University of Alberta

The Effect of Spaceflight on the Patterns of Myosin Heavy Chain and Cross Sectional Area in Rat Crural Diaphragm

by

Gregory Hansen 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

Slow-to-fast fibre transitions and fibre atrophy are typical ultrastructure adaptations of especially slow muscles with short-term (i.e. 4-22 days) exposure to microgravity. However, the effects of spaceflight on respiratory muscles have not been established. The purpose this study was to quantitatively identify potential fibre transitions and muscle atrophy in the diaphragm - the most important respiratory muscle. Five adult rats orbited earth aboard NASA's 14 day Spacelab Life Sciences II (SLS2) mission and were compared to a flight control group of five ground-based rats. Fibre type profile and fibre cross sectional areas (CSA) of the right crural region were immunohistochemically determined. No significant changes to myosin heavy chain (MHC) based fibre type distribution were detected, but there was a non-significant decrease in CSA of all pure fibres. These findings suggest that short duration spaceflight may not dramatically effect neuromuscular patterns of activity in rat diaphragm.

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List of Symbols, Nomenclature or Abbreviations

ABC	Avidin-Biotin-peroxidase Complex
Ar	Argon
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
BF-F3	IgM, anti-MHC_{IIB}
BF-45	IgG, anti-MHC_{emb}
CLFS	Chronic Low Frequency Stimulation
CSA	Cross Sectional Area
CO_2	Carbon dioxide
DAB	3'diaminobenzidine tetrahydrochloride
DNA	Deoxyribonucleic Acid
EDL	Extensor Digitorum Longus muscle
F88	IgG, anti-$MHC_{I\alpha}$
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kDA	kilo-Daltons
O_2	Oxygen
MHC	Myosin Heavy Chain protein
MLC	Myosin Light Chain protein
N_2	Nitrogen
NASA	National Aeronautics and Space Administration

NOQ7.5.4.D	IgG, anti-$MHC_{I\beta}$ and MHC_{Idev}
PAP	Perioxidase-antiperioxidase immunohistochemistry method
RAHF	Rodent Animal Holding Facilities
RNA	Ribonucleic Acid
mRNA	messenger Ribonucleic Acid
SC-71	IgG, anti-MHC_{IIA}
SLS2	Spacelab Sciences II Mission

Chapter One

Introduction

Ever since spaceflight missions have become a possibility, changes induced by weightlessness to mammalian system physiology have been observed. For example, the cardiovascular system declines in both aerobic capacity and orthostatic tolerance; there is a shift in fluid and electrolyte balance, hematocrit, and certain immune parameters; bone mass and muscle strength are reduced; and various neurological responses include space motion sickness and posture and gate alterations (Sulzman, 1996). While many of these physiological responses have been attributed to such factors as the hypokinesia of weightlessness or the unloading of the vestibular system (Sulzman, 1996), specific changes at the molecular level and the exact mechanism to account for these observable alterations has been elusive. However, despite the obvious limitations that exist in obtaining physiological spaceflight data and unavoidable constraints, increases of mission frequency, duration and vast improvements in laboratory techniques, procedures and technology have refined the qualitative research in this field (Roy *et al.*, 1996).

Skeletal muscle atrophy induced by microgravity has long been known, but only recently have molecular adaptations such as muscle protein, amino acid, DNA, RNA and myonuclear content been examined (Sulzman, 1996). These preliminary studies have postulated potential trends, but the mechanism(s) involved largely remains a mystery, and much more information on the muscle profile is required (Sulzman, 1996). Furthermore, while several skeletal muscles such as the soleus, gastrocnemius and tibialis anterior have been closely examined, surprisingly there is currently very little information regarding the diaphragm's plasticity in space.

Purpose

The purpose of this study was to examine some effects of spaceflight on rat crural diaphragm muscle. Five rats orbited earth aboard NASA's 14 day Spacelab Life Sciences II (SLS2) mission and were sacrificed shortly after entry. The right crural fibre type distribution and muscle fibre cross sectional area (CSA) was compared to a flight control group of five ground-based rats that were contained in identical animal holding facilities.

Significance of Study

The significance of examining myosin protein and fibre CSA with spaceflight diaphragm tissue is predominantly linked to two objectives. The first is to investigate musculoskeletal changes during microgravity (Bloomfield, 1997). By quantifying specific physiological adaptations to disuse, the diaphragm spaceflight data could validate known or suggest other regulatory mechanisms. Secondly, with this knowledge, effective countermeasures to minimize functional changes may be further defined (Desplanches, 1997).

While the soleus and other skeletal muscles have been thoroughly examined, little information is available regarding the effects of microgravity on the diaphragm. Any alterations to the diaphragm's ultrastructure could have functional significance, particularly at high work intensities, and may intensify pre-existing medical conditions (Bloomfield, 1997). Consequently, as technological advancements foster long-term mission planning, safety and health issues must be considered (Desplanches, 1997). Furthermore, since the spaceflight model exemplifies other models of disuse like bedrest (Bloomfield, 1997) clinical inferences may be made.

Hypothesis

The effects of spaceflight on MHC based fibre type distribution and CSA in other skeletal muscle tissue are consistent with other forms of disuse models, but are usually magnified. Consequently, as the diaphragm has demonstrated plasticity in previous studies (Sieck, 1994; Yang *et al.*, 1998), and its remarkable activation patterns may make it especially responsive to unloading (Sieck, 1994), the following was hypothesized for the right crural region:

a) decreased pure type I fibre expression at the protein level

b) increased transitional hybrid fibre expression at the protein level

c) decreased CSA, especially in the larger IIB fibres.

Delimitations

This study was essentially composed of two components: 1) NASA's initial experimental protocol with tissue processing, and 2) MHC-based fibre type analysis. Ten male Sprague-Dawley rats were randomly divided into two groups. Five of the rats (FL=14) were subjected to microgravity on a 14-day mission and decapitated shortly after landing. The other five were assigned to a ground-control groups (L=14) and were subjected to near-identical flight protocol. The right crural diaphragm of both groups was examined to identify the muscle fibre type profile, and CSA.

Limitations

Interpretations of the results obtained from spaceflight rats should be cautiously extrapolated, as the typically small sample size from one mission may not be representative, and usually offers a low power for statistical analyses. Generalizing the results to a larger population or to other missions of different duration is often not

recommended, but the results may propose mechanisms or predict future observations that can be later verified. However, in similar studies, small sample sizes i.e. five subjects, have been previously employed to demonstrate a biologically significant phenomenon (Campione *et al.*, 1993; Haddad *et al.*, 1993; Caiozzo *et al.*, 1996). Despite spatial restrictions within NASA's Spacelab, recommendations to increase the number of rats flown have been suggested (Roy *et al.*, 1996).

Secondly, immersion of muscle samples directly into liquid nitrogen creates bubbles, which slows the freezing process and causes ice crystal artifact and consequential micro-tearing (Loughlin, 1993). NASA scientists took some precautions by wrapping the diaphragm in aluminum foil, but preliminary observations through a microscope revealed crystal formation. This was partially counteracted by re-hydrating and re-freezing the muscle, but could not be completely eradicated. Furthermore, although each sample was eventually dehydrated in ascending ethanol, absolute CSA could be influenced by the aforementioned osmotic techniques. Relative CSA however, should not be compromised, as both groups were exposed to identical protocols.

Spaceflight as a model of disuse provides excellent conditions to decrease muscle workloads. Alternative approaches to mimic weightlessness such as water immersion, bed rest or limb suspension have been employed, but the deconditioning of the musculoskeletal system is best studied through lack of gravitational stresses (Stein & Gaprindashvili, 1994). On Earth, it is impossible to remove the tonic forces of gravity except during relatively brief episodes of free fall (Sulzman, 1996). However, because there are so many potential variables in each mission i.e. duration, animal care, time of

tissue processing, erroneous conclusions could be possible with combining data across dissimilar missions (Stein & Gaprindashvili, 1994).

Assumptions

Although it is not essential that the rat model perfectly parallels the human system in microgravity, in this study a subjective degree of homogeneity must be established. In vertebrates, Schiaffino and Reggiani (1996) observed that "the molecular composition and ultrastructure of the sarcomeres is remarkably similar among different types of muscle cells". Small discrepancies do exist between rat and human MHCI, but all myosin II's are structurally alike, possessing similar binding sites, coiled-coil α helix tails and a conserved head sequence (Sellers *et al.*, 1997). Nonetheless, while limited structural and functional data collected in astronauts after weightlessness have been qualitatively similar to those observed in rats, the relevance of examining animals in space has been strongly supported (Desplanches, 1997).

Although, the extent to which direct applications can be made to the human model remains somewhat speculative (Sieck, 1994), a greater understanding of the basic mechanisms and responses should facilitate the development of appropriate preventative strategies that have human implications (Campione, 1993). Ventilatory requirements of the diaphragm are comparable across mammalian species, as both possess a high type II fibre content (approximately 60% in human diaphragm) and most likely do not require the recruitment of fast motor neurons for normal force production during tidal breathing (Sieck, 1994).

Definitions

The operational definitions that will be used in this investigation include:

1. **Disuse, unloading and/or decreased neuromuscular activity**

   Decreased loading due to lack of usual weight bearing forces (immobilization in a shortened position, hindlimb suspension, microgravity) and the decrease in the number and/or magnitude (intensity) of muscle contractions due to decreased neuromuscular activity (denervation, detraining, spinal cord transection and isolation) (Bloomfield, 1997; Pette & Staron, 1997). In most instances, the muscles still receive some neural input, and are capable of isometric contractions (Bloomfield, 1997).

2. **Microgravity**

   The acceleration of gravity experienced in space: 1×10^{-6}g.

Chapter Two

Literature Review

Myosin Protein Physiology in Skeletal Muscle

Skeletal and cardiac muscle cells are primarily characterized by the basic contractile unit of the sarcomere; a highly organized overlapping arrangement of thick and thin protein filaments arranged in series within striated myofibrils (Schiaffino & Reggiani, 1996). During contraction, numerous globular cross-bridges that extend from "thick" myosin proteins bind to "thin" actin filaments after ATP is cleaved, generating a power stroke and a shortening of the sarcomere (Fox, 1993). At the end of the power stroke, each cross-bridge binds to an ATP molecule, breaking its bond with actin, and the resting orientation is again resumed.

Myosin, the molecular motor of skeletal muscle cells, is composed of two heavy chains (MHC) of approximately 200 kDa each (Reiser and Kline, 1998) and two pairs of light chains (MLC) that usually vary between 15 and 22 kDa (Schiaffino & Reggiani, 1996). The molecule is highly asymmetric, with the MHC component consisting of two separated elongated amino-terminal globular heads attached to a long two-coiled α-helix carboxyl-terminal tails (Yanagisawa *et al.*, 1987). Each globular head consists of a heavy chain fragment and two types of MLC: regulatory and essential light chains (Barany, 1996). The essential MLC is in contact with the parts of the molecule constituting the active site region, while regulatory MLC is bound to the C-terminal part of the MHC subfragment (Stepkowski, 1995). Together, they can be called the "regulatory domain" of the molecule, as opposed to the rest of the head – the "motor domain" (Stepkowski, 1995).

Furthermore, skeletal muscle is a very heterogeneous tissue comprised of different fibre types that have different metabolic and contractile properties (Pette & Staron, 1997). These fibre types are generally characterized by a specific MHC protein isoform, but hybrid fibres with more than one MHC isoform exist (Staron & Pette, 1993). In adult rats, for example, four major fibres types have been identified - three fast [type IIA, IIB, and IID/X)] and one slow (type I) – that directly correspond to MHCIIa, MHCIIb, MHCIId/x and MHCI respectively. MHCIβ is the predominant slow isoform in adult mammalian skeletal muscles (Schiaffino & Reggiani, 1996), but two others have also been identified. MHCIα predominates in rat atria and ventricles, muscle spindles (Putman *et al.*, 1999), and is expressed in rabbit diaphragm, while MHCIa has been identified in rabbit plantaris (Pette, 1998). Finally, developmental isoforms such as MHC_{emb} and MHC_{neo} are not only expressed during embryonic or neonatal stages and regeneration, but are also found in intrafusal fibres and specific adult muscles such as extraocular, masseter and tensor typani (Pette & Staron, 1997).

