EFFECTS OF SHORT-TERM LOWER LIMB IMMOBILIZATION ON SKELETAL MUSCLE FUNCTION AND MORPHOLOGY IN MEN AND WOMEN

EFFECTS OF SHORT-TERM LOWER LIMB IMMOBILIZATION ON SKELETAL MUSCLE FUNCTION AND MORPHOROLOGY IN MEN AND WOMEN

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ABSTRACT

The purpose of this study was to determine the effects of short-term (14d) unilateral leg immobilization using a simple knee brace (60° flexion)/crutch-mediated model on muscle function and morphology in men (M, N=13) and women (W, N=14). Isometric and isokinetic (concentric SLOW, 0.52 rad•s^{-1} and FAST, 5.24 rad•s^{-1}) knee extensor peak torque was determined at three time points (PRE, DAY-2, and DAY-14). At the same time points, magnetic resonance imaging was used to measure the cross-sectional area of the quadriceps femoris and DEXA scanning was used to calculate leg lean mass. Muscle biopsies were taken from vastus lateralis at PRE and DAY-14 for myosin ATPase and myosin heavy chain analysis. Women showed greater decreases (PRE vs. DAY-14) compared to men in specific strength (N•cm^{-2}) for isometric (M=3.1±13.3, W=17.1±15.9%; p= 0.055, [mean±SD]) and concentric SLOW (M=4.7±11.3, W=16.6±18.4%; p<0.05) contractions. There were no immobilization-induced sex-specific differences in the decrease in quadriceps femoris cross-sectional area (M=5.7±5.0, W=5.9±5.2%) or leg lean mass (M=3.7±4.2, W=2.7±2.8%). There were no fiber type transformations, and the decrease in Type I (M=4.8±5.0, W=5.9±3.4%), IIa (M=7.9±9.9, W=8.8±8.0%) and IIx (M=10.7±10.8, W=10.8±12.1%) fiber areas was similar between sexes. These findings indicate that immobilization-induced loss of knee extensor muscle strength is greater in women compared to men in spite of a similar extent of atrophy at the myofiber and whole muscle levels after only 14d of unilateral leg immobilization. Furthermore, we have described an effective and safe method of knee

immobilization that results in significant reductions in quadriceps muscle strength and fiber size.

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CHAPTER I

1.1 INTRODUCTION

Skeletal muscle atrophy refers to the wasting or loss of muscle tissue resulting from disease or lack of use. Atrophy can occur for a variety of reasons such as during different disease states (Sandri, 2002), after muscle dennervation (Booth, 1982), absence of weight-bearing activity (Adams et al., 2003; Appell, 1990; Booth, 1982), sarcopenia of aging (Doherty, 2001; Kamel, 2003) and spaceflight (Adams et al., 2003; Fitts et al., 2000, 2001). Upper and lower limb suspension/immobilization is commonly used as a unweighting model to investigate the process and mechanisms of muscle atrophy as well as to characterize the various functional and morphological changes in skeletal muscle after a period of disuse (Adams et al., 2003; Appell, 1990; Booth, 1982; MacDougall et al., 1980; Sale et al., 1982). According to Adams et al. (2003), there are mainly three types of non-weight bearing activities considered to bring about changes in skeletal muscle: 1) bed rest, which involves an essentially complete minimization of weight-bearing activity on all postural body structures and tissues, as well as a significant decrement in activity; 2) limb immobilization via a cast or knee brace, resulting in extensive restriction of motion for the targeted limb with maintained activity via crutches; 3) unilateral lower limb suspension, which results in the unloading of one lower limb without movement restriction and with the maintenance of activity via crutches. In this review, only 2) and 3) will be taken into consideration.

In terms of muscle strength, it has been shown that short-term (10-16 d) as well as relatively long-term lower limb unloading (4-6 weeks) causes a reduction in the ability to voluntarily activate a muscle based on electromyography activities, which indicates that the recruitment of motor units and firing frequencies were altered (Berg and Tesch, 1996; Dudley et al., 1992; Hortobágyi et al., 2000). There is only one study that has looked at possible sex-differences in neural recruitment issues during immobilization (Semmler et al., 1999). This group has reported that regulatory mechanisms between the sense of effort and motor unit recruitment during isometric contraction were disturbed especially in women after 4 weeks of upper limb immobilization (Semmler et al., 1999).

At the morphological level, whether the alterations of whole and/or single fiber size associated with muscle mass occurs are debatable during a short-term lower limb unweighting intervention. For instance, Deschenes et al. (2002) reported no change in either muscle fiber size or distribution after 14 d of a knee brace-mediated immobilization. In contrast, Jones et al. (2004) found a significant decrease in quadriceps lean mass following 14 d of unilateral leg cast immobilization. However, it has been suggested that at least 4 weeks of unilateral leg suspension/immobilization may be required to significantly alter muscle morphology (Berg et al., 1993; Hather et al., 1992). Thus, in this review, different findings will be summarized dependent on each model of lower limb unweighting.

In light of the degree of muscle atrophy, the female sex steroid hormone, 17-β-estradiol, can be considered potentially and indirectly to attenuate muscle

wasting. Previous studies have demonstrated that 17-β-estradiol acts as an antioxidant and a membrane stabilizer and prevents exercise-induced muscle damage in rats and humans (Bär et al., 1988; Kendall and Eston, 2002; Shumate et al., 1979; Tiidus, 2003). Moreover, 17-β-estradiol appears to play a role in preventing creatine kinase leakage (a marker of muscle protein damage) from skeletal muscles at rest (Bär et al., 1988; Shumate et al., 1979). Thus, the effects of sex-steroid hormones will be considered as potential factors involved in differences in muscle function as well as morphology.

1.2 EFFECT OF LOWER LIMB IMMOBILIZATION ON MUSCLE FUNCTION

1.2.1 MUSCLE STRENGTH

In terms of the functional origin of strength loss in the neuromuscular system, previous studies have shown that changes in motor cortical area size occur without spinal excitability or motor threshold during immobilization, although the reduction in area could be quickly reversed by voluntary muscle contraction after immobilization (Liepert et al., 1995; Zanette et al., 1997).

To study strength loss and disuse atrophy, a number of studies have used limb immobilization via a cast as an unweighting model (Figure I-A). When this model is used on the lower limb, common features of this method include the unweighting of one limb, the fixation of the unweighted knee, and the use of crutches enabling ambulatory activity. With this approach, the quadriceps group is fixed in a lengthened position, while a two-joint leg muscle, such as the

gastrocnemious, may experience some degree of shortening (Adams et al., 2003). The force-velocity relationship over non-weight bearing activity is shown in Figure II (Adams et al., 2003).

Most of the performance data from this model have been obtained via measures of knee extension pre- and post-immobilization. Table II (A, B, and C) summarizes previous studies with respect to muscle strength after lower limb unweighting (immobilization and unilateral lower limb suspension). Rozier et al. (1979) found that knee extensor isometric maximal voluntary contraction was decreased by 13% after only 9 d of immobilization, suggesting that performance decrements demonstrate a fairly rapid onset. Hespel et al. (2001) reported a 23% decrease in isometric maximal voluntary contraction after 2 wk of immobilization, whereas Deschenes et al. (2002) found a decline of up to 22% in knee extensor peak torque after 2 wk of knee brace-mediated immobilization. Recently, Jones et al. (2004) have found a 27% decrease in isometric maximal voluntary contraction after 2 wk of cast immobilization. Hortobágyi et al. (2000) reported a 48% decrease in knee extensor maximal voluntary contraction after 3 wk of immobilization. In 4 weeks of immobilization, healthy subjects exhibited a 53% decrease in knee extensor torque (Veldhuizen et al., 1993). Some studies have suggested that immobilization appears to impact the knee extensors to a greater degree than the knee flexors (Deschenes et al., 2002; Veldhuizen et al., 1993).

Unilateral lower limb suspension is another ground-based model for muscle unweighting (Figure I-B). It is essentially a modified immobilization

model that does not involve the constant fixation of the knee joint. To date, two approaches of this model have been applied: a version that involves the use of a support strap to suspend one lower limb (Berg et al., 1991) and thus prevent weight bearing and another version that employs a high-platform shoe on the contralateral limb to prevent weight bearing in the ipsilateral leg (Hather et al., 1992). In each case, ambulatory activity is performed using crutches. In the "strap" model, the knee is maintained in flexion during ambulatory activity whereas in the platform "shoe" model, the suspended limb is in normal anatomic positions at all times and can move freely (Adams et al., 2003). It is possible that the changes in muscle length associated with the strap model might induce altered responses similar to those postulated for immobilization although the degree of changes is dependent on the time spent in ambulatory activity (Adams et al. 2003). However, most studies with the "strap" version of unilateral lower limb suspension reported deep venous thrombosis in a few subjects (Berg and Tesch, 1996; Bleeker et al., 2004a, 2004b; Gamrin et al., 1998), probably because the knee is more extensively flexed in the "strap" suspension model.

Previous studies have found knee extensor maximal voluntary contraction decreased by ~12-22% after short-term unilateral lower limb suspension (10-21 days) (Adams et al., 1994; Berg et al., 1996; Gamrin et al., 1998; Schulze et al., 2002). Furthermore, some have shown a significant decrease by ~13-42% in 1RM leg extension strength even after 10d of suspension intervention (Rozier et al., 1979; Thom et al., 2001). Other studies with relatively longer-term (4-6 weeks) have reported that the maximal vonluntary contraction of the knee

extensor decreased by ~15-21% (Berg et al., 1991, 1993; Dudley et al., 1992). Moreover, several studies have shown significant decreases by ~18-27% in 1RM leg extension strength after 5 wk of unilateral lower limb suspension (Ploutz-Snyder et al., 1995, 1996; Tesch et al., 1994).

Table I. Various kinds of unweighting models

Model	Characteristics
Bed rest	Relatively expensive and logically complex
Immobilization	Usually used via a cast or knee brace
Suspension	Unilateral lower limb suspension with a sling and/or shoe Hindlimb suspension (animal model)

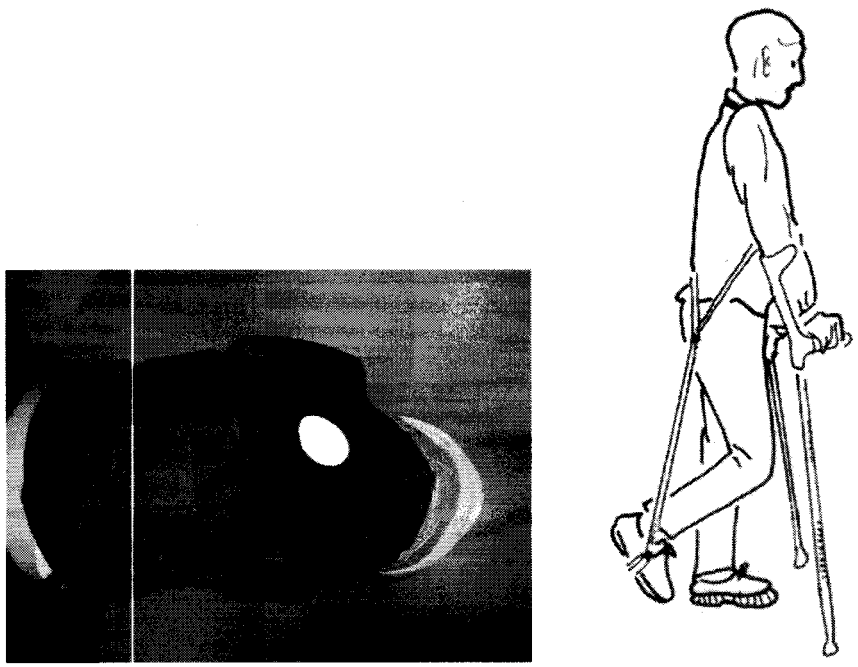
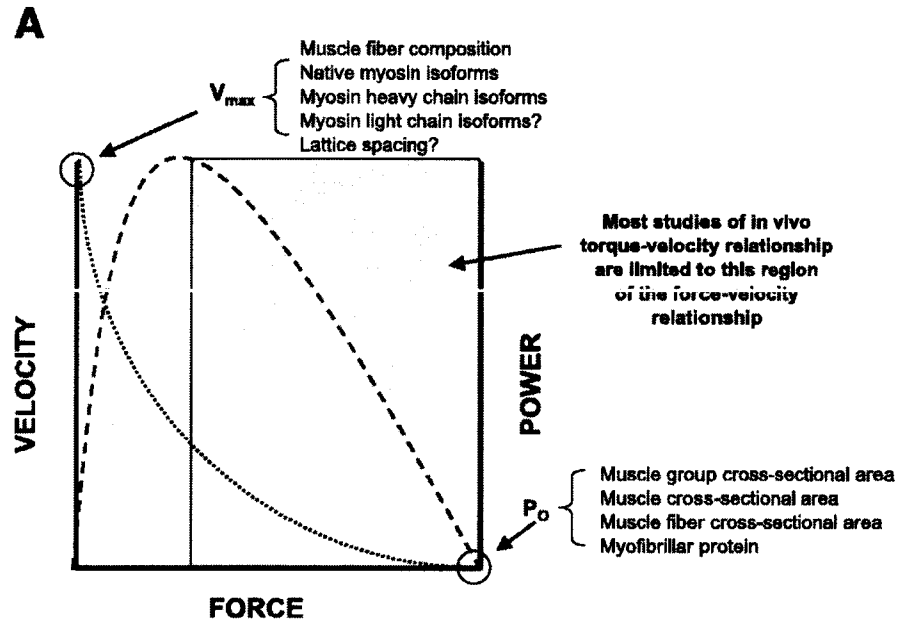


Figure I. Commercially available knee brace (A) and historically used "strap" unilateral lower limb suspension model (B) by Berg et al. (1991).

Figure II.
A: Force-velocity relationship (dotted line) and power curve (dashed line) for hypothetical muscle. The force-velocity relationship represents an important conceptual perspective regarding muscle function. Vmax = maximal shortening velocity, Po = maximal isometric tension.



B: Alterations in the force-velocity relationship and power curves produced by the mechanical unloading of skeletal muscle that is associated with bed rest, immobilization, and unilateral lower limb suspension. Cited from Adams et al. (*J Appl Physiol* 95: 2185-2201, 2003).

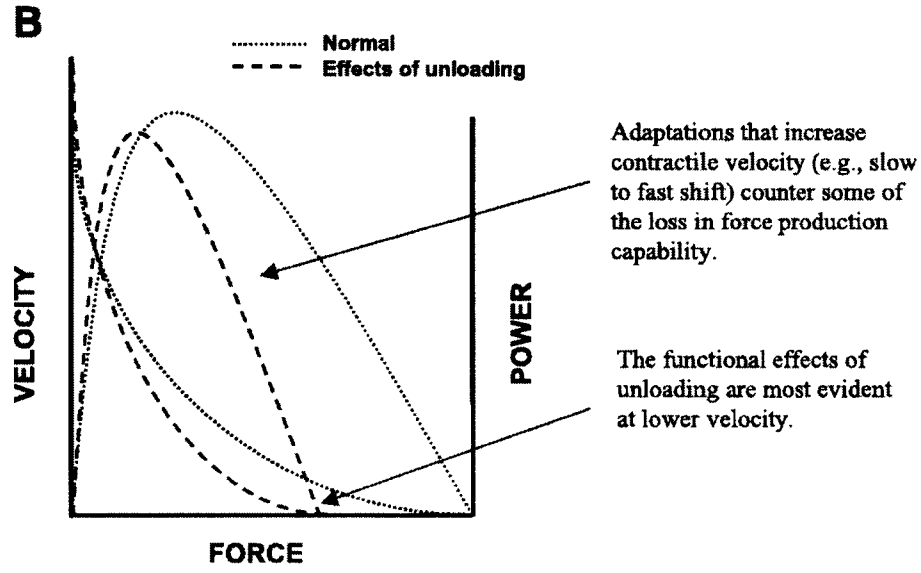


Table II-A. Summary of previous studies with regard to muscle strength after lower limb unweighting

References	Participants	Unweighting model	Duration	Type of measurements	Findings (% decrease)
Rozier et al. (1979)	20 healthy subjects (3 males and 17 females)	IM	9 days	Knee extensor isometric (Isometric MVC)	13%
Berg et al. (1991)	6 healthy males	ULLS	4 weeks	Knee extensor peak torque (Isokinetic - concentric and eccentric at 0.52, 1.57, and 2.62 rad•sec^{-1})	0.52 = 19%, 15% 1.57 = 16%, 16% 2.62 = 16%, 22%
Dudley et al. (1992)	8 healthy subjects (5 males and 3 females)	ULLS	6 weeks	Knee extensor peak torque (Isometric MVC) (Isokinetic - concentric and eccentric at 0.52, 1.75, and 2.97 rad•sec^{-1})	an overall 21% decrease
Veldhuizen et al. (1993)	8 healthy subjects	IM	4 weeks	Knee extensor and flexor peak torque Isokinetic at 1.05 rad•sec^{-1})	53%, 26%
Berg et al. (1993)	6 healthy males	ULLS	4 weeks	Knee extensor peak torque (Isokinetic - concentric at 3.14 rad•sec^{-1})	Average peak torque = 17%
Adams et al. (1994)	10 healthy males	ULLS	16 days	Knee extensor and flexor peak torque (Isometric MVC) (Isokinetic - concentric and eccentric at 0.51, 1.56, and 3.13 rad•sec^{-1})	an overall 12% decrease

IM; immobilization, ULLS; unilateral lower limb suspension, MVC; maximum voluntary contraction, RM; repetition maximum

Table II-B. Summary of previous studies with regard to muscle strength after lower limb unweighting

References	Participants	Unweighting model	Duration	Type of measurements	Findings (% decrease)
Tesch et al. (1994)	7 healthy subjects (4 males and 3 females)	ULLS	5 weeks	1 RM concentric and eccentric extension strength	concentric = 20% eccentric = 19%
Ploutz-Snyder et al. (1995)	7 untrained, healthy subjects (3 males and 4 females)	ULLS	5 weeks	1 RM leg extenstion strength	20%
Berg et al. (1996)	10 healthy males	ULLS	10 days	Knee extensor peak torque (Isometric MVC)	13%
Ploutz-Snyder et al. (1996)	7 untrained, healthy subjects (5 males and 2 females)	ULLS	5 weeks	1 RM concentric and eccentric extension strength	concentric = 18% eccentric = 27%
Gamrin et al. (1998)	5 healthy males	ULLS	10 days	Knee extensor peak torque (Isometric MVC)	17%
Hortobagyi et al. (2000)	Recreationally active, untrained 48 subjects (24 males and 24 females)	IM	3 weeks	Knee extensor peak torque (Isometric MVC) (Isokinetic - eccentric and concentric at 1.05 rad•sec^{-1})	 48% concentric = 45% eccentric = 48%
Hespel et al. (2001)	9 healthy subjects (males and females)	IM	2 weeks	Knee extensor peak torque (Isometric MVC) (Isokinetic at 3.14 rad•sec^{-1})	 23% 24%
Thom et al. (2001)	8 healthy females	ULLS	10 days	1 RM leg extenstion strength	42%

IM: immobilization, ULLS: unilateral lower limb suspension, MVC: maximum voluntary contraction, RM: repetition maximum

Table II-C. Summary of previous studies with regard to muscle strength after lower limb unweighting

References	Participants	Unweighting model	Duration	Type of measurements	Findings (% decrease)
Deschenes et al. (2002)	10 untrained, healthy subjects (6 males and 4 females)	IM	2 weeks	Knee extensor and flexor peak torque (Isometric MVC) (Isokinetic at 0.53, 1.05, 2.09, and 3.14 rad•sec^{-1})	 22% decrease (Knee extensor) 0.53 rad•sec^{-1}=19%, 14% 1.05 rad•sec^{-1}=18%, 12% 2.09 rad•sec^{-1}=14%, 11% 3.14 rad•sec^{-1}=13%, 3%
Schulze et al. (2002)	8 untrained, healthy men	ULLS	3 weeks	Knee extensor peak torque (Isometric MVC) (Isokinetic - eccentric and concentric at 1.05 rad•sec^{-1})	 17% concentric = 22% eccentric = 12%
Jones et al. (2004)	9 untrained, healthy men	IM	2 weeks	Knee extensor peak torque (Isometric MVC) (Isokinetic at 1.57 rad•sec^{-1})	 27% 22%
Bleeker et al. (2004)	7 untrained,healthy subjects (3 males and 4 females)	ULLS	4 weeks	Knee extensor peak torque (Isometric MVC)	 Decrease (No description of actual percentage)

IM: immobilization, ULLS: unilateral lower limb suspension, MVC: maximum voluntary contraction, RM: repetition maximum

1.3. SEX DIFFERENCES IN NEUROMUSCULAR FUNCTION

1.3.1 MUSCLE STRENGTH AND SPECIFIC STRENGTH

Previous results have demonstrated significantly higher absolute voluntary force production in males than females (Hunter and Enoka, 2001; Kanehisa et al., 1996; Maughan et al., 1986; Pincivero et al., 2000, 2003). Normalizing such values to body mass did not attenuate the observed sex-specific difference for knee extensor (quadriceps femoris muscle) and flexor (hamstring muscles) peak torque, work, and power. The sex-specific difference of body-mass-normalized peak torque was also observed by Wretling and Henriksson-Larsen (1998) during maximal-effort contractions at 1.57 $rad \bullet s^{-1}$. However, knee extensor work was not found to be different between males and females following body mass normalization. In a separate investigation, mean torque generated over five consecutive maximal-effort contractions at 3.14 $rad \bullet s^{-1}$ was found to be significantly higher in males when normalized to knee extensor muscle cross-sectional area (Kanehisa et al.,1996). It has been suggested that normalizing force measurements to muscle cross-sectional area may yield a more accurate sex-based comparison (Kanehisa et al., 1994, 1996; Kent-Braun and Ng, 1999; Maughan et al., 1986; Schantz et al., 1983).

Neder et al. (1999) found that young, middle-aged, and older males were stronger than age-matched females for isokinetic knee extensor peak torque in absolute and allometrically scaled, but not body-mass-relative units. Thus, males may possess an inherently greater ability to generate more force than females, which may be related to a significantly greater proportion of fast-twitch muscle

fibers in the quadriceps femoris muscle (Simoneau and Bouchard, 1989; Staron et al., 2000), a greater cross-sectional area of existing fiber types (Henrisksson-Larsen, 1985; Miller et al., 1993), and greater "muscularity" (defined as the ratio of skeletal muscle to adipose tissue-free mass; Wang et al., 2001). Although Kanehisa et al. (1996) found a sex-specific difference when absolute force was normalized to muscle cross-sectional area, other studies observed no differences in specific strength (Gür et al., 2003; Maughan et al., 1983; Schantz et al., 1983). One reason for this discrepancy in those studies may in part be due to the different calculations of specific strength (Schantz et al., 1983). For example, some have used leg length to determine $N \bullet cm^{-2}$ (Kanehisa et al., 1996), whereas other group made calculations using body height (m)(Schantz et al,. 1983).

1.3.2 EFFECT OF SEX STEROID HORMONES

1.3.2.1 TESTOSTERONE

There is agreement that exogenous testosterone (i.e., supplementation) increases maximal voluntary strength (Bhasin et al., 2001). However, the effects of endogenous and exogenous testosterone on other measures of muscle performance such as fatiguability and power (the rate of force generation) are unknown. Previous studies have shown no improvements in performance in endurance events (Casaburi et al., 1996). It has been proposed that testosterone might improve athletic performance in sprint events by decreasing reaction time, as testosterone has been shown to regulate neuromuscular transimission (Leslie

et al., 1991; Blanco et al., 1997). However, unequivocal improvements in measures of athletic performance have not been demonstrated in any study.

1.3.2.2 ESTROGEN AND PROGESTERONE

Evidence that estrogen and progesterone do not have a positive influence on skeletal muscle function is accumulating from previous studies tightly conducted for hormonal analyses (Elliott et al., 2003; Fridén et al., 2003; Janse de Jonge et al., 2001, 2003). However, different results were demonstrated in several studies where hormonal assays for estrogen and progesterone were not carried out (Davies et al., 1991; Phillips et al., 1996; Sawar et al., 1996).

Davies et al. (1991) examined the effect of the menstrual cycle on handgrip and standing long jump. In that study, handgrip and long jump performance was significantly different during different phases of the menstrual cycle. Sawar et al. (1996) found that regularly menstruating women were stronger mid-cycle (defined as day 12-18 of the menstrual cycle) with respect to maximum voluntary isometric force in handgrip and quadriceps. Phillips et al. (1996) reported an increase in adductor pollicis function during the follicular phase, followed by a rapid decrease in strength around mid-cycle, since hormone levels were not measured. These two studies suggested that estrogen may have a strengthening effect on skeletal muscle.

On the other hand, Janse de Jonge et al. (2001) reported no changes over the menstrual cycle for strength and fatigability of the quadriceps (isometric and isokinetic peak torque). Furthermore, in that study, no correlations were found

between the measured muscle contractile characteristics and the hormone concentrations of estrogen and progesterone. Similarly, Elliot et al. (2003) have also reported that mean maximum voluntary isometric force of the first dorsal interosseus muscle did not correlate significantly with the mean concentration of any reproductive hormone measured. Isokinetic strength over the menstrual cycle has been investigated by several studies using hormone concentration measurements to verify menstrual cycle phase (Fridén et al., 2003; Gür, 1997; Janse de Jonge et al., 2001; Lebrun et al., 1995). No changes over the menstrual cycle were found for isokinetic knee flexion and extension (Lebrun et al., 1995; Gür, 1997; Janse de Jonge et al., 2001). Taken together, it appears that the fluctuations in female steroid hormones throughout the menstrual cycle do not affect muscle strength.

1.4 EFFECT OF LOWER LIMB IMMOBILIZATION ON MUSCLE MORPHOLOGY

1.4.1 LEG LEAN MASS

Jones et al. (2004) have determined the effect of 2 weeks of cast immobilization on lean mass of the isolated left thigh region (patella to groin region) using dual energy X-ray absorptiometry (DEXA). Consequently, the authors reported a 4.7% decrease in healthy untrained men (n=9) following 14 days of unilateral leg casting (Table III-B).

1.4.2 CROSS-SECTIONAL AREA OF QUADRICEPS FEMORIS

Table III (A and B) summarizes previous studies with respect to muscle morphology after lower limb unweighting. Previous studies with short-term immobilization (10-14d) have shown ~10-12% reduction in the cross-sectional area (CSA) of knee extensor muscles (Hespel et al., 2001; Thom et al., 2001). Furthermore, Veldhuizen et al. (1993) reported a 21% decrease in knee extensor muscle group CSA after 4 wk of immobilization. The number of immobilization studies conducted with healthy subjects that reported muscle size changes is relatively small, and thus conclusions about this measure are necessarily tenuous (Adams et al., 2003). However, as these studies had a small sample size, interpretation of the data should carefully be considered presumably because of the potential of a Type II error.

