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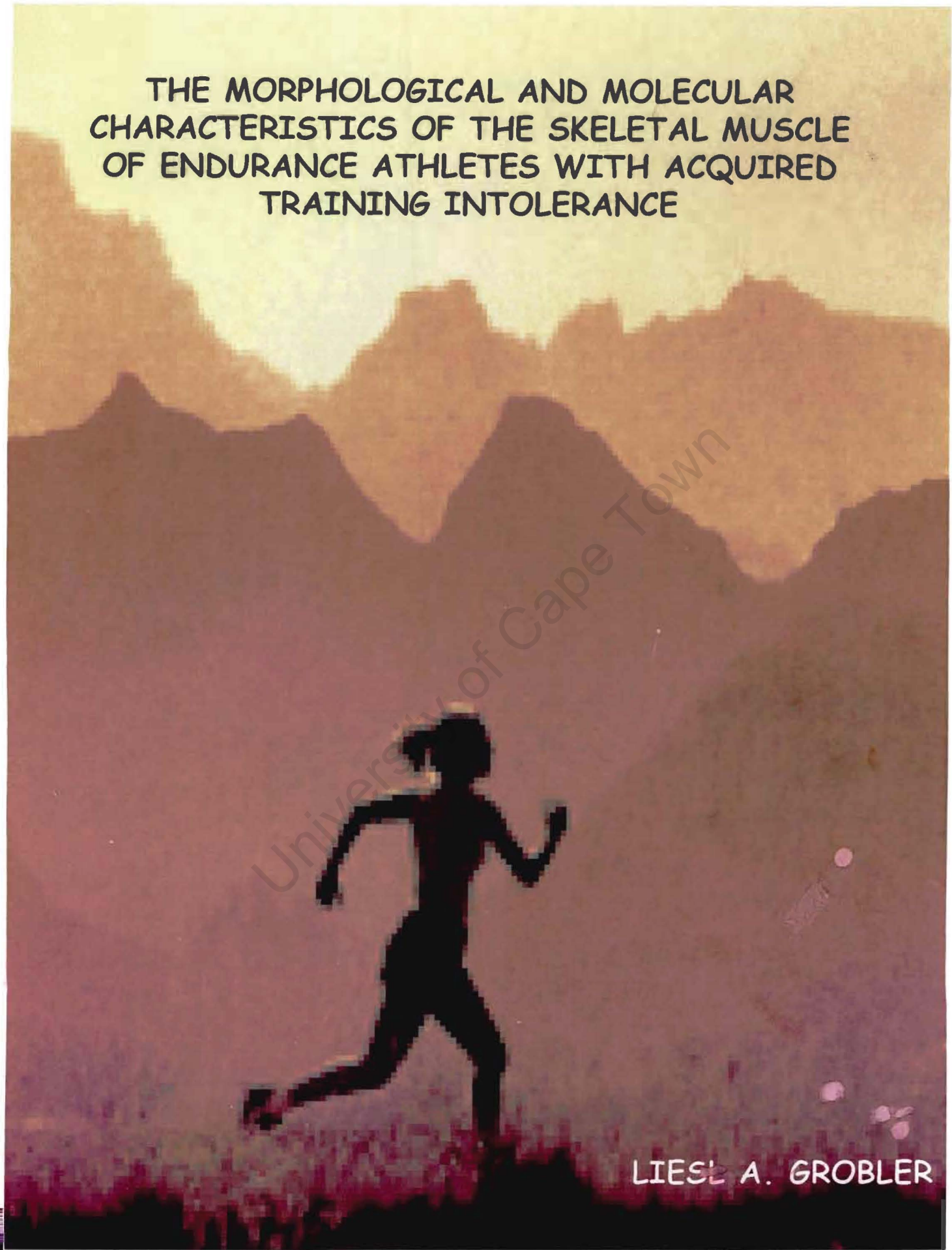
THE MORPHOLOGICAL AND MOLECULAR CHARACTERISTICS OF THE SKELETAL MUSCLE OF ENDURANCE ATHLETES WITH ACQUIRED TRAINING INTOLERANCE



LIESL A. GROBLER

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THE MORPHOLOGICAL AND MOLECULAR CHARACTERISITCS OF SKELETAL MUSCLE IN ATHLETES WITH ACQUIRED TRAINING INTOLERANCE

By

LIESL ANNE GROBLER

This Thesis is presented for the Degree of

DOCTOR OF PHILOSOPHY

In the Department of Human Biology

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This thesis is dedicated to my parents.....for their extensive financial support of this over-aged dependent, but most importantly, for their unconditional love and unwavering faith in me that has been and always will be the foundation as well as the inspiration for my life.

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DECLARATION

I, **Liesl Anne Grobler**, do hereby declare that the experiments presented in this thesis were conceived and executed by myself, except where otherwise indicated.

Neither the substance nor any part of this thesis has been submitted in the past, or is being, or is to be submitted for a degree in the University or any other University. This thesis is presented in fulfilment of the requirements for the degree of PhD.

I hereby grant the University of Cape Town free license to reproduce this thesis in part or whole, for the purpose of research.

Signed: _____

Date: _____

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LIST OF PUBLICATIONS

Published manuscripts

A St Clair Gibson, MBChB, Ph.D., MI Lambert, Ph.D., M Collins, Ph.D., **LA Grobler**, B.Sc. (Hons), KA Sharwood, B.Sc. (Hons), Wayne E Derman, MBChB, Ph.D., TD Noakes, MBChB, MD, FACSM. *Chronic exercise activity and the fatigued myopathic syndrome (FAMS)*. International Sports Medicine Journal, 1(3), July 2000.

Liesl A. Grobler, Malcolm Collins, Michael I. Lambert, Colin Sinclair-Smith, E. Wayne Derman, Alan St Clair Gibson, Timothy D. Noakes (2003). Skeletal muscle pathology in endurance athletes with acquired training intolerance. *British Journal of Sports Medicine (in press)*

Malcolm Collins, Valerie Renault, **Liesl A. Grobler**, Alan St Clair Gibson, Michael I. Lambert, E. Wayne Derman, Gillian S. Butler-Browne, Timothy D. Noakes, Vincent Mouly (2003). Athletes with exercise-associated fatigue have abnormally short muscle DNA telomeres. *Medicine and Science in Sports and Exercise (in press)*

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Liesl A. Grobler, St Clair Gibson, A., Collins, M., Sinclair-Smith, C., Derman, E.W., Lambert, M.I. and Noakes, T.D. (2001) Skeletal muscle ultra structural changes in endurance athletes with chronic fatigue. *The South African Journal of Sports Medicine*, September, pg 33.

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Liesl A. Grobler, Collins, M., Renault, V., St Clair Gibson, A., Sinclair-Smith, C., Derman, E.W., Butler-Browne, G.S., Lambert, M.I., Noakes, T.D. (2003) Ultra structural changes in the skeletal muscle of athletes with acquired training intolerance. *The South African Journal of Sports Medicine*, 15(1), pg 41.

Professional presentations

48th Annual Meeting of the American College of Sports Medicine in Baltimore, Maryland, May 2001 – Poster session: "Skeletal muscle ultra structural changes in endurance athletes with chronic fatigue: A case series."

9th Biennial Congress of the South Africa Sports Medicine Association, Johannesburg, South Africa, September 2001 – Free communication: "Skeletal muscle ultra structural changes in endurance athletes with chronic fatigue".

5th Annual AstraZeneca Medical Research Day, University of Cape Town, October 2002 – Free communication: "Morphological and molecular abnormalities in skeletal muscle of athletes with acquired training intolerance."

10th Biennial Congress of the South Africa Sports Medicine Association, Cape Town, South Africa, February 2003 – Free communication: "Ultra structural changes in the skeletal muscle of athletes with acquired training intolerance."

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ABSTRACT

Background: The hypothesis, upon which this thesis is based, is that repeated bouts of damage-inducing, prolonged, endurance training and racing, over a number of years, may exceed the biological limits of the repair and adaptation process, resulting in maladaptation of the skeletal muscle and malfunctioning of the system.

Aim: To investigate the impact of high volume endurance training and racing on skeletal muscle structure and function in a group of endurance athletes who, after a number of years of high volume endurance training and racing, experience a precipitous decline in their racing performance, that cannot be explained by aging. Another symptom afflicting these athletes is the acquired training intolerance, such that whenever they attempt to increase their training load they experience clinical symptoms of skeletal muscle fatigue, soreness, stiffness and tenderness.

Methods: Performance tests to determine maximal oxygen consumption and maximal voluntary contraction were carried out on these athletes with acquired training intolerance to assess the functional capacity of their skeletal muscle. A muscle biopsy was obtained from the vastus lateralis muscle and the structural and ultra structural morphology of the skeletal muscle was assessed. The control group consisted of endurance athletes who were matched with the experimental athletes for age and years of high volume endurance training, but who were free of any symptoms associated with acquired training intolerance. Furthermore, the

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regenerative history of the athletes' skeletal muscle was investigated by determining the telomeric length of the skeletal muscle DNA.