MLC isoforms have also been identified in mammalian striated muscles for both fast and slow fibres (Wada *et al.*, 1996). In adult rodent, it has been firmly established that fast fibres contain essential MLC-1f and MLC-3f and regulatory MLC-2f isoforms, and that slow fibres express essential MLC-1sa and MLC-1sb and regulatory MLC-2s isoforms (Wada *et al.*, 1996; Biral *et al.*, 1999).

Single muscle fibre experiments have established that type IIB fibres have the highest, type IID/X intermediate and IIA lower maximum shortening velocities (Bottenelli *et al.*, 1994; Li & Larsson, 1996). However, fibres with only one MHC isoform display significant variance in velocities, suggesting that the MHC and MLC

provide a continuum of shortening velocities within fast muscle fibres (Biral *et al.*, 1999). Type I fibres also display a significant variability in shortening velocities, that currently cannot be adequately explained by differences in MLC3f content, nor the relative influence of MLC1sa and MLC1sb isoforms (Biral *et al.*, 1999). In general, the speed characteristics of these fibres decrease in the order of type IIB > type IID/X > type IIA >> type I (Pette, 1998).

Fibre Type Plasticity

Because many biochemical, physiological and morphological characteristics of muscle are capable of transformations, muscle fibre types are usually not considered immutable (Guth, 1990). It is generally accepted, that when neuromuscular patterns are changed, the myonuclei has the ability to alter MHC expression, to meet the requirements of new functional demands (Henriksson *et al.*, 1990). Hybrid fibres express two or more MHC isoforms and are often found in combinations of either MHCIIb + MHCIId/x, MHCIId/x + MHCIIa, or MHCIIa + MHCI (Staron & Pette, 1987). Although these fibres represent a considerable percentage of some normal adult muscle (Pette & Sharon, 1997), they also suggest a continuum between type IIB and type I fibres that are bridged by a transitional phenotype between two pure fibres (Staron & Pette, 1993; Peuker & Pette, 1993).

Research with mammalian muscle fibre under specific exogenous (neural, mechanical and hormonal) conditions appears to support this model of muscle fibre transition. In summarizing the literature on fibre transitions, a general scheme has evolved (Figure 2-1):

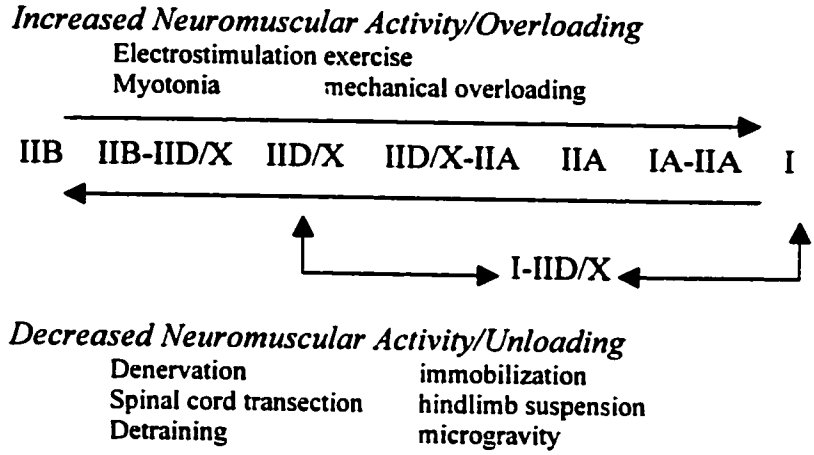

Figure 2-1. Factors influencing the fibre transition scheme (modified from Pette & Staron, 1997; and Talmadge, 2000). Models that increase neuromuscular activity elicit a gradual transformation towards slower MHC isoforms, whereas models that unload the muscle cause a transformation towards faster MHC isoforms. In both instances, the increased expression of hybrid fibres at the protein level represents a transitional phenotype between two pure fibres.

The linear step-wise transformation process occurs in both directions, depending upon the model that is employed. However, after spaceflight, hindlimb suspension, bedrest and spinal cord isolation or transection, fibres containing MHCI+MHCIId/x are found without MHCIIa, suggesting that the MHCIIa isoform is skipped (Talmadge, 2000). More specifically, it also appears that minor changes in loading demands cause limited MHC transformation responses, whereas strong and ongoing loading alterations elicit greater responses (Pette & Staron, 1997).

To a lesser degree, MLC expression is also somewhat responsive. Biral *et al.*, (1999) state that in male rat soleus muscle the MLC1sa content appeared to increase during aging in euthyroid animals, but hyperthyrodism reversed this trend. In other studies, Baumann *et al.*, (1987) found MLC1sb in type IIa vastus lateralis muscle after 8 weeks of bicycle training, while in fast rat muscle the mRNA of MLC1sb was induced after 28 days of electrostimulation (Kirschbaum *et al.*, 1989).

Disuse and Myosin Expression

For this study, the most applicable observations are derived from studies that examined or elicited disuse or unloading. Several models have been utilized to investigate disuse, ranging from a minimal reduction of activity to bedrest, unilateral lower limb suspension, microgravity and finally immobilization due to spinal cord injury and denervation (Bloomfield, 1997). MHC transformations have not been observed with 2 – 6 weeks of bed rest (Andersen *et al.*, 1999; Bamman *et al.*, 1998; Berg *et al.*, 1998) but this may indicate that a transition was not in progress at the protein level. Andersen *et al.*, (1999) found significant increases in the number of fibres expressing mRNA for MHCIId/x, and a decrease in the number of fibres expressing mRNA for MHCI, reflecting perhaps an incomplete transitional process that could be completed after longer periods of bed rest. However, for the implied ethical, scientific and practical issues of these studies, animals are usually utilized despite some MHC content discrepancies.

The effects of hindlimb suspension with rat soleus have been well documented, characterized by an increase in the relative amount of intermediate and fast MHC isoforms, and the emergence of MHC hybrids not seen in the controls (Campione *et al.*, 1993; Diffee *et al.*, 1991; Oishi *et al.*, 1994). Interestingly, while the synthesis of MHCIId/x was clearly observed after suspension and not in controls, it gradually decreased and disappeared after four weeks of recovery (Oishi *et al.*, 1994).

In contrast, hindlimb suspension on adult rat soleus muscle appears to elicit very little change to MLC composition (McDonald *et al.*, 1994). Loughna *et al.*, (1996) observed that muscle disuse has very little effect on mRNA levels of MLC1f in either fast or slow muscles, despite dramatic increases in fast MHC mRNA. However, with juvenile

6-week old rats, changes in MLC isoforms consisted of an increase in MLC1f and MLC2f concomitant with a decrease in MLC2s (Saitoh *et al.*, 1999). Saitoh *et al.*, (1999) hypothesize that MLC transitions may be age dependent, and that the suspended soleus muscles from young rats may acquire intermediate contractile properties between normal soleus and typical fast twitch fibres.

Myosin Protein Responses to Spaceflight

Dramatic adaptations of MHC with microgravity have also been observed as international joint missions have promoted extensive physiological investigations. The first experiment (Caiozzo *et al.*, 1994) on spaceflight MHC observed a slight reduction of MHCI and IIa isoforms with de novo expression of MHCIId/x in rat soleus. A small increase of fast type II and hybrid fibres was also exhibited on the short six-day mission. However, despite an apparently stable fibre profile, a significant increase of the maximal shortening velocity was observed, suggesting either limitations to their immunohistochemical procedures or unknown processes involved in muscle contraction. Furthermore, this "de novo" profile has been recently contested (Allen *et al.*, 1996) as very low levels of MHCIId/x have been observed in controls.

Research (Allen *et al.*, 1996; Caiozzo *et al.*, 1996) from a two-week mission demonstrated other adaptations, indicating perhaps the significance of longer spaceflight. Although both studies were conducted on similar (or identical) rats under very comparable conditions, the investigators utilized different analytical techniques, providing several different quantitative measurements. Through single fibre (~20-50/muscle) electrophoresis on rat soleus, Allen *et al.*, (1996) found a significant decrease in the percentage of pure type I fibres, accompanied an increase in the percentage of

fibres expressing MHCIId/x and MHCIIa - although no pure type IIA fibres were observed after spaceflight. Although the small number of fibres analyzed may not be representative of the entire muscle, similar conclusions were documented by Caiozzi *et al.*, (1996) through immunohistochemical techniques and muscle homogenate electrophoresis. They observed a significant decrease of type I fibres with no relative change of MHCI: an apparent contradiction that was clarified with a significant increase in slow-fast hybrid fibres. A significant increase in relative MHCIId/x was also exhibited together with no relative change in MHCIIa content. Exclusive to the study of Allen *et al.*, (1996) was the "de novo" expression of MHCIIb and MHC_{neo}, both of which were not detected after shorter duration spaceflight.

Together, these adaptations support the transition model, with the significant resurgence of MHC coexpression or hybrid fibres with spaceflight muscle. Allen *et al.*, (1996) found at least five hybrid fibres that could not be identified in controls, primarily due to de novo MHCIIb and MHC_{neo} expressions. These observations are consistent with Caiozzo *et al.*, (1996) who also demonstrated a predictable increase of maximal shortening velocity with soleus muscle. However, the exact mechanism remains unknown (Allen *et al.*, 1996; Caiozzo *et al.*, 1996; Ohira *et al.*, 1992).

In predominantly fast muscles, similar shifts have also been observed in regions of slow fibre concentration. Hadad *et al.*, (1993) examined rat vastus lateralis from a nine day shuttle mission, and found reductions in both the relative and absolute content of MHCI and MHCIIa, small increases in MHCIIb, and possibly MHCIId/x expression in slow high-oxidative red fibres. Significant changes in fast oxidative fibres were not exhibited. Caiozzo *et al.*, (1996) studied only the midsection of the plantaris and tibialis

anterior muscle and postulated that neither exhibited any compositional changes because of their heavy bias to the fast isoforms. In conclusion, slow MHC expression is down regulated and/or fast MHC isoforms are upregulated in slow muscles, and there is a shift towards the faster MHC isoforms in the slow oxidative regions of fast muscle (Roy *et al.*, 1996).

The effect of spaceflight on MLC content has not been extensively examined, but a few observations have been reported. Caiozzo *et al.*, (1994) showed that consistent with the directional shift towards a fast MHC profile, there was an increase in the MLC1f and MLC2f content in the soleus muscle during a six-day space mission. In the adductor longus muscle, 14 days of microgravity revealed increased relative content of fast MLC and decreased amounts of slow MLC (Riley *et al.*, 1992). However, whether these relative changes are attributable to actual increases in fast MLC or to varied atrophy rates of slow vs. fast muscle is still unknown (Roy *et al.*, 1996). Finally, Esser and Hardeman (1995) studied the soleus and extensor digitorum longus (EDL) muscles exposed to 9 days of zero gravity, and found a general increase in fast mRNA levels in both muscles, with unaffected slow mRNA levels in EDL compared to a slight decrease of MLC mRNA in the soleus.

Implications of Changed Myosin Content

The observed changes of MHC isoforms may have significant physiological implications that could effect contractile functioning. Bottinelli *et al.*, (1991) demonstrated that MHC content is an important factor in determining the absolute shortening velocity and hence its work capacity. Reiser *et al.*, (1985) found that the velocity of shortening in slow-fast hybrid fibres was highly correlated and proportional to

MHCII isoforms. And finally, Hilber *et al.*, (1998) showed specific isoform differences between the kinetics of force generating power strokes in the order of MHCIIb > MHCIId/x > MHCIIa >> MHCI for rats, and MHCIIb > MHCIIa >> MHCI in humans. In addition, fibres expressing MHCI have a higher fatigue resistance, while fatigue resistance appears to be inversely correlated with the relative MHCIIb and MHCIId/x fibre content (Sieck, 1994).