Most of the unilateral lower limb suspension studies carried out to date have included magnetic resonance imaging (MRI)- or computed tomography (CT)-based measurements to assess muscle size. Adams et al. (1994) reported an 8% decrease in knee extensor muscle size after 16 d of unilateral lower limb suspension. Some have found ~7% loss in the thigh and leg after relatively longer-term (3 to 4 wk) unilateral lower limb suspension (Berg et al., 1991; Schulze et al., 2002), whereas a 5 wk-unilateral lower limb suspension protocol is reported to have induced a 14% decrease in the CSA of the knee extensor muscles (Ploutz-Snyder et al., 1995, 1996; Tesch et al., 1994). Hather et al. (1992) reported that the CSA of the thigh muscles (combined extensors and flexors) decreased by 12% after 6 wk of unilateral lower limb suspension. In that study, it

was found that knee extensor CSA decreased by 16%, whereas the decrement in the knee flexors was 7%.

It is noteworthy that the lower limb unweighting model may not completely be immobilized as the rectus femoris is a hip flexor as well as a knee extensor (Hather et al., 1992; Thom et al., 2001). In support of this phenomenon, Hortobágyi et al. (2000) has also suggested that the model of lower limb immobilization cannot fully be restricted potentially because unintentional isometric muscle tensing or reflexive postural adjustments with balance-seeking efforts must occur.

1.4.3 CROSS-SECTIONAL AREA OF MYOFIBERS

1.4.3.1 FIBER TYPE AND AREA

Table III (A and B) shows previous studies with respect to muscle morphology after lower limb unweighting. Hespel et al. (2001) reported that muscle-fiber CSA decreased up to 8% for Type I, 11% for Type IIa and 9% for IIb after 2 wk of cast immobilization, indicating that the changes in myofiber size can be detected at this relatively early time point. However, these results were not statistically significant. After 3 wk of cast immobilization, the CSA of myofibers from the vastus lateralis was decreased by 13% for Type I and by 10% for Types IIa and IIx (Hortobágyi et al., 2000). Veldhuizen et al. (1993) found that the CSA decrements of vastus lateralis muscle fibers were as follows: Type I, 26%; IIa, 13%; and IIb(x), 36% after 4 wk of immobilization. The aforementioned studies represent the only reports of muscle fiber size that were found in the

literature for healthy immobilized subjects (Hespel et al., 2001; Hortobágyi et al., 2000; Veldhuizen et al., 1993).

There is little information with respect to measurement of muscle fiber size after unilateral lower limb suspension. Adams et al. (1994) have shown no significant reduction in either fiber size or fiber type distribution after 16 d of unilateral lower limb suspension. Moreover, Berg et al. (1993) showed no changes in fiber size with no fiber type transformation after 4 wk of suspension. However, after 6 wk of the shoe version of unilateral lower limb suspension, the CSA of muscle fibers from the vastus lateralis was decreased by 12 and 15% for Type I and Type II fibers, respectively (Hather et al., 1992).

It has been reported that hindlimb suspended rats show a decreased percentage of Type I fibers (Roy et al., 1991; Talmadge, 2000), whereas several human studies have found no effect of unloading on percent fiber type and area after four to six weeks of lower limb unloading (Berg et al., 1993; Hather et al., 1992). Furthermore, Deschenes et al. (2002) recently found no statistically significant changes in fiber size or fiber type distribution of myofibers from the vastus lateralis after 14 d of knee brace-mediated immobilization. Although athletes who had been immobilized by cast following surgery have a marked increase in percentage of Type II fibers (Häggmark et al., 1986), this may simply be due to a detraining effect because rowers who retired from competition showed essentially the same response (Larsson and Ansved, 1985). Accordingly, it seems unlikely that four to six weeks of lower limb unloading induces the

alterations of muscle fiber type in humans compared to animals partially due to the different degree of protein turnover (Gibson et al., 1987).

1.4.4 MYOSIN HEAVY CHAIN CONTENT

Hortobagyi et al. (2000) examined changes in muscle fiber size and myosin heavy chain (MHC) gene expression after 3 weeks of knee immobilization. After immobilization, Type I MHC mRNA isoform was downregulated 30%, Type IIx MHC mRNA was almost 300% upregulated and the Type IIa MHC mRNA isoform was not altered. These reciprocal changes were correlated (r=0.66, Type I; r=-0.71, Type IIx) with changes in fiber size, suggesting a reasonable synchronization between gene expression and MHC phenotype. That the relationship between gene expression and ATPase-based fiber atrophy is only moderate is most likely due to a mismatch between the MHC mRNA and the protein distribution being augmented by the ATPase method (Anderson et al., 1997). Although previous studies have shown that percent fiber type area and MHC content in human skeletal muscle are highly correlated (Fry et al., 1994; Staron et al., 2000), others have reported high correlations between percent fiber type and MHC content (Adams et al., 1993; Berg et al., 1997; Santána Pereira et al., 1995).

Table III-A. Summary of previous studies with regard to muscle morphology after lower limb unweighting

References	Subjects	Unweighting model	Duration	Sites of measurement	Findings (% decrease)
Berg et al. (1991)	6 healthy males	ULLS	4 weeks	CSA of muscle	6.8%
Hather et al. (1992)	8 subjects (5 males and 3 females)	ULLS	6 weeks	CSA of knee extensors CSA of myofibers	16% Type I-12%, Type II-15%
Berg et al. (1993)	6 healthy males	ULLS	4 weeks	CSA of myofibers	Type I, IIa and IIx-No change No fiber type transformation
Veldhuizen et al. (1993)	8 healthy subjects	IM	4 weeks	CSA of QF CSA of myofibers	21% Type I-26%,Type IIa-13% Type IIx-36%
Adams et al. (1994)	10 healthy males	ULLS	16 days	CSA of QF CSA of myofibers	8% [Type I-10%, Type IIa-1% Type IIx-18.2%, Overall 9%] No significance and no fiber type transformation
Tesch et al. (1994)	7 healthy subjects (4 males and 3 females)	ULLS	5 weeks	CSA of QF	14%
Ploutz-Snyder et al. (1995)	7 untrained, healthy subjects (4 males and 3 females)	ULLS	5 weeks	CSA of QF	14%
Ploutz-Snyder et al. (1996)	7 untrained, healthy subjects (2 males and 5 females)	ULLS	5 weeks	CSA of QF	14%
Hortobagyi et al. (2000)	† 36 recreationally active	IM	3 weeks	CSA of myofibers	Type I-13%, Type IIa-10% Type IIx-10%

CSA: cross-sectional area, QF: Quadriceps femoris, IM: Immobilization, ULLS: Unilateral lower limb suspension. The number of subjects are based on actual assessment, but not for recruitment in the study. *Findings from control groups in the study. †The exact composition of males or females that were assessed were not reported in the study.

Table III-B. Summary of previous studies with regard to muscle morphology after lower limb unweighting

References	Subjects	Unweighting model	Duration	Sites of measurement	Findings (% decrease)
*Hespel et al. (2001)	†9 subjects for CSA of QF and 8 for CSA of myofibers, respectively	IM	2 weeks	CSA of QF CSA of myofibers	10% [Type I-8%, Type IIa-11% Type IIb-9%]-No significance No fiber type transformation
Thom et al. (2001)	8 healthy females	ULLS	10 days	CSA of QF	11.8%
Deschenes et al. (2002)	†8 untrained healthy subjects	IM	2 weeks	CSA of myofibers	[Type I-3.5%, Type IIa-5.2% Type IIb-5.9%]-No significance No fiber type transformation
*Schulze et al. (2002)	8 untrained healthy males	ULLS	3 weeks	CSA of QF	7%
Jones et al. (2004)	9 untrained healthy males	IM	2 weeks	Quadriceps lean mass	4.7%

CSA: cross-sectional area, QF: Quadriceps femoris, IM: Immobilization, ULLS: Unilateral lower limb suspension. The number of subjects are based on actual assessment, but not for the recruitment in the study. *Findings from control group in the study. †The exact composition of males or females to have been assessed are not shown in the study.

1.4.5 OVARIECTOMY

Fisher et al. (1998) examined the effects of ovariectomy and hindlimb unloading on skeletal muscle in female rats. In that study, females rats were randomly placed into intact control and ovariectomized control groups. Two weeks after ovariectomy, animals were further divided into intact 2-wk hindlimb unloaded and ovariectomized hindlimb unloaded groups. Eventually, the authors found a greater degree of atrophy after 2 weeks of hindlimb unloading in the soleus and plantaris muscles of ovariectomized animals compared to intact rats.

1.5 SEX DIFFERENCES IN MUSCLE MORPHOLOGY

1.5.1 LEG LEAN MASS

Although inter-individual variability cannot be excluded, previous studies have shown leg lean mass is greater in men than in women. Recently, Malavolti et al. (2003) have determined leg lean mass for 42 males and 68 females aged 21-82 years using DEXA. The authors reported an average of 9.1 and 6.5 (kg) for men and women, respectively. Furthermore, Roepstorff et al. (2002) have also shown leg lean mass as 10.4±0.2 kg for seven men (aged 25±1.0) and as 8.3±0.5 kg for seven women aged 26±1.0 with DEXA. Therefore, it is obvious that men have a greater leg mean mass compared to women, even with a small sample size.

1.5.2 CROSS-SECTIONAL AREA OF QUADRICEPS FEMORIS

Although MRI and CT have commonly been used to determine the area of the quadriceps femoris in humans, previous studies have shown sex differences in the cross-sectional area of the knee extensor muscle groups. Maughan et al. (1983) determined the cross-sectional area of knee extensor muscles at the mid thigh level for 25 males and 25 females using CT. In the study, males showed a larger area of the muscles (83.2±12.3 cm^2) compared to females (55.4±6.2 cm^2). Furthemore, Schantz et al. (1983) reported the muscle cross-sectional area in 11 males and 10 females with CT imaging. In that study, men showed a larger area of medial extensors and lateral extensors (32.9±1.0 and 55.4±2.2 cm^2, respectively) for men compared to women (24.1± 1.4 and 42.4±1.8 cm^2 for women, respectively). Therefore, it is likely that men have a greater area of the knee extensor muscle groups compared to women.

1.5.3 CROSS-SECTIONAL AREA OF MYOFIBERS

1.5.3.1 FIBER TYPE AND AREA

Sex-specific differences have been reported for fiber size in the vastus lateralis muscle. According to Oertel (1988), muscle fibers are small (12-18 μm diameter) shortly after birth and the Type I fibers are slightly larger than the Type II with no difference between males and females. However, fiber cross-sectional area in males tends to be larger than in females after puberty (Brooke and Engel, 1969; Edström and Nyström, 1969; Saltin et al., 1977; Simoneau et al., 1985; Essén-Gustavsson and Borges, 1986; Simoneau and Bouchard, 1989; Miller

et al., 1993). All three major fiber types (I, IIa and IIb) have been shown to be larger in the vastus lateralis muscle in men compared to women. For example, Simoneau and Bouchard (1989) found that the cross-sectional areas of Type I, IIa and IIb fibers from the vastus lateralis muscle of young men were 14%, 38% and 56% larger compared to young women. Similarly, Staron et al. (2000) reported that Type I, IIa and IIb fibers were approximately 19%, 59% and 66% larger for men compared to women. Furthermore, men showed a larger area of Type II fibers compared to Type I in the muscles (Brooke and Engel, 1969; Saltin et al., 1977; Simoneau et al., 1985; Simoneau and Bouchard, 1989). It has been reported that Type IIa cross-sectional area are larger than Type IIb for both men and women (Simoneau and Bouchard, 1989; Staron et al., 2000). In this regard, Staron et al. (2000) have represented the hierarchy of fiber sizes (from largest to smallest): IIa>IIb=I for men and I=IIa>IIb for women.

Although many studies have demonstrated sex-based differences with respect to fiber size, there are conflicting studies about differences in the overall proportion of fast and slow fibers in the vastus lateralis muscle between men and women. For instance, previous studies have reported a higher percentage of Type I fibers in women compared to men (Simoneau et al., 1985; Simoneau and Bouchard, 1989; Miller et al., 1993), a higher percentage of Type I fibers in men (Komi and Karlsson, 1978; Essén-Gustavsson and Borges, 1986), and no difference between men and women (Saltin et al., 1977; Nygaard, 1981). The reasons for the above conflicting findings may be due to a disparity in age,

hormonal profiles, physical activity level, sample size and methodology (Simoneau and Bouchard, 1989; Staron et al., 2000).

Many studies of human muscle have used small sample sizes and have histochemically delineated only two fiber types, I and II (e.g., Brooke and Engel, 1969; Edström and Ekblom, 1972; Gollnick et al., 1972; Johnson et al., 1973; Komi and Karlsson, 1978; Oertel, 1988; Willan et al., 1990; Miller et al., 1993). Other studies have combined data from both men and women (Gollnick et al., 1974; Edgerton et al., 1975). As there is a large amount of inter- and intra-individual variation in the percentages of fast and slow fibers in the vastus lateralis muscle, it has been suggested not only to use large biopsy samples but also to use large numbers of subjects. Even previous studies with relatively large numbers of subjects have reported no sex-specific differences in Type I and II. Thus, these findings suggest that men and women may be similar with regard to the fiber type distribution in the vastus lateralis muscle.

1.5.4 MYOSIN HEAVY CHAIN CONTENT

It is known that the MHC molecule, the actin-based protein associated with muscle fiber contraction, plays a predominant role in specifying skeletal muscle properties (Talmadge, 2000). In humans and cats, there are three MHC isoforms predominantly expressed in adult limb skeletal muscles (Talmadge, 2000). These isoforms, MHC I, MHC IIa and MHC IIx (Ennion et al., 1995; Smerdu et al., 1994; Talmadge et al., 1996), are found singularly in Type I, IIa and IIx (formerly known as IIb in humans) fibers, respectively. Hybrid fibers that

contain multiple MHC isoforms can exist. In humans and other mammals, the so-called Ic and IIc fibers are examples of hybrid fibers that contain MHC I and MHC IIa in various proportions (Staron and Pette, 1986). Proportions of the hybrid fibers in skeletal muscle are typically low, but their numbers may increase dramatically during times of MHC and fiber transformation (Talmadge et al. 1995; Talmadge et al. 1999).

Staron et al. (2000) examined the relative distribution of MHC content in 95 men (21.2±2.2 yr) and 55 women (21.5±2.4 yr). In that study, the relative content of the three MHC isoforms (MHC I, MHC IIa and MHC IIb) was proportional to the fiber type area. Moreover, the relative percentage of MHC I was significantly smaller and the relative percentage of MHC IIa significantly larger for the men compared to the woman. Likewise, in order from largest to smallest, the relative MHC content for the men was MHC IIa> MHC I> MHC IIb and for the woman was MHC I=MHC IIa>MHC IIb. Correlations between fiber type area and relative MHC percentage were all significant, including correlations for the men and women separately as well as together. These findings suggest that MHC content is associated with specific fiber type.

1.5.5 EFFECT OF SEX STEROID HORMONES

1.5.5.1 TESTOSTERONE

The evidence that a reduction in serum testosterone is associated with decreased fat-free mass is accumulating (Bhasin et al., 2001; Herbst and Bhasin, 2004). For example, previous studies have demonstrated that healthy,

hypogonadal men have lower fat-free mass and higher fat mass compared with those of age-matched eugonadal men (Katznelson et al., 1996; 1998). Mauras et al. (1998) have reported that experimental suppression of serum testosterone by administration of a gonadotropin-releasing hormone (GnRH) agonist analog in healthy young men is associated with a significant reduction in fat-free mass, an increase in fat mass, and decrease in fractional muscle protein synthesis. Thus, these findings suggest that the alterations in serum testosterone have an influence on body composition (Bhasin et al., 2001).

Previous studies have provided important clues to understand the mechanisms of androgen effects on skeletal muscle (Bolanowski and Nilsson, 2001; Bhasin et al., 2003; Ferrando et al., 2002). It is known that skeletal muscles are a syncytium of multinucleated cells or fibers embedded in a matrix of collagen and supplied by motor nerve. If muscle cells are injured, populations of pluripotent satellite cells residing along the muscle fiber differentiate into single nucleated myoblasts lacking myofibrils (Charge and Rudnicki, 2004). These myoblasts are capable of dividing and fusing with each other and existing myofibers to form multi-nucleated myotubes containing myofibrils and other organelles (Hill et al , 2003). In this regard, previous studies suggest that testosterone induces muscle fiber hypertrophy by acting at multiple steps in the pathways that regulate muscle protein synthesis and breakdown (Ferrando et al., 1998, 2003), and pluripotent stem cell commitment and differentiation (Singh et al., 2003). Testosterone administration is associated with a dose-dependent increase in the cross-sectional areas of both Type I and II muscle fibers in men

receiving supraphysiologic doses of testosterone enanthate but not the absolute number nor the relative proportion of these fibers (Sinha-Hikim et al., 2002). According to Herbst and Bhasin (2004), the myonuclear number also correlates significantly with the testosterone concentration and fiber cross-sectional area. Taken together, these findings suggest that the testosterone-induced increase in muscle volume is due to an increase in fusion of myoblasts to existing muscle fibers (Herbst and Bhasin, 2004).

1.5.5.2 ESTROGEN

Although the term estrogen refers to three structurally similar steroid hormones, 17-β-estradiol, estrone and estriol, 17-β-estradiol is the primary estrogen in humans and the one with the greatest estrogenic properties in muscle (Tiidus, 2000), and as such is studied in the majority of investigations (Bunt, 1990). It has been proposed that estrogen has an apparent protective effect on cardiac, smooth and possibly skeletal muscle in terms of damage and inflammation (Kendall and Eston, 2002). For example, the lower incidence of atherosclerosis and other cardiovascular diseases in pre-menopausal females compared with age-matched males is believed to be partially attributable to the protective effect of the female sex hormone estrogen (Stumpf et al., 1977; Bush et al., 1987; Barret-Connor and Bush, 1991; Chisholm, 1991; Tiidus, 2003).

Estrogen is believed to have a high antioxidant capacity and membrane stabilizing properties (Bär et al., 1988; Kendall and Eston, 2002; Shumate et al., 1979; Tiidus, 2003). Estrogen has also been considered to play a role in

preventing creatine kinase leakage (a marker of muscle protein damage) from skeletal muscles at rest (Bär et al., 1988; Shumate et al., 1979). Through all of these interrelated properties, it has been suggested that estrogen could play a role in reducing skeletal muscle damage. The processes involved in muscle damage have already been shown to be complex with many interactions between processes.

1.6 RATIONALE AND HYPOTHESES

Whether or not possible sex differences in muscle strength differentially influence the loss of strength consequent to immobilization has not been evaluated. Furthermore, there is currently no data that has evaluated whether sex attenuates immobilization-induced atrophy in humans. Therefore, The purpose of this study was to determine the effects of 14 d of unilateral leg immobilization on muscle function and morphology in men and women. It was hypothesized that, via a potentially protective effect of estrogen, the degree of immobilization-induced muscle atrophy at both the fiber and whole muscle level would be attenuated in women compared to men. It was also hypothesized that men would show greater decreases in muscle strength compared to women after 14 d of knee brace-mediated immobilization.

CHAPTER II

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Sex-based Differences in Skeletal Muscle Function and Morphology With Short-term Limb Immobilization

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Running head: Leg immobilization in men and women

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ABSTRACT

The purpose of this study was to determine the effects of short-term (14d) unilateral leg immobilization using a simple knee brace (60° flexion)/crutch-mediated model on muscle function and morphology in men (M, N=13) and women (W, N=14). Isometric and isokinetic (concentric SLOW, 0.52 rad•s^{-1} and FAST, 5.24 rad•s^{-1}) knee extensor peak torque was determined at three time points (PRE, DAY-2, and DAY-14). At the same time points, magnetic resonance imaging was used to measure the cross-sectional area of the quadriceps femoris and DEXA scanning was used to calculate leg lean mass. Muscle biopsies were taken from vastus lateralis at PRE and DAY-14 for myosin ATPase and myosin heavy chain analysis. Women showed greater decreases (PRE vs. DAY-14) compared to men in specific strength (N•cm^{-2}) for isometric (M=3.1±13.3, W=17.1±15.9%; p= 0.055, [mean±SD]) and concentric SLOW (M=4.7±11.3, W=16.6±18.4%; p<0.05) contractions. There were no immobilization-induced sex-specific differences in the decrease in quadriceps femoris cross-sectional area (M=5.7±5.0, W=5.9±5.2%) or leg lean mass (M=3.7±4.2, W=2.7±2.8%). There were no fiber type transformations, and the decrease in Type I (M=4.8±5.0, W=5.9±3.4%), IIa (M=7.9±9.9, W=8.8±8.0%) and IIx (M=10.7±10.8, W=10.8±12.1%) fiber areas was similar between sexes. These findings indicate that immobilization-induced loss of knee extensor muscle strength is greater in women compared to men in spite of a similar extent of atrophy at the myofiber and whole muscle levels after only 14d of unilateral leg immobilization. Furthermore, we have described an effective and safe method of knee

immobilization that results in significant reductions in quadriceps muscle strength and fiber size.

Key words: fiber type characteristics, muscle atrophy, muscle strength, neuromuscular adaptation, serum hormone

INTRODUCTION

Skeletal muscle atrophy occurs when muscle protein breakdown exceeds synthesis. Atrophy has been shown to occur as a consequence of aging, dennervation, disease, non weight-bearing activity, and spaceflight (1). From an experimental perspective, lower limb suspension (with a sling and/or shoe) and immobilization (via a cast or knee brace) have commonly been used as the models to qualitatively and quantitatively assess the progress of, and underlying mechanisms contributing to, skeletal muscle atrophy (1).

Previous studies have shown that voluntary knee extensor peak torque dramatically declines in healthy humans after varying durations (4-6 weeks) of lower limb unloading (8, 9, 14). Decrements in knee extensor peak torque were primarily attributed to reductions in muscle and muscle fiber cross-sectional area (8, 9, 14). Significantly decreased knee extensor peak torque in both isometric and isokinetic contraction is observed even with shorter-term (10-16d) unilateral leg unweighting interventions (2, 9, 13, 16, 19, 21). Some have concluded that the loss of muscle strength resulting from short-term muscle unloading is primarily due to reduced neural activation of myofibers (8, 14), given that the decrements in muscle strength following shorter-term lower limb unloading interventions were not associated with significant muscle morphological changes (13). This assumption however may be due to an inability to quantify statistically significant reductions in muscle mass in the short-term due to variability in quantifying muscle mass/fiber area that is compounded by small sample size, possibly leading to a type II error. In animal models, gene expression is

dramatically altered even after 24 hours of hindlimb suspension (35). Furthermore, quadriceps lean mass was significantly reduced by 4.7% following only two weeks of immobilization (21).

It has been shown that absolute knee extensor peak torque is greater in men than in women during isometric (27) and isokinetic contraction (23). However, some (27, 30), but not all (22, 23), have found no differences between males and females when data are expressed as specific strength ($N \bullet cm^{-2}$, voluntary knee extensor peak torque per unit muscle cross-sectional area). Whether or not these possible sex differences in muscle strength differentially influence the loss of strength consequent to immobilization has, to our knowledge, not been evaluated. One possible sex-difference is that women tend to have smaller fibers than men, particularly type II fiber sub-types (34). Furthermore, there appears to be an increase in oxidative stress in atrophying skeletal muscle fibers and 17-β-estradiol acts as an antioxidant and a membrane stabilizer in rats and humans (4, 32, 38). Estradiol can also attenuate creatine kinase appearance (an indicator of muscle membrane damage) from skeletal muscles at rest (4, 32). One study reported a greater degree of atrophy after 2 weeks of hindlimb unloading in the soleus and plantaris muscles of ovariectomized animals compared to intact female rats, implicating a protective effect from female sex hormones (15). There are currently no data regarding potential sex influences on immobilization-induced atrophy in humans.

The purpose of this study was to determine the effects of 14 d of unilateral leg immobilization on muscle function and morphology in men and women. It

was hypothesized that, via a potentially protective effect of estrogen, the degree of immobilization-induced muscle atrophy at both the fiber and whole muscle level would be attenuated in women compared to men. It was also hypothesized that men would show greater decreases in muscle strength compared to women after 14 d of knee brace-mediated immobilization.

METHODS

Subjects

Recreationally active men (N=13) and women (N=14) volunteered as subjects (Table 1). All subjects were screened based on a questionnaire to exclude those who were smokers, highly trained, injured within the previous six months, or taking regular medications. All female participants were eumenorrheic and half of the females were taking low dose estrogen containing birth control pills. This study was approved by McMaster University and the Research Ethics Board of the Hamilton Health Sciences and all subjects provided informed consent.

Experimental procedure

Subjects had one leg immobilized by random assignment using a standard knee brace (epX Knee Control Plus, Smith Orthopedics, Topeka, KS) with a cotton stockinette and were provided with walking crutches. The study period was composed of a total of three testing sessions called PRE (5 days before the immobilization), DAY-2 (48 hours after the onset of immobilization) and DAY-14

(14 days after the onset of immobilization). The PRE timepoint was chosen to be 5 days in advance of immobilization to allow for recovery from the biopsy before immobilization. The subjects were instructed to refrain from any exercise at least 3 days before the first testing session. Each testing session was identical and included dual X-ray absorptiometry (DEXA), magnetic resonance imaging (MRI), muscle strength, muscle biopsy and blood sampling, as described below. Body composition and cross-sectional area (CSA) of knee extensor muscles were completed using DEXA and MRI, respectively, on the night prior to the subsequent testing procedures. The muscle biopsy, blood sampling and strength testing were completed in the morning following an overnight fast, after a standardized nutritional beverage (500 ml; Boost®, Novartis Medical Health, Inc., NY) was consumed 2h prior to the muscle biopsies in order for each subject to be in a similar post-prandial state prior to each biopsy. Strength testing was carried out immediately after biopsies to avoid influencing biopsy sample results. Following the testing, a knee brace was applied with the angle of each knee brace locked at 120° (i.e., 60° from full extension) to provide complete immobilization and yet allow subjects' legs to swing freely while ambulating with crutches, without hitting the ground. A unique identifier was applied to a piece of commercially available standard tape wrapped around to the brace to ensure that the brace was not removed. Compliance and inspection of the immobilized leg and knee brace were monitored via a daily meeting with the subjects. In the presence of this supervision, subjects were allowed to take off their knee braces and stockinette and take a shower in the lab without bearing

weight on the treatment leg. The knee brace was then re-applied and new tape was applied. Subjects spent an overall time of 10-15 minutes with the brace removed.

Dual X-ray absoptiometry (DEXA)

Whole body lean mass, immobilized leg lean mass and body fat percentage were measured at all three time points using DEXA (model QDR-1000/W, Hologic, Waltham MA) as described previously (28). The same investigator carried out scanning for all the images.