Results: Short term, maximal exercise performance of athletes with acquired training intolerance was not impaired in comparison with control endurance athletes who did not present with acquired training intolerance. Secondly, a significantly greater proportion of athletes with acquired training intolerance presented with structural and ultra structural evidence of skeletal muscle pathology, compared to the control athletes. Thirdly, athletes with acquired training intolerance had significantly shorter minimal telomere lengths compared to control athletes. This is most likely indicative of an increased proliferation of satellite cells in the skeletal muscle of these athletes, as a result of the repeated bouts of exercise-induced muscle damage and repair that their muscles have been exposed to. Finally, three of the athletes with acquired training intolerance presented with pathologically shortened minimum, maximum and mean telomere lengths compared to the other athletes with acquired training intolerance.

Conclusions: The presence of chronic skeletal muscle pathology in the skeletal muscle of endurance athletes suggests that the repair process in these athletes' muscle is incomplete. The fact that a greater proportion of the athletes with acquired training intolerance presented with chronic skeletal muscle pathology establishes an association between increased chronic skeletal muscle disruption and skeletal muscle maladaptation (i.e. the inability of these athletes to adapt to endurance training). The fact that the athletes with acquired training intolerance had shorter

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telomere lengths suggests a more prolific regenerative history in the skeletal muscle of these athletes, compared to that of asymptomatic control athletes. We speculate that additional or alternative mechanisms, besides repeated bouts of exercise-induced muscle damage and repair, are responsible for the dramatic shortening of the telomeres in the three athletes with pathologically shortened minimum, maximum and mean telomere lengths.

The results of this thesis provide preliminary evidence to support the proposed hypothesis that repeated bouts of damage inducing endurance exercise, over a number of years, may challenge the biological limits of the regeneration process in some individuals, resulting in incomplete repair of exercise-induced skeletal muscle damage and skeletal muscle maladaptation.

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CHAPTER 1

LITERATURE REVIEW

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1.1. INTRODUCTION

Just for a moment imagine a 70 kg man running a standard marathon (42.2 km) at a pace of five minutes per kilometre (3.3 metres per second) and an average stride frequency of about 90 strides per minute (355). At the end of the marathon, which would take about 3.5 hours to complete, each leg would have made contact with the ground approximately 9500 times. With every heel strike the working limb muscles would be required to absorb vertical ground reaction forces of approximately 2.5 times body mass (314;383). As the supporting muscles (quadriceps) absorb the impact of the vertical ground reaction forces they lengthen. It is these eccentric muscle actions that render the muscles vulnerable to structural damage (5;82;83). Although eccentric muscle actions are not the only cause of exercise-induced muscle damage, they are the most disruptive (306;310;358). In addition to the mechanical damage induced by the eccentric actions of your muscles, the prolonged, exhaustive nature of running a standard marathon at a relatively competitive pace would induce numerous metabolic changes that are also capable of inducing damage in the working muscles (94;126;365;407).

This typical scenario serves to illustrate the potential for profound damage during prolonged, exhaustive exercise of which eccentric muscle actions are a major component. A simple calculation shows that if a serious recreational runner trains 80 km per week and has a running career spanning 10 years, then the muscles of his lower limbs would lengthen under high tension about

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19 million times during his running career (52 weeks x 10 years x 80 km x 450 strides/km).

In a controlled laboratory experiment where skeletal muscle is damaged in a controlled manner and the research subject is required to rest during the recovery phase, complete repair of the exercise-induced muscle damage occurs and normal muscle function and morphology is regained (75;81;117;124;221). In reality, however, anecdotal evidence suggests that most endurance athletes rarely incorporate sufficient time for their muscles to recover after a race or an intense training session (374).

Despite the fact that signs of muscle degeneration were still observed 7 days following a standard marathon (197), that signs of muscle regeneration were noted 12 weeks after a standard marathon (517) and that the time required for athletes to run "pain free" following a 56.6 km road race, ranged from 0 to 10 days for the male athletes and from 1 to 21 days for the female athletes (282), anecdotal evidence shows us that many of the top athletes will run the very next day following a standard or ultra marathon (374). This is summarised by the comments of a former elite distance runner, who still holds the record for being the fastest 18 year old to complete the Comrades Marathon (90 km road race): *"When I was young and fast and thought that more was better, I would go out and train on tired or sore legs; as did the majority of the top runners. It is unfortunate that only when I was past my best did I wake up to the physiological fact that to get faster, sometimes rest is the best training session. I have lots of regrets over that. I think I could have gone faster if I*

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had tempered my enthusiasm somewhat. As I indicated earlier, waiting a bit longer results in an overall faster recovery. It is the elite and want-to-be elite athletes that have a problem. They are so determined and committed that the idea of not training is just a sign of weakness. So they often start training again when they are still sore. I remember when Mark Plaatjies ran a 2 hour:08 minute marathon in the South African Championships and then in the afternoon he went out for a 10km run "to loosen up" as he said." (Dr Andrew Bosch, PhD, 2003, personal communication).

Further anecdotal evidence suggests that the popular concept of "*no pain, no gain*" or "*the harder one trains the greater the training adaptation and the greater the improvement in performance*" is deeply ingrained in the psyche of many social and competitive endurance athletes. This mindset, together with the rigorous training and racing regime of most endurance athletes, could expose the limitations of skeletal muscle in terms of its capacity for regeneration and adaptation (375). The acute effects of prolonged, exhaustive endurance exercise on skeletal muscle is well documented (12;16;117;146;197;203;276;452;453;517). The long-term effects of prolonged, endurance training and racing, particularly in combination with inadequate rest and potentially ineffective/incomplete repair, requires investigation.

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1.1.1. Background to the thesis

Previously Derman et al. (1997) described a group of endurance athletes with exercise-associated chronic fatigue (108). The common characteristics

among these athletes were an inexplicable decline in performance that was not age related, a current intolerance to exercise training and a history of high volume training. This condition was called Fatigued Athlete Myopathic Syndrome (FAMS)(108)(see Appendix xxx for more details on this condition).

These FAMS athletes had consulted numerous physicians, unsuccessfully, and did not respond to long periods of rest or to typical nutritional and psychological support (108). Medical examinations revealed these athletes to be free from the normal physiological (i.e. training -, diet -, travel - or pregnancy – induced) and pathological (haematological or metabolic conditions, classic chronic fatigue syndrome, overtraining syndrome and neurological disorders) conditions associated with fatigue. The physical symptoms were not consistent with the acute consequences of overtraining as in most cases the training loads of the athletes are significantly reduced and extensive rest periods did not alleviate the symptoms, as would occur with overtraining (269).

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These athletes reported skeletal muscle symptoms such as excessive delayed onset muscle soreness, muscle stiffness, tenderness and skeletal muscle cramps. Muscle biopsy of the vastus lateralis revealed evidence of chronic skeletal muscle damage (i.e. atrophic fibres, abnormal lipid and glycogen deposits, abnormal mitochondrial morphology and distribution (subsarcolemmal aggregations), internal nuclei, focal myofibrillar degeneration and muscle fibre necrosis) (108). Although many of the athletes in this study presented with chronic skeletal muscle structural and ultra

structural disruptions, the design of the study did not allow the researchers to determine whether the skeletal muscle disturbances were pathological or merely an unusual form of adaptation by the body to long-term high volume exercise training. It was speculated that the exercise-associated chronic fatigue and training intolerance experienced by these FAMS athletes was related to the skeletal muscle disturbances, which were as a result of the athletes' high volume endurance training and racing regimes. However, the design of the study prevented a cause and effect relationship from being established (108). It was decided that the continued use of the term given to this group of athletes, "fatigued athlete myopathic syndrome" (FAMS) might be somewhat presumptive. Therefore, in light of the fact that the most commonly reported "symptom" that led these athletes to seek medical advice was their inability to tolerate endurance training loads to which they were previously accustomed, it was decided to refer to this group of athletes as athletes with acquired training intolerance (ATI).

1.1.2. Scope and aim of the thesis

The hypothesis, upon which this thesis is based, is that repeated bouts of damage inducing, prolonged, endurance training and racing, over a number of years, may exceed the biological limits of the repair and adaptation process, resulting in maladaptation of the skeletal muscle and malfunctioning of the system. Although many of the athletes in the study by Derman et al. (1997)(108) presented with chronic structural and ultra structural skeletal muscle disruptions, there are no studies that have systematically examined

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the impact of repeated bouts of muscle damage and repair over a number of years on the structure and function of skeletal muscle.

Accordingly, the aim of this thesis was to investigate the functional, morphological and molecular characteristics of the skeletal muscle of a group of endurance athletes who, after a number of years of high volume training and racing, could no longer attain their expected levels of running performance or training load. When attempting to increase their training load they experienced clinical symptoms of skeletal muscle fatigue, soreness, stiffness and tenderness. The functional, morphological and molecular characteristics of these athletes were compared to those characteristics of control athletes who were matched for age and years of endurance training, but who did not have symptoms of acquired training intolerance.