Although our knowledge of MLC in the conversion of chemical energy is growing, little information has been obtained on the functional changes that are associated with MLC plasticity. Increased relative contents of MLC1sa in comparison with MLC1sb have paralleled a decrease in the maximum velocity of unloaded shortening, suggesting that MLC1sa may represent the slower subunit (Biral *et al.*, 1999). This hypothesis is supported by the existence of MLC1sb and not MLC1sa isoforms in type I fibres of muscles transforming towards a faster type (Staron & Pette, 1987). However, more conclusive studies are required to resolve how the relative changes of MLC isoform expression influence contractile velocity and perhaps force.

Fibre Size Adaptations and Spaceflight

Fibre size changes induced by spaceflight has shown variability across various studies, but some of these differences may be attributed to differences in experimental protocol (Roy *et al.*, 1996). In postural muscles, CSA measurements of the slow soleus muscle fibres for example, have demonstrated a rapid period of atrophy within the first two weeks of microgravity, followed by a plateau: the fast soleus fibres also demonstrated an atrophic response, but it was generally smaller (Roy *et al.*, 1996). In fast muscles, specific fibre type atrophic responses have been variable (Roy *et al.*, 1996).

However, one generalization for both muscle types suggests that the largest fibres atrophy the greatest (Roy *et al.*, 1996).

More specifically, Riley *et al.*, (2000) studied human atrophic soleus biopsies after 17 days of spaceflight and reported the loss of contractile proteins was not uniform. Myosin concentrations remained normal while thin actin filament concentrations were significantly decreased, resulting in a net decrease of thick-to-thin filament ratios from 6:1 to ~5:1. Interestingly, the force per CSA (specific tension) of the fibres was only decreased 7 - 9%, indicating atrophic adaptations continued to provide sufficient myofilament binding (Riley *et al.*, 2000).

Although, fibre atrophy appears to account for muscle weakening during spaceflight, fibre damage and necrosis may also be a contributing factor (Riley *et al.*, 1987). Roy *et al.*, (1996) has summarized the most common observations made almost exclusively in slow muscles to include the following: 1) segmental necrosis including extensive cellular infiltration by mononucleated cells; 2) sacromere disorientation to include decreased Z-line width and Z-band streaming; 3) signs of degeneration-regeneration to include centrally placed nuclei and an increased number of satellite cells; and 4) eccentric contraction-like lesions of the sarcomeres. The exact cause of the fibre damage is unknown, but the decreased forces during microgravity and/or the increased forces on a weakened atrophic muscle during the interval between landing and tissue removal have been postulated (Fitts *et al.*, 2000; Roy *et al.*, 1996).

Although exercise programs during space travel have demonstrated only a reduction in the rate of muscular atrophy, other factors besides a reduced generation of active and passive tension forces have been postulated (Vandenburgh *et al.*, 1999).

Atrophy may be attributed to increased levels of catabolic glucocorticoids (Grigor'yev *et al.*, 1990), and/or reduced levels of hormones such as growth hormone (Hymer *et al.*, 1996).

Implications of Atrophy

Disuse related muscle atrophy is associated with decreased total muscle protein, originating from a decreased rate of protein synthesis or an increased rate of protein degradation or both (Goldspink *et al.*, 1986; Booth & Criswell, 1997). Potentially, a loss of contractile protein could increase myofilament lattice spacing and decrease the number of cross bridges per CSA, manifesting in a reduction of force per cross bridge (McDonald & Fitts, 1995). Macroscopically, muscle tension is largely determined by CSA, and a loss in muscle size will decrease maximal muscle force (Booth & Criswell, 1996). Consequently, the susceptibility to fatigue on normal loading is likely to be pronounced during an atrophic state, as more motor neurons must be recruited to complete equal amounts of work (Edgerton *et al.*, 1995).

Atrophic characteristics may be further magnified as unloading mechanisms are removed. Reduced thin filament density and near normal force generation, can amount to a 23 – 30% increase of average stress per remaining thin filament (Riley *et al.*, 2000). Consequently, atrophic fibres may be more susceptible to sarcomere reloading damage (Riley *et al.*, 2000).

Research with spaceflight and hindlimb suspension rats has also found a direct relationship between interstitial fluid volumes and the extent of soleus muscle atrophy. It is hypothesized that a loss of myofibrillar protein is accompanied by a sparing of the sacrolemmal membrane, leading to a wavy membrane structure and consequential

increased extracellular space (Henriksen *et al.*, 1993). Such membrane conservation during atrophy could ultimately affect muscle fibre metabolism (Riley *et al.*, 1990)

Diaphragm

The diaphragm is the most important inspiratory muscle, and in comparison with other skeletal muscles is extremely active (Lumb, 2000). Exclusively innervated by the phrenic nerves, it is in actuality two muscles. While the crural muscle of the diaphragm originates at the anterolateral aspect of the first three lumbar vertebrae and the costal muscle arises from the inner aspects of the lower six ribs and xiphisternum, both regions are inserted at the noncontractile central tendon (Lumb, 2000; Poole *et al.*, 1997). Further divisions may separate the right and left portions as hemidiaphragms, each an elliptical cylinder with a curved dome which can act quite independently (Epstein, 1994). The dome of the diaphragm corresponds to the central tendon, while the cylindrical portion corresponds to the costal muscle directly apposed to the inner aspects of the lower rib cage – the zone of apposition (Mead, 1979). All together, they illustrate the non-uniformity of the muscle, and the need to consider regional heterogeneity when designing experiments (Sugiura *et al.*, 1992).

During inspiration, the diaphragm is activated, causing shortening in the axial length of the apposed diaphragm and lowering of the dome (de Troyer, 1997). The resulting expansion of the thoracic cavity along its craniocaudal axis has two major consequences: 1) intrathoracic pressure decreases and, depending on whether the airways are open or closed, lung volume increases or alveolar pressure decreases; and 2) abdominal pressure increases and the abdominal contents move downwards (de Troyer & Loring, 1986). In addition, because the costal diaphragm fibres insert onto the upper

margins of the lower six ribs and are oriented cephalically, the diaphragm uses the abdominal viscera as a fulcrum to lift the lower rib cage and rotate it outward (de Troyer *et al.*, 1981; de Troyer, 1997). However, aside from insertional forces, the diaphragm also expands the rib cage through appositional forces. Research on dogs has established that during breathing, the pressure changes of the abdominal cavity are very similar to the pressure changes of the pleural recess within the zone of apposition (Urmey *et al.*, 1988). Surprisingly, pressure in this pleural recess increased during inspiration, indicating that the "increase in abdominal pressure is truly transmitted through the apposed diaphragm to expand the rib cage" (de Troyer, 1997).

Although the two aforementioned forces influence the rib cage expansion, the descent of the diaphragm is largely determined by the resistance provided by abdominal contents (de Troyer, 1997). When compliance is low (high abdominal resistance), descent of the diaphragm is restricted and the zone of apposition remains significant in size throughout inspiration and the increase in abdominal pressure is greater (Epstein, 1994; de Troyer 1997). Consequently, the expansion of lower ribs is greater. Conversely, when compliance is high (low abdominal resistance), the dome of the diaphragm descends more easily, the zone of apposition decreases more, abdominal pressure is smaller, and rib cage expansion is reduced (Epstein, 1994; de Troyer 1997).

Functionally, the costal and crural muscles of the diaphragm act differently on the lower rib cage. The costal part has a direct inspiratory action on the lower rib cage, while the crural part has no direct effect on lower rib cage dimensions, which is largely due to differences of muscle insertion (de Troyer *et al.*, 1981; de Troyer *et al.*, 1982; de Toyer & Estenne, 1988). The costal muscle is inserted into the ribs and directed upward, parallel

to the rib cage axis, and is in series with the intercostal and accessory muscles of inspiration (de Troyer *et al.*, 1981; de Troyer & Estenne, 1988). In contrast, the fibres of the crural muscle have no insertions into the ribs, and thus cannot have a direct effect on them (de Troyer *et al.*, 1982; de Troyer & Estenne, 1988). It does however, displace the central tendon caudal, and compress the abdominal cavity (de Troyer & Loring, 1986).

The functional "two muscle" concept is further supported by two physiological differences. Although the phrenic nerve innervates both sections of the diaphragm, several studies on cats and dogs demonstrate that the muscles are primarily innervated by different cervical nerve roots (de Troyer *et al.*, 1982; Sant'ambrogio *et al.*, 1963). Secondly, Powers *et al.*, (1990) found in rat diaphragm that the crural diaphragm contained significantly less protein than the costal region and differed in enzyme activity. Gosselin *et al.*, (1992) examined MHC content in rats, and found that the isoform composition in the crural region had a significantly higher fast-to-slow ratio compared with the costal region. Sugiura *et al.*, (1992a) agree and observed that the crural region contains less MHCI and greater MHCIIb protein compared with the costal region. In addition, with the exception of the crural region, the fibre distribution in rat diaphragm is similar in all regions (Metzger *et al.*, 1985). Furthermore, both areas of the muscle are characterized by an abundance of MHCIId/x isoforms (Sugiura *et al.*, 1990; Sugiura *et al.*, 1992). In humans, type II fibres comprise approximately 60% of the diaphragm, suggesting that the muscle may be quite vulnerable to models of disuse (Sieck, 1994).

In comparison with peripheral skeletal muscles, the diaphragm is very unique. Characterized by increased fatigue resistance, increased maximal blood flow, greater oxidative capacity, and higher capillary density, it contracts against elastic loads, rather

than inertial forces, and does not have an antagonist muscle (Epstein, 1994; Syme & Stevens, 1989). The diaphragm's activation pattern is also very distinct, as its duty cycle (ratio of active to inactive times) is between 40 – 45% in most species, in contrast to an approximate 2 – 14% for most slow and fast twitch muscles (Sieck, 1994).

Finally, the cross sectional shape of the rib cage in humans is larger along the its transverse than dorsoventral diameter, whereas in quadrupeds it is larger along its dorsoventral diameter (de Troyer & Estenne, 1988). As well, the adult human rib cage is less distortable than the rib cage of many small animals (de Troyer & Loring, 1986). However, despite this shape difference, the effects of the muscle contraction are qualitatively very similar (de Troyer & Estenne, 1988).

Diaphragmatic MHC and CSA Adaptations

While research has shown that fibre type composition greatly determines contractile functioning and is responsive to exogenous factors, it is not surprising that the diaphragm muscle demonstrates a degree of plasticity through conditions of altered use. Studies that have induced >4weeks of hemidiaphragm denervation in rats have demonstrated a massive shift towards the MHCII isoform expression, resulting in a change of the characteristics normally associated with the MHCI phenotype (Carraro *et al.*, 1982; Carraro *et al.*, 1985). In studies that assessed rat MHC composition after a two-week period of compensatory loading induced by paralysis of the contralateral hemidiaphragm, only slight changes were exhibited (Sieck, 1994). Ten weeks of endurance training induced a significant decrease in the percentage of MHCIIb (Sugiura *et al.*, 1992) and an increase of MHCI (Vrabas *et al.*, 1999) in the costal diaphragm. Finally, respiratory overloads produced by tracheal banding in rats demonstrated an

increase in the proportion of slow twitch fibres (Keens *et al.*, 1978). In summation, in agreement with other muscles, it appears that loading and unloading cause a transition towards slow and fast MHC isoforms, respectively.