Magnetic resonance imaging (MRI)

MRI was completed in a 1.5 Tesla scanner with superconducting magnets (Symphony Quantum, Siemens, Erlangen, Germany) to determine the CSA of the vastus area (vastus lateralis, vastus intermedius and vastus medialis) and rectus femoris. MRI was carried out using T1-weighted spin-echo sequences in the axial plane [repetition time/echo time (TR/TE) = 400/15; field of view (FOV) = range from 20 x 14 to 20 x 18 cm; matrix size = range from 180 x 256 to 232 x 256; slice thickness = 5 mm] and was taken at 70 percent of the distance from the top of the greater trochanter to the lateral joint space of the knee where a mark was maintained over the course of the study with indelible marker. Imaging was performed with subjects in the supine position with heels fixed on a nonmetallic support to control joint and scan angle.

MRI scans were transferred electronically from the scanner to a personal computer (Windows XP) with eFilm (Version 1.5.3 for Windows, Aycan Digitalsysteme GmbH, Wuerzburg, Germany) and analyzed with Image Pro Plus (V4.0 for Windows, Media Cybernetics, Silver Spring, MD) using manual planimetry. The immobilized leg of each subject was analyzed for CSA. As it was difficult to distinguish the fascial planes between vastus lateralis, vastus intermedius and vastus medialis in the images, those three muscle groups were combined as the "vastus area." The average CSA (cm^2) was determined for vastus and rectus femoris and subsequently summed for the total quadriceps femoris. The same investigator carried out all measurements on five separate occasions with a low coefficient of variation (<1% for vastus area, <1% for rectus femoris).

Blood sampling

At the three time points, blood samples were taken from an antecubital vein. The samples were immediately put into 10-ml nontreated tubes and allowed to clot for serum. After centrifugation, serum was stored at -80°C for subsequent hormonal assays.

Muscle biopsies

Muscle biopsies were obtained from the vastus lateralis muscle of the immobilized leg under local anesthesia (2% lidocaine) with a modified Bergström biopsy needle with manual suction. Incisions were made at the

randomly chosen proximal, distal or mid (15 cm proximal to lateral joint space) site of the vastus lateralis separated by at 3.5-5.0 cm. There were 7 days between biopsies from PRE to DAY-2 and 12 days from DAY-2 to DAY-14. Each sample was immediately dissected of fat and connective tissue. Subsequently, the sample was placed into optimum cutting temperature embedding medium (OCT Tissue-Tek, Sakura Finetek Inc., Torrance, CA), with the orientation of the fibers perpendicular to the horizontal plane, and was quickly frozen in isopentane cooled by liquid nitrogen, and stored at -80 °C until subsequent histochemical analysis (5).

Muscle strength testing

Muscle strength testing was conducted at each testing session (PRE, DAY-2 and DAY-14). Isometric and isokinetic (concentric at an angular velocity of 0.52 $rad \bullet s^{-1}$: Concentric-SLOW; concentric at an angular velocity of 5.24 $rad \bullet s^{-1}$: Concentric-FAST) knee extensor peak torques were determined for all subjects using a dynamometer (Biodex-System 3, Biodex Medical Systems Inc., New York) to evaluate the relative changes in maximal force generating capacity over the time course of immobilization. The order of testing for each mode was randomly assigned during each testing session. All subjects had familiarization sessions at least 2wk prior to testing baseline muscle strength measurements. In order to standardize the testing, subjects had their shoulders strapped to the chair with adjustments for the back and hips and performed each protocol in the sitting position (37). Each subject performed three repetitions (5 sec x 3) with 90

sec rest between the repetitions for the isometric contraction, with the subject's knee at an angle of 70°. For Concentric-SLOW and Concentric-FAST contractions, subjects carried out ten repetitions throughout the complete 65° range of motion, respectively. The subjects were given more than two min of recovery time between each exercise mode. All subjects were verbally encouraged to voluntarily produce their maximal forces. Furthermore, subjects were given visual feedback of their force production to encourage maximal effort during the exercise testing. Subsequently, the highest peak torque value was considered as the maximal value after recording for all repetitions. To determine the voluntary force generating capacity of the muscle, specific strength was calculated as voluntary peak force per unit CSA of total quadriceps femoris ($N \bullet m/(cm^2 \times m)$), as described previously (29, 30).

Histochemical analysis

Histochemical analyses were conducted as described by Brooke and Kaiser (12), with the following modifications. The OCT mounted muscle samples were serially sectioned to 10 μm thickness, and slides were preincubated at a pH value of 4.60 (in 50 mM potassium acetate, 17.5 mM calcium chloride) for 7 min. Slides were rinsed with distilled, deionized water (ddH_20) between each of the following steps. Slides were incubated in 3 mM ATP using an alkaline solution (75 mM glycine, 40.5 mM calcium chloride, 75 mM NaCl, 67.5 mM NaOH, pH 9.4) for 45 min at 37 °C with agitation at regular intervals. They were incubated

consecutively in 1% $CaCl_2$ and 2% $CoCl_2$ for 3 min, then incubated in 1% ammonium sulphide for 30 seconds at room temperature.

Sections were photographed at 20x magnification with a microscope (Olympus America Inc., Melville, NY) in conjunction with a SPOT digital camera (Model: SP401-115, SPOT Diagnostic Instruments Inc., MI). Based on the staining intensity at pH 4.60 after the enzymatic reaction, the three fiber types were classified as Type I (dark), IIa (light) and IIx (intermediate). Cross-sectional areas of the muscle fibers (μm^2) were determined using an image analysis program (Image Pro, V6.0, Media Cybernetics Inc., Silver Spring, MD). Total fiber numbers counted to distinguish the three fiber types and determine CSA were 294±73 for men and 317±77 for women at PRE and 320±67 for men and 311±92 for women at 14-DAY. For Type I, total circled fiber numbers averaged 126±29 for men and 146±12 for women at PRE and 129±24 for men and 130±42 for women at DAY-14. For Type IIa, 122±50 for men and 132±52 for women were used at PRE and 140±59 for men and 135±59 for women were taken into account at DAY-14. Finally, for Type IIx, 46±10 and 41±14 for men and women, respectively, at PRE and 48±6 and 46±7 were counted for men and women, respectively, at DAY-14.

Myosin heavy chain analysis

Mixed muscle myosin heavy chain (MHC) analysis was performed to examine the percentage of MHC content in a modification of previous studies (3, 34). The muscle samples (seven to eight serial sections) were cut at 20 µm using a

cryostat and placed into microcentrifuge tubes including 250 µl of chilled lysis buffer (10% glycerol (wt/vol), 5% 2-merocaptoethanol (vol/vol), and 2.3% sodium dodecyl sulphate (SDS) (wt/vol) in 62.5 mM Tris (hydroxymethol) aminomethane at pH 6.8). The samples were immediately placed into a 60°C water bath for 10 min and subsequently stored at –80 °C. Samples were mixed in a 1:1 ratio with 2x SDS-sample buffer and were boiled at 100°C for 2mins. 20µl of the lysed muscle extract was loaded onto a 8 cm x 7.3 cm x 1.5 mm SDS polyacrylamide gel consisting of a 4% stacking layer and an 8% separating layer, both containing 30% glycerol. To minimize variability, samples were loaded in duplicate and PRE and DAY-14 samples were loaded in adjacent lanes for each subject. Samples were stained with Coomassie Blue after electrophoresis overnight (17-18 h) at 10mA in an ice-packed Styrofoam cooler. The three MHC isoforms (Type I, IIa and IIx) were visually identified according to each isoform's unique molecular weight (IIx > IIa > I) being inversely proportional to the electrophoretic mobility pattern (IIx < IIa < I). The gels were then scanned using a laser densitometer and the relative staining intensity (number of arbitrary densitometric units) of each band was calculated. Finally, the intensity of each band was described as a percentage of the summed staining intensity for all three bands.

Serum hormone assays

Serum testosterone (free and total), estradiol, cortisol and progesterone concentrations were determined at three time points using commercially

available solid phase radioimmunioassay kits (TKTT1, TKE21 and TKPG1, respectively, Diagnostic Products Corporation, Los Angeles, CA). The intra-assay CVs were <3.5 and 1.4% for men and women, respectively, for free testosterone, <4.8 and 2.8% for men and women, respectively, for total testosterone, <3.1 and 2.3% for men and women, respectively, for estradiol, <2.0 and 2.3% for men and women, respectively, for cortisol and <2.2% for women for progesterone.

Statistical procedures

An independent Student's t-test was applied to assess significant sex-specific differences in age, body height and body mass index. A two-way (gender and time) analysis of variance (ANOVA) with mixed design was performed to test for significant differences in all dependent variables regarding body composition, muscle function, morphology and hormonal levels (for progesterone, one-way ANOVA) with a computerized statistical package (Statistica V5.1, Statsoft, Tulsa, OK). Pearson's product-moment correlations were computed to examine the relationships between the percent loss of muscle strength and the atrophy of quadriceps and fiber area by using StatView statistical software (V5.0.1, SAS Institute Inc., Cary, NC). The level of significance was set at $p \leq 0.05$. All data are presented as mean ± SD.

RESULTS

Physical and hormonal characteristics of the subjects

Table 1 shows the physical and hormonal features of the subjects over 14 days of unilateral leg immobilization. Men were significantly taller ($p<0.001$), and had greater body mass ($p<0.01$) than women, whereas percent body fat was significantly greater in women ($p<0.01$). The body mass and fat free mass were similar in both men and women over three time points. With respect to free testosterone and total testosterone, men showed a higher concentration compared to women over 14d immobilization ($p<0.001$, respectively), whereas no significant changes were found over the immobilization period in both men and women. Additionally, no changes were found in estradiol for both men and women over 14d immobilization, whereas there was a tendency for women to have a higher concentration of estradiol compared to men ($p=0.109$). Cortisol significantly decreased between PRE and DAY-14 in both men and women ($p<0.05$), whereas there was a trend for women to have higher cortisol values ($p=0.054$) over the immobilization period. Similar progesterone levels were found in women over the time course.

CSA of quadriceps

In terms of CSA of knee extensor muscle groups (cm^2), both men and women showed significant decreases between PRE and DAY-14 in vastus, rectus femoris and total quadriceps femoris ($p<0.001$, $p<0.05$, $p<0.001$, respectively; Figure 1A, 1B and 1C). Men showed a larger CSA of vastus ($p<0.001$; Figure 1A) and total quadriceps femoris ($p<0.001$; Figure 1C) compared to women, whereas there were no sex-based differences in CSA of rectus femoris ($p=0.149$; Figure

1B). The percent decreases in vastus, rectus femoris and total quadriceps femoris between PRE and DAY-14 for men and women were similar with 5.9±5.3%, 3.7±7.0% and 5.7±5.0% for men and 6.4±5.6%, 2.8±6.0% and 5.9±5.2%, respectively.

Leg lean mass

There were significant decreases between PRE and DAY-14 in leg lean mass (kg) in both men and women ($p<0.01$, Figure 2). Furthermore, men showed greater values of leg lean mass compared to women over the time course ($p<0.001$, Figure 2). The percent decreases between PRE and DAY-14 for men and women correspond to 3.7±4.2% and 2.7±2.8%, respectively.

Muscle strength

Both men and women showed significant decreases in absolute peak torque (N•m) between PRE and DAY-14 and DAY-2 and DAY-14 for isometric ($p<0.001$, $p<0.01$, respectively; Figure 3A). With respect to concentric-SLOW and concentric-FAST, there were significant decreases between PRE and DAY-2, PRE and DAY-14 and DAY-2 and DAY-14 in both men and women ($p<0.01$, $p<0.001$, $p<0.01$ for concentric-SLOW, $p<0.05$, $p<0.001$, $p<0.05$ for concentric-FAST, respectively; Figure 3B and 3C). Men showed significantly greater peak torque values (N•m) compared to women for isometric ($p<0.001$; Figure 3A), concentric-SLOW ($p<0.001$; Figure 3B) and concentric-FAST ($p<0.001$; Figure 3C) contractions, at all times.

When muscle strength was expressed as specific strength (N•m/(cm^2×m)), there was a trend for women to show a greater loss in isometric mode between PRE and DAY-14 compared to men (interaction; gender × time, p=0.055, Figure 3D). With respect to concentric-SLOW mode, women showed a significant loss between PRE and DAY-2 and PRE and DAY-14 ($p<0.01$, $p<0.001$, respectively) but men did not (Figure 3E). Both men and women showed significant decreases in the concentric-FAST mode between PRE and DAY-2 and PRE and DAY-14 ($p<0.05$, $p<0.001$, respectively; Figure 3F). The percent decreases in specific strength at isometric, concentric-SLOW and concentric-FAST modes between PRE and DAY-14 for men and women were; 3.1±13.3%, 4.7±11.3% and 9.2±18.8% for men, and 17.1±15.9%, 16.6±18.4% and 15.6±14.9% for women, respectively.

Histochemical analysis

Both men and women showed significant changes between PRE and 14-DAY in CSA of Type I, Type IIa and Type IIx fibers ($p<0.001$, $p<0.01$ and $p<0.01$, respectively; Figure 4A, 4B and 4C). Moreover, men showed significantly larger CSAs (μm^2) of Type IIa and IIx ($p<0.01$, $p<0.05$, respectively; Figure 4B and 4C), but not for Type I ($p=0.354$; Figure 4A) at all time points. In terms of fiber type distribution, no significant changes were found in either men (M) or women (W) for Type I (M: 45.2±10.6%, W:47.8±9.5% for PRE and M:44.1±9.0%, W: 48.3±7.5% for DAY-14, respectively), Type IIa (M:37.0±6.7%, W: 35.0±3.4% for PRE and M: 38.2±11.7%, W: 36.3±7.1% for DAY-14, respectively) and Type IIx (M: 17.8±8.3%, W: 17.2±10.2% for PRE and M: 17.7±7.7%, W: 15.4±9.4% for DAY-14, respectively)

during the immobilization. Men had a greater Type II (a+x) fiber area percentage compared to women ($p<0.05$, M: 72.8±3.3%, W: 68.6±4.6% for PRE and M: 71.8±2.8%, W: 67.6±4.4% for DAY-14, respectively), whereas women showed a higher Type I area percentage compared to men ($p<0.05$, M: 27.2±3.3%, W:31.4±4.6% for PRE and M: 28.2±2.8%, W: 32.4±4.4% for DAY-14, respectively).

The percent atrophy in CSA of Type I, IIa and IIx between PRE and DAY-14 for men and women corresponds to 4.8±5.0%, 7.9±9.9% and 10.7±10.8% for men and 5.9±3.4%, 8.8±8.0% and 10.8±12.1%, respectively.

Myosin heavy chain content

Both men and women showed a similar percent distribution of MHC I isoforms over 14 days of lower limb immobilization (M:41.1±11.9%, W: 46.9±11.4% for PRE and M: 38.1±10.4%, W: 44.7±9.7% for DAY-14, respectively), IIa (M: 40.1±6.9%, W: 37.5±8.9% for PRE and M: 43.9±14.0%, W: 40.7±9.6% for DAY-14, respectively) and IIx (M: 18.8±10.2%, W: 15.6±11.1 for PRE and M: 18.0±9.0%, W: 14.6±11.0% for DAY-14, respectively), with no sex-specific differences.

Correlation coefficients

Significant correlations were found between the percent loss of isometric and concentric-SLOW peak torque and the percent atrophy of quadriceps for men ($r=0.548$, $p\leq0.05$ for isometric; $r=0.734$, $p<0.01$ for concentric-SLOW). Furthermore, there was a significant correlation between percent loss of

isometric peak torque and Type IIx fibers for men ($r=0.727$, $p<0.05$). However, women did not show any correlation between the percent loss of muscle strength for any mode and the percent atrophy in CSA of quadriceps or fibers.

In terms of correlation coefficients between percent fiber type distribution and MHC content, men showed significant correlations between percent fiber type distribution for Type I, IIa and IIx and overall MHC content at PRE ($r=0.809$, 0.845, and 0.895, respectively, $p<0.01$) and DAY-14 ($r=0.889$, 0.956, and 0.897, respectively, $p<0.01$), and women for Type I and IIx only at PRE ($r=0.773$ and 0.796, respectively, $p<0.05$) and DAY-14 ($r=0.744$ and 0.845, respectively, $p<0.05$).

DISCUSSION

The novel finding in the present study was that the reduction in specific strength of the knee extensors (concentric-SLOW and isometric) was attenuated in men compared to women in spite of similar atrophy in CSA of total quadriceps femoris and myofibers (Type I, IIa and IIx). There were no fiber transformation and similar reductions in leg lean mass for both men and women following 14 d of knee brace-mediated leg immobilization.

There are historically two types of lower limb unweighting models considered to bring about changes in skeletal muscle. One model is regarded as lower limb immobilization via a cast or knee-brace, and another is unilateral lower limb suspension with a sling and/or shoe (1). In the present study, subjects did not have to remove the brace for sitting, sleeping, or other daily activities, in contrast to unilateral sling-suspension. The knee brace used in the

present study was light (~650 g total weight) and well tolerated. It is noteworthy that the unilateral lower limb suspension model may also alter blood flow in the treatment leg (10). For example, several studies with unilateral lower limb suspension have reported the incidence of deep venous thrombosis for a few subjects (8, 10, 16). In the present study, the degree of flexion during bracing (60° from full extension) did not occlude femoral or popliteal artery blood flow (assessed using Doppler ultrasound, results not shown). Thus, our lower limb immobilization model and protocol minimized the possibility of developing deep vein thrombosis, as opposed to the sling- suspension model in which the knee is more extensively flexed.

In association with the aforementioned aspects of our immobilization model, it has been reported that the lower limb unloading model may not be a model of complete immobilization since the limb is still able to move at the hip joint (18, 36). Hence, since the rectus femoris is a hip flexor as well as a knee extensor it is perhaps not surprising that this muscle showed less atrophy since it would likely have been active to a small degree in hip flexion (18, 36). Hortobágyi et al. (20) has also suggested that the model of lower limb immobilization cannot fully be restricted since unintentional isometric muscle tensing or reflexive postural adjustments with balance-seeking efforts must occur. In line with those data (18, 36), we had similar results in that the rectus femoris showed less atrophy compared to the vastus in both men and women. In spite of the preceeding arguments, we still observed significant morphological reductions in the CSA of both the vastus and rectus femoris as well as the total

quadriceps femoris as a whole in both men and women with short-term knee brace-mediated immobilization.

Our unweighting model was similar to what Deschenes et al. (13) has reported. In the present study, the angle of knee flexion from full extension was set at 60° from full extension and Deschenes et al. (13) set the angle at 70°. Interestingly, Deschenes et al. (13) did not find statistically significant changes in fiber size or fiber type distribution of myofibers from the vastus lateralis in a total of 8 subjects including males and females after 14 days of leg immobilization with a knee brace. However, Jones et al. (21) have recently shown significant reductions in quadriceps lean mass (patella to groin region) in 9 males following 14 days of lower limb casting. In addition, Hespel et al. (19) showed significant decreases up to ~10% in CSA of quadriceps by MRI (a total of 9 subjects composed of males and females) after 14 days of leg immobilization with a cast, whereas there were no significant decreases in CSA of any fiber type (a total of 8 subjects composed of males and females). The findings in the present study showed significant atrophy of CSA of total quadriceps femoris (a total of 27 subjects), myofibers (Type I, IIa and IIx, a total of 17 subjects, respectively), and reductions in leg lean mass (a total of 22 subjects) for men and women after 14d of immobilization.

Variables such as differences in hormonal profiles, physical activity levels, methodology and inter- and intra-individual variability may explain why some have not reported significant reductions in the CSA of myofibers (13, 19). Furthermore, the sample size of our study was 2-to-3 fold greater than many of

the other studies which will significantly reduce the risk of a Type II error. Subtle differences in study design could also explain why some have not found significant atrophy after 14d of immobilization. For example, in the study by Deschenes et al. (13), subjects removed their knee braces and performed range of motion exercises before retiring at night, whereas the subjects in our study were required to wear the brace while sleeping. Given the potent effect of physical activity on gene expression and protein synthesis (11), it is important to avoid weight-bearing and range of motion activity in an immobilization model.

It has been suggested that 17-β-estradiol functions as an antioxidant and a membrane stabilizer and protects against exercise-induced muscle damage in rats and humans (4, 32, 38). In addition to this, it has been proposed that 17-β-estradiol plays a role in preventing creatine kinase leakage from skeletal muscles at rest (4, 32). Moreover, Fisher et al. (15) have shown a greater atrophy in the soleus and plantaris muscles of ovariectomized rats compared to controls after 2 weeks of hindlimb unloading. In the present study, there was a tendency for women to show higher 17-β-estradiol concentration over the immobilization period. However, both men and women showed a similar extent of muscle atrophy at both the fiber and whole muscle level following 14 days of unilateral immobilization. Therefore, it seems unlikely that 17-β-estradiol had a preventive effect on skeletal muscle atrophy in humans, following 14 days of a unilateral leg immobilization.

Similarly to previous studies (7, 18), we observed no fiber type transitions for men or women in either histochemical assessments or in the relative

proportion of MHC determined in the same serial sections after short-term immobilization. In accordance with previous data (8), the results of the present study suggest that a fiber type-specific atrophy is unlikely to alter the relative CSA occupied by a certain fiber type, with unaltered MHC proportions across myofibers over the time course of immobilization. According to Berg et al. (8), specific MHC contents are influenced by not only fiber type but also the relative fiber size. To sum up, it appears unlikely that fiber type transformations or qualitative changes in MHC proportion in knee extensor muscles occurs for men and women in response to 14 days of lower limb immobilization. However, advanced fiber type classifications in conjunction with histochemical and MHC analyses may reveal the more subtle regulatory pathways and mechanisms that affect the multiple genes which are associated with different modules of fiber type function (33).

In terms of the functional origin of strength loss in the neuromuscular system, previous studies have shown that changes of motor cortical area size occur without spinal excitability or motor threshold during immobilization, although the reduction in area could be quickly reversed by voluntary muscle contraction after immobilization (24, 39). It has been shown that short-term (10-16 days) as well as relatively long-term lower limb unloading (4-6 weeks) causes a reduction in the ability to voluntarily activate a muscle, which indicates that the recruitment of motor units and firing frequencies were altered (9, 14, 20). In association with these phenomena, previous studies have demonstrated that the

loss of voluntary muscle strength is greater than that of muscle CSA as a consequence of lower limb unloading (6, 14, 18).

In contrast to the research showing a clear role for neural factors mediating a proportion of the early immobilization-induced strength loss, there are conflicting results regarding whether or not sex-specific differences exist in baseline (i.e. non-immobilized) values with minimal evidence to support a sex-difference during immobilization. For instance, several studies reported sex-specific differences in knee extensor peak torque (22, 23), whereas some studies have shown that there were no sex differences in knee extensor isometric (27) or isokinetic peak torque (30). One reason for the disparity in those studies may in part be due to the different calculations of specific strength (30). For example, some have used leg length to determine $N \bullet cm^{-2}$ (23), whereas other group made calculations using body height (m) (30). Although our calculations were also based upon body height (30), we found that women showed greater losses of specific strength (concentric-SLOW and isometric) between PRE and DAY-14 compared to men. To our knowledge, there is only one study that has looked at possible sex-differences in neural recruitment issues during immobilization (31). This group found that women displayed an intermittent electromyogram recruitment pattern during post-immobilization (4 weeks) submaximal contractions that was not apparent before immobilization or after recovery or at all in men (31). Taken together with the data from the current study, it appears that there are minimal to no sex-based differences in specific strength or neural activation patterns at baseline, but that women display an immobilization-

induced loss of specific strength that is related more to neural vs. atrophy-based factors.

Different fiber type characteristics between men and women and the contractile properties of the fiber types have been suggested as a potential reason for sex-related discrepancies of muscle strength (17, 25). Our findings demonstrated that men showed a greater percentage of Type II fiber (fast-oxidative-glycolytic) area in the vastus lateralis compared to women, and women had a higher percentage of Type I fiber (slow-oxidative) area compared to men that was maintained over the immobilization period. Furthermore, a similar fiber type percentage (Type I, IIa and IIx) was found in both men and women throughout the immobilization protocol. Our PRE value findings were consistent with another large study evaluating sex differences in both fiber type distribution and fiber area percentage (34). Some (37) but not all (26) studies have shown that Type II fibers may be able to generate a high force, particularly at higher angular velocities (37). Interestingly, in the present study, strength loss in isometric and concentric-SLOW was highly correlated with the atrophy in CSA of the entire quadriceps muscles for men, but not for women. Moreover, even when men had a higher Type II area percentage compared to women, a similar loss in concentric-FAST specific strength was found for both men and women over the 14 d of immobilization. Therefore, sex-related differences in specific loss strength following immobilization cannot fully be explained by sex-specific differences in fiber type composition.

In conclusion, the findings from the present study indicate that immobilization-induced loss of specific strength at isometric and slower angular velocity concentric contractions are attenuated in men compared to women. This sex-specific voluntary strength loss occurred in spite of a similar amount of muscle atrophy at the fiber and whole muscle level, with no fiber type transitions following only 14 d of unilateral lower leg immobilization. Overall, these findings indicate that the immobilization-induced specific strength loss for women has a proportionately greater neural activation component as compared to that observed in men. Future studies will be required to explore whether this sex-based difference is occurring at the level of central recruitment, neuromuscular transmission, or excitation-contraction coupling.

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FIGURE LEGENDS

Figure 1.

Cross-sectional area (CSA, cm^2) of vastus (A), rectus femoris (B), and total quadriceps femoris (C) for men (N=13) and women (N=14) before (PRE) and after 2 wk (DAY-14) of unilateral leg immobilization. Values are mean ± SD. Main effects for gender (*) and time (†)($p<0.05$). No interaction (gender × time) found in CSA of vastus, rectus femoris and total quadriceps femoris.

Figure 2.

Leg lean mass (kg) for men (N=11) and women (N=11) before (PRE) and after 2 wk (DAY-14) of unilateral leg immobilization. Values are mean ± SD. Main effects for both gender (*) and time (†)($p<0.01$, respectively). No interaction (gender × time) found.

Figure 3.

Isometric peak torque (A), concentric peak torque (B) at a velocity of 0.52 $rad \bullet sec^{-1}$ (Con-SLOW) and concentric peak torque (C) at a velocity of 5.24 $rad \bullet sec^{-1}$ (Con-FAST). Specific strength ($N \bullet cm^{-2}$) in isometric peak torque (D), concentric peak torque (E) at a velocity of 0.52 $rad \bullet sec^{-1}$ (Con-SLOW) and concentric peak torque (F) at a velocity of 5.24 $rad \bullet sec^{-1}$ (Con-FAST) at PRE, DAY-2 and DAY-14. Values are mean ± SD (N=13 men and 14 women). Main effects for gender (*) and time (†)($p<0.05$). ‡ Significant loss between PRE and

DAY-2 and PRE and DAY-14 in women compared to men. No main effect for gender and no interaction (gender × time) found in specific strength at isometric (a trend for interaction, p=0.055) and Con-FAST.

Figure 4.

Mean fiber area (μm^2) in Type I (A), Type IIa (B) and Type IIx (C) fibers for men (N=9) and women (N=8) before (PRE) and after 2 wk (DAY-14) of unilateral leg immobilization. Values are mean ± SD. Main effects for gender (*) and time (†) ($p<0.05$). No interaction (gender × time) found in either Type I, Type IIa or Type IIx.