1.1.3. Summary of the approach to the thesis

Information that is relevant to the research question will be discussed in a review of the literature. The review will focus on the proposed factors responsible for exercise-induced muscle damage. The regeneration process, including the infiltration of immune cells, revascularisation of the damaged area, the activation and proliferation of satellite cells, the fusion of myoblasts and the maturation of myotubes will also be discussed. Furthermore, the process of skeletal muscle adaptation and the repeat bout effect associated with exercise-induced muscle damage are also reviewed. Finally, evidence for maladaptation in skeletal muscle, in the form of the aging process and the overtraining syndrome, are discussed. The experimental section of the thesis,

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in which the questions outlined above are addressed, will follow the review of the literature.

1.2. EXERCISE-INDUCED SKELETAL MUSCLE DAMAGE

1.2.1. Introduction

Unaccustomed, eccentric exercise, whereby the activated muscle is forced to lengthen while producing tension (5;306;310;358), as well as prolonged, exhaustive, endurance exercise have been shown to induce damage in the active skeletal muscle (166;197;230;283;452;453;517).

1.2.2. Physiological symptoms of exercise-induced muscle damage

Physiological evidence of exercise-induced skeletal muscle damage is a reduced ability of the muscle to produce force (5;535), muscle stiffness, swelling and pain (78;95;143;145;321).

1.2.2.1. Reduced force production

The greatest decrease in strength occurs immediately after damage-inducing exercise. This reduction in muscle strength may persist for up to 10 days post exercise (84;85;160;418). Possible mechanisms to explain this immediate and dramatic reduction in force are i) cellular damage to muscle cells and whole fibres, rendering them unexcitable, ii) disruption of the excitation-contraction coupling mechanics, leading to reduced calcium release and reduced force

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production, iii) disorganisation and changes in the calcium sensitivity of the contractile machinery, iv) changes in the central nervous system, motor nerve or neuromuscular junction, v) failure of the thick and thin filaments of overstretched sarcomeres to reinterdigitate on relaxation (5;124;250;281;358).

Warren et al. (1999) concluded in a review article that isometric maximal voluntary contraction torque and range of motion measurements were the most reliable and practical methods for quantifying muscle injury in humans (519). Since this critical review, more dynamic measures of muscle function, which more accurately reflect impairment in athletic performance, have been used to assess the effect of exercise-induced muscle damage on force production (59-61).

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1.2.2.2. Muscle swelling and stiffness

Muscle swelling following exercise-induced muscle damage is assessed either by measuring the change in the circumference of the affected muscle or by means of ultrasound or MRI imaging of the damaged muscle compartment (78;377). The precise mechanism of action that causes the muscle swelling is not known. However, it is postulated that the increased synthesis of prostaglandin E_2 within 24 hours post exercise and the degranulation of mast cells 48 hours post exercise may cause vasodilation and increased vascular permeability in the area of damage (457;474). The increased vascular permeability allows plasma proteins to leak into the interstitial space, further increasing colloid osmotic pressure. The movement of creatine kinase and other cytoplasmic proteins from the muscle cell into the interstitial space may

also alter the interstitial osmotic pressure, leading to an influx of fluid into the area (78;474).

The extent of muscle stiffness following exercise-induced muscle damage may be assessed by three different means. Namely, changes in the relaxed joint angle (83), the external force required to straighten the affected joint (240) and change in the slope of the torque-angle curve produced by the joint in the horizontal plane (214). When assessed by the first two methods, muscle stiffness follows a similar time course to that of muscle swelling, whereby it increases immediately post eccentric exercise, peaking at 3 days and dissipating by 7-10 days post exercise (78;81). Accordingly, it was suggested that muscle swelling is responsible for the increase in muscle stiffness and the decrease in range of motion (78). However, when measuring muscle stiffness by assessing the change in the slope of the passive torque-angle curve, the initial, immediate increase in muscle stiffness was not related to the increase in muscle swelling (78). The time course of the decrease in muscle stiffness did, however, appeared to be related to the decrease in muscle swelling (78). It was initially thought that the increased muscle stiffness might be associated with an increase in the resting EMG activity. This notion was dispelled by the observation that the resting EMG levels of the damaged muscle were not different to those of the undamaged muscle (78). Furthermore, Jones et al (1987) noted that stiff and painful eccentrically damaged muscle was electrically silent at rest and therefore increased EMG activity could not account for the stiffness of the damaged muscle (240). The altered calcium homeostasis associated with exercise-induced muscle

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damage is the most likely cause of the initial increase in muscle stiffness (78;214).

1.2.2.3. Muscle pain (Delayed onset muscle soreness)

Delayed onset muscle soreness (DOMS) is the most frequently reported physiological symptom of muscle damage. The pain appears between 8 and 24 hours post exercise and peaks between 24 and 48 hours post exercise. The pain may still be present for up to 7 days post exercise (250;379).

The precise cause of the pain is not known. Myelinated group III and unmyelinated group IV afferent neurons, known transmitters of pain sensation, are found throughout the muscle and are particularly dense in the regions of connective tissue (117). While the myelinated group III fibres transmit sharp pain, the unmyelinated group IV fibres transmit dull, diffuse pain. It is believed that the group IV afferents are primarily responsible for the sensation of DOMS because dull, diffuse pain is more readily associated with DOMS and because there is an increased presence of these afferents in skeletal muscle and connective tissue (250).

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Prostaglandin E_2 (PGE_2), bradykinin, acetylcholine, histamine, serotonin and potassium are some of the substances released as a result of exercise-induced muscle damage and the subsequent immune response (250;457). These substances may be responsible for sensitising the pain receptors into a state of hyperalgesia in which increased tissue oedema together with

additional mechanical pressure (movement or palpation) provide sufficient stimulus to cause the sensation of pain (12;117;250;327;457).

Another possibility is that increased tissue pressure resulting from tissue swelling may be associated with the perception of soreness (145;327;457). Although studies have shown that prostaglandin-inhibiting drugs do not alleviate the perception of soreness (112;277), and that tissue pressure itself is not sufficient to induce pain, the pain may result from a combination of these and other factors (457). This is supported by the fact that the soreness associated with DOMS is primarily sensed when the injured muscle is palpated or activated, either eccentrically or concentrically.

Delayed onset muscle soreness (DOMS) can leave the athlete relatively incapacitated and thus it seems that pain would be an obvious mechanism whereby the body might protect itself from further muscle damage (327). However, in the case of exercise-induced muscle damage, DOMS is not a good reflection of the structural or ultra structural muscle damage or the muscle dysfunction that results from a bout of muscle damaging exercise (379). DOMS is initially observed about 24 hours after a bout of damaging exercise. By 48 hours after the bout of exercise the pain is at its worst. DOMS dissipates by about 72 hours after the exercise bout (321). In contrast to this, the extent of skeletal muscle dysfunction and structural and ultra structural disruption is most profound immediately after a bout of damage inducing exercise (5;81;125). While skeletal muscle function is restored within approximately 10-14 days (81;125;160), studies have shown that structural

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and ultra structural skeletal muscle disruptions can take anywhere between 7 (75) and 70 (517) days to be completely repaired.

1.2.3. Cellular markers of exercise-induced muscle damage

Fridén et al. (1981) were the first to show clear evidence of morphological changes in human skeletal muscle following the eccentrically biased activity of running down a flight of stairs (148). While they did not observe any signs of muscle fibre necrosis or inflammation, electron microscopic analyses revealed Z disc streaming and broadening of the Z lines (148). Apart from disruptions to the Z discs and signs of muscle fibre necrosis and inflammation, other cellular markers of exercise-induced muscle damage include the presence of internal nuclei, variations in muscle fibre size (atrophy and hypertrophy), focal deletions of myofibrils, enlarged mitochondria, subsarcolemmal aggregations of mitochondria and an accumulation of lipid and glycogen droplets within the intermyofibrillar spaces (15;79;117;140;142;369).

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1.2.3.1. Disruptions to the Z disc

The Z disc (also known as the Z line or Z band) is the region that connects one sarcomere to the next (397). Each actin filament is attached via the α–actinin protein to four other actin filaments in the region of the Z disc (397). During eccentric contractions, when the muscle is required to produce force while lengthening, a great deal of tension develops within the area of the Z disc. When the sarcomeres are stretched beyond the length that produces optimal force they become vulnerable to damage. The region of the Z disc is

often one of the first areas to be disrupted as a result of an eccentric muscle contraction (141). Although a certain degree of Z disc streaming may be present in normal muscle (472), extensive Z disc streaming is indicative of either exercise-induced ultrastructural muscle damage (140;141;160;264;477) or a non-specific myopathy (114).

1.2.3.2. Internal nuclei

The presence of internal nuclei may be indicative of various neuromuscular disorders as well as muscle degeneration/regeneration (32;114;159;532). The presence of internal nuclei within more than 3% of the muscle fibres is generally considered to be abnormal (114).

1.2.3.3. Muscle fibre size variation and focal deletions of myofibrils

In a normal muscle sample the fibres should all be similarly sized and it is generally believed that the presence of a certain degree of variation in the size of the muscle fibres is abnormal (114). Similarly, focal deletions in isolated fibres are a normal occurrence in healthy muscle. However, extensive loss of myofibrils in several muscle fibres is considered abnormal. Increased muscle fibre size (hypertrophy) is characteristic of skeletal muscle adaptation to increased workload (refer to Chapter 1.3.7.1 for further details) (63;507). A decrease in the size of the muscle fibres (atrophy) is associated with skeletal muscle myopathies, dystrophies, denervation or immobilisation (11;114;387).