The adaptability of costal CSA has also been examined with use/disuse models. In a study utilizing intermittent inspiratory resistive load training, CSA of all muscle fibres increased in the moderate resistance group (Rollier *et al.*, 1998). Surprisingly, endurance training seems to elicit a decrease in CSA of costal type I, IIA and IIB fibres in both adult and developing rats (Powers *et al.*, 1992; Powers *et al.*, 1992a; Green *et al.*, 1989; Tamaki, 1987). Although the regulatory mechanism is unclear, it appears that atrophy of type I and IIA fibres improves their relative oxidative function by increasing their oxidative capacities, as reflected by an increased activity of the mitochondrial reference enzyme - succinate dehydrogenase (SDH) (Powers & Criswell, 1996). Unloading models utilizing long term (>4 weeks) hemidiaphragm denervation also elicit muscle atrophy (Carraro *et al.*, 1985).

The aforementioned studies examining diaphragmatic adaptations have concentrated on either the costal or hemidiaphragm. Since rat costal and crural differ in their ventilatory responses, data on the costal diaphragm should not be directly applied to the crural diaphragm (Powers *et al.*, 1996). Research has shown no significant changes of MHC expression in crural diaphragm after 10 weeks of endurance training in rats (Sugiura *et al.*, 1992; Gosselin *et al.*, 1992), but obesity in rats resulted in a decrease in %MHCIIb and an increase in %MHCI expression (Powers *et al.*, 1996a). Although severe obesity impairs pulmonary function and increases respiratory muscle load, it is "tenuous to speculate that these alterations are primarily a result of increased work loads"

(Poole *et al.*, 1997). Regardless, crural plasticity was confirmed. Very little has been documented on crural CSA modifications.

Adaptive responses of the diaphragm to models of use and disuse may not parallel changes observed in peripheral skeletal muscles, as diaphragmatic regulatory myofibril gene expression may differ due to its unique physiological demands (i.e. a constant duty cycle) and characteristics (Yang *et al.*, 1998). Furthermore, Sieck (1994) hypothesized that while ventilation requires the recruitment of primarily type I fibres, that appear to be less adaptive, disuse responses to type II fibres may only reduce the functional reserve capacity of the diaphragm without decreasing ventilatory performance. This hypothesis is quite probable as the force production during tidal breathing is estimated at approximately 10% of maximum (Sieck, 1994). Vrabas *et al.*, (1999) postulate that although a change in MHC composition may be statistically significant, the change could be of insufficient magnitude to influence specific force and shortening velocity. Unfortunately, limited ventilatory performance under intense workloads and potential magnifications of ventilatory pathologies may persist.

Diaphragm and Spaceflight

Although the documented effects of spaceflight on skeletal muscle has increased significantly over the last decade, the ultrastructure of the diaphragm muscle has only been examined in a few studies with rats aboard Russian biosatellites. Holy *et al.*, (1996) observed no change of maximal mechanical activity in the diaphragm after Biocosmos 1514 and 1667 spaceflights. However, whether microgravity induces MHC and CSA adaptations within the diaphragm has yet to be determined.

Summary

Studies have demonstrated the plasticity of skeletal muscle, as relative MHC and MLC content and fibre CSA adapt to both models of use and disuse. It has been hypothesized that neural activity in part induces these changes, and studies have demonstrated that overloading induces a fast-to-slow phenotypic transformation while disuse elicits a slow-to-fast transition and atrophy. These adaptations may have significant functional implications, since the composition and size of a muscle fibre influences the speed and power of contraction. While both slow and fast muscles have been studied, little is known about the effects of spaceflight on the diaphragm muscle. In being the most important respiratory muscle, an alteration to its phenotype may lead to a decrease in functioning, suggesting that the other models of disuse (i.e., chronic bedrest or immobilization) or prolonged spaceflight may be detrimental for normal respiration.

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Chapter Three

Methods and Procedures

NASA Protocol and Subjects Overview

Adult male Sprague-Dawley rats were used in NASA's Spacelab Life Sciences-2 project (October 16 – 30, 1993) conducted from the Kennedy Space Center. They were selected from the Taconic Farms laboratories, and randomly assigned into three groups of five. The flight animals were individually housed in Rodent Animal Holding Facilities (RAHF), which had very restrictive dimensions (two body lengths long, one body width wide [(10 x 11 x 25 cm)] that prevented the animals from standing on their hindlimbs. They were decapitated 5.3 – 6.3 hours upon landing after two weeks of spaceflight [FL (flight) = 14 (days)]. The remaining ten rats were assigned into a two ground-control groups. One vivarium group was killed at the time of launch [L (launch) + 0 (days)], while the other was housed in identical RAHF cages and killed after 14 days [L (launch) + 14 (days)]. For this study only the FL=14 and L+14 groups were examined: at sacrifice, they weighed 319.3±10.3g and 319.8±24.1g respectively. Both groups were fed water and food ad libitum, caged at constant temperature (25°C) with 12h:12h light-dark cycles, and dissected immediately after death. During flight the Spacelab and shuttle are maintained at approximate sea level atmospheric pressure (760 torr) and gas composition. The diaphragm tissue was then wrapped in aluminum foil and snap frozen in liquid nitrogen before being stored and delivered in a dry ice shipper (-70°C) to the University of Alberta. Upon arrival, the tissue was stored at -80°C.

Study Design

The study design was a modified posttest-only randomized experiment with a control and spaceflight treatment group.

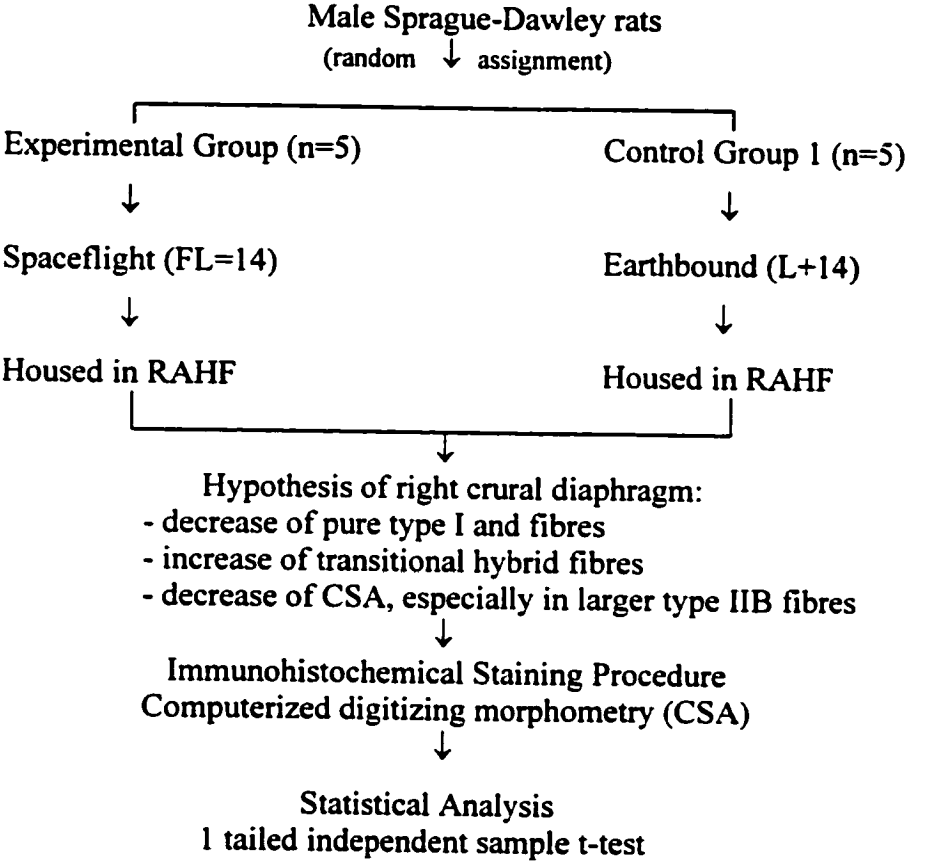

Figure 3-1. Spaceflight study procedure for MHC and CSA.

Treatment

The spaceflight treatment group was housed in RAHF and contained in the Spacelab module – a pressurized cylindrical laboratory (7 meters long and 3.9 meters wide) located in the cargo bay of the shuttle transport system (STS-58) (Sulzman, 1996). Sea level atmospheric pressure was maintained throughout the 14 day mission, with the following approximate normoxic ambient gas composition; 0.2 – 0.4% CO_2, ~21% O_2,

~0.9% Ar and ~78% N_2. The rats were killed 5.3 – 6.3 hours after landing by decapitation (Riley *et al.*, 1996).

Muscle Sample Preparation

Preliminary observations revealed ice crystal artifact and micro-tearing in the crural: a typical consequence of the liquid nitrogen tissue freezing process utilized (Loughlin, 1993). This was partially counteracted by re-hydrating and re-freezing the muscle. The right crural diaphragm was carefully excised at -20°C and dropped in a 0.9% saline bath for 10 seconds. The rehydrated samples were immediately frozen in melting isopentane (-159°C) and mounted in embedding medium (OCT compound, Miels Tissue Tek). Serial cross sections (10 μm) were collected on poly-L-lysine coated slides in a cryostat maintained at -20°C, and stored at -80°C for immunohistochemical staining.

Myosin Heavy Chain Immunohistochemistry

Immunohistochemistry targets particular epitopes on specific antigens with the use of monoclonal or polyclonal antibodies. Among the several staining procedures available, the use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques results in high staining intensity and specificity (Hsu *et al.*, 1981). Briefly, the sequence of reagent application for MHC includes primary antibody, biotinylated secondary antibody, avidin-biotin horse radish peroxidase complex (ABC) and the final staining substrate of 3'diaminobenzidine tetrahydrochloride (DAB) (Naish, 1989; Peuker *et al.*, 1999). Open avidin sites from ABC bind to the secondary antibody, that has been covalently bonded to biotin (Naish, 1989). The strong affinity of avidin to biotin make this method more sensitive (Hsu *et al.*, 1981; Naish, 1989) than previous peroxidase-antiperoxidase (PAP) methods (Gorza *et al.*, 1986) previously employed for MHC.

However, like all immunohistochemical procedures, the sensitivity of the ABC stain depends to a large degree on antibody quality (Naish, 1989).

One limitation of the ABC method is that it "cannot distinguish between high and low concentrations of tissue antigens" (Naish, 1989). Consequently, absolute MHC content cannot be estimated from staining patterns.

To determine a potential MHC transition, four monoclonal antibodies against adult rat MHC isoforms were employed: NOQ7.5.4D (IgG, anti-$MHC_{I\beta}$ and MHC_{Idev}) (Wehrle *et al.*, 1994), F88 (IgG, anti- $MHC_{I\alpha}$) (Putman *et al.*, 1999), SC-71 (IgG, anti-MHC_{IIA}) (Schiaffino *et al.*, 1989) and BF-F3 (IgM, anti-MHC_{IIB}) (Schiaffino *et al.*, 1989). Unfortunately an antibody specific to MHCIId/x is not presently available, so pure type IID/X fibres were identified by an "unstained" default. In addition, a monoclonal IgG directed against MHC_{emb} (BF-45) was also utilized (Schiaffino *et al.*, 1988), to identify a possible emergence of embryonic fibres in spaceflight tissue.

Preparation of the frozen sections involved air drying at room temperature with no fixation applied. For IgG antibodies, samples were blocked with a 1% bovine serum albumin (BSA), 0.1% Tween-20 in PBS (pH 7.4) and 10% horse serum solution. For IgM antibodies, goat serum replaced the horse serum. Primary antibodies, diluted in blocking solution (NOQ 1:4000, SC-71 1:50, F88 1:20, BF-45 1:1000, IgM BF-F3 1:50), were applied. Secondary antibodies, biotinylated horse anti mouse IgG and biotinylated goat anti mouse IgM, were diluted 1:400 in blocking solution. After several washings, sections were incubated with ABC (Vector Laboratories Inc, USA), and finally stained for six minutes with 0.07% diaminobenzidine, 0.05% H_2O_2, 0.03% $NiCl_2$ in 50mM Tris-HCl (pH 7.5) (Vector Laboratories Inc, USA). Control samples were stained in parallel

with either the omission of the primary IgM antibody, or a nonspecific control mouse-IgG antibody (Santa Cruz, CA, USA). Dehydration with sequential ethanol solutions followed by xylene prepared the section for the final mount with Entellan (Merck, Darmstadt, Germany).