Table 1. Physical and hormonal characteristics of subjects

Characteristic		Men (n = 13)	Women (n = 14)	
Age (yr)		20.8±1.9	21.3±2.6	
Height (cm)		178.9±8.2	164.9±5.4*	
Body mass index		24.5±3.5	22.8±2.1	
Body mass (kg)	PRE	78.8±14.3	62.3±8.7	†
	DAY-2	79.4±14.2	62.5±8.9	
	DAY-14	78.9±14.0	62.4±9.1	
Body fat (%)	PRE	17.5±4.2	25.7±4.4	†
	DAY-2	18.4±3.7	26.4±4.1	
	DAY-14	18.0±4.2	26.0±4.2	
Lean body mass (kg)	PRE	64.1±10.7	45.2±5.0	†
	DAY-2	63.9±10.5	45.0±5.2	
	DAY-14	63.7±10.0	45.1±5.6	
Free Testosterone ($pmol \cdot l^{-1}$)	PRE	51.3±10.2	3.1±1.6	†
	DAY-2	48.6±10.6	3.3±1.8	
	DAY-14	50.2±11.8	2.8±1.6	
Total Testosterone ($nmol \cdot l^{-1}$)	PRE	14.3±2.5	0.8±0.3	†
	DAY-2	14.1±3.0	0.7±0.4	
	DAY-14	14.2±3.9	0.7±0.3	
Estradiol ($pmol \cdot l^{-1}$)	PRE	105.1±22.4	161.7±172.6	
	DAY-2	104.4±30.6	163.5±140.8	
	DAY-14	103.9±30.5	153.7±135.5	
Progesterone ($nmol \cdot l^{-1}$)	PRE	n/a	5.5±8.8	
	DAY-2	n/a	6.5±10.0	
	DAY-14	n/a	8.3±14.4	
Cortisol ($nmol \cdot l^{-1}$)	PRE	585.8±162.2	705.2±208.9	‡
	DAY-2	502.2±175.6	643.8±136.0	
	DAY-14	457.5±128.6	638.8±214.7	

Body mass index was calculated based on baseline values (PRE). All values are mean±SD. For free and total testosterone, 13 men and 13 women, and for cortisol 12 men and 11 women were assessed. *Significantly different from men ($p<0.05$). †Main effect for gender ($p<0.05$). ‡Main effect for time ($p<0.05$).

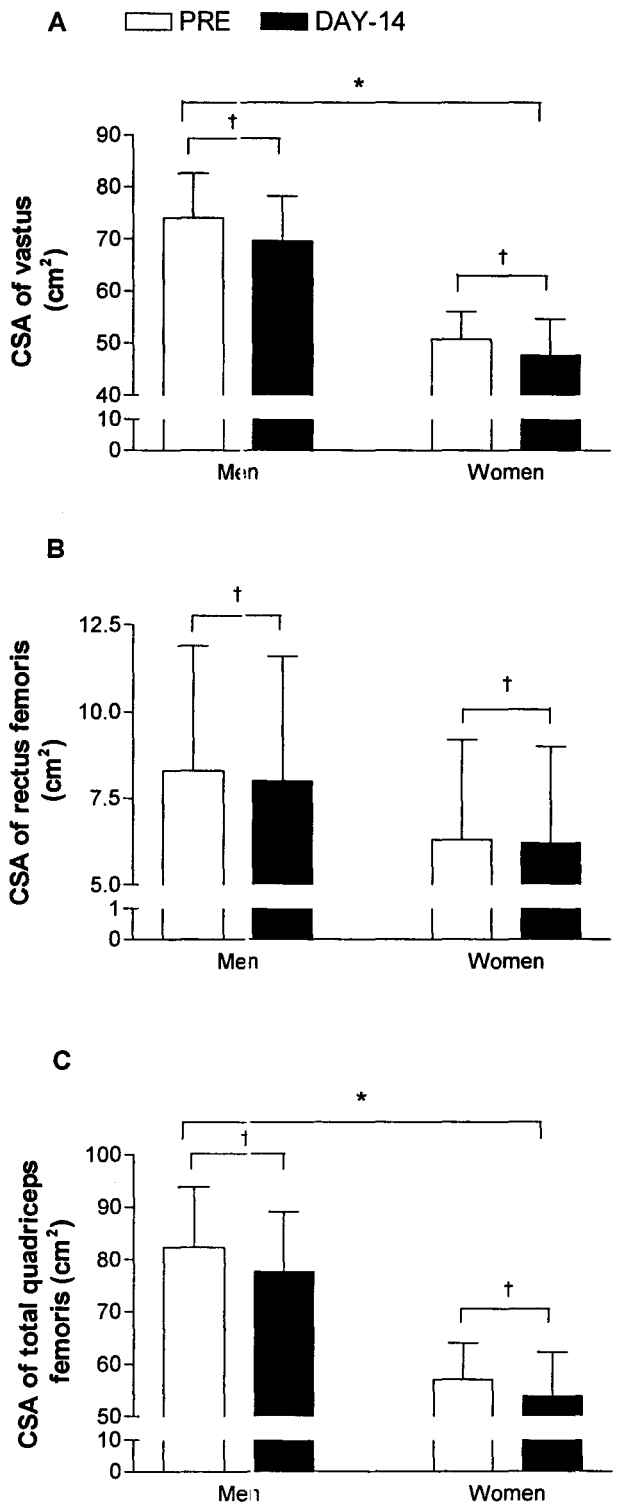


Figure 1A, 1B, and 1C.

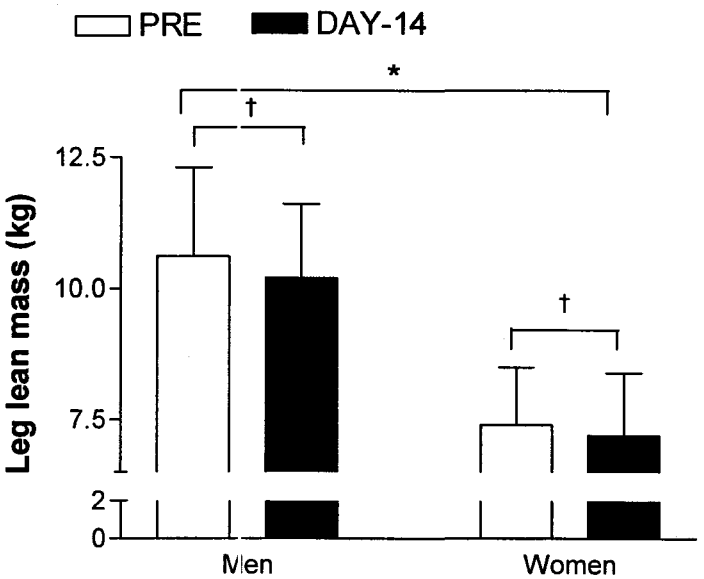


Figure 2.

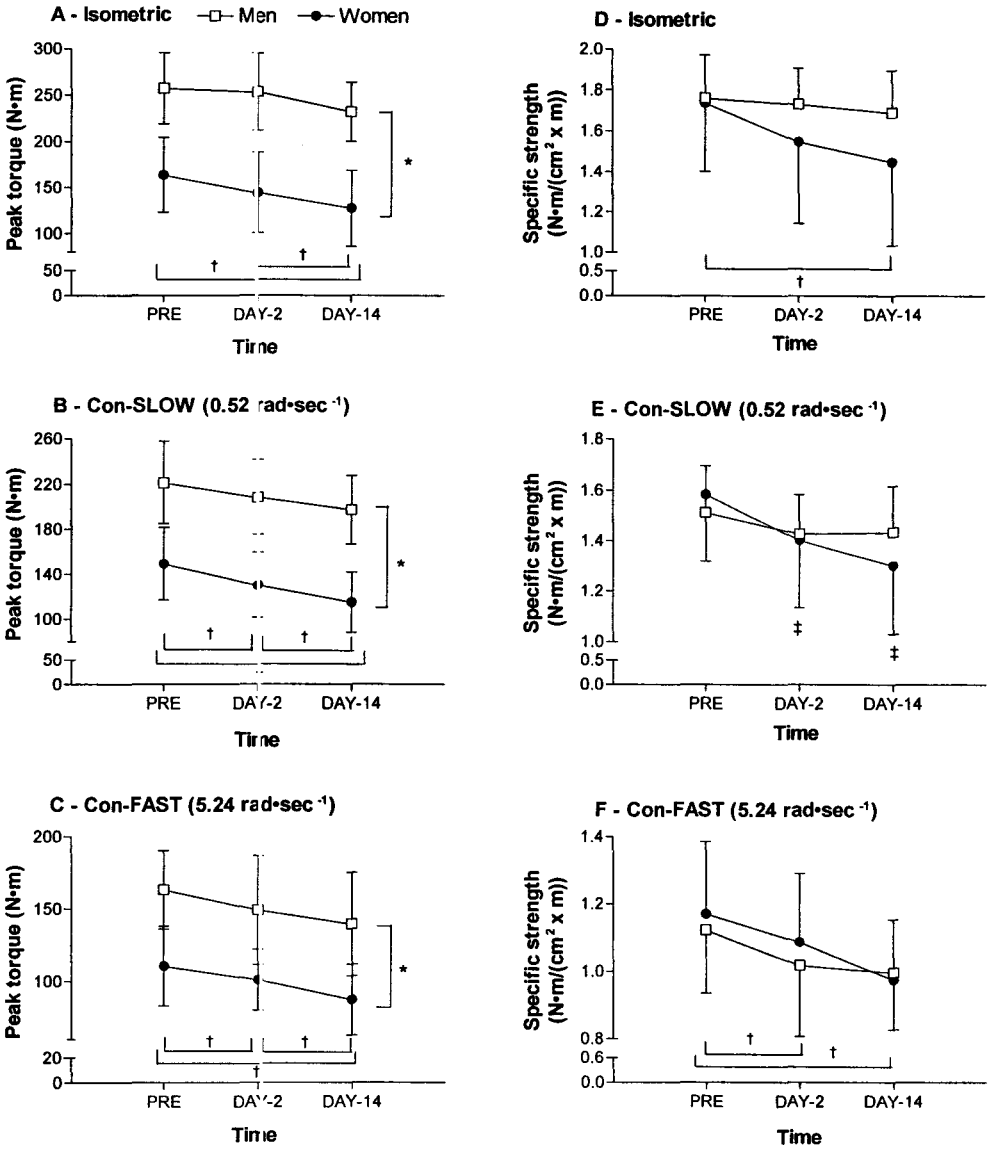
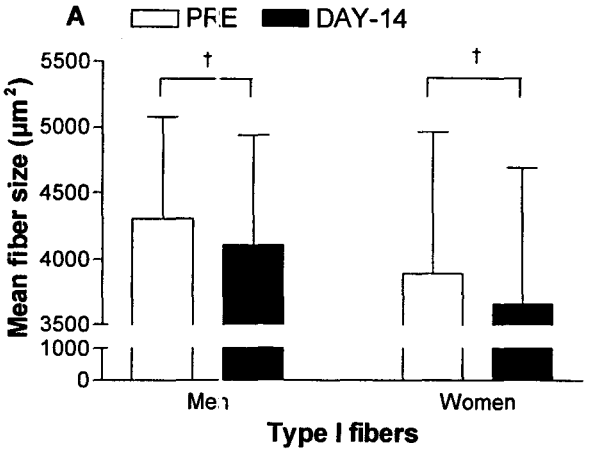
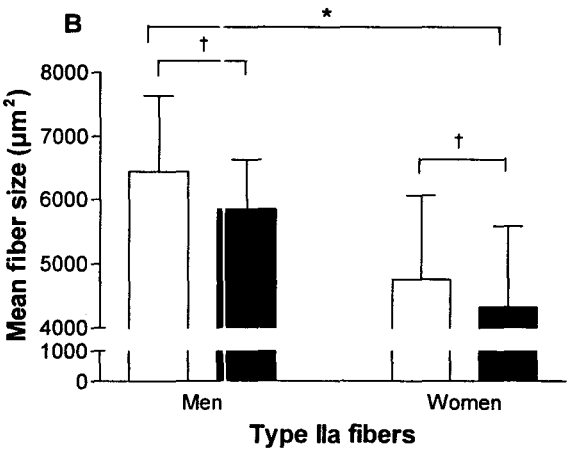


Figure 3A, 3B, 3C, 3D, 3E, and 3F.





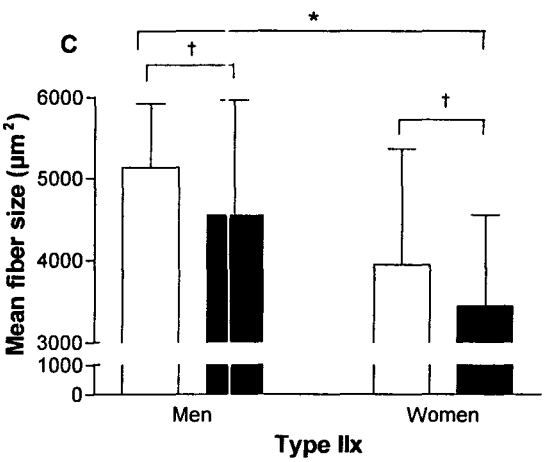


Figure 4A, 4B and 4C.

OVERALL SUMMARY

The findings from the present study indicate that immobilization-induced loss of specific strength at isometric and slower angular velocity concentric contractions are attenuated in men compared to women. This sex-based voluntary strength loss occurred in spite of a similar amount of muscle atrophy at the fiber and whole muscle level, with no fiber type transformation after only 14 d of unilateral lower leg immobilization. The data from the present study may be useful in designing future strategies to combat muscle wasting (i.e. atrophy) in clinical situations such as bed rest, injury and/or sarcopenia of aging. Furthermore, the present study highlighted sex-specific responses to lower limb immobilization, which is very important for designing sex-specific strategies to combat muscle wasting or therapies (such as estrogen analogues).

There are mainly three types of ground-based models considered to bring about changes in skeletal muscle: 1) bed rest, 2) limb immobilization via a cast or knee brace, and 3) unilateral lower limb suspension (Adams et al. 2003). Particularly, in the unilateral lower limb suspension model originally developed by Berg et al. (1991), subjects use crutches for support while one leg is unloaded from weight bearing. This model has been used extensively to study neuromuscular and muscle adaptations to unweighting. Recently, Deschenes et al. (2002) have used a modified version of this model for "knee brace-mediated immobilization."

However, there are a couple of limitations in unweighting models such as unilateral lower limb suspension and cast/knee brace immobilization. For

example, it is inconvenient to complete activities of daily living while employing those models. Especially, these models increase the risk of falls, as body movement is relatively restricted compared to bed rest. Moreover, the biggest limitation and concern in these models is the incidence of deep venous thrombosis as most studies have reported deep venous thrombosis, particularly in a sling suspension model with the knee extensively flexed (Berg and Tesch, 1996; Bleeker et al. 2004a, 2004b; Gamrin et al. 1998). During unilateral lower limb suspension, it is possible for gravity to lead to pooling of blood in the legs and this may affect DVT susceptibility with restricted knee angle (Bleeker et al. 2004a, 2004b). Therefore, it has been proposed that precautionary measures should be taken into consideration to prevent thromboembolism (Bleeker et al. 2004a, 2004b).

In the present study, a knee brace/crutch-mediated immobilization model was used and this model was similar to what Deschenes et al. (2002) have reported. For instance, the angle of knee flexion from full extension was set at 60° from full extension in the present study and Deschenes et al. (2002) set the angle at 70°. The knee brace used in the present study was light (~650 g total weight) and well tolerated (i.e., not too hot, does not rub or cause irritation or itching). Furthermore, subjects did not have to remove the brace for sitting, sleeping, or other daily activities, in contrast to unilateral sling-suspension. The degree of flexion during bracing (60° from full extension) did not occlude femoral or popliteal artery blood flow (assessed using Doppler ultrasound, results not shown). Thus, the incidence of developing deep vein thrombosis was

minimized in our lower limb immobilization model and protocol, as compared to the sling- suspension model in which the knee is more extensively flexed.

In light of these aspects of the immobilization model, the model used in the present study was very effective at all functional and morphological levels, as shown in the findings of a decrease in absolute muscle strength (N•m) and CSA at the whole and myofiber levels in both men and women. Furthermore, the findings of the present study were in a range comparable to the results of previous studies (Figure 5, 6, 7 and 8). However, women showed a greater loss of specific strength in isometric and concentric-SLOW modes compared to men after 14 d of immobilization, probably because neural factors largely contribute to these sex-specific differences (Semmler et al. 1999).

In this study, it was hypothesized that the degree of immobilization-induced muscle atrophy, at both the fiber and whole muscle level, would be attenuated in women compared to men after 14d of immobilization. However, a similar amount of muscle atrophy was found between men and women for CSA of myofiber and total quadriceps femoris and leg lean mass. Although it has been suggested that 17-β-estradiol functions as an antioxidant and a membrane stabilizer in rats and humans (Bär et al. 1988; Shumate et al. 1979; Tiidus, 2003) and plays a role in preventing creatine kinase leakage from skeletal muscles at rest (Bär et al. 1988; Shumate et al. 1979), the results of the present study indicate that 17-β-estradiol may not have a protective effect on muscle atrophy.

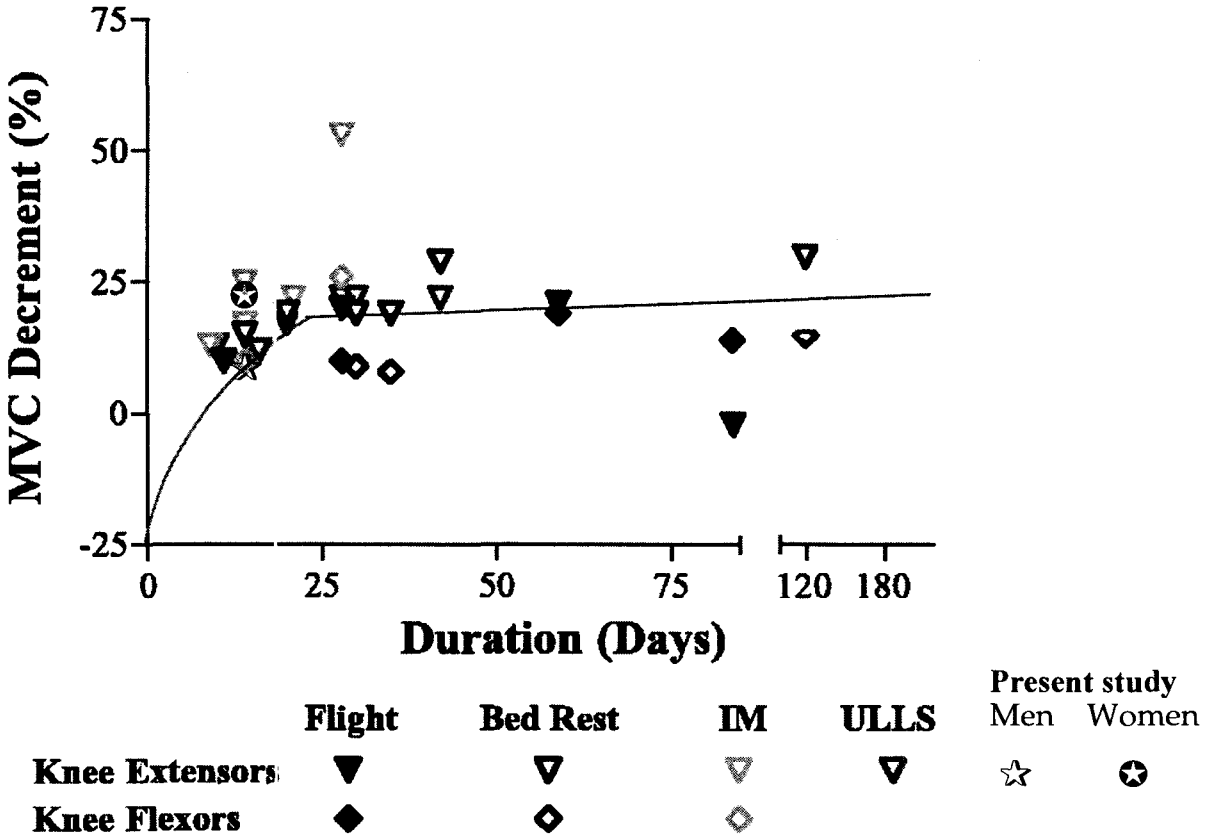


Figure 5. Comparison of previous studies with the present study regarding decrease in maximum voluntary contraction with different kinds of unweighting models. Modified from Adams et al. (*J Appl Physiol* 95: 2185-2201, 2003). MVC: Maximum voluntary contraction, IM: Immobilization, ULLS: Unilateral lower limb suspension. Projected mean trend line inserted.

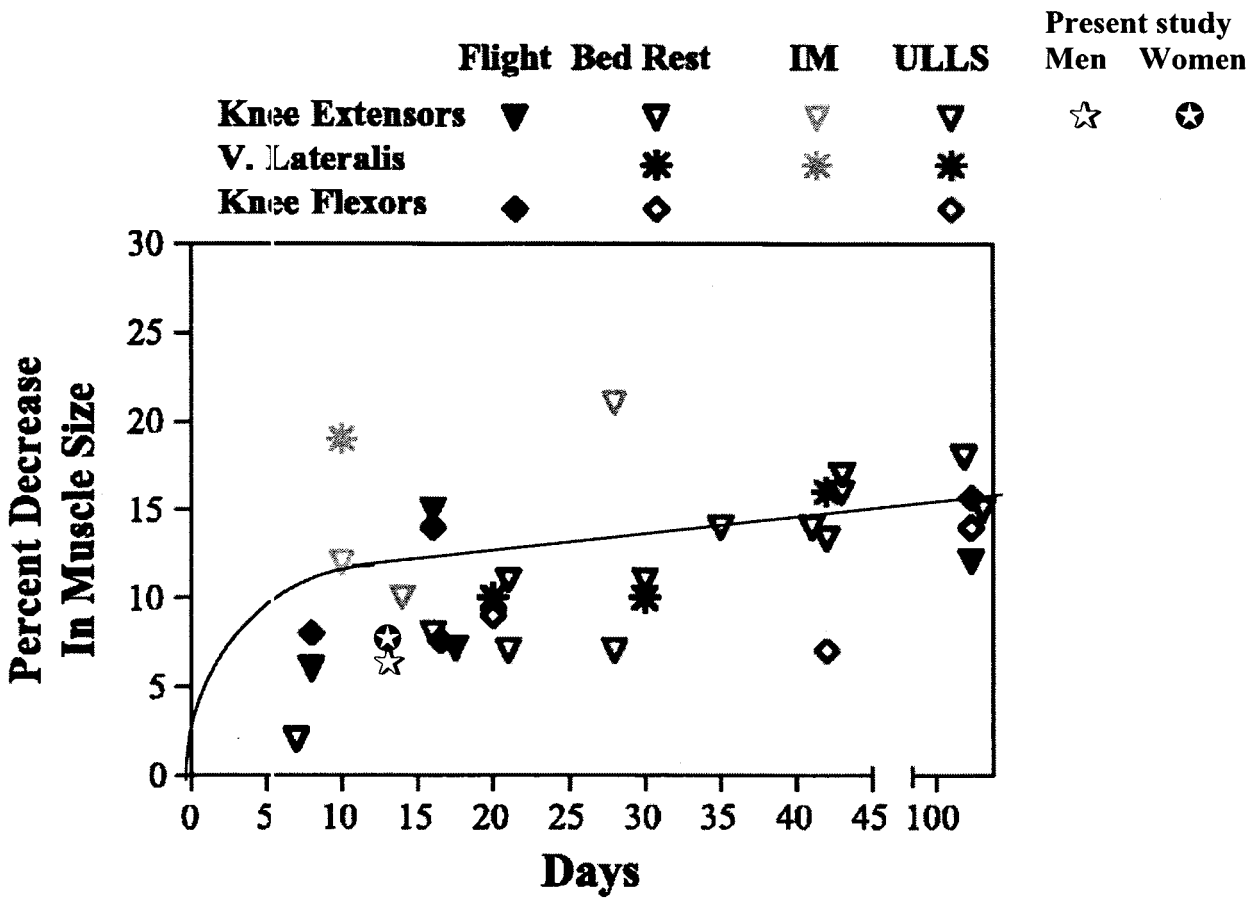


Figure 6. Comparison of previous studies with the present study regarding percent decrease in whole CSA of the muscle in various kinds of unweighting models. Modified from Adams et al. (*J Appl Physiol* 95: 2185-2201, 2003). IM: Immobilization, ULLS: Unilateral lower limb suspension. The line shows theoretical trend for atrophy.

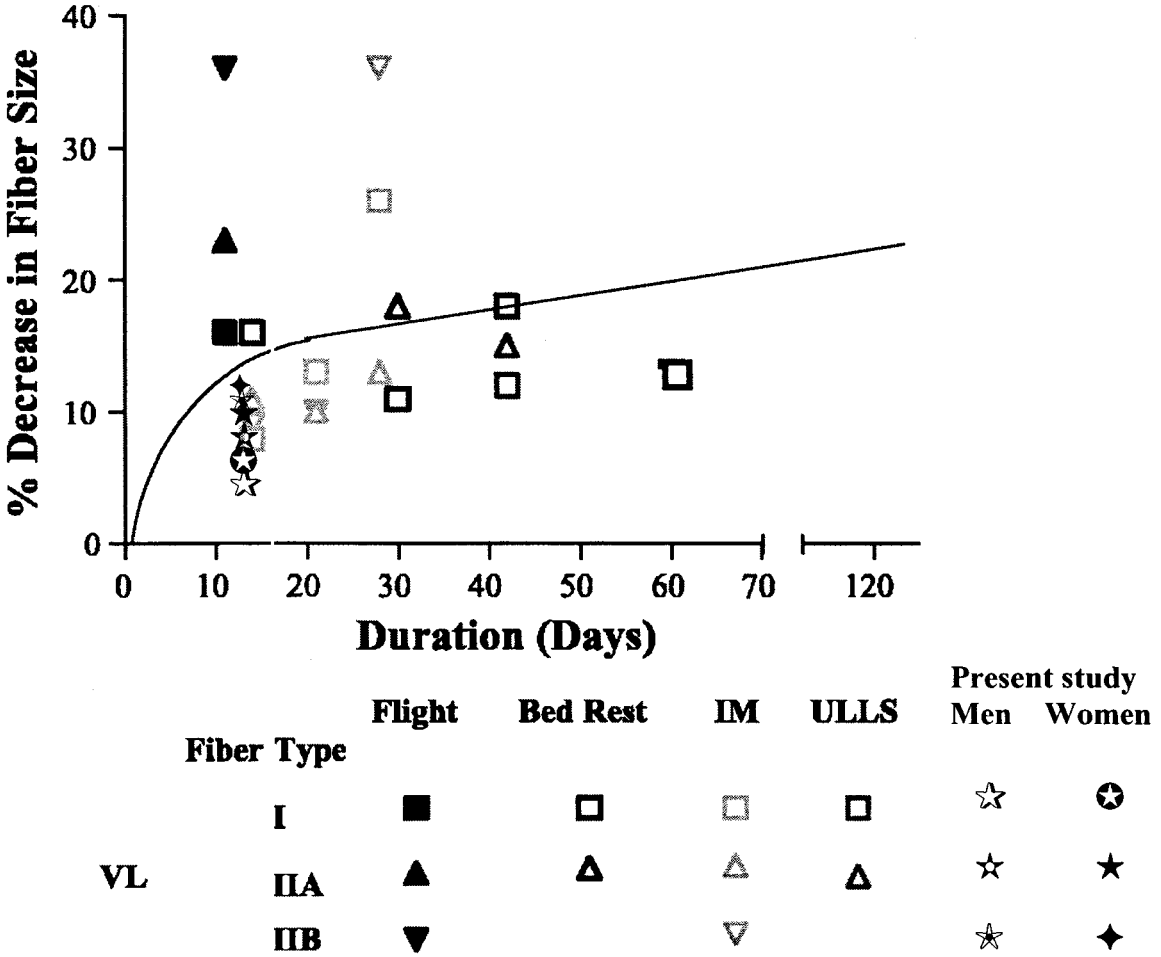


Figure 7. Comparison of pervious studies with the present study regarding percent decrease in CSA of myofibers in various kinds of unweighting models. Modified from Adams et al. (*J Appl Physiol* 95: 2185-2201, 2003). VL: Vastus lateralis, IM: Immobilization, ULLS: Unilateral lower limb suspension. The line shows theoretical trend for atrophy.

