a strength-training program that consists of total body exercises, to help promote overall health and fitness for the women who are part of the study. Another recommendation would be to look at the changes in muscle strength and morphology over a longer period of time, for instance 12 months. Since several of the investigations that saw effects of HRT were on smaller muscle groups (*adductor pollicis*) [31, 37], when looking at a larger muscle group (quadriceps) it is possible that the HRT needs a longer period of time to effect the strength and/or morphology of the muscle. Lastly, due to the variance in the hormonal status of postmenopausal women, it is recommended that some measures of estrogen and progesterone are made at baseline, during the training, and following the training protocol.

To our knowledge, this is the first time a study like this has been done, looking at early postmenopausal women and how HRT can alter muscle strength and morphology with and without ST. Based on the findings from the baseline analysis, we reject our first hypothesis and conclude that HRT alone does not have beneficial effect on the muscle strength and morphology of early postmenopausal women. In addition, since the training program had not altered the muscle strength or morphology, it is inconclusive as to whether HRT interacts with ST. Therefore, we can neither accept nor reject our second hypothesis. However, due to the limitations of this study, it is recommended that further research is done before conclusive decisions are made about HRT and ST for early postmenopausal women.

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APPENDICES

Appendix A

OREGON STATE UNIVERSITY

Report of Review by the Institutional Review Board for the Protection of Human Subjects

TO: Gianni Maddalozzo
Exercise and Sport Science

COPY: Laura Lincoln

RE: The combined effects of moderate intensity weight training and estrogen therapy on bone mass in early postmenopausal women

The referenced project was reviewed under the guidelines of Oregon State University's institutional review board (IRB), the Committee for the Protection of Human Subjects, and the U.S. Department of Health and Human Services. The IRB has approved your application. The approval of this application expires upon the completion of the project or one year from the approval date, whichever is sooner. The informed consent form obtained from each subject should be retained in program/project's files for three years beyond the end date of the project.

Any proposed change to the protocol or informed consent form that is not included in the approved application must be submitted to the IRB for review and must be approved by the committee before it can be implemented. Immediate action may be taken where necessary to eliminate apparent hazards to subjects, but this modification to the approved project must be reported immediately to the IRB.

Anthony Wilcox Date: 1/12/01

Anthony Wilcox, Chair
Committee for the Protection of Human Subjects
Langton 214
anthony.wilcox@orst.edu; 737-6799

Appendix B

Department of Exercise and Sport Science

INFORMED CONSENT DOCUMENT

A. Title of the Research Project: Cellular adaptations to resistance exercise training

B. Principal investigator: Jeffrey Widrick, Ph.D.
Assistant Professor
Dept. of Exercise and Sport Science

Co-investigator: Gianni Maddalozzo, Ph.D.
Instructor
Dept. of Exercise and Sport Science

C. Purpose of the Research Project.

There is a gradual reduction in muscular strength as people become older. Resistance exercise training may be capable of preventing, slowing, or even reversing this loss. It is not clear whether this improvement in muscular strength is due to alterations occurring within the muscle itself or is the result of changes in muscle recruitment, increased skill, or greater motivation. This study will investigate the effects of resistance exercise training on the properties of individual muscle cells or fibers independently of other changes that may affect muscular function.

D. Procedures.

1. Pre-study screening. I have been invited to volunteer for this study because, I am a current participant in a study conducted by Dr. Gianni Maddalozzo, titled "The combined effects of moderate intensity weight training and estrogen therapy on bone mass in early postmenopausal women." I have received an oral and a written explanation of this study and I understand that as a participant in this study the following things will happen:

2. What I will be asked to do during the study. If I agree to participate in this study, I will be asked to do the following:

   a) *Muscle biopsies.* A small muscle sample will be obtained from my left thigh muscle (vastus lateralis) at the start of the study and again 6 and 12 months later. The method used to obtain these samples (percutaneous needle biopsy technique) is used by the medical community to obtain muscle samples for the assessment of neuromuscular diseases. Muscle samples obtained in this way are approximately the size of one grain of rice. A physician will perform the procedure.