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1.2.3.4. Muscle fibre necrosis and inflammation

Although there are studies to contradict this (327;329;517;537), a typical, acute response to exercise-induced muscle damage is an infiltration of immune cells and necrosis of the damaged muscle fibre (82;197;275;320;452;457;493). The presence of lipofuscin granules, indicative of chronic muscle fibre necrosis and inflammation, has been observed in the undamaged and damaged muscle sample of experienced endurance athletes (102;140;166;517).

1.2.3.5. Enlarged and subsarcolemmal aggregations of mitochondria

In a normal muscle fibre the mitochondria are evenly dispersed throughout the cytoplasm. In the case of various myopathies, dystrophies or metabolic disturbances, abnormalities in the number, size, structure and distribution of mitochondria within the cytoplasm may occur (114). Alternatively, it is well established that endurance exercise training induces changes in the number, size, structure and distribution of mitochondria in the cytoplasm (2;39;77;206;242;256;489;496;540).

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1.2.3.6. Accumulation of lipid and glycogen molecules

An increase in lipid and glycogen deposits, particularly surrounding the mitochondria, is a well-described adaptation to endurance training (19;55;173;270;272;289). However, an increased accumulation of lipid and glycogen molecules in the intermyofibrillar space may be a consequence of a metabolic abnormality or a state of inactivity in a previously well-trained endurance athlete. The sarcoplasm surrounding internal nuclei normally

contain aggregations of mitochondria and glycogen droplets. In addition to this, glycogen and lipid droplets tend to accumulate in the areas where myofibres have been deleted (114).

Studies have shown exercise-induced muscle damage to be characterized by the presence of disruptions to the sarcolemma, sarcoplasmic reticulum, t-tubules and contractile and cytoskeletal proteins (as discussed in greater detail in section 1.2.7)(15;79;80;82;117;140;369;410;483). Furthermore, exercise-induced muscle damage is associated with elevated levels of muscle proteins, such as creatine kinase (CK), myoglobin (Mb), lactate dehydrogenase (LDH), myosin heavy chain (MHC), troponin I (sTnI) and proteolytic enzymes, in the blood (144;265;317;367;368;464).

While the physiological symptoms and cellular disruption associated with exercise-induced skeletal muscle damage are well described, the underlying cause and mechanisms responsible for these symptoms and disruptions are not yet fully understood. The following aspects of exercise-induced muscle damage will be discussed in this section of the literature review:

- The mechanical and metabolic factors implicated in the initiation of the muscle damage.
- Exercise-induced muscle damage and the sarcomeric proteins.
- Exercise-induced muscle damage and the sarcomeric membranes.
- The consequences of increased intracellular calcium concentrations in the perpetuation of the damage and the initiation of the regeneration process.

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1.2.4. Mechanical factors associated with exercise-induced muscle damage

1.2.4.1. Eccentric muscle action

During a concentric muscle contraction, tension is produced while the muscle shortens. During an isometric muscle action, tension is produced while the muscle remains a constant length (281). As mentioned previously, during an eccentric muscle action, tension is developed while the active muscle is forcibly lengthened (281;410).

During a maximal voluntary eccentric action the muscle is able to resist 30% more force than it is able to produce during a maximal voluntary concentric contraction (250). In addition to this, fewer muscle fibres are recruited during an eccentric action and thus the mechanical strain experienced per muscle fibre is greater during an eccentric action compared to a concentric contraction (120). While the tension generating mechanics involved in an eccentric muscle action enables the muscle to develop and resist great forces, it also renders the muscle vulnerable to extensive muscle damage. Mechanical damage to the skeletal muscle is caused primarily by unaccustomed, eccentric muscle actions.

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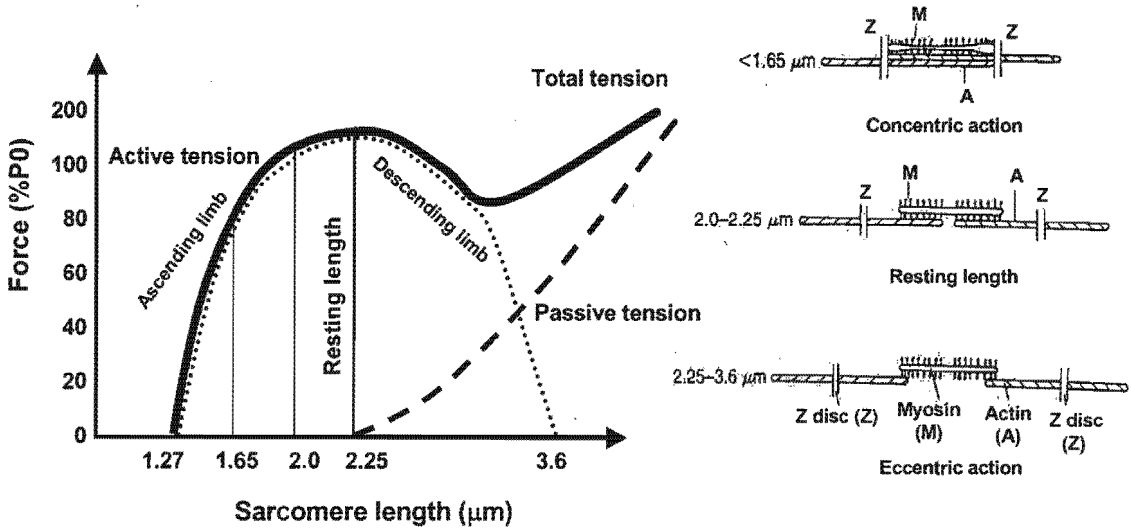

Figure 1.2.1. The relationship between sarcomere length and the tension developed in the active muscle. (Gordon,A.M., Huxley,A.F. & Julian,F.J. *J Physiol*, 1966; 184: 170-192). Z = Z disc, M = myosin, A = actin. Dotted line = active tension, dashed line = passive tension, solid line = total tension.

The tension generating mechanics of an eccentric contraction occurs in two phases. Initially, as the sarcomere lengthens from about 1.65 μm to 2.25 μm, the muscle actively produces tension through cross bridge cycling (active tension; Figure 1.2.1) (167;358). As the sarcomere lengthens further beyond this point there is a gradual reduction in the extent of interaction between the contractile filaments and a consequent decrease in the total tension produced by the sarcomere (358). This is known as the descending limb of the length-tension curve produced by a classic eccentric muscle action (5;357;358;410). The sarcomeres are most vulnerable to mechanical disruption at lengths that fall upon this portion of the curve (5;356-358;410). Upon further lengthening of the sarcomere, a point is reached at which the contractile filaments no longer interact with one another. Beyond this point, tension is produced in the

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sarcomere by the passive stretching of the contractile and cytoskeletal proteins (passive tension)(5;248;281;358;473).

1.2.4.2. Variability in sarcomere length

In an early study investigating the force-velocity relationship of concentric and eccentric muscle actions in isolated frog muscle, a discontinuity in the force-velocity relationship between the two muscle actions was observed (248). It was noted that proportionally greater force was required for a given rate of stretch or lengthening (eccentric muscle action) than for the same rate of shortening (concentric muscle action; Figure 1.2.2) (248). An alternative interpretation would be that for a given rate of lengthening, a greater force or tension is developed in the muscle compared to the force or tension produced by the muscle for a similar rate of shortening.

In addition to this, it was also observed that the lengths of the sarcomeres vary by about 1% along the length of the muscle fibre (223). This difference in sarcomere length affects the velocity of lengthening, whereby the shorter sarcomeres lengthen more quickly than the longer sarcomeres (140). Due to the steep nature of the lengthening portion of the force-velocity curve (Figure 1.2.2), the force produced in adjacent sarcomeres with slightly differing sarcomere lengths may vary by more than 50 % P_o (P_o being the peak isometric force)(140). This mismatch in force production in the individual sarcomeres in series would place undue directional stress on the Z discs separating the sarcomeres (140). This inconsistent production of force in neighbouring sarcomeres is the most likely cause of the Z disc streaming that

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is frequently observed after damage inducing exercise (140;141;160;264;477).

1.2.4.3. The "popping sarcomere" theory

In addition to the difference in the force velocity relationship between eccentric and concentric muscle actions, it was also observed that when the muscle was required to resist (eccentric action) a force greater than 180 $\%P_o$ (P_o being the peak isometric force), the muscle suddenly yielded and lengthened at a very high velocity without any additional force being applied to it (point (c) in Figure 1.2.2.) (248). The length of the sarcomere at which this yield point occurs, corresponds with the sarcomere lengths that fall within the region of the descending limb of the length-tension curve (Figure 1.2.1). In accordance with this observation, Morgan et al. (1990) postulated that as active muscles lengthen, the shorter, weaker sarcomeres lengthen preferentially and reach their yield point on the descending limb on the length-tension curve sooner (5;356;358;410). Once these sarcomeres reach their yield point they lengthen instantaneously, in a rapid and uncontrolled manner (356). This is known as the "popping sarcomere theory" (5;356;358;410).