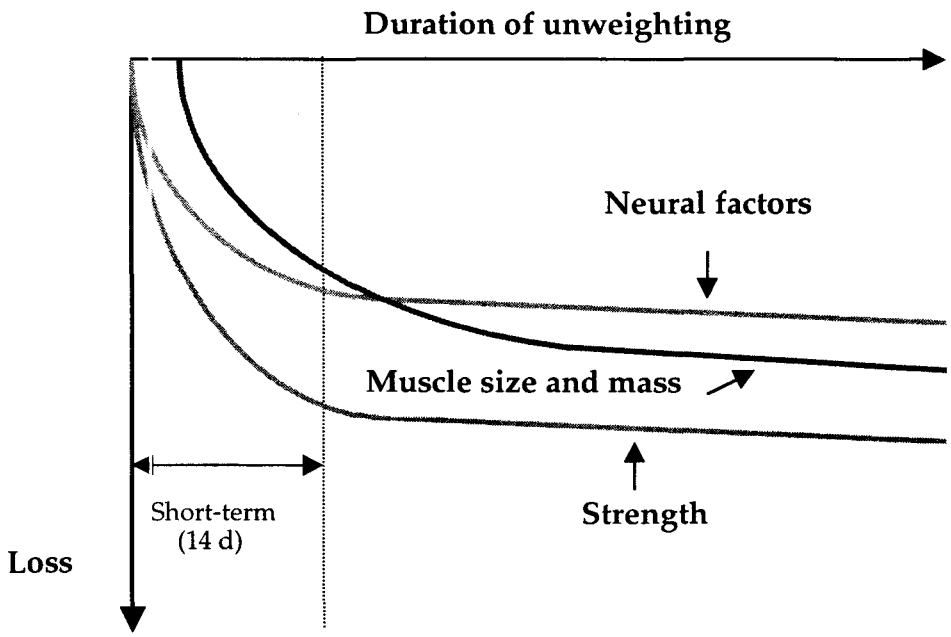


Figure 8. Predicted time course of factors in neural and muscular reductions to immobilization. Modified from Sale (*Med Sci Sports Exerc*, 20: S135-S145, 1988).

FUTURE DIRECTIONS

Semmler et al. (1999) has examined whether sex-differences exist in neural recruitment during 4 wk of immobilization. The authors found that women displayed an intermittent electromyogram recruitment pattern in submaximal contractions after 4 wk of immobilization, which was not apparent before immobilization or after recovery (Semmler et al. 1999). In line with the data, in the present study, women showed immobilization-induced greater losses of specific strength (relative units expressed as $N \bullet m/(cm^2 \times m)$) at isometric and concentric-SLOW that was not correlated with any measure of muscle mass. These results suggests that a proportionately greater attenuation of the neural activation component occurred in women as compared to that observed in men in response to immobilization. However, it still remains to be elucidated whether sex-specific differences exist in central (motor drive) and peripheral (loss of motor units) changes, taking into consideration underlying mechanisms that are distal to the neuromuscular junction, such as excitation-contraction coupling, and some that are located within the central nervous system, such as a decline in cortical output and an increase in the inhibitory effects of sensory feedback. Therefore, the assessment of electromyography and percent motor unit activation will be required to clarify immobilization-induced sex-based differences, in light of specific strength (maximum voluntary force per unit CSA of the muscle) and specific tension ("true" force per unit CSA of the muscle).

Adaptations to strength training are most often specific to the movement pattern, velocity, contraction type, and contraction force during training (Sale

and MacDougall, 1981; Behm and Sale, 1993). Secondary neural adaptation observed with strength training is the increase in strength commonly seen in an untrained limb after a period of training with the contralateral limb (Hortobágyi et al., 1997; Moritani and deVries, 1979). This neural adaptation is referred as "cross-education" (Moritani and deVries, 1979). Taking into account this phenomenon, the findings of the present study showed that men showed a gradual increase in the non-immobilized leg in isometric and concentric-SLOW peak torque compared to women over the time course of immobilization, whereas there were no changes in women over the immobilization period (Figure 9A, 9B, and 9C). Although CSA of total quadriceps femoris in the non-immobilized leg (a total of 8 subjects composed of 6 men and 2 women - data not shown) were determined using MRI, muscle hypertrophy was not found only after 14 days as it may be too short to induce hypertrophy. Therefore, the sex-specific difference in the non-immobilized leg may in part be accounted for due to neural factors with a different degree of the cross education effect. However, the reason why such a sex-specific difference occurred over the time course still remains to be clarified. Therefore, in non-treatment leg, a relatively longer-term unweighting model will be required to examine the changes at the functional and morphological levels.

Previous studies have observed a difference in various biomarkers of oxidative stress between men and women. However, the observed differences do not provide a clear indication as to whether oxidative stress is higher in men or women (Ide et al. 2002; Lacy et al. 2000; Trevisan et al. 2001). Furthermore, the

findings in the present study showed similar muscle atrophy in both men and women after only short-term lower limb immobilization in spite of the hypothesis that 17-β-estradiol may have a protective effect on muscle atrophy. Therefore, oxidative stress with immobilization may be on epiphenomenon. In future studies, careful evaluation of whether sex influences muscle atrophy should be considered to confirm the relationship between muscle atrophy and oxidative stress with a longer period of immobilization intervention.

In conjunction with the aforementioned considerations, the molecular analyses may also deeply clarify the overall regulatory mechanisms of muscle atrophy between men and women (i.e., microarray analyses of expressed mRNAs during atrophy) (Jackman and Kandarian, 2004). Although progress has been made during the last several years in gene expression, there is currently no sex-comparative data. Furthermore, the mechanisms underlying changes in protein turnover with immobilization (Gibson et al. 1987) need to be further investigated, with special attention to potential sex-specific differences. Thus, further research from functional to molecular levels is needed to elucidate sex-specific mechanisms of muscle wasting.

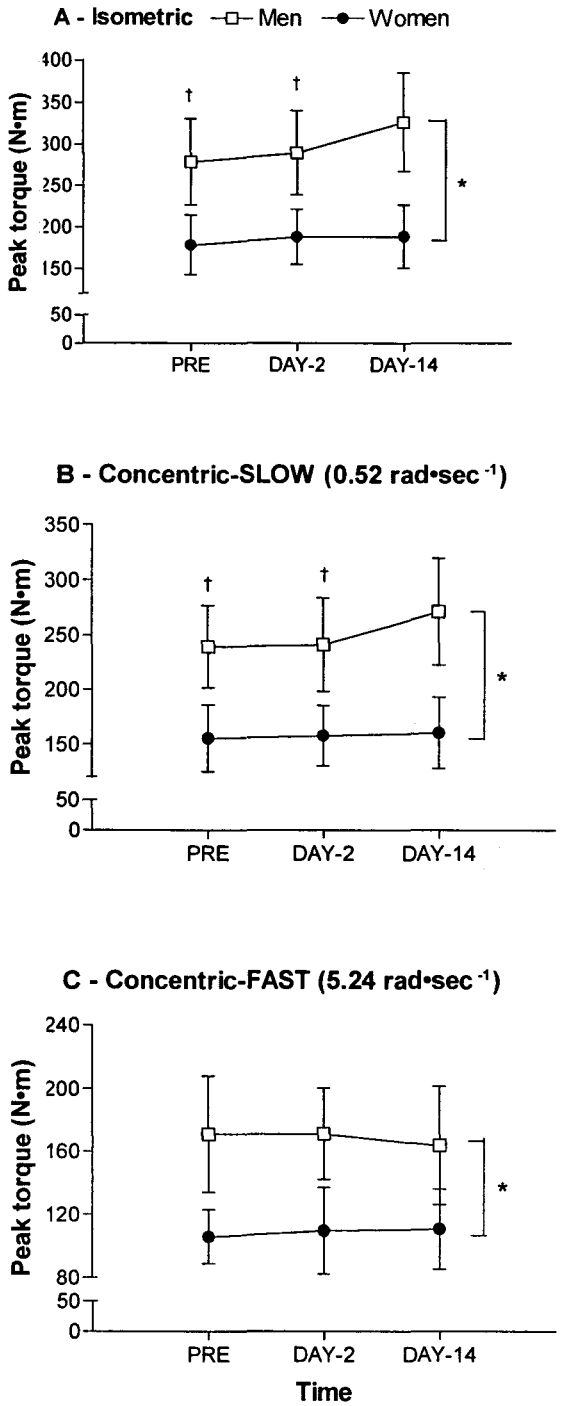


Figure 9. Peak torque (N•m) in the non-immobilized leg for isometric (A), concentric-SLOW (B), and concentric-FAST (C). Values are mean± SD. *Significantly different from men ($p<0.001$). †Significantly different from DAY-14 ($p<0.001$).

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APPENDIX 1

Subject Consent Form

Subject Screening Questionnaire

Letter of Informed Consent Form

Gender Differences in Muscle Atrophy Following Single Leg Immobilization

Mark A. Tarnopolsky, M.D., Ph.D. FRCP(C)
Stuart M. Phillips, Ph.D.
Department of Medicine and Kinesiology, McMaster University
Nobuo Yasuda, M.Sc., M.Ed. and Elisa Glover, M.Sc.
Department of Kinesiology, McMaster University

OUTLINE

Muscle atrophy regularly occurs as a consequence of immobilization or disuse. The loss of muscle strength is the most evident response. In addition, a dramatic decrease in skeletal muscle mass and fiber size can also be observed. Whether there are any gender specific differences remains to be elucidated despite of the fact that the atrophic process has been widely investigated.

In the present study, twenty-four healthy men (n=12) and women (n=12) will serve as subjects. You will complete a two-week study with immobilization of the dominant leg using a knee immobilization splint and walking crutches. You will be tested in the morning following an overnight fast and cannot perform any sports or formal physical activity for 3 days before the first testing session. There will be a total of 3 testing sessions called PRE (before the immobilization), + 2 d (48 h after immobilization), and +14 d (14 days after immobilization). The testing will be identical for each trial.

Trial Outline:

At the end of each of the three testing sessions, you will report to the neuromuscular laboratory to undergo testing consisting of body composition analysis, determination of quadriceps muscle cross-sectional area (CSA), evaluations of maximal force generating capacity and isometric torque of the knee extensors, blood testing, and muscle biopsy (outlined below):

Explanation of Tests

1. Immobilization

Immobilization of the leg will be completed using a knee immobilization splint that extends from the groin to the ankle. Crutches will be given to you and a seal will be applied to the splint. You will return to the clinic every day and the seal will be removed and you will be allowed to shower. The splint will then be re applied and a new seal placed on the splint.

2. Nutritional Intake Assessment

You will complete 4-day dietary intake records (including 1 weekend day) for calculation of mean daily energy and protein intake. This information will be used to design individualized diets that you will consume for 3 days before both experimental trials. On the study day, you will consume a prepackaged diet, which will contain no meat products and will be provided on the basis of their habitual energy intake.

3. Body Composition Analysis

DEXA - This test will require you to lie completely still for a period of less than 5 minutes. During this time a low intensity radioactive beam will scan your entire body to determine the body composition (fat and muscle content). Low levels of radiation are emitted during this test. However, this calculation is about 10X less than a chest X-ray, and is similar to the amount of radiation that you would be exposed to in a 4 hour airplane flight.

BIA - This test will require you to lie on his/her back while 2 electrodes are placed on each of his/her foot and hand. These electrodes will measure how quickly a pulse moves from one electrode to the other and will help determine body water content. This procedure is completely painless and there are no known side effects.

3. Determination of Quadriceps Muscle Cross-Sectional Area (CSA)

Magnetic resonance imaging (MRI) will be performed to the quadriceps muscle cross-sectional area (CSA) of the anterior compartment. MRI is a type of diagnostic radiography using electromagnetic energy. There are no known side effects in healthy individuals although MRI is contraindicated in patients with cardiac pacemakers.

3. Evaluations of Maximal Force Generating Capacity and Isometric Torque of The Knee Extensors

Maximal force generating capacity and isometric torque of the knee extensors will be evaluated on an isokinetic dynamometer (Biodex). The exercise test will consist of unilateral knee extensions performed while sitting. You will perform three repetitions while being verbally encouraged to sustain an isometric maximal voluntary contraction (MVC). Each contraction will be 5 s in duration. A 60 s rest will be given between each repetition, and all repetitions will be performed with the your knee at an angle of 90°. The peak torque exerted will be recorded for all three repetitions with the highest value being considered the MVC torque.

3. Blood Sampling

A single needle stick will be used to take blood at the site of insertion of the plastic venous catheters. There is a possibility of a small bruise at the site where the needle stick is administered. The total amount of blood taken over the course of the study will be 3 X 30 milliliters (3 X 2 tablespoons), which is about 12 X less than a normal blood donation and should have no negative effects.

3. Muscle Biopsy

A muscle biopsy will be taken from the outer thigh (2 in one leg, and 1 in the other). Dr. Tarnopolsky or a 4th-year Ph.D. student under the direct supervision of Dr. Tarnopolsky will perform the biopsy. Dr. Tarnopolsky has performed over 10,000 of these in patients and healthy people ranging in age from 1 week to 90 years with the following complications:

- 4/10,000 -- local skin infection
- 1/200 – fibrous lump at the site of the biopsy (connective tissue); all disappeared with massage after < 1 week.
- 1/1,000 – small patch of numbness just past the biopsy incision (size of a quarter) due to cutting a small sensory nerve branch. In all cases complete recovery occurred in < 3 months.
- The muscle usually has a dull ache for 24-48 hours (reduced with ice and Tylenol or Advil)
- In theory, it is possible to damage a small motor nerve of the muscle on the outer thigh, which would weaken the lower aspect of the muscle. This should not affect function since this muscle is 1 of 4 involved in extending the knee. To date, this has never been observed in any of the research participants biopsied by Dr. Tarnopolsky.

Potential Risks and Discomforts

Please refer to the attached form entitled "Description of Medical Procedures " for a complete description of the medical procedures to be performed during the study and the potential risks associated with these procedures. If trial-induced injury occurs at any time during the investigation, appropriate first aid will be administered and you will be advised to seek necessary medical help.

I consent to participate in a study designed by the above individual examining the effects of unilateral leg immobilization on muscle atrophy in young, healthy men and women.

I understand that the results of this study will be made available to the scientific community, although neither my name nor any reference to me will be used in compiling or publishing these results.

I understand that I may withdraw from the study at any time without any negative repercussions. I understand that I will receive $500.00 for my time commitment upon completions of the study.

I have had the study explained to me by one of the investigators and I have read the documents entitled, "medical procedures" and "subject medical screening". I am aware that Dr. Tarnopolsky will be available on a 24 hour basis if I have any questions or concerns during the study. I will be given a signed copy of this form to keep in case I have any questions during the study.

Dr. Mark Tarnopolsky:	521-2100 ext. 76593, or pager 2888
Nobuo Yasuda or Elisa Glover	525-9140 ext. 22427

Printed Name	Signature	Date

Witness Name	Signature	Date

NEUROMUSCULAR AND METABOLIC RESEARCH LABORATORY
DEPARTMENTS OF MEDICINE AND KINESIOLOGY, MCMASTER UNIVERSITY

SUBJECT SCREENING QUESTIONNAIRE

Your responses to this questionnaire are confidential and you are asked to complete it for your own health and safety. If you answer "YES" to any of the following questions, please give additional details in the space provided and discuss the matter with one of the physician investigators (Dr. Tarnopolsky unless noted otherwise). You may refuse to answer any of the following questions, however, for your own safety, this may result in your not being allowed to participate in the study.
Before we start, are you a smoker? If so, for safety and scientific reasons we will not permit you to participate in the study but appreciate your interest.

Name:———————————— Date:————————————

1. Have you ever been told that you have a 'heart problem'?

   YES NO

2. Have you ever been told that you have a breathing problem, such as asthma?

   YES NO

3. Have you ever been told that you sometimes experience seizures?

YES NO

4. Have you ever had any major joint instability or ongoing chronic pain such as in the knee or back?

YES NO

5. Have you ever been told that you have 'kidney problems'?

YES NO

6. Have you had any allergies to any medications (including 'over-the-counter' medicines such as aspirin or Tylenol™)?

YES NO

7. Have you had any allergies to food (fish or nuts) or environmental factors (dust, pollen, or mold)?

YES NO

8. Have you had any stomach problems, such as ulcers?

YES NO

9. When you experience a cut do you take a long time to stop bleeding?

YES NO

10. When you receive a blow to your muscle, do you develop bruises easily?

YES NO

11. Are you currently taking any medication (including aspirin) or have you taken any medication in the last two days?

YES NO

11a. Please list any prescription medications:

11b. Please list any over the counter medications and dietary supplements:

12. Is there any medical condition with which you have been diagnosed and are under the care of a physician (e.g. diabetes, high blood pressure)?

YES NO

13. Have you previously participated in a study under the supervision of Dr.'s Mark Tarnopolsky, Stuart Phillips, or Martin Gibala that involved having muscle biopsies taken

YES NO

I have read and understood the above questions and have discussed any medical concerns that I have about participating in the study with one of the investigators.

_____ _____

Participant Date

I have reviewed the above questionnaire and addressed any questions that the above mentioned participant had regarding the medical safety of their participation in the stated study.

_____ _____

Dr. M. Tarnopolsky, MD, PhD, FRCP(C), or other physician member of the research team Date

APPENDIX 2

Medical Procedures

Neuromuscular and Neurometabolic Research Laboratory
Departments of Pediatrics and Medicine,
McMaster University

DESCRIPTION OF MEDICAL PROCEDURES – Addendum to the Information Sheet

The study in which you are invited to participate involves three procedures which require medical involvement: immobilization, muscle biopsy sampling and venous blood sampling. Prior to any involvement, you are asked to read this form, which outlines the potential medical risks inherent to these procedures. In addition, you must also complete the "Subject Screening Questionnaire" which is designed to identify any medical reason, which might preclude your participation as a subject.

NOTE: If you have participated in another research study that has involved muscle biopsies then you may not be allowed to participate in this study. We have taken a conservative approach that no more than six muscle biopsies will be taken from the outer leg muscle and no more than three from any other muscle, over a life-time.

Immobilization

In terms of immobilization, you should not feel major discomfort during the period of wearing the leg brace. Leg muscles should completely recover from any muscle or strength loss that occurs within a few weeks. In a 3-week leg immobilization study, leg strength returned to 89% and muscle fibre cross-sectional area to 95% of pre-immobilization values with two weeks of spontaneous recovery (Hortobagyi et al., *J Physiol*, 2000 524 Pt 1:293-304). Deep vein thrombosis is the only real risk of any significance and an expert has determined that this is very low. We will be removing the immobilizer every day and checking the leg for signs of DVT (redness, swelling, positive Homan's test). Any person with signs of DVT will be sent for IPG testing and assessment by thromboembolism group and will not be allowed to continue in the study.

Muscle Biopsy

This procedure involves the removal of a small piece of muscle tissue using a sterile hollow needle. A medical doctor will clean an area over your quadriceps muscle (Vastus lateralis) and inject a small amount of local anesthetic ("freezing") into and under the skin. He will then make a small incision (~4-5

mm) in the skin in order to create an opening through which to put the biopsy needle into your thigh. There is a small amount of bleeding from the incision, but this is minimal. He will then quickly cut off a very small piece of muscle (~50-100 mg; about the size of the eraser on the end of a pencil) and remove the needle from your leg. During the time that the sample is being taken (~5 sec), you may feel the sensation of deep pressure in your thigh and on some occasions this is moderately painful. However, the discomfort very quickly passes and you are quite capable of performing exercise and daily activities.

Following the biopsies, the incisions will be closed with sterile suture (stitch), and wrapped with a tensor bandage. You should refrain from excessive muscle use for the remainder of the day. Once the anesthetic wears off, your leg may feel tight and often there is the sensation of a deep bruise or "Charlie Horse". Analgesics (pain killers) such as Tylenol or Ibuprofen (such as Advil or Motrin) are acceptable if you experience significant pain associated with the biopsy. It is also beneficial to periodically apply an ice pack to the biopsy site the following day, as this will help to reduce any swelling and any residual soreness. The following day your leg may feel uncomfortable when going down stairs. The tightness in the muscle usually disappears within 2 days and subjects routinely begin exercising at normal capacity within a day. In order to allow the incisions to heal properly and minimize any risk of infection, you should avoid prolonged submersion in water for 2-3 days. Daily showers are acceptable, but baths, swimming, saunas, etc. should be avoided for at least 4 days following the biopsy procedure.

Potential Risks. The biopsy technique is routinely used in physiological research, and complications are rare provided that proper precautions are taken. However, there is a risk of internal bleeding at the site of the biopsy, which can result in bruising and temporary discoloration of the skin. On occasion, a small lump may form under the site of the incision, but this normally disappears within 2-3 months, or within a few days to a week if massaged. As with any incision, there is also a slight risk of infection, however this risk is virtually eliminated through proper cleansing of the area and daily changing of wound coverings. If the incision does not heal within a few days or you are in any way concerned about inflammation or infection, please contact us immediately. In very rare occasions, there can be damage to a superficial sensory nerve, which will result in temporary (up to 4 months is the longest) numbness in an area. There is also an extremely remote chance (1 in 1,000,000) that you will be allergic to the local anesthetic.

In past experience with healthy young subjects, approximately 1 in 2,000 have experienced a local skin infection; 1 in 500 have experienced a small lump at the site of the biopsy (in all cases this disappeared within ~1-2 weeks using local massage); 1 in 1,500 have experienced a temporary loss of sensation in the skin

at the site of incision (an area of numbness about the size of a Loonie which lasted up to 4 months), and 1 in 100 have experienced bruising around the site of incision which lasted for ~4-5 days. While there is also a theoretical risk of damage to a small motor nerve (that is used to allow your muscle to move) branch of the outer leg muscle, this has never been seen in over 10,000 biopsies performed by Dr. Tarnopolsky. Hence, the risk of damaging a small motor nerve branch is impossible to truly estimate, but in the extremely unlikely chance that it did occur, only about 20 % of the lower part of one of four large muscles that moves the knee would be affected (hence, it would not impact on function in daily activities) and even this small area of muscle would likely recover in 6 – 9 months.

Venous Blood Sampling

This procedure is identical to that performed when you go to the doctor's office for a "blood test". A new, sterile needle with a yellow shield will be inserted into a vein on the forearm by a physician or a medically trained and certified member of the laboratory group. A sterile tube with a rubber cap is placed in the yellow shield and the blood flows into the tube by vacuum. Occasionally, a few attempts must be made to get the needle into a suitable vein for sampling. Once the sample volume is taken (at most 2 tablespoons), the needle is removed and pressure is applied followed by a band-aid or gauze and tape. Mild discomfort at the site and/or a small bruise may occur but resolve spontaneously.

Potential Risks. Blood sampling is a common medical practice and involves few risks if proper precautions are taken. The blood is taken under completely sterile conditions, however there is a theoretical risk of infection (unknown for we have never seen, nor heard of, nor read a report of, anyone getting an infection). There is a chance of a slight leakage of blood (up to 1 teaspoon) if adequate pressure is not maintained upon removal of the catheter or a bruise up to the size of a Loonie that could induce minor discomfort and could result in bruising/skin discoloration which could last up to a few weeks.