3. Foreseeable risks or discomforts.

   a) *Muscle biopsy.* I may experience a stinging sensation as a local anesthetic is injected at the sample site. A small incision, approximately 3/8 inch long, is made through the skin and the sheath that surrounds the muscle. I understand that I will have a small scar as a result of this incision. To obtain the muscle sample, a biopsy "needle", ~ 2/10 of an inch in diameter, is inserted through the incision and into the muscle. Most people report feeling an odd, "pressure" sensation as this is done. Following the procedure, I may experience muscle soreness or tenderness near the biopsy site. This soreness usually disappears within 24-48 hours. Like any surgical procedure, there are certain risks. These include the risk of infection and the development of an intramuscular heamotoma. The following precautions will be taken to minimize these risks: 1) the biopsies will be performed by a physician, 2) all procedures will be performed using sterile technique, 3) I will be given directions as to how to care for the incision, and 4) the incision will be periodically examined by one of the investigators. I will be referred to a physician if there is evidence of any unusual response.

4. Benefits to be expected from the research. I will learn about the muscle fiber type composition of my thigh muscle (i.e. % slow and % fast fibers). It is expected that the results from this research may improve the health and quality of life for others.

E. Confidentially. Any information collected from me will be kept confidential. The only persons who will have access to this information will be the investigators. A code number will be used to identify test results or other information provided by the investigators. My name will never be used in any data summaries or publications.

F. Compensation for injury. I understand that the University does not provide a research subject with compensation or medical treatment in the event that the subject is injured as a result of participation in the research project.

G. Voluntary participation statement. I understand that my participation in this study is completely voluntary and that I may either refuse to participate or withdraw from the study at any time without penalty, loss of benefits or jeopardizing my standing in the primary research study I am participating in with Dr. Gianni Maddalozzo.

H. If I have questions. I understand that any questions I have about the research study and/or specific procedures should be directed to: Jeffrey Widrick, 105 Women's Bldg., Oregon State University, (541) 737-5923. Any other questions that I have should be directed to: Sponsored Programs Director, OSU Research Office, (541) 737-0670.

I. Understanding and compliance. My signature below indicates that I have read and I understand the procedures described above and give my informed and voluntary consent to participate in this study. I understand that I will receive a signed copy of this consent form.

Signature of subject	Name of subject
Date signed	
Subjects present address	Subjects phone number
Signature of Principal Investigator	Date signed

Appendix C

Oregon State University Bone Research Laboratory
Department of Exercise and Sport Science
College of Health and Human Performance

Estrogen Replacement: Effects on the Musculoskeletal System.

- Most individuals find that the questionnaire can be completed in approximately 20-30 minutes.
- Replies are important from all Oregon State University Bone Research Laboratory; exercisers or non-exercises
- Be as accurate as possible, but provide your best estimate if you do not remember precisely.
- All responses will be kept strictly confidential like other Oregon State University Bone Research records.

If you wish to comment on any questions or to qualify your answers, please write in the margins. Your comments are welcome and will be taken into account.

A summary of this research will be sent to all participants.

Thank you for your help!

Name: ______________________________ Date: ______________

Subject ID: _____________

In this section we would like to ask you about your current physical activity and exercise habits that you perform regularly, at least once a week. Please answer as accurately as possible. Circle your answer or supply a specific number when asked.

EXERCISE/PHYSICAL ACTIVITY

1. **For the last three months, which of the following moderate or vigorous activities have you performed regularly?** ***(Please circle YES for all that apply and NO if you do not perform the activity; provide an estimate of the amount of activity for all marked YES. Be as complete as possible.)***

Walking
NO YES ⟶ How many sessions per week? ______
How many miles (or fractions) per session? ______
Average duration per session? ______(minutes)

What is your usual pace of walking? ***(Please circle one)***

CASUAL or STROLLING (< 2 mph)	AVERAGE or NORMAL (2 to 3 mph)	FAIRLY BRISK (3 to 4 mph)	BRISK or STRIDING (4 mph or faster)

Stair Climbing
NO YES ⟶ How many flights of stairs do you climb UP each day? ______
(1 flight = 10 steps)

Jogging or Running
NO YES ⟶ How many sessions per week? ______
How many miles (or fractions) per session? ______
Average duration per session? ______(minutes)

Treadmill
NO YES ⟶ How many sessions per week? ______
Average duration per session? ______(minutes)
Speed? _____(mph) Grade? _____(%)

Bicycling
NO YES ⟶ How many sessions per week? ______
How many miles per session? ______
Average duration per session? ______(minutes)