Only when the passive tension in the rapidly lengthened sarcomeres equals the active tension in the adjacent sarcomeres, will the uncontrolled elongation of the weaker sarcomeres cease (5;358;410). At this stage there is little or no overlap of the contractile filaments in these overstretched sarcomeres (5;358;410).

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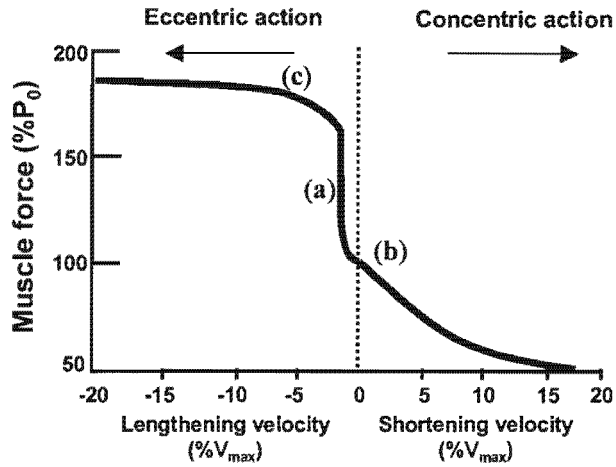

Friden, J. and Lieber,R.L. *Medicine and Science in Sports and Exercise*, 24(5):521-530, 1992.

Figure 1.2.2. The differences in the force-velocity relationships during a concentric muscle action (shortening) and an eccentric muscle action (lengthening). P_0 = the peak isometric force. Muscle force is expressed as a percentage of (P_0). V_{max} = the maximal unloaded shortening velocity. The lengthening and shortening velocity is expressed as a percentage of V_{max}. Note the differences in the slope of the curve at point (a) and (b). At point (a), the muscle force developed per rate of lengthening velocity ($\%V_{max}$) is far greater than the muscle force developed per rate of shortening velocity ($\%V_{max}$), point (b)). (Woledge,R.C., Curtin,N.A. & Homsher,E. Energetic Aspects of Muscle Contraction, Academic Press, London, 1985: pp 1-329).

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It is presumed that on relaxation of the muscle fibre, after a single eccentric contraction, the contractile filaments in the majority of the overstretched sarcomeres reinterdigitate and thus remains undamaged (410). The sarcomeres in which the contractile filaments fail to reinterdigitate become disrupted and are unable to develop tension in a subsequent contraction (358;410;484). However, during repeated eccentric contraction it is proposed that there is an increase in the number of sarcomeres in which the contractile filaments fail to reinterdigitate. Furthermore, the previously disrupted sarcomeres place additional load on neighbouring sarcomeres, making them more prone to disruption during subsequent disruptions (358;410). The

increasing number of disrupted sarcomeres eventually places excessive strain on the sarcolemma, the sarcoplasmic reticulum and the t-tubules, leading to disruption of these membranes (410). In addition to this, increased strain may be placed on the Z discs as well as on particular cytoskeletal proteins involved in stabilising the sarcomeres, such as desmin and titin. This strain may overwhelm the cytoskeletal proteins and so result in the disruptions observed by researchers, such as Z disc streaming, broadening and disruption as well as hypercontraction and disorganisation of the contractile proteins (149).

Studies involving the rapid-fixation technique, developed by Brown and Hill (1991), allowed researchers to study muscle ultra structure during or immediately after stretch-induced damage to the muscle. This methodology provided evidence for the "popping sarcomere" hypothesis (48). This study showed that when active fibres were stretched to sarcomere lengths longer than the optimum (i.e. on the descending limb of the length-tension curve; Figure 1.2.1.), there were occasional sarcomeres in which the thin filaments were either partially or completely pulled out of the thick filament array in either one or both halves of the sarcomere (48). A study by Talbot and Morgan (1996)(484) used a similar technique on toad sartorius muscle and showed direct evidence of overstretched sarcomeres that were randomly scattered throughout the stretched muscle (484).

The muscle mechanics involved in force production while the muscle lengthens, exposes the inherent instability of the sarcomere on the descending limb of the length-tension curve (140;358;410). Sarcomeres are

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vulnerable to overstretching and loss of interdigitation of the contractile filaments increases beyond a certain length during an eccentric muscle action (5;358). In addition to this, inhomogeneity in sarcomere length, within the length of the myofibrils, affects the velocity of lengthening of the sarcomeres (140;248). Minor differences in lengthening velocity relate to major differences in the tension produced in adjacent sarcomeres, further rendering the sarcomere vulnerable to damage (140;223). Thus the contractile mechanics of the eccentric contraction and inherent sarcomere inhomogeneities appear to be responsible for the initial damage to the muscle cell (410).

1.2.5. Metabolic factors associated with exercise-induced muscle damage

Although eccentric muscle actions are believed to be the major factor responsible for the damage induced by exercise, the contribution of metabolic factors, particularly during prolonged, exhaustive exercise cannot be ignored. Metabolic events such as the depletion of high energy phosphates (430), the production of reactive oxygen and nitrogen species (371;373), increase in body temperature (76;333) and the accumulation of hydrogen ions (291;509) are likely to occur during prolonged, exhaustive exercise. A common consequence of these events is a disruption of cytoplasmic calcium homeostasis, which is associated with muscle damage. Each of these events will be discussed in more detail.

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1.2.5.1. *Depletion of high-energy phosphates*

During low to moderate intensity exercise the production of ATP and other high-energy phosphates via various metabolic pathways matches the cellular demand for ATP hydrolysis (271). During prolonged, exhaustive exercise there will be a reduction, albeit small, in the availability of high-energy phosphates such as phosphocreatine and ATP in the cytoplasm of the muscle cell (16;271). Furthermore, a small decrease in the availability of high-energy molecules in a key area of the cell can profoundly affect the functioning of the cell (16). For example, a slight reduction in the concentration of ATP and other high-energy molecules at the level of the sarcoplasmic reticulum might impair the function of the Ca^{2+}-ATPase pump (56). Impairment of the Ca^{2+}-ATPase pump would subsequently hinder the reuptake of calcium into the sarcoplasmic reticulum, resulting in an increased calcium concentration in the cytoplasm (16;56).

1.2.5.2. *Production of reactive oxygen and nitrogen species*

Tissues that have a high metabolic rate produce an increased quantity of oxygen and nitrogen free radicals (250). These substances cause irreversible damage to many cellular constituents (56). Increased free radical production will result in the oxidation of phospholipids, DNA, carbohydrates and proteins (372).

The mitochondrial electron transport chain, membrane bound oxidases, infiltrating immune cells and the interaction of myoglobin and haemoglobin

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with peroxides are all possible sources of free radical production during exercise (89;250).

Peroxidation of membrane phospholipids may disrupt the normal permeability of the sarcolemma, permitting abnormal diffusion of molecules (i,e. calcium) down concentration gradients (116;250). In addition to this, free radical oxidation of sulfhydryl groups of the Ca^{2+}-ATPase pump is highly correlated with a reduction in the rate of calcium reuptake by the sarcoplasmic reticulum, resulting in an increase in cytoplasmic calcium (56;116). Free radicals may also cause oxidative damage to DNA and other structural proteins (305;338;435;523).

1.2.5.3. Increased body temperature

It is commonly observed that the post-race rectal temperature of marathon runners is elevated (76;333). It has been reported that the lipid membrane surrounding the Ca^{2+}-ATPase pump in the sarcoplasmic reticulum is altered when the body temperature reaches 38 °C. Although the precise mechanism is not known, alteration of the membrane in this region impairs the pumps ability to resequester calcium from the cytoplasm (56;225), further contributing to an increased concentration of calcium within the cytoplasm.

1.2.5.4. Lowered pH (accumulation of hydrogen ions)

Strenuous exercise and an increased metabolic rate result in an accumulation of hydrogen ions in the cytoplasm of the cell (291;509). An increased concentration of hydrogen ions in the cytoplasm affects the ability of the

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sarcoplasmic reticulum to resequester calcium (56). This has been attributed to calcium and hydrogen competing for the calcium-binding site on the Ca^{2+}-ATPase pump (301). This will further increase the levels of calcium in the cytoplasm of the muscle cell.

The end result of the exercise-induced muscle damage be it caused by mechanical, structural or metabolic factors, is a disruption of cytoplasmic calcium homeostasis. This has profound consequences on the structure and function of the cell, as will be discussed in section 1.2.9.

1.2.6. Exercise-induced muscle damage and other sarcomeric proteins

1.2.6.1. Cytoskeletal muscle proteins

In addition to the contractile proteins, muscles possess a number of cytoskeletal proteins that stabilize the contractile proteins and allow for the transmission of tension both laterally and longitudinally (397). Three cytoskeletal proteins in particular, titin, desmin and dystrophin, due to their positions in the cytoskeleton, their role in force transmission and their intrinsic characteristics, are believed to have integral functions in the process of exercise-induced muscle damage (5).