APPENDIX 3

Physical Characteristics

SUBJECT CHARACTERISTICS

SUBJECT N	STUDY CODE	GENDER	IMMOBILIZED LEG	GROUP	AGE (yr)	HEIGHT (cm)
1	#1	Men	Right	1	20	175.0
2	#2	Men	Right	1	20	177.0
3	#4	Men	Left	2	20	177.0
4	#5	Men	Left	2	21	173.0
5	#6	Men	Right	2	22	176.0
6	#7	Men	Right	3	18	193.0
7	#8	Men	Right	3	21	179.0
8	#9	Men	Right	3	19	171.0
9	#10	Men	Left	4	25	174.0
10	#11	Men	Right	4	20	189.0
11	#12	Men	Left	4	24	185.0
12	#55	Men	Left	4	20	191.0
13	#66	Men	Left	4	20	166.0
Mean	-	-	-	-	20.8	178.9
SD	-	-	-	-	1.9	8.2
14	#13	Women	Right	1	19	167.0
15	#14	Women	Right	1	19	170.0
16	#15	Women	Right	1	19	159.0
17	#16	Women	Right	1	23	157.0
18	#17	Women	Left	2	21	165.0
19	#18	Women	Left	2	20	160.0
20	#19	Women	Right	2	18	163.0
21	#20	Women	Left	2	22	156.0
22	#21	Women	Right	3	21	167.0
23	#23	Women	Left	3	23	163.0
24	#24	Women	Left	3	28	172.0
25	#25	Women	Right	4	21	168.0
26	#26	Women	Left	4	20	172.0
27	#27	Women	Left	4	24	170.0
Mean	-	-	-	-	21.3	164.9
SD	-	-	-	-	2.6	5.4

APPENDIX 4

Body Composition Raw Data and ANOVA Tables

BODY MASS (kg)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	61.7	61.4	61.3
2	#2	Men	87.4	88.1	89.1
3	#4	Men	78.4	78.3	78.4
4	#5	Men	72.2	72.5	71.3
5	#6	Men	74.2	75.3	74.7
6	#7	Men	76.7	77.8	75.8
7	#8	Men	66.8	68.2	68.3
8	#9	Men	69.4	71.2	70.1
9	#10	Men	91.2	92.0	91.3
10	#11	Men	84.9	86.2	86.6
11	#12	Men	91.6	92.3	94.3
12	#55	Men	111.1	110.2	106.6
13	#66	Men	59.0	58.2	57.8
Mean	-	-	78.8	79.4	78.9
SD	-	-	14.3	14.2	14.0
14	#13	Women	61.3	62.5	61.4
15	#14	Women	63.7	64.5	63.5
16	#15	Women	50.0	50.2	50.2
17	#16	Women	52.1	52.3	52.0
18	#17	Women	59.9	59.5	58.5
19	#18	Women	66.1	65.5	64.8
20	#19	Women	61.7	61.9	62.8
21	#20	Women	48.6	47.9	48.0
22	#21	Women	65.0	65.0	65.0
23	#23	Women	60.0	59.7	59.0
24	#24	Women	71.5	71.5	71.5
25	#25	Women	61.6	61.4	62.1
26	#26	Women	81.2	81.8	82.5
27	#27	Women	70.1	71.4	71.9
Mean	-	-	62.3	62.5	62.4
SD	-	-	8.7	8.9	9.1

PERCENT BODY FAT

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	12.2	11.6	11.3
2	#2	Men	26.4	25.2	24.9
3	#4	Men	16.5	17.4	16.4
4	#5	Men	14.7	15.5	14.8
5	#6	Men	19.6	20.7	20.1
6	#7	Men	14.9	16.7	16.6
7	#8	Men	16.9	17.3	17.4
8	#9	Men	9.8	14.4	10.8
9	#10	Men	22.5	24.0	24.4
10	#11	Men	18.2	19.3	18.7
11	#12	Men	18.9	19.2	19.6
12	#55	Men	18.8	19.4	19.9
13	#66	Men	18.1	18.9	19.0
Mean	-	-	17.5	18.4	18.0
SD	-	-	4.2	3.7	4.2
14	#13	Women	26.5	25.3	25.6
15	#14	Women	25.8	25.9	24.9
16	#15	Women	21.1	21.0	21.3
17	#16	Women	27.8	26.1	26.7
18	#17	Women	20.0	20.1	19.5
19	#18	Women	31.4	33.0	32.6
20	#19	Women	19.8	21.1	20.1
21	#20	Women	26.7	29.2	29.4
22	#21	Women	26.5	26.5	26.5
23	#23	Women	19.0	25.8	22.8
24	#24	Women	26.2	26.2	26.2
25	#25	Women	24.8	25.7	25.4
26	#26	Women	33.4	34.0	32.8
27	#27	Women	30.5	30.3	30.6
Mean	-	-	25.7	26.4	26.0
SD	-	-	4.4	4.1	4.2

LEAN BODY MASS (kg)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	54.2	54.3	54.4
2	#2	Men	64.4	65.9	66.8
3	#4	Men	65.5	64.7	65.5
4	#5	Men	61.6	61.2	60.7
5	#6	Men	59.7	59.7	59.7
6	#7	Men	62.2	61.6	60.1
7	#8	Men	53.4	54.3	54.3
8	#9	Men	60.1	58.4	59.9
9	#10	Men	70.6	69.9	69.0
10	#11	Men	69.4	69.6	70.4
11	#12	Men	74.3	74.6	75.8
12	#55	Men	90.2	88.8	85.3
13	#66	Men	48.3	47.2	46.8
Mean	-	-	64.1	63.9	63.7
SD	-	-	10.7	10.5	10.0
14	#13	Women	45.1	46.7	45.7
15	#14	Women	47.3	47.8	47.7
16	#15	Women	39.5	39.6	39.5
17	#16	Women	37.6	38.7	38.1
18	#17	Women	48.0	47.6	47.1
19	#18	Women	45.4	43.9	43.7
20	#19	Women	49.5	48.8	50.2
21	#20	Women	35.6	33.9	33.9
22	#21	Women	43.3	43.3	43.3
23	#23	Women	44.2	41.4	42.1
24	#24	Women	48.4	48.4	48.4
25	#25	Women	46.3	45.6	46.3
26	#26	Women	54.1	54.0	55.5
27	#27	Women	49.0	49.7	49.9
Mean	-	-	45.2	45.0	45.1
SD	-	-	5.0	5.2	5.6

BODY MASS (kg)

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	5583.139	25	412.5129	13.53446	**0.001124**
2	2	0.991	50	0.6245	1.58634	0.214802
12	2	0.291	50	0.6245	0.46664	0.629810

PERCENT BODY FAT

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	1318.812	25	50.34317	26.19644	**0.000027**
2	2	4.842	50	0.91528	5.29014	**0.008241**
12	2	0.055	50	0.91528	0.0596	0.942212

LEAN BODY MASS (kg)

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	7162.809	25	198.0059	36.17472	**0.000003**
2	2	0.68	50	0.7467	0.91016	0.409025
12	2	0.153	50	0.7467	0.20548	0.814938

APPENDIX 5

Serum Hormone Raw Data and ANOVA Tables

FREE TESTOSTERONE ($pmol \cdot l^{-1}$)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	61.4	60.4	54.5
2	#2	Men	44.4	47.4	43.9
3	#4	Men	56.1	67.3	51.6
4	#5	Men	44.9	44.8	68.9
5	#6	Men	37.3	33.3	35.7
6	#7	Men	48.5	40.6	37.6
7	#8	Men	63.9	48.5	40.0
8	#9	Men	48.0	29.8	41.3
9	#10	Men	33.5	49.8	38.8
10	#11	Men	62.7	58.6	69.7
11	#12	Men	54.2	50.9	51.8
12	#55	Men	46.8	44.4	61.2
13	#66	Men	64.6	56.1	58.2
Mean	-	-	51.3	48.6	50.2
SD	-	-	10.2	10.6	11.8
14	#13	Women	2.7	3.5	3.6
15	#15	Women	2.1	2.9	1.7
16	#16	Women	5.9	6.6	3.1
17	#17	Women	2.6	1.5	2.5
18	#18	Women	3.0	1.8	2.5
19	#19	Women	4.8	5.7	5.3
20	#20	Women	1.0	2.3	1.6
21	#21	Women	1.6	2.9	1.7
22	#23	Women	3.7	3.1	2.3
23	#24	Women	1.5	2.0	2.3
24	#25	Women	5.7	6.6	6.8
25	#26	Women	2.3	1.6	1.0
26	#27	Women	3.6	2.2	2.6
Mean	-	-	3.1	3.3	2.8
SD	-	-	1.6	1.8	1.6

TOTAL TESTOSTERONE ($nmol \cdot l^{-1}$)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	16.4	18.9	15.3
2	#2	Men	11.7	12.5	10.7
3	#4	Men	14.7	14.6	13
4	#5	Men	13.0	12.0	21.7
5	#6	Men	12.2	8.6	10.6
6	#7	Men	13.5	11.9	10.4
7	#8	Men	15.7	16.6	14.1
8	#9	Men	12.5	11.8	13.5
9	#10	Men	11.1	11.5	10.9
10	#11	Men	14.7	16.0	14
11	#12	Men	20.0	17.6	22.7
12	#55	Men	16.9	15.7	15.2
13	#66	Men	13.8	15.9	12.7
Mean	-	-	14.3	14.1	14.2
SD	-	-	2.5	3.0	3.9
14	#13	Women	0.8	0.8	0.7
15	#15	Women	1.1	0.7	1.3
16	#16	Women	1.4	1.8	0.9
17	#17	Women	0.8	0.5	0.6
18	#18	Women	0.7	0.3	0.5
19	#19	Women	1.1	0.8	0.7
20	#20	Women	0.4	0.5	0.5
21	#21	Women	0.4	0.7	0.6
22	#23	Women	1.1	0.8	0.7
23	#24	Women	0.4	0.6	0.5
24	#25	Women	0.7	1.0	1.1
25	#26	Women	0.6	0.5	0.5
26	#27	Women	0.5	0.6	0.6
Mean	-	-	0.8	0.7	0.7
SD	-	-	0.3	0.4	0.2

ESTRADIOL (pmol•l^{-1})

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	84.4	88.8	68.0
2	#2	Men	98.1	106.8	125.5
3	#4	Men	101.0	135.3	110.5
4	#5	Men	89.3	84.3	86.8
5	#6	Men	88.7	82.6	86.0
6	#7	Men	76.8	51.7	63.4
7	#8	Men	131.1	148.5	131.7
8	#9	Men	92.0	71.4	107.9
9	#10	Men	102.1	140.2	104.0
10	#11	Men	122.1	110.6	75.8
11	#12	Men	158.3	136.8	166.8
12	#55	Men	102.9	122.1	139.2
13	#66	Men	120.1	78.1	85.6
Mean	-	-	105.1	104.4	103.9
SD	-	-	22.4	30.6	30.5
14	#13	Women	72.2	107.4	152.7
15	#14	Women	54.9	58.7	100.7
16	#15	Women	50.5	53.4	38.5
17	#16	Women	300.6	413.5	142.9
18	#17	Women	130.4	61.1	63.6
19	#18	Women	72.3	80.7	51.8
20	#19	Women	698.5	297.3	177.7
21	#20	Women	35.7	25.4	39.8
22	#21	Women	57.9	91.7	62.4
23	#23	Women	121.7	85.8	47.6
24	#24	Women	106.8	152.3	295.3
25	#25	Women	183.1	188.7	238.8
26	#26	Women	135.3	483.0	212.4
27	#27	Women	243.3	190.2	528.2
Mean	-	-	161.7	163.5	153.7
SD	-	-	172.6	140.8	135.5

CORTISOL ($nmol \cdot l^{-1}$)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	620.0	500.7	250.0
2	#2	Men	550.1	362.2	390.6
3	#4	Men	516.1	550.1	384.1
4	#5	Men	747.2	335.2	653.9
5	#7	Men	742.2	638.4	522.7
6	#8	Men	791.4	867.1	634.7
7	#9	Men	841.2	319.2	586.6
8	#10	Men	447.8	375.2	335.2
9	#11	Men	315.7	326.1	366.9
10	#12	Men	502.9	499.3	461.6
11	#55	Men	447.4	512.7	365.6
12	#66	Men	507.0	741.2	538.4
Mean	-	-	585.8	502.3	457.5
SD	-	-	162.2	175.6	128.6
13	#13	Women	723.2	685.0	670.3
14	#14	Women	744.5	688.8	604.1
15	#17	Women	679.4	787.6	906.0
16	#18	Women	1014.6	683.3	615.3
17	#19	Women	461.2	557.9	407.5
18	#20	Women	940.9	749.1	916.0
19	#23	Women	651.8	550.1	523.0
20	#24	Women	381.2	366.6	221.1
21	#25	Women	551.3	658.2	627.8
22	#26	Women	993.7	839.5	900.2
23	#27	Women	615.0	516.2	635.9
Mean	-	-	705.2	643.8	638.8
SD	-	-	208.9	136.0	214.7

PROGESTERONE ($nmol \cdot l^{-1}$)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#13	Women	1.9	1.9	1.6
2	#14	Women	1.2	1.4	1.2
3	#15	Women	3.2	1.5	3.0
4	#16	Women	3.2	31.7	10.7
5	#17	Women	2.5	2.2	3.0
6	#18	Women	2.3	1.4	1.5
7	#19	Women	6.3	25.7	2.1
8	#20	Women	1.2	1.6	1.4
9	#21	Women	2.4	2.1	2.1
10	#23	Women	2.2	1.7	1.2
11	#24	Women	2.6	0.8	46.0
12	#25	Women	12.1	3.4	36.9
13	#26	Women	1.9	1.7	2.4
14	#27	Women	34.5	13.6	2.5
Mean	-	-	5.5	6.5	8.3
SD	-	-	8.8	10.0	14.4

FREE TESTOSTERONE (pmol•l^{-1})

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	42984.18	24	119.6437	359.2681	**0.000000**
2	2	10.08	48	30.5385	0.33	0.720511
12	2	13.74	48	30.5385	0.45	0.640294

TOTAL TESTOSTERONE (nmol•l^{-1})

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	3544.431	24	10.89677	325.2735	**0.000000**
2	2	0.093	48	2.20892	0.042	0.958885
12	2	0.05	48	2.20892	0.0225	0.977743

ESTRADIOL (pmol•l^{-1})

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	61491.23	25	22197.35	2.770206	0.108520
2	2	212.51	50	7148.92	0.029726	0.970728
12	2	155.52	50	7148.92	0.021754	0.978490

CORTISOL (nmol•l^{-1})

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	289846	21	69263.26	4.184701	0.053520
2	2	55508.5	42	12499.81	4.440747	**0.017802**
12	2	4466.4	42	12499.81	0.357317	0.701657

MAIN EFFECT: TIME

	{1} 649.8416	{2} 578.0342	{3} 554.7911
PRE {1}		0.086862	**0.016777**
DAY-2 {2}	0.086862		0.761970
DAY-14 {3}	**0.016777**	0.76197	

PROGESTERONE (nmol•l^{-1})

1-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	2	26.73643	26	120.9931	0.220975	0.803227

APPENDIX 6

Strength Test Raw Data and ANOVA Tables

PEAK TORQUE (N•m) -ISOMETRIC

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	234.6	204.2	249.0
2	#2	Men	221.4	293.6	245.8
3	#4	Men	226.7	216.8	249.4
4	#5	Men	266.7	250.7	213.3
5	#6	Men	283.0	286.1	287.0
6	#7	Men	212.2	217.6	219.0
7	#8	Men	287.2	256.4	186.5
8	#9	Men	296.7	296.4	241.6
9	#10	Men	233.8	217.6	227.8
10	#11	Men	314.0	286.5	276.9
11	#12	Men	254.4	254.0	210.5
12	#55	Men	311.2	323.5	238.4
13	#66	Men	204.2	191.7	178.3
Mean	-	-	257.4	253.5	232.6
SD	-	-	38.2	41.5	31.5
14	#13	Women	211.9	167.1	169.2
15	#14	Women	169.9	145.5	136.0
16	#15	Women	151.5	173.4	161.8
17	#16	Women	126.4	129.9	117.3
18	#17	Women	108.3	75.9	79.7
19	#18	Women	151.9	97.1	104.8
20	#19	Women	238.4	225.0	212.6
21	#20	Women	100.6	97.5	81.2
22	#21	Women	168.1	141.3	102.1
23	#23	Women	137.8	129.0	80.5
24	#24	Women	166.0	173.0	137.0
25	#25	Women	155.8	129.6	131.0
26	#26	Women	184.4	118.0	93.2
27	#27	Women	225.4	221.4	182.7
Mean	-	-	164.0	144.6	127.8
SD	-	-	40.8	43.9	41.6

PEAK TORQUE (N•m) -CONCENTRIC SLOW

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	197.3	184.7	193.9
2	#2	Men	186.5	234.9	210.5
3	#4	Men	201.0	177.4	210.5
4	#5	Men	245.4	236.4	206.9
5	#6	Men	214.0	221.2	219.7
6	#7	Men	197.0	177.0	144.8
7	#8	Men	227.1	198.1	158.9
8	#9	Men	246.2	246.9	179.1
9	#10	Men	219.0	192.0	200.3
10	#11	Men	304.8	232.4	238.8
11	#12	Men	209.1	187.1	204.9
12	#55	Men	262.8	267.0	243.4
13	#66	Men	167.5	155.4	156.1
Mean	-	-	221.4	208.5	197.5
SD	-	-	36.3	33.3	30.5
14	#13	Women	187.5	168.1	162.0
15	#14	Women	150.1	138.4	117.6
16	#15	Women	149.7	149.0	142.7
17	#16	Women	91.8	103.5	107.0
18	#17	Women	114.0	109.4	105.2
19	#18	Women	139.8	105.6	105.9
20	#19	Women	186.2	178.7	152.6
21	#20	Women	101.0	99.9	75.9
22	#21	Women	170.2	158.9	97.1
23	#23	Women	116.2	86.5	62.8
24	#24	Women	173.0	144.8	130.7
25	#25	Women	153.6	123.9	117.6
26	#26	Women	176.3	103.9	118.7
27	#27	Women	180.9	151.9	114.0
Mean	-	-	149.3	130.2	115.0
SD	-	-	32.4	29.1	27.0

PEAK TORQUE (N•m) -CONCENTRIC FAST

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	133.4	136.0	108.8
2	#2	Men	157.2	149.0	124.6
3	#4	Men	142.7	114.7	101.7
4	#5	Men	166.0	164.9	133.2
5	#6	Men	139.8	120.0	144.8
6	#7	Men	163.1	169.2	135.2
7	#8	Men	211.9	173.0	121.8
8	#9	Men	175.9	104.8	117.3
9	#10	Men	149.7	163.1	174.1
10	#11	Men	219.4	212.6	213.0
11	#12	Men	173.7	152.3	177.6
12	#55	Men	152.6	198.5	170.2
13	#66	Men	134.9	79.1	86.8
Mean	-	-	163.1	149.0	139.2
SD	-	-	27.1	37.6	35.7
14	#13	Women	133.4	105.6	111.2
15	#14	Women	127.5	138.0	105.9
16	#15	Women	80.5	105.2	78.8
17	#16	Women	64.3	69.8	59.0
18	#17	Women	102.8	89.6	47.3
19	#18	Women	85.8	80.1	63.6
20	#19	Women	103.5	103.5	107.0
21	#20	Women	84.1	70.9	54.0
22	#21	Women	144.8	125.3	107.4
23	#23	Women	98.2	86.1	67.4
24	#24	Women	156.1	107.0	107.4
25	#25	Women	102.8	100.2	94.2
26	#26	Women	125.3	97.1	116.2
27	#27	Women	138.4	133.4	101.7
Mean	-	-	110.5	100.8	87.2
SD	-	-	27.4	21.1	24.4

SPECIFIC STRENGTH - ISOMETRIC [N•m/(cm² x m)]

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	2.0	1.7	2.2
2	#2	Men	1.5	2.0	1.7
3	#4	Men	1.9	1.8	2.1
4	#5	Men	1.8	1.7	1.5
5	#6	Men	1.6	1.6	1.6
6	#7	Men	1.4	1.4	1.7
7	#8	Men	2.1	1.9	1.6
8	#9	Men	1.8	1.8	1.6
9	#10	Men	1.6	1.5	1.6
10	#11	Men	1.8	1.7	1.7
11	#12	Men	1.6	1.7	1.5
12	#55	Men	1.9	2.0	1.5
13	#66	Men	1.9	1.8	1.7
Mean	-	-	1.8	1.7	1.7
SD	-	-	0.2	0.2	0.2
14	#13	Women	2.0	1.6	1.6
15	#14	Women	1.8	1.6	1.5
16	#15	Women	1.8	2.1	2.0
17	#16	Women	1.5	1.6	1.6
18	#17	Women	1.1	0.8	0.9
19	#18	Women	2.1	1.4	1.7
20	#19	Women	2.2	2.0	2.0
21	#20	Women	1.4	1.4	1.4
22	#21	Women	1.7	1.4	1.1
23	#23	Women	1.5	1.4	0.9
24	#24	Women	1.6	1.7	1.4
25	#25	Women	1.7	1.4	1.5
26	#26	Women	1.6	1.0	0.8
27	#27	Women	2.4	2.3	2.1
Mean	-	-	1.7	1.6	1.5
SD	-	-	0.3	0.4	0.4

SPECIFIC STRENGTH - CONCENTRIC SLOW [N•m/(cm^2 x m)]

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	1.7	1.6	1.7
2	#2	Men	1.3	1.6	1.4
3	#4	Men	1.7	1.5	1.8
4	#5	Men	1.6	1.6	1.5
5	#6	Men	1.2	1.2	1.3
6	#7	Men	1.3	1.2	1.1
7	#8	Men	1.7	1.5	1.4
8	#9	Men	1.5	1.5	1.2
9	#10	Men	1.5	1.3	1.4
10	#11	Men	1.8	1.3	1.4
11	#12	Men	1.3	1.3	1.5
12	#55	Men	1.6	1.6	1.5
13	#66	Men	1.6	1.5	1.5
Mean	-	-	1.5	1.4	1.4
SD	-	-	0.2	0.2	0.2
14	#13	Women	1.8	1.6	1.6
15	#14	Women	1.6	1.5	1.3
16	#15	Women	1.8	1.8	1.8
17	#16	Women	1.1	1.3	1.4
18	#17	Women	1.2	1.2	1.1
19	#18	Women	1.9	1.5	1.7
20	#19	Women	1.7	1.6	1.4
21	#20	Women	1.4	1.5	1.3
22	#21	Women	1.7	1.6	1.0
23	#23	Women	1.2	0.9	0.7
24	#24	Women	1.6	1.4	1.3
25	#25	Women	1.7	1.4	1.3
26	#26	Women	1.5	0.9	1.0
27	#27	Women	1.9	1.6	1.3
Mean	-	-	1.6	1.4	1.3
SD	-	-	0.3	0.3	0.3

SPECIFIC STRENGTH - CONCENTRIC FAST [N•m/(cm² x m)]

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	1.1	1.1	0.9
2	#2	Men	1.1	1.0	0.9
3	#4	Men	1.2	0.9	0.9
4	#5	Men	1.1	1.1	1.0
5	#6	Men	0.8	0.7	0.8
6	#7	Men	1.1	1.1	1.0
7	#8	Men	1.6	1.3	1.1
8	#9	Men	1.1	0.6	0.8
9	#10	Men	1.0	1.1	1.2
10	#11	Men	1.3	1.2	1.3
11	#12	Men	1.1	1.0	1.3
12	#55	Men	0.9	1.2	1.1
13	#66	Men	1.3	0.8	0.8
Mean	-	-	1.1	1.0	1.0
SD	-	-	0.2	0.2	0.2
14	#13	Women	1.3	1.0	1.1
15	#14	Women	1.4	1.5	1.2
16	#15	Women	1.0	1.3	1.0
17	#16	Women	0.8	0.9	0.8
18	#17	Women	1.1	1.0	0.5
19	#18	Women	1.2	1.2	1.0
20	#19	Women	0.9	0.9	1.0
21	#20	Women	1.2	1.0	0.9
22	#21	Women	1.4	1.2	1.1
23	#23	Women	1.0	0.9	0.8
24	#24	Women	1.5	1.0	1.1
25	#25	Women	1.1	1.1	1.0
26	#26	Women	1.1	0.8	1.0
27	#27	Women	1.5	1.4	1.2
Mean	-	-	1.2	1.1	1.0
SD	-	-	0.2	0.2	0.2

PEAK TORQUE (N•m) - ISOMETRIC

1-GENDER, 2-TIME

	df Effect		MS Effect	df Error	MS Error	F	p-level
1		1	211861.8	25	3889.897	54.46465	**0.000000**
2		2	6395.1	50	440.815	14.50746	**0.000011**
12		2	437.6	50	440.815	0.99267	0.377771

MAIN EFFECT: GENDER

		{1} 247.8128	{2} 145.4571
Men	{1}		**0.000149**
Women	{2}	**0.000149**	

MAIN EFFECT: TIME

		{1} 210.7104	{2} 199.0096	{3} 180.1849
PRE	{1}		0.111561	**0.000129**
DAY-2	{2}	0.111561		**0.005184**
DAY-14	{3}	**0.000129**	**0.005184**	

PEAK TORQUE (N•m) - CONCENTRIC SLOW

1-GENDER, 2-TIME

	df Effect		MS Effect	df Error	MS Error	F	p-level
1		1	121891.8	25	2335.393	52.19327	**0.000000**
2		2	5719.3	50	319.94	17.87612	**0.000001**
12		2	187.6	50	319.94	0.58621	0.560210

MAIN EFFECT: GENDER

		{1} 209.1282	{2} 131.4905
Men	{1}		**0.000149**
Women	{2}	**0.000149**	

MAIN EFFECT: TIME

		{1} 185.3343	{2} 169.3393	{3} 156.2544
PRE	{1}		**0.005317**	**0.000125**
DAY-2	{2}	**0.005317**		**0.026029**
DAY-14	{3}	**0.000125**	**0.026029**	

PEAK TORQUE (N•m) - CONCENTRIC FAST

1-GENDER, 2-TIME

	df Effect		MS Effect	df Error	MS Error	F	p-level
1		1	52376.1	25	1992.343	26.2887	**0.000027**
2		2	3762.77	50	289.106	13.01518	**0.000028**
12		2	38.05	50	289.106	0.13162	0.876973

MAIN EFFECT: GENDER

		{1} 150.4256	{2} 99.53333
Men	{1}		**0.000171**
Women	{2}	**0.000171**	

MAIN EFFECT: TIME

		{1} 136.8179	{2} 124.9291	{3} 113.1915
PRE	{1}		**0.034837**	**0.000136**
DAY-2	{2}	**0.034837**		**0.037681**
DAY-14	{3}	**0.000136**	**0.037681**	

SPECIFIC STRENGTH - ISOMETRIC [N•m/(cm^2 x m)]

1-GENDER, 2-TIME

	df Effect		MS Effect	df Error	MS Error	F	p-level
1		1	0.446925	25	0.23613	1.892708	0.181098
2		2	0.219687	50	0.027707	7.928889	**0.001021**
12		2	0.085405	50	0.027707	3.082432	0.054655

MAIN EFFECT: TIME

		{1} 1.749313	{2} 1.641539	{3} 1.57
PRE	{1}		0.054471	**0.0008**
DAY-2	{2}	0.054471		0.264099
DAY-14	{3}	**0.0008**	0.264099	

SPECIFIC STRENGTH - CONCENTRIC SLOW [N•m/(cm² x m)]

1-GENDER, 2-TIME

	df Effect		MS Effect	df Error	MS Error	F	p-level
1		1	0.015549	25	0.117471	0.13236	0.719053
2		2	0.234084	50	0.019394	12.06972	**0.000053**
12		2	0.069486	50	0.019394	3.58281	**0.035146**

MAIN EFFECT: TIME

		{1} 1.548297	{2} 1.416374	{3} 1.368352
PRE	{1}		**0.003055**	**0.00017**
DAY-2	{2}	**0.003055**		0.420344
DAY-14	{3}	**0.00017**	0.420344	

INTERACTION: 1 x 2

		{1} 1.512308	{2} 1.428462	{3} 1.433846	{4} 1.584286	{5} 1.404286	{6} 1.302857
Men	1 {1}		0.643902	0.705053	0.760478	0.349324	**0.003751**
Men	2 {2}	0.643902		0.999999	0.057728	0.99756	0.197256
Men	3 {3}	0.705053	0.999999		0.073335	0.993688	0.161862
Women	1 {4}	0.760478	0.057728	0.073335		**0.01514**	**0.000167**
Women	2 {5}	0.349324	0.99756	0.993688	**0.01514**		0.398448
Women	3 {6}	**0.003751**	0.197256	0.161862	**0.000167**	0.398448	

SPECIFIC STRENGTH - CONCENTRIC FAST [N•m/(cm² x m)]

1-GENDER, 2-TIME

	df Effect		MS Effect	df Error	MS Error	F	p-level
1		1	0.020419	25	0.077915	0.262073	0.613192
2		2	0.176019	50	0.018021	9.767532	**0.000263**
12		2	0.015681	50	0.018021	0.870161	0.42513