Fibre Analysis

MHC fibre analysis was completed with a Leitz Diaplan microscope (Enrst Leitz Wetzlar GmbH, Germany) housed with a Pro Series High Performance CCD camera (Media Cybernetics, USA) and two separate analytical imaging software programs: Image Pro Plus 4.0 and another developed by Dr. Dirk Pette (Constance, Germany). Three regions for a total mean of 423±68 fibres in each sample were analyzed. Briefly, Image Pro Plus video images from a x10 microscope objective were transferred to Dr. Pette's image processing system, and fibres were circled with a computer mouse. The enclosed area was determined from the number of pixels within each outlined fibre. The camera was calibrated with a known object, and a correction factor of 0.6241 was established.

Internal Validity

The posttest-only randomized experimental design is a valid design for assessing cause-effect relationships (Trochim, 1999). Pretest data was not crucial to assess comparability between groups, as the random assignment of the Sprague-Dawley rats were assumed to be probabilistically equivalent (Trochim, 1999). Selection-mortality threats among orbiting research animals is always an issue as keeping them alive and healthy in space remains an uncertain business (Reichhardt, 1998), but fortunately all five rats survived the mission.

Statistical Analysis

The percentage of fibres expressing specific MHC isoforms, fibre area, and percentage of fibre area was independently analyzed in both groups and presented as group means and standard deviations. Differences among the groups was determined with a 1 tailed independent sample t-test, and statistical significance was considered at the $P<0.05$ level.

Chapter Four

Results

MHC Expression

The expression of various MHC isoforms at the protein level was analyzed immunohistochemically (Fig. 4-1). Unstained fibres were designated as type IID/X. The proportions of different fibre types in the right crural muscle are reported in Table 4-1. The mean percentage of fibre type expression was similar in both groups. There were no significant changes in pure fibres expressing only one isoform (MHCI, MHCIIa and MHCIIb). Pure type I and IIA fibres represented the largest fractions of approximately 30% each, whereas pure type IIB and IID/X each accounted for approximately 20%. Hybrid fibre coexpression was limited to only one isoform combination (MHCI + MHCIIa), and was also similar in both groups representing minor fractions of less than 2%. Together, total fibres expressing either fast (MHCII) or slow (MHCI) isoforms was unchanged at approximately 70% and 30%, respectively. MHC_{emb} was not expressed in either group.

Fibre Cross Sectional Areas

The cross sectional areas (CSA) of different fibre types are summarized in Table 4-2. No significant changes were observed through the spaceflight treatment. The mean CSA in both groups was established in the order of type IIB > IID/X > IIA > IA ≈ I-IIA. A general CSA reduction was found in all pure spaceflight fibres, but this difference was not statistically significant. In addition, type IIB fibres in both groups accounted for approximately 35% of total CSA, while type IIA, I and IID/X represented fractions of approximately 25%, 20% and 20%, respectively (Table 4-3).

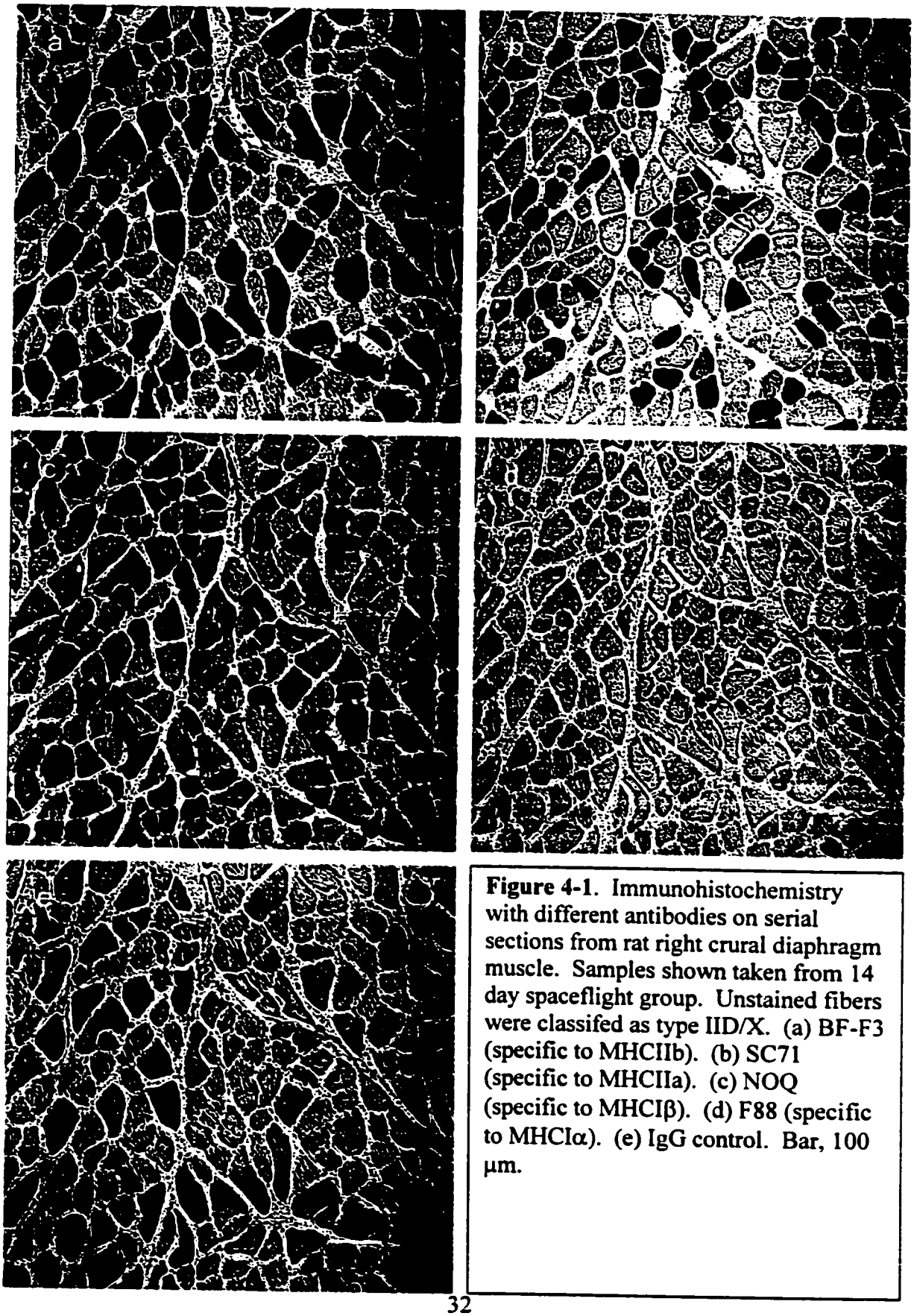

Figure 4-1. Immunohistochemistry with different antibodies on serial sections from rat right crural diaphragm muscle. Samples shown taken from 14 day spaceflight group. Unstained fibers were classifed as type IID/X. (a) BF-F3 (specific to MHCIIb). (b) SC71 (specific to MHCIIa). (c) NOQ (specific to MHCIβ). (d) F88 (specific to MHCIα). (e) IgG control. Bar, 100 μm.

Table 4-1. Proportions (%) of right crural diaphragm fibres.

Fibre Type	Control Group	Spaceflight Group
Pure I	27.3±3.4	28.9±4.0
Pure IIA	30.6±3.1	30.4±4.1
Pure IIB	19.8±3.5	22.0±5.5
Pure IID/X	20.5±3.9	16.6±2.7
Embryonic Hybrid	0	0
Hybrid I-IIA	1.3±1.0	2.0±1.2
Total I	28.3±3.5	30.0±4.3
Total II	71.7±3.5	70.0±4.3

Values are means±SD in percent of samples from five rats in each group. Proportions based on 423±68 fibres for each rat from three separate regions within the crural diaphragm.

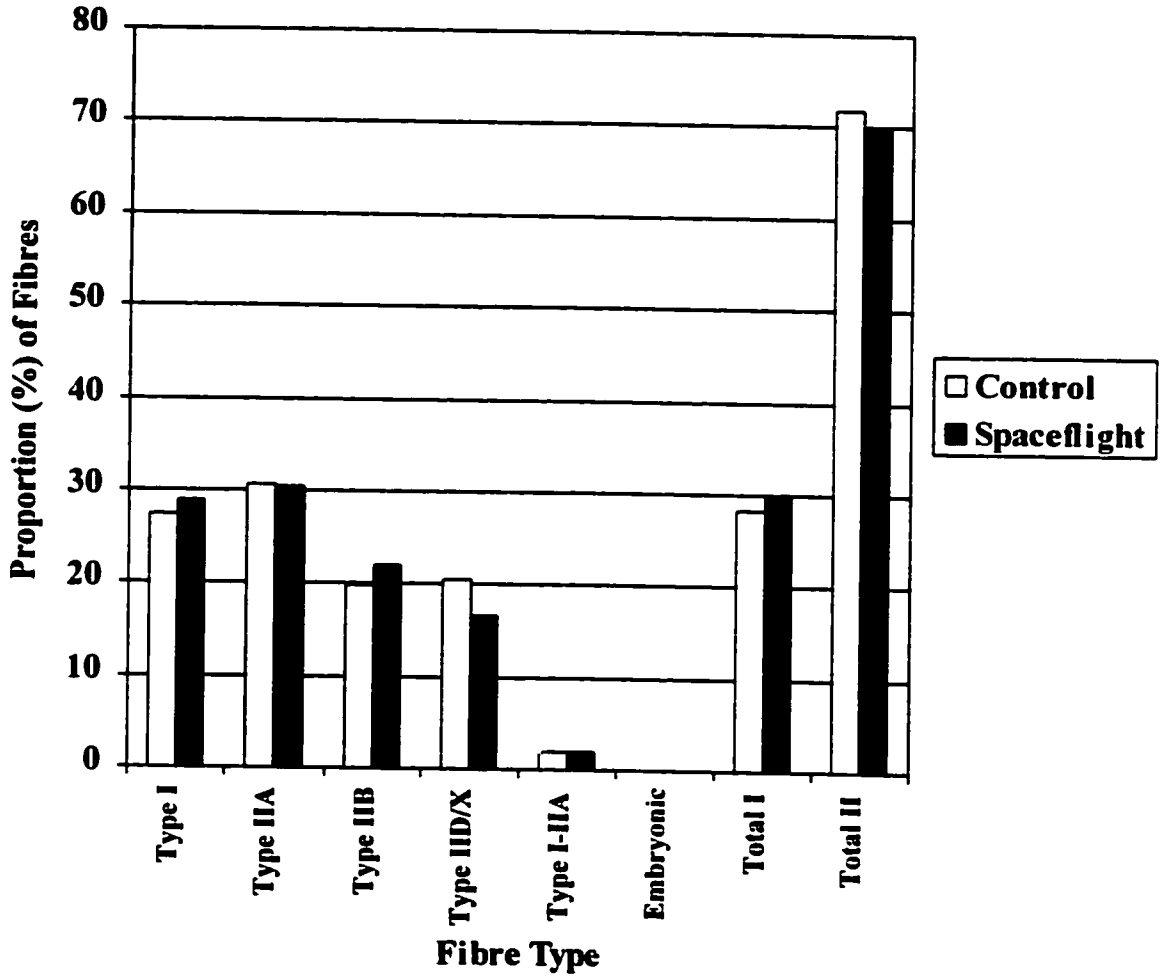

Figure 4-2. Effects of 14 days of microgravity on the proportions (%) of right crural diaphragm fibres. Muscle fibre type profile was determined immunohistochemically with monoclonal antibodies specific to MHCI, MHCIIa, MHCIIb and MHC_{emb}. Type IID/X fibres were identified by an "unstained" default. Embryonic represents embryonic hybrid fibres not observed in either group.