Titin

Titin is a giant (~ 3.5 MDa) intrasarcomeric protein that spans half the length of the sarcomere from the Z line to the M line. Titin is orientated in parallel with the contractile filaments and it transmits force from the myosin filament to

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the Z discs. Studies investigating the source of passive tension in muscle have revealed a direct relationship between muscle elasticity and the mechanical properties of the titin molecule (498).

The various regions of the titin molecule possess different elastic properties. The section of titin in the I-band region is highly elastic (312). This portion of the molecule consists of two main structural elements, serially linked immunoglobulin-like domains and a unique segment that is rich in proline (P), glutamate (E), valine (V) and lysine (K) residues, the so-called PEVK segment (171;312). The A-band section of the titin molecule is functionally stiff as it is firmly bound to various myosin proteins (312). The relative tension that develops in I-band titin, on extension of the molecule, determines the passive tension of the muscle fibre (208;414).

As the sarcomere is stretched, the tandem immunoglobulin repeats extend, this is followed by extension of the PEVK region and then finally by the unfolding of the tandem immunoglobulin domains (171). If the sarcomere is required to stretch beyond this point, it appears that the A-band titin becomes unbound and enters the I-band region of the sarcomere. The sarcomere length at which this occurs correlates to the yield point of the sarcomere, whereby further extension of the sarcomere fails to produce a further increase in passive tension (as discussed in section 1.2.4) (208).

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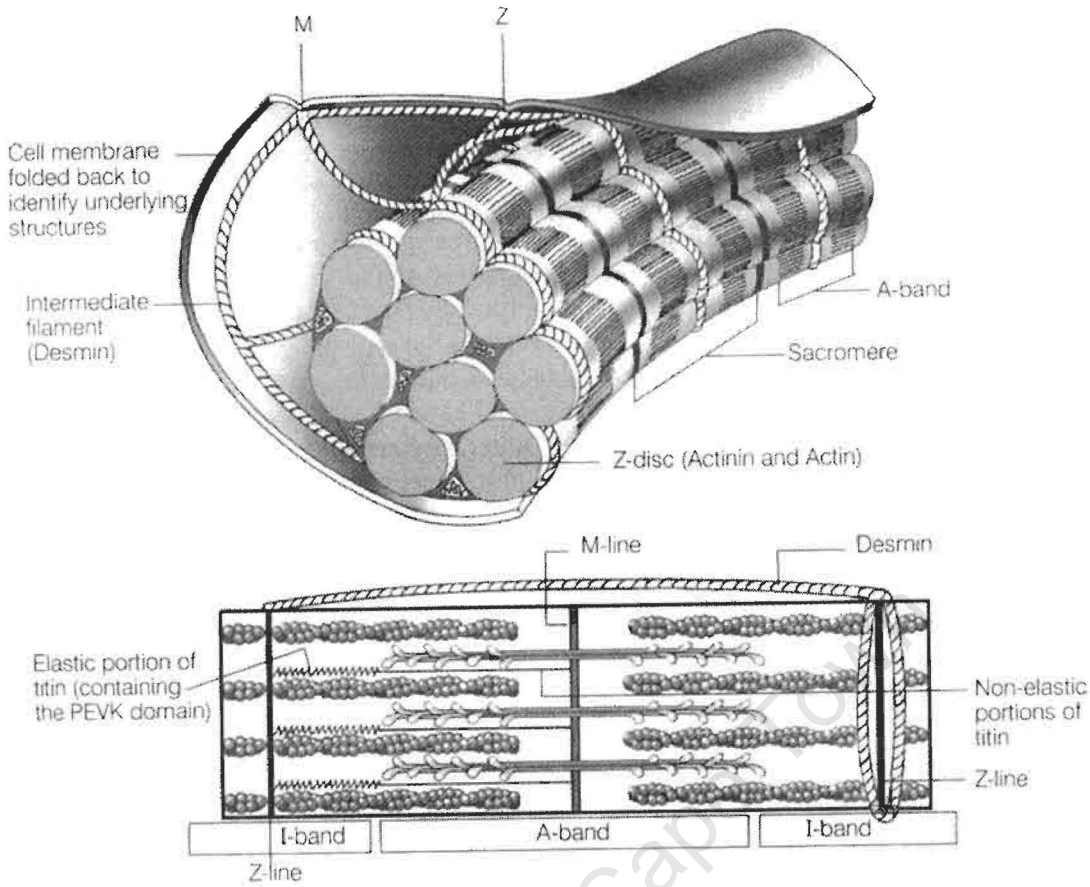

Figure 1.2.3. A detailed diagram of the sarcomere showing the contractile filaments (actin and myosin), the intrasarcomeric protein, titin and the intermediate filament, desmin. Other proteins such as nebulin, talin, vinculin and dystrophin are not shown. (T.D. Noakes, Lore of Running, 4th edition, 2001).

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Furthermore, it is proposed that if the yield point of the sarcomere is not exceeded, the structure and function of the titin molecule allows for similar tensions to be produced in the muscle during repeated cycles of sarcomere extension and relaxation (208). In this way, titin may play an important role in the reinterdigitation of the contractile filaments after stretch. However, if the yield point of the sarcomere is exceeded, the dislodgement of the A-band titin from the myosin filament may prevent or hinder the reinterdigitation of the contractile filaments (208;358).

Researchers have observed different isoforms of the titin molecule in various skeletal muscle fibres (208). In addition to this it has also been observed that muscle fibres with longer titin isoforms develop passive tension at longer sarcomere lengths (208). The findings of a study by McHugh et al. (1999) show that stiffer or less compliant muscles, as determined by straight-leg-raise stretch, experience greater symptoms of muscle damage follow eccentric exercise (342). This study provides experimental evidence of an association between flexibility and muscle injury. It is interesting to speculate that this is where the functional importance of the titin molecule and the role it plays in determining skeletal muscle elasticity may be evidenced.

The phenomenon whereby concentrically trained muscle is more susceptible to exercise-induced muscle damage appears to be related to muscle stiffness (164;405;527). It is thought that concentric training induces a reduction in the number of sarcomeres in series thereby increasing muscle stiffness and hence susceptibility to damage following eccentric exercise (516;528).

Desmin

The extrasarcomeric protein, desmin, connects adjacent Z discs to one another and peripheral Z discs to the costamere in the surface membrane (5;358;397). In addition to maintaining the alignment of the Z discs across the fibre, desmin is also responsible for lateral transmission of sarcomere tension (358).

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Previous studies investigating the potential role of desmin in muscle damage induced by repeated eccentric muscle action, noted a loss of desmin staining as early as 5-15 minutes following a bout of eccentric exercise (309). From these results it was concluded that disruption of the desmin protein is an early manifestation of muscle damage induced by eccentric exercise (309). Studies have also shown that the number of fibres staining negative for desmin increases for up to 3 days following a bout of eccentric exercise (308;309). A recent study investigated the effect of muscle damaging eccentric exercise on the integrity of the sarcolemma and the structural protein, desmin, in humans. The study showed that there was no loss of desmin staining or muscle fibre degeneration or necrosis present in the eccentrically damaged muscle samples (537).

Researchers are not sure whether disruption of desmin is a cause or consequence of exercise-induced muscle damage. It is thought that strain activated calcium channels in the membrane (309) or damage to the sarcoplasmic reticulum (56) and/or t-tubular system (483), caused by the overextension of sarcomeres (410), induces an increase in intracellular calcium. The increased intracellular calcium concentration consequently activates proteases such as calpain, which are known to hydrolyse proteins, including desmin (29). The loss of desmin exacerbates the structural instability and further contributes to the disruption of the sarcomere (307). Calpain activity and calcium sensitivity increase following exhaustive exercise (29). The disruption of desmin is most likely a consequence of exercise-induced muscle damage that contributes to further sarcomeric instability and

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cellular disruption. However, it is not known whether the reported increase in intracellular calcium concentration can activate calpain to cause the desmin disruption that has been seen 5-10 minutes after an eccentric contraction (5).

1.2.6.2. Subsarcolemmal proteins

Dystrophin

Dystrophin forms part of a complex of glycoproteins that is located on the cytoplasmic side of the sarcolemma and is attached to actin filaments (402). Numerous studies have been conducted on the muscular dystrophic (mdx) mouse, which lacks the subsarcolemmal protein dystrophin, with the aim of understanding the precise function of dystrophin and its role in exercise-induced muscle damage and regeneration (91;130;345;351). Dystrophin-deficient muscle fibers of the mdx mouse exhibit an increased susceptibility to contraction-induced sarcolemmal rupture (402). Furthermore, the level of sarcolemmal damage appears to be directly correlated with the magnitude of mechanical stress placed upon the membrane during contraction (402). These findings suggest that dystrophin reinforces the sarcolemma, protecting it from mechanical strain that is developed during concentric and eccentric muscle actions (402). The mdx mouse model represents a genetic homologue of human Duchenne's muscular dystrophy and as a result similar conclusions may be drawn regarding the role of dystrophin in human skeletal muscle.