MAIN EFFECT: TIME

		{1} 1.145769	{2} 1.052418	{3} 0.9848626
PRE	{1}		**0.036032**	**0.000276**
DAY-2	{2}	**0.036032**		0.164484
DAY-14	{3}	**0.000276**	0.164484	

APPENDIX 7

Leg Lean Mass Raw Data and ANOVA Table

LEG LEAN MASS (kg)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#2	Men	11.3	11.5
2	#4	Men	9.7	9.8
3	#5	Men	10.4	10.1
4	#6	Men	10.5	10.2
5	#7	Men	11.3	10.5
6	#8	Men	9.5	8.8
7	#9	Men	10.7	10.4
8	#10	Men	10.9	10.4
9	#11	Men	11.2	11.2
10	#55	Men	14.4	12.5
11	#66	Men	7.3	7.0
Mean	-	-	10.6	10.2
SD	-	-	1.7	1.4
12	#13	Women	7.8	7.8
13	#14	Women	8.0	8.1
14	#15	Women	6.4	6.3
15	#16	Women	6.2	6.1
16	#17	Women	8.3	8.0
17	#18	Women	7.6	7.0
18	#19	Women	8.1	8.0
19	#20	Women	5.3	5.0
20	#23	Women	7.3	6.8
21	#25	Women	7.4	7.3
22	#26	Women	9.2	9.1
Mean	-	-	7.4	7.2
SD	-	-	1.1	1.2

LEG LEAN MASS (kg)

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	106.1712	20	3.613596	29.38103	**0.000026**
2	1	1.0492	20	0.091129	11.51309	**0.002887**
12	1	0.1561	20	0.091129	1.713	0.205437

APPENDIX 8

CSA of Knee Extensors Raw Data and ANOVA Tables

VASTUS (cm^2)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	62.0	61.8
2	#2	Men	74.2	74.1
3	#4	Men	65.4	63.4
4	#5	Men	79.4	72.9
5	#6	Men	85.3	82.2
6	#7	Men	69.8	60.6
7	#8	Men	68.6	57.0
8	#9	Men	84.0	75.1
9	#10	Men	78.0	75.5
10	#11	Men	82.1	78.4
11	#12	Men	76.1	69.2
12	#55	Men	78.7	77.5
13	#66	Men	58.2	56.9
Mean	-	-	74.0	69.6
SD	-	-	8.6	8.6
14	#13	Women	56.7	56.5
15	#14	Women	48.5	48.4
16	#15	Women	46.0	44.7
17	#16	Women	46.4	40.3
18	#17	Women	55.8	53.7
19	#18	Women	44.9	39.1
20	#19	Women	58.4	56.9
21	#20	Women	43.2	36.2
22	#21	Women	50.1	45.8
23	#23	Women	49.9	44.7
24	#24	Women	53.1	48.5
25	#25	Women	45.9	45.8
26	#26	Women	59.7	59.6
27	#27	Women	50.8	45.9
Mean	-	-	50.7	47.6
SD	-	-	5.3	7.0

RECTUS FEMORIS (cm^2)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	4.6	4.5
2	#2	Men	8.7	8.3
3	#4	Men	3.5	3.6
4	#5	Men	7.4	7.0
5	#6	Men	18.0	17.9
6	#7	Men	8.8	8.0
7	#8	Men	7.5	7.4
8	#9	Men	11.3	11.1
9	#10	Men	7.1	7.1
10	#11	Men	10.1	10.0
11	#12	Men	8.1	6.1
12	#55	Men	7.5	7.5
13	#66	Men	5.1	5.1
Mean	-	-	8.3	8.0
SD	-	-	3.6	3.6
14	#13	Women	5.4	5.9
15	#14	Women	6.0	5.9
16	#15	Women	5.9	5.9
17	#16	Women	7.3	7.0
18	#17	Women	2.8	2.5
19	#18	Women	0.6	0.5
20	#19	Women	9.5	8.9
21	#20	Women	2.2	2.1
22	#21	Women	10.6	10.5
23	#23	Women	8.6	8.6
24	#24	Women	8.2	8.2
25	#25	Women	8.2	8.1
26	#26	Women	7.8	7.5
27	#27	Women	5.4	5.5
Mean	-	-	6.3	6.2
SD	-	-	2.9	2.8

TOTAL QUADRICEPS FEMORIS (cm^2)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	66.6	66.3
2	#2	Men	82.9	82.4
3	#4	Men	68.9	67.0
4	#5	Men	86.8	79.9
5	#6	Men	103.3	100.1
6	#7	Men	78.6	68.6
7	#8	Men	76.1	64.4
8	#9	Men	95.3	86.2
9	#10	Men	85.1	82.6
10	#11	Men	92.2	88.4
11	#12	Men	84.2	75.3
12	#55	Men	86.2	85.0
13	#66	Men	63.3	62.0
Mean	-	-	82.3	77.6
SD	-	-	11.5	11.4
14	#13	Women	62.1	62.4
15	#14	Women	54.5	54.3
16	#15	Women	51.9	50.6
17	#16	Women	53.7	47.3
18	#17	Women	58.6	56.2
19	#18	Women	45.5	39.6
20	#19	Women	67.9	65.8
21	#20	Women	45.4	38.3
22	#21	Women	60.7	56.3
23	#23	Women	58.5	53.3
24	#24	Women	61.3	56.7
25	#25	Women	54.1	53.9
26	#26	Women	67.5	67.1
27	#27	Women	56.2	51.4
Mean	-	-	57.0	53.8
SD	-	-	6.9	8.4

VASTUS (cm^2)

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	6922.177	25	105.7185	65.47747	**0.000000**
2	1	189.222	25	5.1674	36.61859	**0.000003**
12	1	5.759	25	5.1674	1.11443	0.301216

RECTUS FEMORIS (cm^2)

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	46.41485	25	21.03493	2.206561	0.149927
2	1	0.58154	25	0.09114	6.380824	**0.018243**
12	1	0.15635	25	0.09114	1.715558	0.202175

TOTAL QUADRICEPS FEMORIS (cm^2)

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	8102.244	25	181.177	44.72004	**0.000001**
2	1	210.784	25	5.6345	37.40936	**0.000002**
12	1	7.813	25	5.6345	1.3866	0.250069

APPENDIX 9

Muscle Fiber Size Raw Data and ANOVA Tables

TYPE I (μm^2)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	3463.5	3155.8
2	#2	Men	4162.1	4042.3
3	#4	Men	4851.2	4691.2
4	#5	Men	3964.1	3589.8
5	#6	Men	4158.1	4152.6
6	#10	Men	6037.0	5958.9
7	#11	Men	3531.9	3446.4
8	#12	Men	4370.9	3738.6
9	#55	Men	4198.2	4180.5
Mean	-	-	4304.1	4106.2
SD	-	-	772.8	831.2
10	#13	Women	4622.8	4319.1
11	#15	Women	3648.6	3266.0
12	#16	Women	4151.2	3996.7
13	#17	Women	5983.7	5767.0
14	#21	Women	4026.6	3564.9
15	#24	Women	2695.9	2572.4
16	#25	Women	2960.4	2823.1
17	#27	Women	3039.1	2965.0
Mean	-	-	3891.0	3659.3
SD	-	-	1074.4	1036.3

TYPE IIa (μm^2)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	4279.1	4278.7
2	#2	Men	5462.1	5184.2
3	#4	Men	7422.7	6625.5
4	#5	Men	8474.5	5907.4
5	#6	Men	6654.0	6613.0
6	#10	Men	6940.6	6666.4
7	#11	Men	5953.9	5951.4
8	#12	Men	6686.1	5606.8
9	#55	Men	6081.6	5801.8
Mean	-	-	6439.4	5848.4
SD	-	-	1196.5	777.6
10	#13	Women	4904.5	4713.1
11	#15	Women	5320.7	4060.2
12	#16	Women	6134.6	5559.5
13	#17	Women	6718.2	6435.2
14	#21	Women	3857.4	3178.7
15	#24	Women	2648.7	2444.0
16	#25	Women	4187.5	4157.2
17	#27	Women	4256.1	4125.4
Mean	-	-	4753.5	4334.2
SD	-	-	1304.5	1260.2

TYPE IIx (μm^2)

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	3535.6	3402.8
2	#2	Men	4824.4	4724.4
3	#4	Men	5597.0	5462.3
4	#5	Men	6115.8	4100.0
5	#6	Men	5400.0	5372.9
6	#10	Men	5315.3	4502.3
7	#11	Men	4362.1	4056.5
8	#12	Men	5794.8	4590.4
9	#55	Men	5250.4	4675.1
Mean	-	-	5132.8	4543.0
SD	-	-	788.9	644.3
10	#13	Women	4127.9	4052.6
11	#15	Women	5483.6	3624.3
12	#16	Women	4342.7	4144.8
13	#17	Women	6022.5	5182.0
14	#21	Women	3550.6	2717.6
15	#24	Women	1499.1	1449.5
16	#25	Women	3042.7	3039.3
17	#27	Women	3474.7	3286.8
Mean	-	-	3943.0	3437.1
SD	-	-	1416.6	1109.0

TYPE I (μm^2)

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	1566328	15	1711213	0.91533	0.35387
2	1	390898	15	15613	25.03703	**0.000157**
12	1	2431	15	15613	0.15573	0.69867

TYPE IIa (μm^2)

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	21686476	15	2400351	9.03471	**0.008868**
2	1	2161686	15	220972	9.782613	**0.006914**
12	1	62462	15	220972	0.282671	0.602741

TYPE IIx (μm^2)

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	11160519	15	1850186	6.0321	**0.026723**
2	1	2542443	15	213576	11.90418	**0.003570**
12	1	14940	15	213576	0.06995	0.795009

APPENDIX 10

Muscle Fiber Type Distribution Raw Data and ANOVA Tables

TYPE I % DISTRIBUTION

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	30.6	39.9
2	#2	Men	61.1	57.3
3	#4	Men	50.6	35.9
4	#5	Men	42.2	36.6
5	#6	Men	29.6	29.9
6	#10	Men	44.0	49.7
7	#11	Men	43.4	52.0
8	#12	Men	49.1	50.2
9	#55	Men	56.4	45.8
Mean	-	-	45.2	44.1
SD	-	-	10.6	9.0
10	#13	Women	58.2	51.8
11	#15	Women	56.1	53.7
12	#16	Women	42.3	41.4
13	#17	Women	49.6	44.7
14	#21	Women	58.4	59.4
15	#24	Women	35.2	48.6
16	#25	Women	47.4	35.7
17	#27	Women	35.4	51.3
Mean	-	-	47.8	48.3
SD	-	-	9.5	7.5

TYPE IIa % DISTRIBUTION

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	50.0	48.0
2	#2	Men	36.6	38.0
3	#4	Men	39.1	50.1
4	#5	Men	37.7	52.3
5	#6	Men	40.2	47.0
6	#10	Men	32.4	20.7
7	#11	Men	40.0	24.5
8	#12	Men	26.9	30.0
9	#55	Men	30.2	33.2
Mean	-	-	37.0	38.2
SD	-	-	6.7	11.7
10	#13	Women	32.5	40.2
11	#15	Women	36.3	33.9
12	#16	Women	41.2	46.0
13	#17	Women	36.7	45.5
14	#21	Women	36.0	33.6
15	#24	Women	33.4	35.3
16	#25	Women	29.8	28.9
17	#27	Women	33.4	26.7
Mean	-	-	34.9	36.3
SD	-	-	3.4	7.1

TYPE IIx % DISTRIBUTION

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	19.4	12.1
2	#2	Men	2.3	4.7
3	#4	Men	10.3	14.0
4	#5	Men	20.1	11.1
5	#6	Men	30.3	23.1
6	#10	Men	23.5	29.5
7	#11	Men	16.6	23.5
8	#12	Men	24.0	19.8
9	#55	Men	13.4	21.1
Mean	-	-	17.8	17.7
SD	-	-	8.3	7.7
10	#13	Women	9.2	8.0
11	#15	Women	7.5	12.4
12	#16	Women	16.4	12.6
13	#17	Women	13.7	9.8
14	#21	Women	5.6	7.0
15	#24	Women	31.4	16.1
16	#25	Women	22.8	35.4
17	#27	Women	31.2	22.0
Mean	-	-	17.2	15.4
SD	-	-	10.2	9.4

TYPE I % DISTRIBUTION

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	97.44059	15	131.558	0.740666	0.402994
2	1	0.70693	15	39.9279	0.017705	0.895914
12	1	5.27163	15	39.9279	0.132029	0.721407

TYPE IIa % DISTRIBUTION

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	34.49688	15	94.15946	0.366367	0.554039
2	1	13.65026	15	32.51363	0.419832	0.526815
12	1	0.05497	15	32.51363	0.001691	0.967745

TYPE IIx % DISTRIBUTION

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	16.42167	15	129.5258	0.126783	0.726749
2	1	7.83589	15	29.1426	0.268881	0.611648
12	1	6.13	15	29.1426	0.210345	0.653072

APPENDIX 11

Muscle Fiber Area Distribution Raw Data and ANOVA Tables

% TYPE I AREA

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	30.7	29.1
2	#2	Men	28.8	29.0
3	#4	Men	27.1	28.0
4	#5	Men	21.4	26.4
5	#6	Men	25.6	25.7
6	#10	Men	33.0	34.8
7	#11	Men	25.5	25.6
8	#12	Men	25.9	26.8
9	#55	Men	27.0	28.5
Mean	-	-	27.2	28.2
SD	-	-	3.3	2.8
10	#13	Women	33.9	33.0
11	#15	Women	25.2	29.8
12	#16	Women	28.4	29.2
13	#17	Women	32.0	33.2
14	#21	Women	35.2	37.7
15	#24	Women	39.4	39.8
16	#25	Women	29.1	28.2
17	#27	Women	28.2	28.6
Mean	-	-	31.4	32.4
SD	-	-	4.6	4.4

% TYPE II (a+x) AREA

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	69.3	70.9
2	#2	Men	71.2	71.0
3	#4	Men	72.9	72.0
4	#5	Men	78.6	73.6
5	#6	Men	74.4	74.3
6	#10	Men	67.0	65.2
7	#11	Men	74.5	74.4
8	#12	Men	74.1	73.2
9	#55	Men	73.0	71.5
Mean	-	-	72.8	71.8
SD	-	-	3.3	2.8
10	#13	Women	66.1	67.0
11	#15	Women	74.8	70.2
12	#16	Women	71.6	70.8
13	#17	Women	68.0	66.8
14	#21	Women	64.8	62.3
15	#24	Women	60.6	60.2
16	#25	Women	70.9	71.8
17	#27	Women	71.8	71.4
Mean	-	-	68.6	67.6
SD	-	-	4.6	4.4

% TYPE I AREA

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	150.4606	15	27.26529	5.518394	**0.032934**
2	1	8.4824	15	1.63859	5.176626	**0.037999**
12	1	0.0012	15	1.63859	0.00072	0.978940

% TYPE II (a+x) AREA

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	150.4606	15	27.26529	5.518394	**0.032934**
2	1	8.4824	15	1.63859	5.176626	**0.037999**
12	1	0.0012	15	1.63859	0.00072	0.97894

APPENDIX 12

Myosin Heavy Chain Isoform Contents Raw Data and ANOVA Tables

MHC I %

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	28.6	38.5
2	#2	Men	56.8	52.3
3	#4	Men	55.4	27.5
4	#5	Men	37.3	30.9
5	#6	Men	21.9	22.9
6	#10	Men	36.4	53.5
7	#11	Men	51.8	42.4
8	#12	Men	39.5	40.1
9	#55	Men	42.2	34.5
Mean	-	-	41.1	38.1
SD	-	-	11.9	10.4
10	#13	Women	47.5	44.6
11	#15	Women	57.3	50.5
12	#16	Women	36.1	34.9
13	#17	Women	43.3	40.8
14	#21	Women	64.4	62.0
15	#24	Women	43.7	52.8
16	#25	Women	53.5	36.8
17	#27	Women	29.2	35.4
Mean	-	-	46.9	44.7
SD	-	-	11.4	9.7

MHC IIa %

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	52.3	56.0
2	#2	Men	40.8	41.7
3	#4	Men	38.9	52.7
4	#5	Men	39.7	59.2
5	#6	Men	49.9	55.7
6	#10	Men	33.7	17.9
7	#11	Men	37.0	28.2
8	#12	Men	31.8	37.8
9	#55	Men	37.0	45.8
Mean	-	-	40.1	43.9
SD	-	-	6.9	14.0
10	#13	Women	45.7	51.4
11	#15	Women	38.4	39.0
12	#16	Women	45.1	40.8
13	#17	Women	45.2	54.2
14	#21	Women	28.8	28.9
15	#24	Women	41.7	38.6
16	#25	Women	21.4	27.5
17	#27	Women	33.9	45.4
Mean	-	-	37.5	40.7
SD	-	-	8.9	9.6

MHC IIx %

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-14
1	#1	Men	19.2	5.4
2	#2	Men	2.4	6.1
3	#4	Men	5.7	19.8
4	#5	Men	23.0	9.9
5	#6	Men	28.2	21.4
6	#10	Men	29.9	28.5
7	#11	Men	11.3	29.4
8	#12	Men	28.7	22.1
9	#55	Men	20.8	19.8
Mean	-	-	18.8	18.0
SD	-	-	10.2	9.0
10	#13	Women	6.8	4.0
11	#15	Women	4.3	10.6
12	#16	Women	18.8	24.3
13	#17	Women	11.5	5.0
14	#21	Women	6.8	9.1
15	#24	Women	14.6	8.6
16	#25	Women	25.2	35.7
17	#27	Women	36.9	19.2
Mean	-	-	15.6	14.6
SD	-	-	11.1	11.0

MHC I %

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	327.3624	15	180.4467	1.814178	0.198006
2	1	56.8947	15	57.5833	0.988041	0.335987
12	1	1.6524	15	57.5833	0.028695	0.867749

MHC IIa %

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	70.2856	15	170.0663	0.413283	0.530013
2	1	102.7788	15	38.7053	2.655418	0.124013
12	1	0.68	15	38.7053	0.017569	0.896314

MHC IIx %

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	94.1961	15	159.3332	0.591189	0.453897
2	1	6.90359	15	52.1941	0.132268	0.721167
12	1	0.18359	15	52.1941	0.003518	0.953489

APPENDIX 13

Peak Torque (Non-immob-leg) Raw Data and ANOVA Tables

ISOMETRIC PEAK TORQUE (N•m) - NON-IMMOBILIZED LEG

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	254.0	245.8	300.9
2	#2	Men	255.3	262.4	317.6
3	#4	Men	263.5	240.6	283.7
4	#5	Men	276.6	279.5	408.4
5	#6	Men	269.8	302.0	387.1
6	#7	Men	267.0	279.0	301.3
7	#8	Men	246.5	250.0	262.1
8	#9	Men	305.2	351.9	384.3
9	#10	Men	279.1	287.9	322.9
10	#11	Men	343.3	374.1	365.3
11	#12	Men	269.6	320.0	318.9
12	#55	Men	406.9	362.7	386.9
13	#66	Men	185.1	206.0	201.6
Mean	-	-	278.6	289.4	326.2
SD	-	-	52.3	51.1	59.1
14	#13	Women	206.9	206.2	188.2
15	#14	Women	191.5	217.9	219.7
16	#15	Women	169.9	153.6	163.5
17	#16	Women	138.9	175.2	169.2
18	#17	Women	137.0	168.1	149.1
19	#18	Women	161.1	160.3	177.6
20	#19	Women	220.8	229.3	218.3
21	#20	Women	146.9	145.5	145.1
22	#21	Women	202.0	220.1	219.0
23	#23	Women	130.7	145.5	122.9
24	#24	Women	216.1	228.2	213.3
25	#25	Women	158.2	166.7	178.0
26	#26	Women	172.8	185.1	216.6
27	#27	Women	245.4	230.7	260.4
Mean	-	-	178.4	188.0	188.6
SD	-	-	35.7	32.8	37.6

CONCENTRIC-SLOW PEAK TORQUE (N•m) - NON-IMMOBILIZED LEG

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	193.2	202.5	226.7
2	#2	Men	225.6	237.0	263.5
3	#4	Men	230.7	224.3	260.6
4	#5	Men	265.2	241.9	314.7
5	#6	Men	250.7	239.9	345.8
6	#7	Men	208.0	205.0	246.5
7	#8	Men	215.5	221.2	223.9
8	#9	Men	255.7	282.2	307.7
9	#10	Men	260.6	254.7	271.3
10	#11	Men	302.7	310.5	295.6
11	#12	Men	235.5	240.1	254.7
12	#55	Men	293.8	314.3	339.4
13	#66	Men	173.7	161.1	175.9
Mean	-	-	239.3	241.1	271.3
SD	-	-	37.4	42.8	48.6
14	#13	Women	177.4	191.5	192.8
15	#14	Women	198.1	203.4	209.5
16	#15	Women	141.6	137.0	145.5
17	#16	Women	112.7	138.0	137.8
18	#17	Women	119.7	121.5	128.1
19	#18	Women	153.6	138.4	133.8
20	#19	Women	204.2	175.2	186.9
21	#20	Women	126.4	133.4	143.1
22	#21	Women	174.8	184.0	175.5
23	#23	Women	115.1	126.4	92.2
24	#24	Women	177.6	168.1	172.3
25	#25	Women	134.5	147.9	150.8
26	#26	Women	172.8	153.2	187.9
27	#27	Women	168.4	195.3	192.1
Mean	-	-	155.5	158.1	160.6
SD	-	-	30.7	27.6	32.6

CONCENTRIC-FAST PEAK TORQUE (N•m) - NON-IMMOBILIZED LEG

SUBJECT N	STUDY CODE	GENDER	PRE	DAY-2	DAY-14
1	#1	Men	136.3	153.0	118.7
2	#2	Men	145.9	171.7	158.2
3	#4	Men	141.6	151.5	133.8
4	#5	Men	160.7	161.1	163.1
5	#6	Men	154.0	150.8	145.1
6	#7	Men	187.5	179.4	198.1
7	#8	Men	171.9	171.3	144.8
8	#9	Men	176.3	196.3	174.8
9	#10	Men	174.1	186.5	175.9
10	#11	Men	216.6	230.0	250.5
11	#12	Men	222.9	185.4	184.0
12	#55	Men	232.8	185.1	181.8
13	#66	Men	104.1	106.3	103.1
Mean	-	-	171.1	171.4	164.0
SD	-	-	37.0	29.2	37.7
14	#13	Women	104.8	110.9	118.9
15	#14	Women	115.8	146.6	135.6
16	#15	Women	93.2	94.2	87.6
17	#16	Women	69.6	61.4	61.0
18	#17	Women	124.6	119.1	97.5
19	#18	Women	92.9	83.7	85.4
20	#19	Women	89.4	131.7	122.9
21	#20	Women	105.6	84.3	86.8
22	#21	Women	113.8	113.0	120.4
23	#23	Women	110.5	91.1	108.3
24	#24	Women	125.7	156.8	141.6
25	#25	Women	103.1	91.1	105.2
26	#26	Women	98.9	111.2	127.2
27	#27	Women	135.6	140.9	152.6
Mean	-	-	106.0	109.7	110.8
SD	-	-	17.1	27.4	25.3

ISOMETRIC PEAK TORQUE (N•m) - NON-IMMOBILIZED LEG

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	258382.5	25	5413.567	47.72869	**0.000000**
2	2	5797.4	50	390.987	14.82761	**0.000009**
12	2	3051.6	50	390.987	7.80481	**0.001122**

MAIN EFFECT: GENDER

	{1} 298.0718	{2} 185.0357
Men {1}		**0.000149**
Women {2}	**0.000149**	

MAIN EFFECT: TIME

	{1} 228.5253	{2} 238.7027	{3} 257.4332
PRE {1}		0.151899	**0.000129**
DAY-2 {2}	0.151899		**0.003056**
DAY-14 {3}	**0.000129**	**0.003056**	

INTERACTION: 1 x 2

	{1} 278.6077	{2} 289.3769	{3} 326.2308	{4} 178.4429	{5} 188.0286	{6} 188.6357
Men {1		0.733606	**0.000143**	**0.000142**	**0.000142**	**0.000142**
Men {2	0.733606		**0.000369**	**0.000142**	**0.000142**	**0.000142**
Men {3	**0.000143**	**0.000369**		**0.000142**	**0.000142**	**0.000142**
Women {4}	**0.000142**	**0.000142**	**0.000142**		0.79302	0.747961
Women {5}	**0.000142**	**0.000142**	**0.000142**	0.79302		1
Women {6}	**0.000142**	**0.000142**	**0.000142**	0.747961	1	

CONCENTRIC-SLOW PEAK TORQUE (N•m) - NON-IMMOBILIZED LEG

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	173033.9	25	3699.752	46.76905	**0.000000**
2	2	2760.2	50	211.541	13.0481	**0.000028**
12	2	1668.1	50	211.541	7.88528	**0.001056**

MAIN EFFECT: GENDER

	{1} 250.5615	{2} 158.0595
Men {1}		**0.000149**
Women {2}	**0.000149**	

MAIN EFFECT: TIME

	{1} 197.3964	{2} 199.6118	{3} 215.9234
PRE {1		0.842076	**0.000182**
DAY-2 {2}	0.842076		**0.000517**
DAY-14 {3}	**0.000182**	**0.000517**	

INTERACTION: 1 x 2

	{1} 239.3	{2} 241.1308	{3} 271.2538	{4} 155.4929	{5} 158.0928	{6} 160.5928
Men {1		0.99956	**0.000151**	**0.000142**	**0.000142**	**0.000142**
Men {2	0.99956		**0.000174**	**0.000142**	**0.000142**	**0.000142**
Men {3	**0.000151**	**0.000174**		**0.000142**	**0.000142**	**0.000142**
Women {4}	**0.000142**	**0.000142**	**0.000142**		0.996934	0.937495
Women {5}	**0.000142**	**0.000142**	**0.000142**	0.996934		0.997454
Women {6}	**0.000142**	**0.000142**	**0.000142**	0.937495	0.997454	

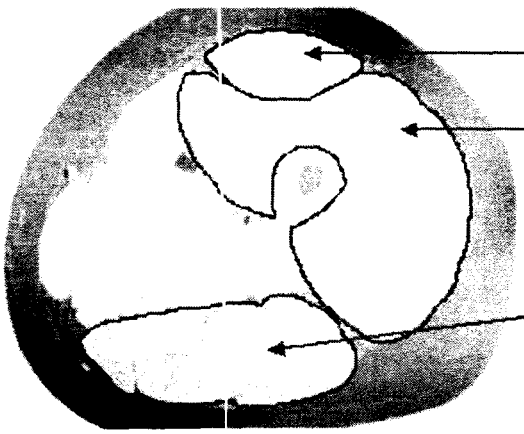
CONCENTRIC-FAST PEAK TORQUE (N•m) - NON-IMMOBILIZED LEG

1-GENDER, 2-TIME

	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	72860.02	25	2284.055	31.89941	**0.000007**
2	2	69.64	50	170.101	0.40942	0.666239
12	2	255.25	50	170.101	1.50061	0.232865

APPENDIX 14

Circling Area in MRI scans



Rectus femoris

Vastus (vastus lateralis, vastus intermedius, vastus medialis)

Hamstrings

APPENDIX 15

ATPase Histochemical Protocol

ATPase HISTOCHEMICAL ANALYSIS

REFERENCE: Dubowitz, V. Muscle Biopsy: A practical approach 2ed. London: Bailliere Tindall, 1985.