Table 4-2. Fibre cross sectional area (μm^2).

Fibre Type	Control Group	Spaceflight Group
I	1354.4±255.7	1093.4±276.2
IIA	1449.8±246.4	1413.4±106.5
IIB	3144.6±692.8	2841.8±284.8
IID/X	1900.7±326.7	1796.3±222.4
I-IIA	1143.3±111.9	1167.1±309.2

Values are means±SD in absolute area of samples from five rats in each group.

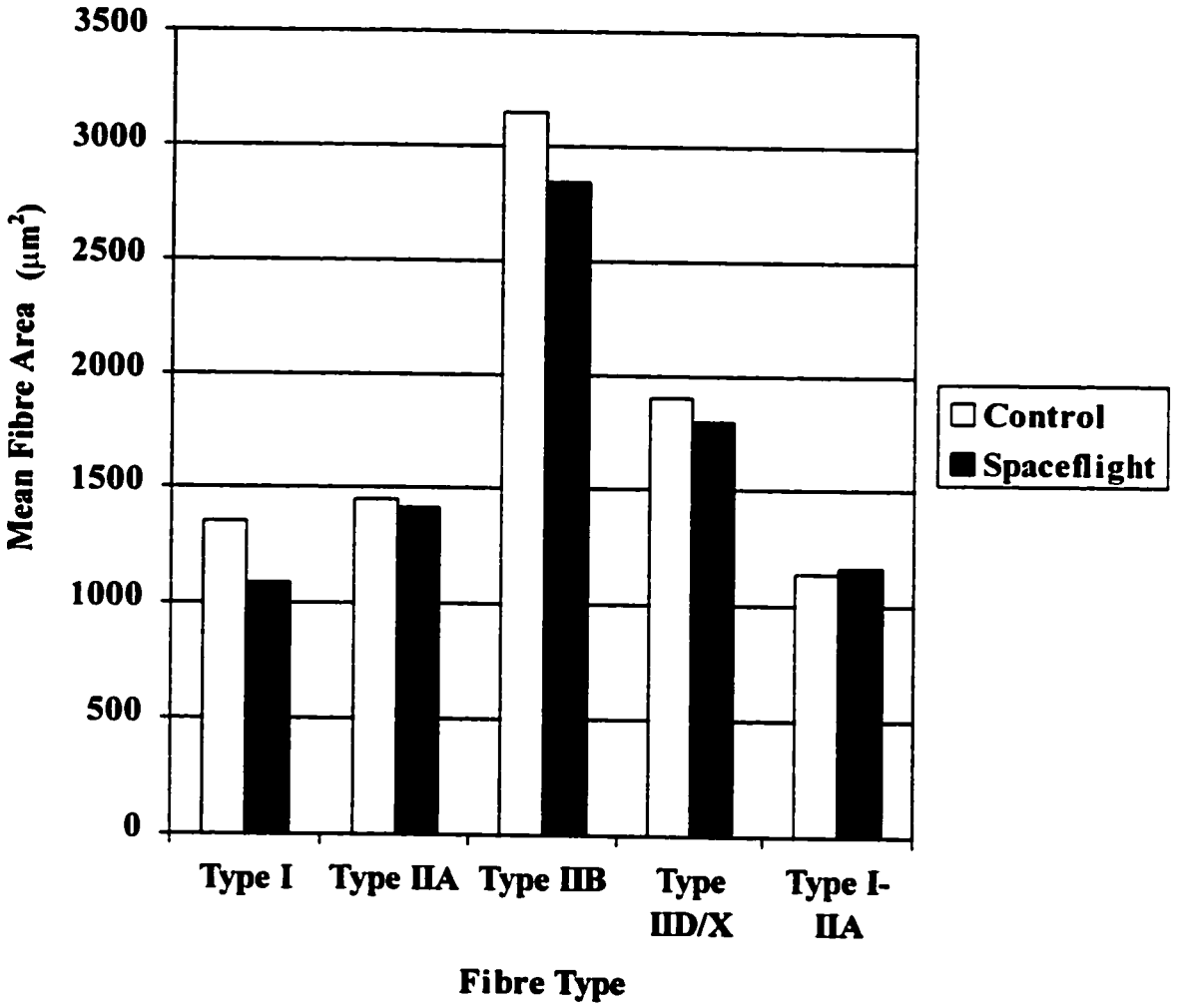

Figure 4-3. Effects of 14 days of microgravity on muscle fibre cross sectional area (μm^2). CSA was determined by computerized digitizing morphometry following immuno-histochemical fibre type identification.

Table 4-3. Relative contribution (%) of fibre cross sectional area (μm^2).

Fibre Type	Control Group	Spaceflight Group
I	20.2±2.5	18.8±5.9
IIA	24.1±3.7	25.5±4.9
IIB	33.4±4.9	36.4±7.3
IID/X	21.4±4.4	17.8±4.5
I-IIA	0.9±0.8	1.5±1.2

Values are means±SD in percent of samples from 5 rats in each group.

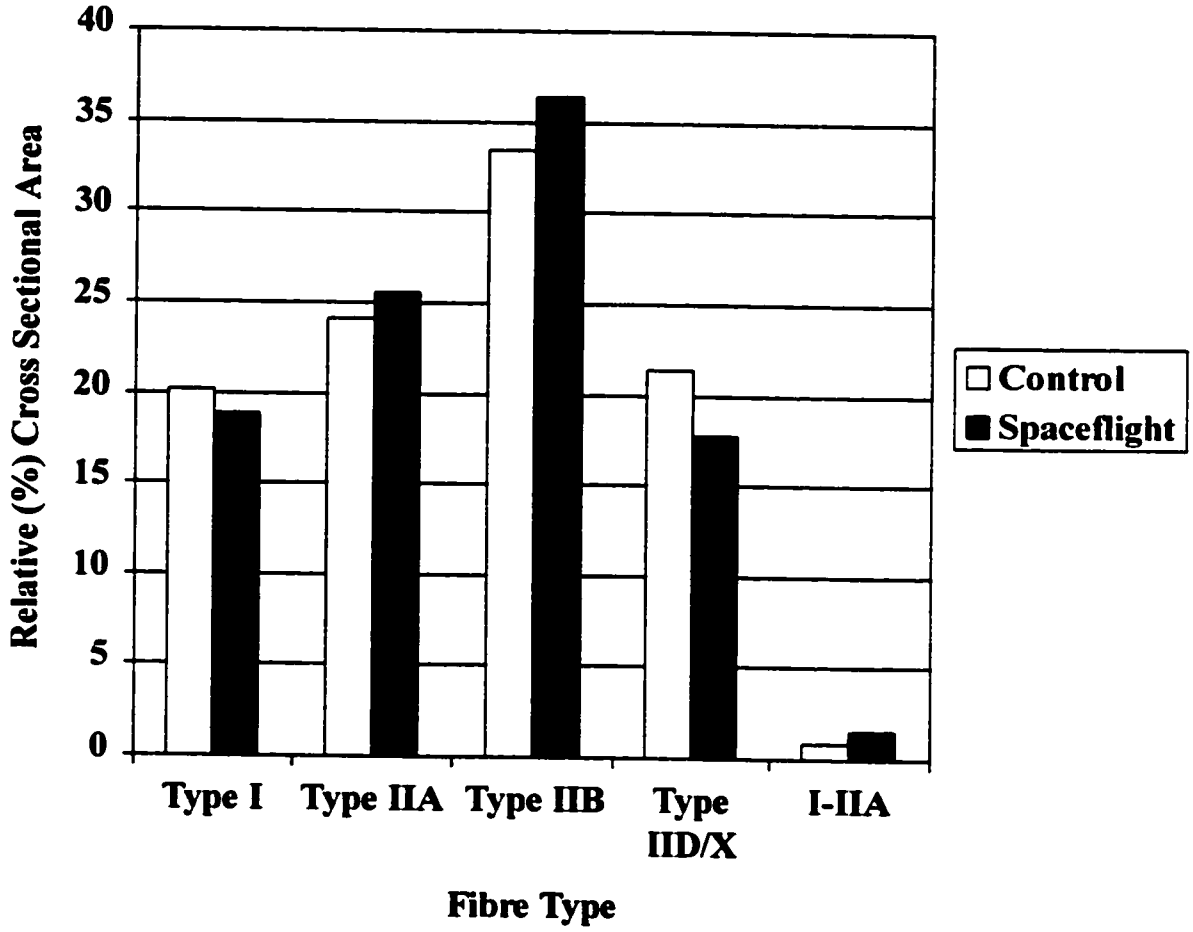

Figure 4-4. Effects of 14 days of microgravity on the relative (%) contribution of fibre cross sectional area (μm^2). Proportions were determined by assimilating fibre type profile and CSA data together.

Chapter Five

Discussion

Crural MHC Isoform Expression

To my knowledge, this is the first study to investigate the fibre type profile of rat crural diaphragm through immunohistochemical methods. Traditionally, research has either focused on costal plasticity, or utilized ATPase histochemical classification and electrophoresis for the crural region. However, incompatibilities exist between metabolic enzyme-based classification and immunohistochemcial labeling, especially with type II fibres (Pette & Staron, 1997). Furthermore, since electrophoresis measurements are limited to assessing the fractions of specific MHC isoform content within muscle homogenates, fibre type profiles cannot be directly observed.

The large percentage of type II fibres (~70%) exhibited in control rat diaphragm agrees with high MHCII% distributions (~78 - 88%) through electrophoretic observations (Powers *et al.*, 1997, Sugiura *et al.*, 1992a). All three type II fibres were observed in large proportions (IIA~30%, IIB~20% and IID/X~20%), reflecting perhaps, a greater potential for varied responses. This agrees with the proposal that the diaphragm has multiple functions: oxidative fibres (I and IIA) are necessary to sustain respiration at rest and exercise, while fast fibres are required for increased respiratory rates in exercise, or for high power output in sneezing and coughing (Edward & Faulkner, 1985). Hybrid fibres signified a very small percentage (~2%) of the profile, as only one type of coexpression (MHCI+MHCIIa) was observed. Unfortunately, hybrid fibres coexpressing MHCIId/x could not be detected, as an antibody specific to the isoform is not currently available. However, it seems reasonable to speculate that MHCIId/x hybrids are more

common, as MHCIId/x is the predominate isoform (~32 - 40%) of the total MHC pool (Powers *et al.*, 1997, Sugiura *et al.*, 1992a) while pure IID/X fibres account for only ~20% of the profile.

The present study found the crural diaphragm to be invariable in spaceflight, as no significant changes were observed. The apparent immutable profile of the group does not support the hypotheses of decreased pure type I fibre and increased transitional hybrid expression during spaceflight, indicating limitations of the immunohistochemical procedures and/or unique characteristics or mechanisms that may extend beyond typical skeletal muscle adaptations.

Potential procedural limitations can be considered in conjunction with a previous study that examined several muscles from the identical SLS-2 mission (Ciaozzo *et al.*, 1996). Two monoclonal antibodies were employed with specificity to only slow and fast MHC isoforms; consequently, only slow, fast and slow-fast hybrids could be detected. Furthermore, electrophoresis was also utilized to measure relative MHC isoform contents. The vastus intermedius, for example, only showed a significant decrease ($p<0.05$) of slow muscle, but electrophoretically demonstrated both significant ($p<0.001$) decreases of MHCIIa and increases of MHCIId/x. Conversely, with the plantaris muscle, immunohistochemistry expressed significant changes, while electrophoresis did not. Finally with tibialis anterior, neither method detected any adaptations. Together these observations demonstrate that MHC plasticity may not be detected unless electrophoresis and immunohistochemistry are together employed.