In general, mechanical disruption of the sarcolemmal proteins may result in further structural instability of the sarcomeres, thereby increasing the potential

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for damage during prolonged, exhaustive exercise, particularly if the exercise is primarily eccentric in nature.

1.2.7. Exercise-induced muscle damage and the sarcomeric membranes

1.2.7.1. Sarcoplasmic reticulum, t-tubules and sarcolemma

Many studies have reported exercise-induced muscle damage to the sarcoplasmic reticulum, the t-tubules and/or the sarcolemma following damage inducing exercise. Exhaustive exercise of both moderate and high intensity reduces the ability of the sarcoplasmic reticulum to resequester Ca^{2+} following excitation of the cell (56-58). In addition to this, a loss of sarcoplasmic reticulum vesicle membrane integrity was observed in rat muscle following electrically stimulated eccentric muscle actions (534). Furthermore, a study by Takekura et al. (2001) showed dramatic changes in the organisation of the membrane system involved in excitation-contraction coupling following eccentric contractions (483). In particular, an increase in the number of longitudinally oriented t-tubules was observed (483). The authors concluded that the t-tubular disruption was a consequence of the high mechanical forces imposed by eccentric contractions (483).

Damage or functional alterations to the sarcoplasmic reticulum, the t-tubules and the sarcolemma would cause leakage of calcium from the lumen of the sarcoplasmic reticulum and the extracellular space into the cytoplasm of the cell, so elevating the cytoplasmic $[Ca^{2+}]$. In addition to this, it has been suggested that forced lengthening of the muscle fibres triggers strain-

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activated calcium channels in the membrane, resulting in a further influx of calcium into the cytoplasm (309). The consequences of an increased concentration of calcium in the cytoplasm of the muscle cell will be discussed in section 1.2.9.

The metabolic factors implicated in exercise-induced muscle damage will also impact on the sarcomeric membranes. As a result of its role in the active transport of calcium, the sarcoplasmic reticulum consumes one third of the total energy requirements of the activated cell, with the remaining two thirds being used in the contractile apparatus (203). The insufficient mitochondrial respiration and subsequent reduction in high-energy phosphates that occurs as a result of strenuous exercise may hinder or prevent the active transport of calcium back into the sarcoplasmic reticulum, resulting in an increased concentration of calcium in the cytoplasm of the muscle cell (203).

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1.2.8. Intracellular calcium homeostasis

1.2.8.1. Excitation-contraction coupling mechanism

Action potentials are propagated from the motor centre in the brain via the spinal cord to the motor neuron. The motor neuron innervates the muscle cell via the neuromuscular junction. The action potential continues along the surface membrane of the muscle cell and down the t-tubules. The action potential induces the voltage sensors to trigger the release of calcium into the cytoplasm, from the stores in the sarcoplasmic reticulum. The interaction of calcium with troponin initiates cross bridge cycling and the subsequent

shortening of the sarcomere. Restoration of the sarcomere to its original length (i.e. relaxation) occurs by the active transport of calcium back into the sarcoplasmic reticulum (46;358).

The maintenance of specific levels of calcium within the muscle cell is imperative for efficient contractile function of the muscle cell (46;358). As a result of this, there are numerous cellular mechanisms that maintain calcium homeostasis, such as calcium-ATPase and sodium: calcium exchangers, calcium channels and electrophoretic uniporters (16). The importance of maintaining intracellular calcium homeostasis is further evidenced by the increased presence of cytoplasmic calcium in diseased or dysfunctional muscle (31;116;332).

Although a transient increase in intracellular calcium is important as it induces stress protein gene transcription and so provides the cell with increased tolerance to future stressful situations (315), a prolonged increase in cytoplasmic calcium levels results in muscle cell lysis and necrosis (116;119;183). Disruption of the sarcolemma, the sarcoplasmic reticulum and the other Ca^{2+} homeostatic mechanisms, by exercise-induced mechanical and metabolic factors results in an increased cytoplasmic concentration of calcium (391). Once a critical level of intracellular calcium is reached and remains elevated for a sufficient amount of time, numerous degradative pathways and/or mechanisms within the cell are activated (14;16;250). This is known as the autolytic or autogenic phase of skeletal muscle damage (14;16;250).

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1.2.9. Consequences of increased cytoplasmic calcium concentration

1.2.9.1. Autolytic phase

Calcium sensitive neutral proteases (e.g. calpain)

Calcium activates calcium sensitive neutral proteases, such as calpain (16;28;163;250). It has been suggested that the activation of calpain results in the degradation of Z-discs, contractile filaments, intracellular membranes and cytoskeletal proteins (28;29;215). In recent studies it was found that calpain activity in rat hind limb was increased by 26% during 60 minutes of level running (17;28). Calpain I is activated at calcium concentrations as low as 1μM (28) or 5 μM (103). These concentrations of calcium are likely to be reached during muscle contractions (28;163).

It has been proposed that the peptide fragments resulting from the proteolytic actions of the calcium-sensitive neutral proteases, assist in the initiation of the repair process by attracting the immune cells to the damaged area. This will be discussed in more detail in section 1.3.2.

Phospholipase A_2 (PLA_2) pathway

Increased cytoplasmic calcium also activates the phospholipase A_2 pathway (228). Phospholipase A_2 is a membrane bound enzyme that is dependent upon calcium for activation. Membrane phospholipids are the substrates upon which phospholipase A_2 acts. The end products of the enzymatic interaction are arachidonic acid (and subsequent production of prostaglandins, leukotrienes and thromboxanes) and lysophospholipids (16;250). Arachidonic

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acid and lysophospholipids degrade muscle cell membranes resulting in "leaky" membranes (16;250). The "leaky" state of the membrane has been implicated in the appearance of myoglobin and intramuscular enzymes, such as creatine kinase and lactate dehydrogenase, in the plasma after a bout of damage inducing exercise (250;391). In addition to this, increased damage to the sarcolemma will allow further influx of extracellular calcium into the cytoplasm of the cell, resulting in a vicious cycle of increased cytoplasmic calcium-activated damage (163).

1.2.9.2. Mitochondria

The mitochondria accumulate cytoplasmic calcium either as a result of, or in an attempt to compensate for the increased cytoplasmic calcium concentration (16;163). Mitochondria have the capacity to accumulate large amounts of calcium (up to 3 $\mu mol.mg^{-1}$ mitochondrial protein), both in a diseased state (16;31) and as a consequence of exhaustive exercise (113;163). However, increased concentration of calcium within the mitochondria is extremely destructive as it depresses mitochondrial function, induces degradation of mitochondrial membranes and results in reduced respiration and further decreased production of ATP (163;529).

1.2.9.3. Contractures

Elevated levels of cytoplasmic calcium may also result in muscle contractures or uncontrolled, hypercontractions of the muscle fibres (16). The occurrence of such contractures will result in a further depletion of high-energy phosphates and an increase in lactate production (16). In addition to this, the

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contractures may further contribute to any mechanical damage to structural components of the already unstable muscle cell (16).

1.2.10. Summary

Just as different physical activities are composed of a variety of muscle contractions (i.e. isometric, concentric and eccentric) and movements, so the events that initiate the cascade of disruptions in skeletal muscle following prolonged exhaustive exercise are complex and numerous. It is most likely that the muscle damage induced by prolonged, exhaustive endurance exercise has both a mechanical and/or a metabolic origin, the consequences of which result in an increased concentration of calcium within the cytoplasm of the muscle cell. Increased concentrations of calcium within the cytoplasm triggers the proteolysis of various sarcomeric structures, impairs the functioning of the mitochondria and may even induce uncontrolled contractions of the sarcomere, all of which further contribute to the extent of the damage within the muscle cell.

It is hypothesised that the resultant cellular debris acts as a wound hormone to attract the circulating immune cells to the site of damage. In addition to this, cytokines are released from the contracting or damaged muscle, which mobilise the various stages of the regeneration process, as discussed in the following section.