ADAPTED BY: Snow, R. J. School of Health Sciences, Deakin University, Australia.

PRINCIPLE

The tissue section is incubated in a solution containing ATP and calcium. The enzyme ATPase splits off the terminal phosphate from ATP and, because of the presence of calcium in the solution, this phosphate immediately combines to form calcium phosphate. At an alkaline pH, calcium phosphate is insoluble and is therefore deposited at the site of enzyme activity. The tissue section is then placed in a solution of cobalt chloride. The cobalt is exchanged for the calcium, forming cobalt phosphate at the sites where calcium phosphate was previously present.

The tissue is then placed in ammonium sulphide and this results in the formation of black, insoluble cabaltous sulphide. The site of enzyme activity originally present is thus demonstrated.

PART A: CUTTING MUSCLE

1) Store OCT mounted muscle at -80°C.
2) Prior to cutting muscle, place mounted muscle in cyostat for at least 15 to reach -20°C.
3) Trim the OCT covered portion of the sample at 30µm/cut. Once muscle sample is exposed, reduce thickness of cut to 10µm.
4) Cut 2-3 samples per slide.
5) Cover slides with paper towel and allow slides to dry overnight at 4°C.
6) Once dried, wrap slides in aluminum foil and store at -80°C until further analysis.

PART B: PREPARATION OF SOLUTIONS

Alkaline Stock Solution, pH 9.4

Reagent	*Manufacturer*	*Quantity*
① Glycine	BioShop Biotechnology Grade – GLN 001	2.8163 g
② $CaCl_2 \bullet 2H_2O$	BDH 10070/EM Science 10070 – 34	3.00 g

③ NaCl	Bioshop Reagent Grade – SOD 002	2.1938 g
④ NaOH	BDH Analytical Reagent ACS 816	1.3500 g
⑤ Distilled H_2O		500 mL

1) Dissolve reagents in distilled H_2O and bring to volume.
2) Calibrate pH meter prior to adjusting pH to 9.4 with conc. HCl/5M KOH.
3) Store stock solution in fridge (4°C).

Acid Preincubation Stock Solution, pH 4.6

Reagent	*Manufacturer*	*Quantity*
① Potassium Acetate	EM PX 1330-1	2.45 g
② $CaCl_2 \bullet 2H_2O$	BDH 10070/EM Science 10070 – 34	1.30 g
③ Distilled H_2O		500 mL

1) Dissolve reagents in distilled H_2O and bring to volume.
2) Calibrate pH meter prior to adjusting pH to 4.6 with glacial acetic acid.
3) Store stock solution in fridge (4°C).

* 5M NaOH (MW: 40.00 g/mol) – Dissolve 20.00 g in 100 mL
* 5M KOH (MW: 56.11 g/mol) – Dissolve 28.055 g in 100 mL

(I) Alkaline Preincubation Solution
1) Remove alkaline stock solution from fridge and allow stock solution to reach room temperature.
2) Adjust pH of an appropriate volume (50 mL) of alkaline stock solution to 10.50 using 5M NaOH (This should be done with continuous mixing and a pH meter sensitive to 0.001 pH units).

(II) Acid Preincubation Solution
1) Remove acid stock solution from fridge and allow stock solution to get to reach room temperature.
2) Adjust pH of an appropriate volume (50 mL) of acid stock solution to 4.30, 4.54, 4.60 with glacial acetic acid (This should be done with continuous mixing and a pH meter sensitive to 0.001 pH units).

(III) ATP Preincubation Solution, pH 9.4 (PREPARE FRESH ONLY)
1) Add 170 mg of ATP (SIGMA A2383) to 100 mL volumetric flask and bring up to volume using ALKALINE STOCK SOLUTION.
2) Adjust pH to 9.4.
3) Keep in fridge (4°C) until ready for use.

(IV) 1% Calcium Chloride Stock Solution
1) Dissolve 10 g of $CaCl_2 \bullet H_2O$ in 1000 mL volumetric flask distilled H_2O and bring up to volume.
2) Store at room temperature.

(V) 2% Cobalt Chloride
1) Dissolve 5 g of $CoCl_2 \bullet 6H_2O$ in 250 mL volumetric flask using distilled H_2O and bring up to volume.
2) Cover in aluminum foil and store at room temperature.

(VI) 1% Ammonium Sulfide (PREPARE FRESH DAILY)
1) Add 5 mL of 20% ammonium sulfide solution to 100 mL volumetric flask.
2) Bring to volume.
3) Store in fume hood until ready to use.

PART C: FIBER TYPE STAINING PROCEDURE

1) Incubate the sections in acid preincubation solutions adjusted to a pH of 4.30, 4.54 and 4.60; and alkaline preincubation solution adjusted to pH of 10.50 at the following time periods:

pH	Incubation time (min)
10.50	25
4.54	7.5
4.60	6.5
4.30	5.0

2) Transfer slides into plastic staining trough.

3) Rinse slides in distilled water 3 times.

4) Incubate slides in ATP incubation solution for 45 minutes at 37°C. This should be done in a temperature-controlled shaker.

5) Rinse slides in distilled water 2 times.

6) Incubate slides in 1% $CaCl_2 \bullet 2H_2O$ (Calcium Chloride) for 3 minutes at room temperature.

7) Rinse slides in distilled water 5 times.

8) Incubate slides in 2% $CoCl_2 \bullet 6H_2O$ (Cobalt Chloride) for 3 minutes at room temperature.

9) Rinse slides with distilled water 5 times.

10) Incubate slides in 1% ammonium sulfide for 1 minute at room temperature.

11) Rinse slides in distilled water 5 times.

12) Dehydrate tissue for 2 minutes in each alcohol concentrations (70, 80, 90, 95

and 100% ethanol).

13) Clear sections with xylene. Do twice in clean xylene @ 2 minutes.

14) Blot off excess xylene using Kimwipes. Mount the coverslips on slides using Permount (Fisher SP15-100). Allow Permount to dry (~ 1h). Store slides in the dark when not in use.

PART D: CAPTURING IMAGES AND IMAGE ANALYSIS

1) Turn on camera and microscope and allow warming up for 5 min.

2) Focus image at 4x magnification.

3) Refocus image at 20x magnification to calculate fiber area (μm^2).

4) Open SPOT Advanced software.

5) Click "Get Image" icon to capture image.

6) Click "Focus" icon to refocus image.

7) Save image as jpg file.

8) Capture 3-4 images per sample.

9) When finished with microscope and camera:
   a) Remove slide, lower platform, turn off camera, then microscope.
   b) Replace lens and dust covers.

10) Use Image Pro Plus to determine fiber area (μm^2).

Appendix 16

MHC Protocol

RESOLUTION OF MYOSIN HEAVY CHAIN ISOFORMS USING SDS-PAGE

References:

1) Staron, R.S., Hagerman, F.C., Hikida, R.S., Murray, T.F., Hostler, D.P., Crill, M.T., Ragg, K.E. and Toma, K. Fiber type composition of the vastus lateralis muscle of young men and women. *J. Histochem. Cytochem. 48(5):623-629, 2000.*

2) Talmadge, R.J. and Roy, R.R. Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. *J. Appl. Physiol. 75:2337-2340, 1993.*

3) Bamman, M.M., Clarke, M.S.F., Talmadge, R.J. and Feeback, D.L. Enhanced protein electrophoresis technique for separating human skeletal muscle myosin heavy chain isoforms. *Electrophoresis. 20:466-468, 1999.*

4) BIO-RAD Mini-PROTEAN 3 Cell Instruction Manual.

5) BIO-RAD PowerPac HC Power Supply Instruction Manual.

Principle:

Myosin heavy chain proteins (MHCs) are the predominant protein in human skeletal muscle. There are three MHC isoforms in human muscle: MHCI, MHCIIa and MHCIIx. Each isoform has a slightly different molecular weight (IIx > IIa > I) which allows for separation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). With clear resolution, the relative amounts of each isoform can be quantified using laser densitometry and pre/post samples can be analyzed for fibre type shifting.

Reagents:

1) Glycerol
   Sigma G-8733 (MW 92.09)

2) 2-Mercaptoethanol
   Sigma M-6250 (MW 78.13)

3) Lauryl Sulfate (SDS)
   Sigma L-3771 (MW 288.4)

4) Trizma Base
   Sigma T-6066 (MW 121.1)

5) Glycine
   Sigma G-8898 (MW 75.07)

6) EDTA
   Sigma ED2SS (MW 372.2)

8) Acetic Acid
   Stores

9) Brilliant Blue R
   Sigma B-0149 (MW 826.0)

10) Bromphenol blue
    Sigma B-8026 (MW 691.9)

11) TEMED
    Sigma T-9281 (MW 116.2)

12) Ammonium Persulfate (APS)
    Sigma A-3678

13) ProtoGel
    (37.5:1 Acrylamide to

Bisacrylamide)

7) Methanol — National Diagnostics EC-890
   Stores

Equipment:

1) BIO-RAD Mini-PROTEAN 3 Cell:
   a) 1mm Spacer Plates
   b) Short Plates
   c) Casting Frame
   d) Casting Stand
   e) 1mm Combs
   f) Buffer Dam
   g) Gel Releaser
   h) Electrode Assembly
   i) Clamping Frame
   j) Mini Tank and Lid
2) BIO-RAD PowerPac HC Power Supply
3) Epson Expression 1600Pro Colour Image Scanner & GeneTools Software
4) Promega Gel Drying Kit

Stock Solutions:

1) **1.5M Tris-HCl (pH 8.8)**
   - Add 18.165g Trizma base to 80mL of Milli-Q water (MQW)
   - Adjust to pH 8.8 with concentrated HCl
   - Bring volume to 100mL with MQW
   - Store at 4°C for upto one month

2) **0.5M Tris-HCl (pH 6.8)**
   - Add 6.055g Trizma base to 80mL MQW
   - Adjust pH to 6.8 with concentrated HCl
   - Bring volume to 100mL with MQW
   - Store at 4°C for upto one month

3) **1M Glycine**
   - Add 7.507g glycine to MQW and bring volume to 100mL
   - Store at 4°C for upto one month

4) **100mM EDTA (pH 7.0)**
   - Add 3.722g EDTA to 80mL MQW
   - Adjust to pH 7.0 with NaOH
   - Bring volume to 100mL with MQW
   - Store at 4°C for upto one month

5) **10% Ammonium Persulfate (APS)**
   - Dissolve 0.1g of APS in 1mL MQW
   - Store at 4°C for upto one week

6) **10% SDS**
   - Dissolve 10g SDS in 100mL MQW
   - Store at room temperature

7) **10X SDS-PAGE Running Buffer**
   - Add 30.275g Trizma base, 144.13g glycine and 10g SDS to 900mL MQW
   - Bring volume to 1L using MQW
   - Store at room temperature

8) **Brilliant Blue Stain Solution**
   - Mix 2g of Brilliant blue with 500mL methanol and 30mL acetic acid
   - Bring volume to 1L using MQW
   - Store at room temperature

9) **Gel Destain Solution**
   - Mix 400mL methanol with 140mL acetic acid
   - Bring volume to 2L using MQW
   - Store at room temperature

10) **Gel Drying Solution**
   - Mix 400mL methanol, 100mL glycerol and 75mL acetic acid
   - Bring volume to 1L using MQW
   - Store at room temperature

Lysis Buffer:

10% glycerol
5% 2-mercaptoethanol
2.3% SDS
62.5mM Tris-HCl buffer (pH 6.8)
- Mix 10mL glycerol, 5mL of 2-mercaptoethanol, 23mL of 10% SDS (or 2.3g of 100% SDS powder), 12.5mL of 0.5M Tris-HCl pH 6.8 and bring volume to 100mL using MQW
- Store at 4°C for upto one month

Dyed Sample Buffer:

30% glycerol
5% 2-mercaptoethanol
2.3% SDS
62.5mM Tris-HCl buffer (pH 6.8)
0.01% Bromphenol blue
- Mix 3mL glycerol, 500µL of 2-mercaptoethanol, 2.3mL of 10% SDS (or 0.23g of 100% SDS powder), 1.25mL of 0.5M Tris-HCl pH 6.8, 1mg bromphenol blue and bring volume to 10mL using MQW
- Aliquot 1mL samples into eppendorf tubes, keeping one tube as a working solution at 4°C and store the rest at -20°C

Sample Sectioning and Lysing Procedure:

1) Place eppendorph tubes in cryostat to cool.

2) Cut 7-8 sections at 20µm thick for each sample and place in bottom of tube using a toothpick. Keep the sectioned tissue cool in cryostat until ready to lyse.

3) Add 300µL of chilled lysis buffer to each sample tube. Quickly vortex and place immediately into a water bath at 60°C for 10 minutes.

4) Snap freeze in liquid nitrogen and store at -80°C.

Glass Plate and Casting Stand Assembly:

NB: For further details including figures, refer to the BIO-RAD Mini-PROTEAN 3 Cell Instruction Manual, pg. 5-7.

1) Clean glass spacer plates and glass short plates with MQW and dry with a Kimwipe. A single gel requires one spacer plate and one short place. If preparing two gels, clean two spacer plates and two short plates.

2) Place the casting frame upright on a flat surface with the pressure cams in the open position (facing outwards).

3) Orient the spacer plate so that the labelling is "up" and place a short plate on top of it. Slide the two glass plates into the casting frame, keeping the short plate facing the front of the frame (side with pressure cams). **Ensure that both plates are flush on a level surface (i.e. bench top) to prevent leaking.**

4) When glass plates are in place, engage the pressure cams to secure the plates in the casting frame. This assembly is referred to as the gel cassette. Re-check that both plates are flush at the bottom.

5) Engage the spring loaded lever and place the gel cassette on the grey casting stand gasket. Ensure the horizontal ribs on the back of the casting frame are flush against the face of the casting stand and the glass plates are perpendicular to the level surface. Release the lever to push the spacer plate down against the grey rubber gasket.

6) For a second gel, repeat steps 1 – 5.

Gel Casting:

Note: Acrylamide is a known neurotoxin and should be handled carefully with gloves at all times.

1) Place a clean 1mm comb between plates of the assembled gel cassette. Measure 1cm down from the comb teeth and mark with permanent marker. This is the level to which the separating gel is poured. Remove the comb.

2) Mix the components of the 8% separating gel and 4% stacking gel in separate 15mL plastic screw-cap tubes. Add the components in the order listed below. At this point **do not add TEMED or 10% APS** to either gel mixture. Additionally, since acrylamide is light sensitive, do not add it, or SDS, to the 4% stacking gel mix until step 7. Keep the 4% mix on ice until ready to use. The recipe below is adequate to pour two gels:

Solution	8% Separating Gel (mL)	4% Stacking Gel (mL)
100% Glycerol	3.000	3.000
Milli-Q Water	1.565	3.396
(Vortex thoroughly until glycerol mixes uniformly with water)		
1.5M Tris (pH 8.8)	1.333	----------
0.5M Tris (pH 6.8)	----------	1.400
1M Glycine	1.000	---------
100mM EDTA (pH 7.0)	----------	0.400
~30.8% Acrylamide-bis	2.597	1.299
10% SDS	0.400	0.400
TEMED	0.005	0.005
10% APS	0.100	0.100
Total	10.00	10.00

3) Add TEMED and 10% APS to the **8% separating gel mixture** and invert the tube a couple of times to mix. Use a long 5mL or 10mL disposable pipette to

draw up the gel mixture and carefully pipette the mix between the glass plates of the gel cassette assembly on the casting stand. Try to avoid air bubbles. Pour the gel mix until it reaches the mark made in step one.

4) Immediately overlay the freshly poured gel mixture with MQW using a pre-loaded syringe. Be sure to apply the MQW gently and evenly by moving the syringe back and forth along the length of the plate. Ensure that a smooth even surface is obtained (you can see a line separating the MQW from the gel).

5) Allow the gel to polymerize for approximately **1 hour**. Use the left-over gel in the plastic tube as a guide (this should be sealed and sitting at room temperature).

6) Once gel is set, pour off the MQW and rinse with additional MQW. Then, slide a Kimwipe between the glass plates to ensure that plates above the gel are as dry as possible. Do not touch the surface of the gel.

7) Add TEMED and 10% APS to the **4% stacking gel mixture** and invert tube a couple of times to mix. Use a long 5mL or 10mL disposable pipette to draw up the gel mixture and carefully pipette the mix into the gel cassette assembly containing the polymerized 8% separating gel. Pour the gel mix until it reaches the top of the short glass plate.

8) Immediately, insert a 10-well, 1mm comb between the glass plates. It is easiest to insert the comb starting at an angle by inserting well 1 first, then 2, 3, and so on until the comb is completely inserted.

9) Allow gel to polymerize for approximately **45 mins**, again using the left over gel as a guide. If desired, at this point, the gel can be stored overnight and run the following day. To store the gel, remove it from casting stand and casting frame and wrap in wet paper towel, place in a plastic bag and store overnight at 4°C.

10) Once gel is polymerized, carefully pull comb straight out. Wipe any pieces of residual gel from plates using a Kimwipe. Immediately flush out wells with 1X SDS-running buffer using a 22G syringe. **Do not use too much force** or wells will break. Continue to flush until wells are **completely free** from residual glycerol.

Electrophoresis Module Assembly:

Note: All running buffers should pre-chilled at 4°C prior to use

1) Remove gel cassette assembly from casting stand. Rotate pressure cams of the casting frame inward to release the gel cassette containing the polymerized gel.

2) Place the gel cassette into the slots at the bottom of each side of the electrode assembly. **Be sure that the short place of the gel cassette faces inward**

toward the notches of the green u-shaped gaskets. *Note: If only running one gel, insert buffer dam plate on one side of the electrode assembly.*

3) With the gel cassettes flush against the green gasket, place the electrode assembly into the clamping frame. Press down on the electrode assembly while closing the two cam levers of the clamping frame to form the inner chamber. **Ensure that the short place is firmly aligned with the gasket.**

4) Fill the inner chamber with ~125mL of 3X SDS running buffer until the level reaches halfway between the tops of the tall and short plates. **Ensure that the inner chamber is not leaking** by leaving it on a dry section of the lab bench for a few minutes to monitor for any leaks.

5) Lower the inner chamber assembly into the mini tank. Add ~200mL of 1X SDS running buffer to the mini tank (lower buffer chamber).

Sample Preparation:

Note: For both methods A and B, once samples are thawed on ice they should be vortexed to mix and briefly spun down to remove air bubbles. After the addition of dyed sample buffer to the diluted samples, the mixtures should be boiled at 100°C for 2mins and then briefly spun down. .

Method A: Identical protein loads

NB: If desired, samples can be prepared so that protein loads are identical. This requires use of the Bradford Assay to determine the protein concentration of each sample (see Bradford Assay Protocol). This, however, is not essential because the percentage expression of MHCs is a relative measurement.

The desired amount of protein to load per well/lane is approximately **2µg**. Using the results from the Bradford protein assay, determine the volume of sample that will give 2µg of total protein.

In most cases, samples will be loaded in **duplicate**. To reduce variability between duplicates make up a single mixture at a volume sufficient for both duplicates. A sample calculation is provided below:

[Sample] = 1.31µg/µL

Want to have 2µg of total protein in each of two 20µL sample aliquots
2µg / 20µL = 0.1µg/µL (desired concentration)

Want to make up sample in 50µL total to give enough for 2x20µL (with 10µL extra)
50µL x 0.1µg/µL = 5µg of protein

Now determine how much of the 1.31µg/µL sample is needed to give 5µg:

5µg / 1.31µg/µL = 3.82µL of sample

To make a total of 50µL mix as follows:
- 3.82µL sample
- 21.18µL lysis buffer
- 25µL dyed sample buffer

Note that dyed sample buffer comprises 50% of the total volume (i.e 25µL) and the remainder of volume is filled with lysis buffer (i.e. 25µL – 3.82µL = 21.18µL)

The final 50µL mix contains 5µg of total protein at a concentration of 0.1µg/µL. Thus, a 20µL aliquot contains 2µg of total protein.

Method B: Non-identical protein loads

NB: This is a more empirical method and in some cases samples that have extremely high or low concentrations may have to be re-run due to protein overloading or insufficient protein, respectively.

As above in method A, prepare a single mixture at a volume sufficient for duplicate samples:
- 15µL sample
- 10µL MQW
- 25µL dyed sample buffer

Sample Loading:

1) Before loading, flush out the wells again using 1X SDS running buffer to ensure there is **no glycerol in the wells**.

2) Rinse each well again immediately prior to loading (i.e. rinse well 1 then load well 1, rinse well 2 then load well 2). Load 20µL of each sample per well (in duplicate) using gel-loading tips. It is essential to use a gentle pipetting technique to avoid spilling sample into the adjacent wells. Also, be careful not to puncture the bottom of the well with the tip. If desired, use Bio-Rad's patented sample loading guide to aid with loading. Place the guide between the two gels in the electrode assembly and use the marked lanes as guides. *(NB: do not load samples in wells one and ten; instead load 20µL of dyed sample buffer).*

3) Once samples are loaded, check the buffer level of the inner chamber and adjust accordingly.

Gel Electrophoresis:

1) Place the lid on the mini tank. Make sure to align with colour coded plus and jacks, with red going to red and black to black.

5µg / 1.31µg/µL = 3.82µL of sample

To make a total of 50µL mix as follows:
- 3.82µL sample
- 21.18µL lysis buffer
- 25µL dyed sample buffer

Note that dyed sample buffer comprises 50% of the total volume (i.e 25µL) and the remainder of volume is filled with lysis buffer (i.e. 25µL – 3.82µL = 21.18µL)

The final 50µL mix contains 5µg of total protein at a concentration of 0.1µg/µL. Thus, a 20µL aliquot contains 2µg of total protein.

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As above in method A, prepare a single mixture at a volume sufficient for duplicate samples:
- 15µL sample
- 10µL MQW
- 25µL dyed sample buffer

Sample Loading:

1) Before loading, flush out the wells again using 1X SDS running buffer to ensure there is **no glycerol in the wells**.

2) Rinse each well again immediately prior to loading (i.e. rinse well 1 then load well 1, rinse well 2 then load well 2). Load 20µL of each sample per well (in duplicate) using gel-loading tips. It is essential to use a gentle pipetting technique to avoid spilling sample into the adjacent wells. Also, be careful not to puncture the bottom of the well with the tip. If desired, use Bio-Rad's patented sample loading guide to aid with loading. Place the guide between the two gels in the electrode assembly and use the marked lanes as guides. *(NB: do not load samples in wells one and ten; instead load 20µL of dyed sample buffer).*

3) Once samples are loaded, check the buffer level of the inner chamber and adjust accordingly.

Gel Electrophoresis:

1) Place the lid on the mini tank. Make sure to align with colour coded plus and jacks, with red going to red and black to black.

2) Insert the electrical leads with the proper polarity into the PowerPac HC power supply. Select amperes (A) as the constant parameter.

3) Run the gels at 20mA (0.02A) for 10-15mins to allow the samples to enter the stacking gel.

4) Transfer the unit to an ice packed styrofoam cooler and seal closed with tape.

5) Run the gels overnight at 10mA (0.01A) for approximately 17 -18 hours.

Gel Staining:

1) After 17-18 hours, turn off power supply and remove inner chamber assembly. Pour off and discard running buffer.

2) Open cams of the clamping frame and pull electrode assembly out of the clamping frame and remove gel cassette assemblies. Using the green, wedge-shaped plastic gel releaser, carefully pry open glass plates. The gel will adhere to one of the plates. Trim off the stacking gel using the gel releaser and mark the top right corner of the gel to note orientation.

3) Scoop the gel off the glass plate (using gel releaser) and allow it to slide into a pipette-tip box containing enough Brilliant Blue stain to cover the gel. Place on rocker for about 45 minutes (time not crucial). *(NB: Alternatively, if it is the end of the day, make a solution of half stain and half destain and allow gel to rock in it over night then use 100% destain the next day).*

4) After sufficient incubation, pour off the stain into a bottle for re-use. Cover the gel with destain solution and place on rocker. For the first 3 washes, change destain approximately every 3-5mins. After that, change destain about every 30mins until bands are a prominent blue / purple colour and the background has minimal colour.

5) After sufficient destaining, pour off destain, rinse gel with MQW and store in MQW until ready to scan or dry. At this point, there should only be three bands present. From top (lowest electrophoretic mobility) to bottom (greatest electrophoretic mobility) the bands are: IIx, IIa and I.

Gel Analysis using GeneTools Software:

NB: For gel analysis, ensure that reflective document mat has been removed from the scanner

1) Place gel on scanner's document table and gently close lid.

2) Open *GeneTools* software from desktop. To scan the gel, select *File menu* and *Acquire (Twain).* Two windows will appear. One window contains the settings for

the document to be scanned and other window contains a preview of the scan. Select the following settings: *Document Source* – TPU for Pos. Film, *Image Type* – 24bit colour (Stc), *Destination* – Epson Stylus Printer (Photo), *Resolution* – 300. Select *Preview*. A preview of the gel will now appear in the second window. To crop the image and focus only on the 3 desired bands, use the cursor to draw a box around the desired area and select *Preview* in the second window. Once the desired image is obtained, return to the first window and select *Scan*.

3) Once scan is complete a *Sample Properties* window will appear. The software will automatically detect the *Image Type* as absorption. The direction of electrophoresis should be checked as *down* and the type of analysis should be checked as *gel*. Enter the *number of tracks* (wells / lanes in gel). If a number is not entered here, the software will automatically detect the number of lanes on its own. Select *OK*.

4) The scanned gel image will now appear with tracks and bands defined. It is often necessary to adjust the location and size of the tracks. Do this by using the toolbar on the top left of the screen. Once the tracks are appropriately set, select *Lock All Tracks*. After doing so, data and graphs for the tracks will appear. The software should detect the 3 desired bands in each lane. If not, select the track and double click on a band to add it. In the results section, select *Results for all Tracks*. Right click on the data and add *Raw Volume Percentage*.

5) Click the *Excel* icon on the toolbar and all data will automatically be exported to an Excel worksheet for statistical analysis.

Gel Drying Procedure:

NB: It is most efficient to dry 4 – 6 gels at one time.

1) Soak stained gel in gel drying solution for 3 – 5 minutes.

2) Moisten one sheet of gel drying film in gel drying solution and tightly clamp to gel drying frame.

3) Place gel on clamped gel drying film and remove any air bubbles.

4) Moisten a second sheet of gel drying film in gel drying solution and tightly clamp to gel drying frame over gel, trying to avoid air bubbles.

5) Hang frame in vertical position and allow to dry completely.

6) Cut out dried gel and tape in lab book.