The immunohistochemical methods of this study were somewhat superior, as anti-MHCII antibodies could further delineate the fast fibres. However, in considering the

expected crural fibre transition (I → IIA → IID/X → IIB), the unavailability of a specific MHCIId/x antibody, and lack of supplemental electrophoretic data, potential discrepancies do emerge. Any intermediate MHCIId/x hybrid such as IIA-IID/X or IID/X-IIB would be erroneously classified as pure IIA or IIB, respectively. Despite these concerns, the stable I, IIA and I-IIA fibre profiles and I-II fibre ratios strongly suggest either an immutable or very gradual slow-to-fast fibre transition. However, an observed decrease of type IID/X fibres and increase of type IIB fibres may be attributed to an increase of undetected IID/X-IIB hybrids. Consequently, a transition within the fast isoforms (namely MHCIId/x hybrids) cannot be excluded.

Any changes of the fibre profile were expected to predominantly stem from isoform conversions, but the observed fibre damage and necrosis of primarily slow muscles previously reported (Roy *et al.*, 1996) also presented a possibility for fibre regeneration. Regenerating muscle fibres mimic developing fibres in their sequential expression of embryonic, neonatal, and adult isoforms of the myosin molecule (d'Albis et al., 1989). Studies that induced gracilis fibre damage by denervation for example, found maximal MHC_{emb} expression following two and three weeks of denervation (Yoshimura & Harii, 1999). However, it is not generally thought that denervated fibres are replaced with regenerated fibres, but rather activated satellite cells fuse to the denervated maternal fibres in order to repair them (Yoshimura & Harii, 1999). Allen *et al.*, (1996) found ~19% of spaceflight soleus fibres expressed some neonatal MHC, but hypothesize that these were adult fibres re-expressing neonatal MHC in the absence of regeneration. They postulate that this re-expression may be a prerequisite for reprogramming adult MHC isoform expression. Since spaceflight fibres expressing neonatal MHC are larger than

fibres that do not express them, and are similar in size to ground controls, they contend that the re-expression is an early event that precedes the decrease in fibre size. Regardless, MHC_{emb} was not immunohistochemically expressed in spaceflight crural, signifying minimal fibre regeneration, repair, damage and/or future atrophy.

Cross Sectional Area

To my knowledge, this is the first study to summarize rat crural CSA. The CSA order of type I < IIA < IID/X < IIB exhibited in the control group is consistent with orders of other studies on rat costal (Miyata *et al.*, 1995, van Balkom *et al.*, 1997), but considerable absolute CSA discrepancies exist. For example, the mean costal CSA of type IIB fibres in adult male Wistar (van Balkom *et al.*, 1997) and Sprague-Dawley (Miyata *et al.*, 1995) control rats measured 5828±599 μm^2 and 2288±145 μm^2 respectively, compared to 3144.6±692.8 μm^2 in the present study. These differences may be attributed to disparities between rat species, specimen handling, staining methods and perhaps regional diaphragmatic variations. Type IIB fibres were ~2.5 larger than type I fibres, and although they accounted for only ~20% of the fibres expressed, their relative contribution to total crural area was the greatest at ~33%.

It was hypothesized that spaceflight would induce crural atrophy, especially in the larger type IIB fibres. Although the fibre type specific atrophic response in predominately fast muscle has been quite variable, in general, larger fibres appear to atrophy the greatest (Roy et al., 1996). Results from the spaceflight group in the present study suggested no significant changes to crural CSA and relative contribution, although small reductions in all pure fibres were observed. This may indicate the development of a gradual atrophic response, but only a longer duration spaceflight could substantiate this.

Conversely, hybrid fibres appeared unaffected, which may be a reflection of their smallest relative size.

Three Alternate Hypotheses

The effects of spaceflight on skeletal rat muscle has generally demonstrated rapid atrophy and fibre type transformations, but these changes are very specific to the muscle and to the region of the muscle (Roy *et al.*, 1996). Although the observed data supports crural specific adaptability, three plausible hypothesizes regarding the apparent immutable character of the crural diaphragm in microgravity need to be considered.

1) Very Gradual Transformation Hypothesis

The first explanation reasons that a small fibre transition was indeed evoked, but could not be detected at the protein level. Sugiura *et al.*, (1992) found that 10 weeks of moderate-intensity endurance training on rats induced only significant changes to costal MHC and not to crural diaphragm. In conclusion, they suggested that a more intense training regimen or a longer duration program may have been necessary to induce significant crural changes. In summary, the study demonstrated the stability of crural ultrastructure, and perhaps the prescription of transition measurement devices other than at the protein level.

Andersen and Schiaffino (1997) examined human muscle biopsies in which MHC changes were expected to occur with greater frequency, and found a minor proportion of fibres showing a mismatch in the relative proportion of mRNA and protein. They proposed that this mismatch corresponds with a transformation process, and that the nature of the mismatch offers a sign to the direction in which the fibres are changing. Furthermore, studies of chronic low frequency stimulated (CLFS) rat muscles found that

changes at the mRNA level occur more rapidly and precede those at the protein level (Termin *et al.*, 1989: Jaschinski *et al.*, 1998). The delay at the protein level may relate to a slow turnover period of MHC isoforms, as decay periods of 11 – 14.7 days have been approximated for MHCIIb exposed to CLFS (Termin & Pette, 1992; Pette & Staron, 1997). Consequently, protein degradation appears to exert a post-translational regulatory role in fibre remodeling, as newly synthesized isoforms can only be inserted in the sarcomere after the pre-existing isoform is no longer expressed and degraded (Termin & Pette, 1992). Together, the research indicates that while mRNA changes occurs more rapidly and precede protein changes, slight fibre transitions may be undetected by solely measuring MHC expression at the protein level. Furthermore, although spaceflight induced fibre changes in other muscles, possible differences in crural diaphragmatic post-translational regulation and/or protein degradation may have been a factor.

2) Spaceflight Does Not Unload the Crural Diaphragm Hypothesis

Secondly, perhaps spaceflight does not unload the crural diaphragm, and consequently no changes in its ultrastructure should be expected. Conventionally, microgravity decreases muscle workloads and neuromuscular activity, but the diaphragm is atypical in comparison to weight bearing skeletal muscles. Therefore, an insight towards the diaphragm's activation may be provided through direct and indirect associations with other respiratory structures and pulmonary function in space.

Unfortunately, our knowledge is only limited to the adult human model, as research has not yet evaluated the respiratory mechanics or responses of quadrupeds in space. Baranov *et al.*, (1992) attributed reductions of peak expiratory flow and forced vital capacity to the deconditioning of secondary respiratory muscles. This explanation

seems plausible, because the postural muscles of the thorax, abdomen, shoulder and neck are unloaded in gravity and serve as accessory breathing muscles during a maximal output (Linnarsson, 1996). Consequential loading of the diaphragm may ensue at maximal exertion, but is probably insignificant during normal tidal breathing.

Researchers utilizing parabolic flight patterns also observed several rib cage adaptations to brief periods of microgravity: cranial displacement, more circular shape at end expiration, and a reduction of tidal expansion of the ventral rib cage (Estenne *et al.*, 1992). However, because the tidal expansion of the rib cage was not uniformly decreased, it should not significantly influence appositional forces of the diaphragm, as the ventral portion of the apposed diaphragm is generally small (Estenne *et al.*, 1992).

Perhaps the most important diaphragmatic implications arise from decreases of functional residual capacity and tidal volumes in space (Prisk *et al.*, 1994; Elliott *et al.*, 1996). Both have been attributed to a cephalic shift of the diaphragm, as the weight of abdominal organs have been eliminated, causing lung volume to decrease (Linnarsson, 1996; Prisk *et al.*, 1995). Hence, because of the length-tension behavior of muscle, by contracting from a greater initial length, for a given neural activation a greater force and transdiaphragmatic pressure would be produced; thus, the diaphragm is able to contract more effectively (Nunn, 1993; de Troyer & Loring, 1986). However, a significant compensatory increase in the respiratory frequency was found, and coupled with tidal volume reductions, accounted for a 4% reduction of total ventilation (Prisk *et al.*, 1995). If the diaphragm is therefore capable of contracting at a greater force, and the respiratory frequency has increased, it would seem plausible to suggest that spaceflight induces a loading phenomenon. However, an increase of abdominal compliance, resulting

primarily from the release of passive tension in the ventral abdominal wall, was also observed (Wantier *et al.*, 1998), insinuating that a decrease of the abdominal fulcrum should decrease the insertional force (Estenne *et al.*, 1992). Consequently, the inspiratory action of the diaphragm on the rib cage should be decreased (de Troyer, 1997)

Research on rats aboard Russian biosatellites provides a brief perspective on the neuromuscular junction adaptations in space. In the adductor longus, for example, changes included: 1) a decrease in the number of synaptic vesicles; 2) degeneration of axon terminals; 3) axonal sprouting (i.e., a sign of regeneration) and 4) a presence of Schwann cell processes between pre- and post synaptic sites (Roy *et al.*, 1996). Babakova *et al.*, (1992) found similar signs of regeneration most pronounced in soleus muscle, and much less in gastrocnemius and diaphragm. They concluded that the diaphragm (of unspecified location) demonstrated some signs of synaptic reconstruction: expansion of the synaptic zone due to the increased number of axon terminals, and hypertrophied post-synaptic folding, but analysis problems do exist. Roy *et al.*, (1996) indicated that abnormal neuromuscular junctions are also observed in controls, and while the examination of the junctions is a very tedious and difficult task, sampling errors may persist. Nevertheless, these adaptations may suggest a compensatory effort to improve neuromuscular transmission (Prakash *et al.*, 1995).

Together the literature demonstrates that microgravity imposes many indirect and direct factors on the diaphragm, that may influence normal functioning. Isolated observations may suggest either loading (i.e., increased respiration rate) or unloading (i.e., increased abdominal compliance, abnormal neuromuscular junctions) mechanisms, which makes it difficult to assess the net activation of the diaphragm. This presents

numerous possibilities and challenges the central assumption of this thesis that spaceflight unloads the diaphragm. Further complications arise when the crural is specifically analyzed, as generalizations concerning the diaphragm may not extend to unique crural mechanics. Although the data seems to indicate a very gradual unloading mechanism, further research studying diaphragm activation through electromyograms (EMG), and kinetics induced by spirometry through m-mode sonography (Ayoub *et al.*, 1997) could provide invaluable insight regarding microgravity as a model of inactivation for the diaphragm.

3) Cranial Stretch Could Partially Oppose Unloading Adaptations Hypothesis

Finally, it is possible that a cranial shift of the diaphragm during microgravity could actually oppose the expected spaceflight muscle adaptations of atrophy and slow-to-fast fibre phenotype transitions through the stretch phenomenon. Although prolonged diaphragm denervation induces myofiber atrophy and a loss of MHCI expression (Yang *et al.*, 1998), short periods of hemidiaphragm denervation have induced significant muscle hypertrophy of the inactivated side (Feng & Lu, 1965; Yang *et al.*, 1998) and a MHCII-to-I phenotype transformation (Yang *et al.*, 1998). Yang *et al.*, (1998) concluded that the diaphragmatic hypertrophy was "associated with reciprocal changes in type I and type II MHC isoforms that are directly opposed to the type I-to-type II MHC phenotype transformation reported in the diaphragm denervation model".

Hypertrophic responses have been attributed to the passive stretching of the paralyzed side by the continual rhythmic contractions of the normally innervated contralateral hemidiaphragm (Feng & Lu, 1965), and muscle stretch has been reported to repress MHCII and induce MHCI gene expression (Goldspink *et al.*, 1992). However,

the degree to which the myofibres respond to the passive stretch mechanism, as opposed to the other consequences associated with spaceflight, is presently uncertain (Yang *et al.*, 1998). Nevertheless, these adaptations are directly opposed to unloading responses, and consequently may hinder atrophic and fibre type transformations.