Swimming Laps
NO YES ⟶ How many sessions per week? ______
How many miles per session?
(880 yds = 0.5 miles) ______
Average duration per session? ______(minutes)

Aerobic Dance/Calisthenics/Floor Exercise
NO YES ⟶ How many sessions per week? ______
Average duration per session? ______(minutes)

Moderate Sports
(e.g. Leisure volleyball, golf (not riding), social dancing, doubles tennis)
NO YES ⟶ How many sessions per week? ______
Average duration per session? ______(minutes)

Vigorous Racquet Sports
(e.g. Racquetball, singles tennis)
NO YES ⟶ How many sessions per week? ______
Average duration per session? ______(minutes)

Other Vigorous Sports or Exercise Involving Running (e.g. Basketball, soccer)
NO YES ⟶ Please specify:______________________
How many sessions per week? ______
Average duration per session? ______(minutes)

Other Activities
NO YES ⟶ Please specify:______________________
How many sessions per week? ______
Average duration per session? ______(minutes)

Weight Training
(Machines, free weights)
NO YES ⟶ How many sessions per week? ______
Average duration per session? ______(minutes)

Household Activities (Sweeping, vacuuming, washing clothes, scrubbing floors)
NO YES ⟶ How many hours per week? ______

Lawn Work and Gardening
NO YES ⟶ How many hours per week? ______

2. How many times a week do you engage in vigorous physical activity long enough to work up a sweat? ______ *(times per week)*

Appendix D

Solutions for ATPase Procedures

1) 0.1M Sodium Barbital (need 50 mL)
   - 2.06g/100mL MQ water or 1.03g/50mL MQ water
   - make fresh, store in frig. up to 5 days

2) 0.18M $CaCl_2$ (need 24 mL)
   - 2.6469g/100mL MQ water
   - lasts, store in frig.

3) Sodium Acetate/Sodium Barbital Buffer (need 4.34 mL)
   - Sodium Acetate, 0.97g/50mL MQ water
   - Sodium Barbital, 1.47g/50mL MQ water
   - lasts 1 week, store in frig.

4) 1% $CaCl_2$ (need for 3 changes)
   - 1g/100mL MQ water
   - lasts, store in frig.

5) 2% $CoCl_2$ (need for 1 change)
   - 2g/100mL MQ water
   - lasts, store in frig.

6) 0.01M Sodium Barbital (need for 6 changes)
   - 1.03g/500mL MQ water
   - lasts up to 1 week, store in frig.

7) 1% Ammonium Sulfide (100 mL) (light sensitive)
   - 1g/100mL MQ water
   - make during run, immediately before use, under hood

Solutions for procedure

	0.1M Sodium Barbital	0.18M $CaCl_2$	Double Dist. water	other
1) Alkaline solution (pH 10.0)	2 mL	2 mL	6 mL	-
2) Wash Solution (pH 9.40)	24 mL	12 mL	84 mL	-
3) ATP Incubating Solution (pH 9.40)	24 mL	12 mL	84 mL	**.30g ATP Disodium Salt** (in dessicator in freezer)

4) Acid solutions (pH 4.35 & 4.65)
   - 2.17 mL Sodium Acetate/Barbital Buffer
   - 4.35 mL 0.1M HCL
   - 3.48 mL Double Dist. Water

5) Set-up wells of descending alcohols (70%, 80%, 95%, & 100%) and xylene, under hood

Appendix E

ATPase Procedures

1) Incubate in Alkaline solution (pH 10.0) for **15 mins.**

2) After 10 mins. start Acid (pH 4.35 & 4.65) incubation for **5 mins.**

3) Transfer to carrier & rinse in wash solution

4) Place in ATP incubating solution for **10 mins.** at 37°C (in oven)

5) Wash in 3 changes of 1% $CaCl_2$ for **10 mins.**

6) Transfer to 2% $CoCl_2$ for **3 mins.**

7) Wash well in 6 changes of 0.01M Sodium Barbital for **8-10 mins.**

8) Wash well in running water for **45 sec.**

9) Place in 1% $(NH_4)_2S$ for **45 sec.**

10) Wash well in running water for **90 sec.**

11) Dehydrate rapidly in ascending alcohols & clear in xylene

12) Mount in permount & put on glass slides