Fibre Transition and Atrophy Mechanisms

In adult skeletal muscle, it is clear that the activation history (i.e., the total activity and activity pattern) exerts the greatest impact on muscle contractile and metabolic properties (Sieck, 1994). Convincing studies have shown that cross-reinervation of a fast twitch muscle with a slow nerve, or a slow twitch muscle with a fast nerve lead to phenotype adaptations that ultimately make the fast muscle slower or the slow muscle faster (Pette & Staron, 1997). In agreement, studies that have diminished diaphragmatic activity when accompanied by an intact neuromuscular unit, have demonstrated dramatic slow-to-fast fibre transitions, leading to a more homogeneous fast muscle (Talmadge, 2000). Since increased fast MHC expression is typically accompanied by increased levels of corresponding mRNA, MHC regulation may be primarily via transcriptional control mechanisms (Talmadge, 2000). The exact mechanism however, is currently unknown. Furthermore, aside from neural activity, when subjected to stretch, skeletal muscle apparently adapts to a more posture type of role, by expressing the slow myosin isoform and repressing the fast isoform genes (Yang *et al.*, 1997)

The mechanism of muscle atrophy has also been elusive, as changes of protein mass have been attributed to alterations in the relative rates of protein synthesis and degradation (Loughna *et al.*, 1986; Booth & Criswell, 1997). Unloading the soleus for example, has been shown to decrease protein synthesis before increasing protein

degradation, indicating modulation of protein synthesis through translation occurs before transcription and protein degradation (Thomason *et al.*, 1996). Interestingly, in rat soleus-stretch models, the extent of atrophy in response to hypokinesia and hypodynamia was greatly reduced (Loughna *et al.*, 1986).

In contrast to most peripheral skeletal muscles, the crural diaphragm in this study has demonstrated remarkably stable fibre type profiles and CSA during spaceflight. Although muscle activation and kinetics data were not collected in either study, the results could insinuate that crural activation does not dramatically change under models that would normally be defined as loading or unloading. The immutable characteristics of the crural may be further attributed to passive muscle stretch. Short periods of hemidiaphragm denervation (2 weeks) lead to significant muscle hypertrophy, which has generally been ascribed to rhythmic passive stretching of the paralyzed myofibres by contractions of the normally innervated contralateral hemidiaphragm (Feng & Lu, 1965). Unlike many skeletal muscle, the diaphragm is not motionless when chronically inactive (Howell *et al.*, 1997). Therefore, while the diaphragm sacromeres are normally passively stretched with reductions in lung volume (Poole *et al.*, 1997), its cranial displacement during spaceflight may further increase the stretch phenomenon, and consequently repress fast myosin genes and atrophy.

Whether little changes to activation patterns, increased passive stretch, or neither primarily account for fibre profile and CSA stability, the diaphragm nevertheless demonstrates a tremendously heterogeneity and uniqueness with respect to structure (e.g., fibre length, thickness, profile) and function (Poole *et al.*, 1997). For example, the larger motor units comprising type IID/X and IIB muscle fibres are probably recruited only in

rare instances involving expulsive behaviors (i.e. gagging, coughing, vomiting), but do not display significant adaptations to inactivity under normal conditions (Miyata *et al.*, 1995). Perhaps these complexities are only an indication of the tremendous intricacies within the muscle, and demonstrate a great functional plasticity potential with structural stability under extreme circumstances like endurance training or spaceflight.

Countermeasures

Ayas *et al.*, (1999) observed that human diaphragmatic atrophy from prolonged inactivation may be prevented by brief periods of phrenic nerve electrical stimulation. Research on rats and humans has also demonstrated that the diaphragm can adapt to long term respiratory resistive loading by increasing mass, decreasing contractility and increasing endurance (Prezant *et al.*, 1993; Darnely *et al.*, 1999). However, while these countermeasures appear very effective, the relatively immutable character of the crural phenotype during 14 days of microgravity suggest that specifically targeting the diaphragm may not be necessary. Furthermore, because astronauts utilize various exercise modalities (i.e. resistance training, continuous endurance exercise), and exogenous anabolic agents (i.e. growth hormone, insulin-like growth factor-I) to attenuate skeletal muscle atrophy, the diaphragm should also indirectly benefit (Booth & Criswell, 1997). However, Space Shuttle flights are limited to short duration missions, and only the acute phases of crural adaptations can be studied; missions of longer duration may prioritize the need and importance of effective crural countermeasures.

Applications

Spaceflight has been shown to cause skeletal muscle atrophy; reductions of muscle power, force, and capacity for fat oxidation; and, increased expression of fast type

II fibres, fatigability and susceptibility of muscle damage (Fitts *et al.*, 2000). These negative complications, among others, provide scientists with opportunities to elucidate complicated biological mechanisms, but present several health and safety issues to the astronauts. The observed ultrastructural stability of the right crural diaphragm muscle is not a physiological concern over short duration spaceflight, and may offer insight into more effective countermeasures. While cycling, treadmill, resistance training and other mechanical overloading mechanisms have largely been used, the potential of passive stretching and electrical stimulation have not been fully explored.

Valid clinical applications are difficult to make, as other models of inactivation (i.e. denervation) are more conclusive with consequential activation patterns. The aforementioned countermeasure suggestions have therapeutical implications, and have already been successfully utilized to various degrees. Further development is needed in this area. As well, although fibre damage was not studied, spaceflight may be an excellent model to study fibre necrosis during chronic hypoinflation.

Chapter Six

Conclusion

Summary

The purpose of this study was to examine the effects of spaceflight on rat crural diaphragm muscle. By examining potential physiological adaptations to disuse, further insight into biological regulatory mechanisms and effective countermeasures may be obtained.

The study design was a modified posttest-only randomized experiment with a control and spaceflight treatment group. Five rats (319.3±10.3g) orbited earth aboard NASA's 14 day Spacelab Life Sciences II (SLS2) mission and were decapitated shortly after entry. Their right crural myosin heavy chain (MHC) based fibre type distribution and muscle fibre cross sectional area (CSA) were compared to a flight control group (319.8±24.1g) of five ground-based rats that were contained in identical rodent animal holding facilities. Expression of MHC isoforms at the protein level was analyzed immunohistochemically, and fibre CSA was determined by a computer imaging pixel program. In control rats, type I and IIA fibres each represented the largest fractions of approximately 30% each, whereas type IIB and IID/X each accounted for approximately 20% each; hybrid fibre coexpression (only MHCI+MHCIIa) was limited to minor fractions. The mean CSA was established in the order of type IIB > IID/X > IIA > I ≈ I-IIA. Spaceflight induced no significant changes, but an increase in type IIB fibres and a decrease in type IID/X may suggest a gradual transition. In addition, a general CSA reduction was found in all pure spaceflight fibres.

Conclusions

The present study was a novel analysis on the ultrastructure of spaceflight diaphragm. The data and current research indicate that the following conclusions on the crural diaphragm may be made:

a) Spaceflight induced no significant changes to the fibre type profile, but a gradual transition, especially with fast MHC isoforms, may have occurred.

b) No significant changes to fibre CSA were observed, but there was a small decrease in all pure fibres.

c) The results suggest that either neuromuscular activation patterns or passive stretching, or a combination of the two may, have been responsible for the lack of plasticity observed in the fibres.

Recommendations

As with most spaceflight studies, small sample sizes are characteristic and difficult to overcome. High mission mortality rates (Reichhardt, 1998), spatial limitations, and numerous scientific projects present tangible restrictions to ideal research designs. Consequently, in most studies of this nature, the power is very low, and type 2 errors are very possible. Thus, strong conclusive statements should be avoided, and must be validated through future research. With the insurgence of international space missions and the collaborative development of the space station, perhaps a greater accommodation to research designs is possible.

The immunohistochemical analysis employed was a useful method in determining fibre profile, but its limitation in being unable to measure relative MHC content and

MHCIId/x isoforms may have prevented the observation of a gradual MHC transformation. Electrophoresis alone also has limitations, but together may provide the most adequate measurements possible at the protein level.

Finally, future research in this area that should clarify issues presented in the present study. The following proposals could be:

a) Measure mRNA and utilize electrophoresis on spaceflight crural to demonstrate whether a transformation occurred at the MHC isoform or RNA level. However, it may be possible that the 6 hour delay from re-entry to sacrifice could be large enough to influence spaceflight mRNA levels.

b) Perform the identical analysis on costal diaphragm, to determine if its adaptations mirror those of the crural. This information may provide further insight to the two-muscle model of the diaphragm, and any differences may have unloading implications.

c) Measure diaphragm muscle activation patterns and kinetics in spaceflight.

d) Utilize electrical stimulation and passive stretch protocols as skeletal muscle countermeasures in spaceflight.

Soli Deo Gloria

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Appendices

Appendix A

Relative (%) Fiber Type Profile

Control Group

Rat #	Type I	Type IIA	Type IID/X	Type IIB	Hybrid I-IIA
I6	28.804	30.163	17.120	23.370	0.543
I7	24.877	35.714	22.167	14.532	2.709
I8	32.530	29.518	18.976	18.072	0.904
I9	23.990	27.273	26.515	21.717	0.505
I10	28.117	30.562	17.604	21.516	2.000

Spaceflight Group

Rat #	Type I	Type IIA	Type IID/X	Type IIB	Hybrid I-IIA
H16	31.127	23.284	19.363	22.304	3.922
H17	31.949	32.130	16.430	18.051	1.444
H18	31.316	32.105	19.211	15.000	2.368
H19	22.269	33.613	14.706	27.731	1.681
H20	28.008	30.966	13.412	26.824	0.789

Appendix B

Fiber Cross Sectional Area (μm^2)

Control Group

Rat #	Type I	Type IIA	Type IID/X	Type IIB	Hybrid I-IIA
I6	1785.137	1655.371	1969.058	3350.816	1065.535
I7	1174.206	1401.345	1787.872	2830.381	1281.845
I8	1388.903	1752.156	2398.628	4210.436	1248.197
I9	1183.363	1244.963	1502.510	2362.658	1052.720
I10	1240.144	1195.257	1845.524	2968.475	1068.204

Spaceflight Group

Rat #	Type I	Type IIA	Type IID/X	Type IIB	Hybrid I-IIA
H16	1485.251	1341.418	2016.473	2584.592	1622.937
H17	831.887	1416.323	1967.369	2875.036	1042.101
H18	1072.792	1277.016	1578.448	2545.420	781.926
H19	842.527	1499.98	1539.863	2971.613	1126.823
H20	1234.431	1532.245	1879.530	3232.219	1261.815

Appendix C

Relative Contribution (%) of Fiber Cross Sectional Area (μm^2)

Control Group

Rat #	Type I	Type IIA	Type IID/X	Type IIB	Hybrid I-IIA
I6	24.034	23.338	15.756	36.601	0.271
I7	17.866	30.611	24.241	25.157	2.124
I8	20.571	23.548	20.723	34.644	0.514
I9	18.150	21.708	26.997	32.805	0.340
I10	20.499	21.475	19.099	37.546	1.382

Spaceflight Group

Rat #	Type I	Type IIA	Type IID/X	Type IIB	Hybrid I-IIA
H16	25.610	17.302	21.629	31.933	3.526
H17	16.610	28.838	20.479	32.887	0.954
H18	23.177	28.285	20.919	26.341	1.278
H19	10.653	28.627	12.857	46.788	1.075
H20	17.737	24.341	12.932	44.479	0.511

Appendix D

Rat Masses (g) at L-6 (Launch - 6 days) and R+0 [Recovery + 0 (rat termination)]

Control Group

Rat #	L - 6	R + 0
I6	206	333
I7	201	345
I8	205	318
I9	203	281
I10	210	322

Spaceflight Group

Rat #	L - 6	R + 0
H16	216	330
H17	214	309
H18	212	312
H19	221	326
H20	Mass Not Measured	Mass Not Measured