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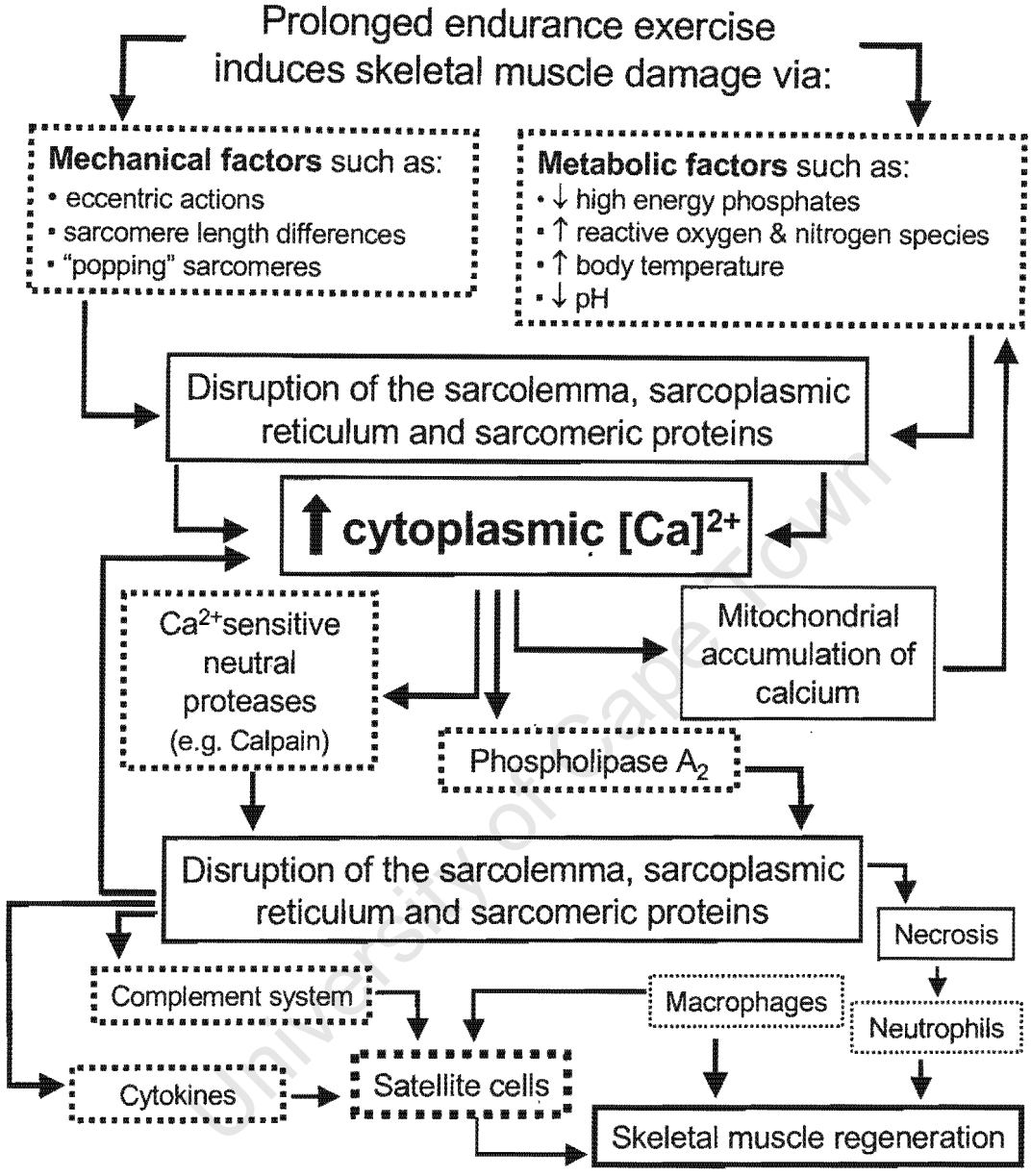

Figure 1.2.4. Flow diagram illustrating the sequence of events that occurs following muscle damage induced by prolonged, exhaustive endurance exercise. The dotted boxes indicate activation of the various pathways or factors.

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1.3. SKELETAL MUSCLE REGENERATION

1.3.1. Introduction

Constant mechanical activity, on a daily basis, throughout an individual's life, renders skeletal muscle prone to sporadic, yet continual, bouts of damage and degeneration (539). As discussed in the previous section (section 1.2), training to improve physical fitness and competitive performance exacerbates the muscle damage and degeneration.

Mature skeletal muscle fibres are highly specialised cells. The fibres achieve this specialised state through terminal differentiation. This involves withdrawal of the muscle cell from the mitotic cell cycle (post mitotic; Figure 1.3.1) and the establishment of a specific set of genes and proteins that define the identity and function of the muscle cell (539). Due to the high degree of specialization and post mitotic state, the muscle fibre itself is unable to meet the chronic demands of growth, repair and adaptation. This task is mediated by the satellite cells (75;92;191;439;442;539).

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The experimental models used to study the regeneration process include contusion (220;221), crush (180;465;526), freeze (96), chemically induced damage (156) and transplantation of whole muscle grafts (87). It is reasonable to assume that the damage induced by these experimental models is likely to be far more extensive than the damage induced by exercise. However, the fact that the process of skeletal muscle regeneration

follows a similar sequence of biological events regardless of the type of injury incurred (75;174), justifies the use of these models to study the process of skeletal muscle regeneration following exercise-induced muscle injury. Damage to the muscle fibre results in the infiltration of immune cells into the damaged area, revascularisation of the damaged area, phagocytosis of damaged necrotic muscle tissue, activation and proliferation of satellite cells, differentiation and fusion of myoblasts into multinucleated myotubes and finally maturation and reinnervation of myofibres (see Figure 1.3.1) (75;174;191). Each of these steps will be discussed in more detail in this section.

1.3.2. Infiltration of immune cells

Damage to the muscle fibre results in the release of substances from the injured tissue, which act to attract immune cells from the blood stream to the damaged site (327;493). The local immune response to the muscle damage occurs within minutes whereas a systemic (acute phase response) immune response occurs 3-4 days after the muscle has been damaged (389;493). Prior to regeneration, all necrotic tissue must be phagocytosed and cleared from the damaged area as regeneration is inhibited by persisting necrotic tissue (75;174;327;457;493).

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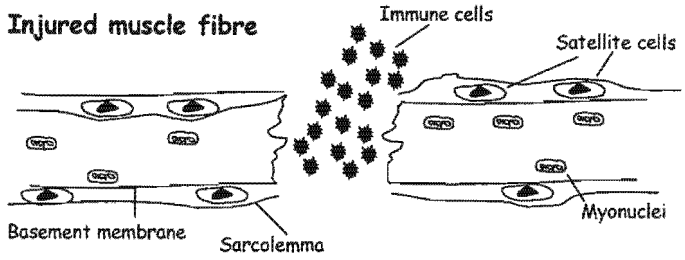
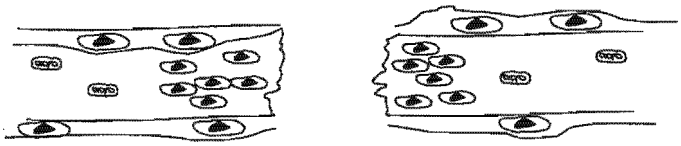
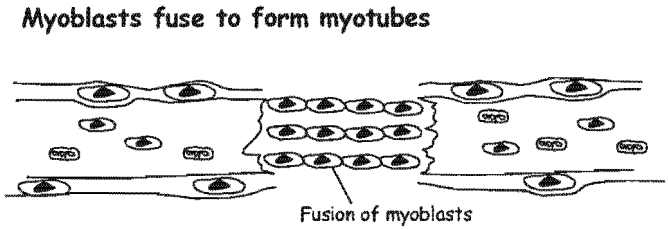


Within 24 hours after the damaging stimulus (e.g. strenuous exercise) **immune cells** infiltrate the damaged area. **Revascularisation** of the area occurs at about the same time.

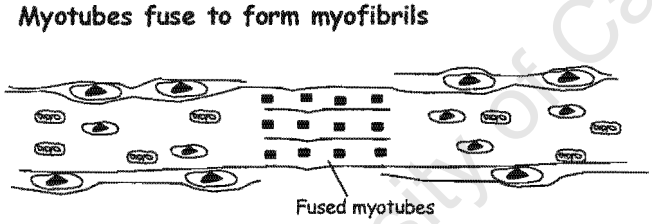


Between 24-48 hours after the damaging stimulus (e.g. strenuous exercise) **satellite cells** are **activated**, they **proliferate** and migrate to the damaged area.

During this time the activated satellite cells (myogenic precursor cells) begin to **differentiate** and express myogenic regulatory factors **MyoD** and **Myf5**.

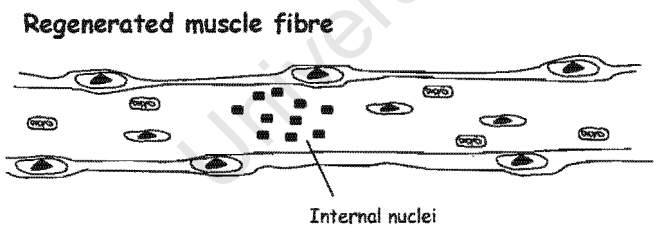


By **48 hours myogenin** and **MRF4** are expressed by the myogenic precursor cells, committing them to terminal differentiation.



Fusion of myoblasts and myotubes occurs between **3-7 days** after the damaging incident.

Between 7-10 days after the damaging stimulus, the myotubes fuse to form myofibrils. The myofibrils **mature** into myofibres.



During this time the mature myofibril/muscle fibre is **reinnervated** with the end result being a mature muscle fibre

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Figure 1.3.1. Diagrammatic representation of the various stages involved in the regeneration process and an approximate time course of the events. (Adapted from slide presentation of Fred Kolkhorst, San Diego State University,USA)

In addition to phagocytosis of the necrotic tissue, the infiltrating immune cells also release important growth factors and cytokines that attract satellite cells to the damaged area and stimulate their proliferation (75;174;421).

1.3.2.1. Initiation of the immune response

Wound hormone hypothesis

Exercise, particulary strenuous, unaccustomed or eccentric exercise that is prone to induce muscle damage elicits an immune response (129;327;457;482;493). The magnitude of the immune response is dependent upon the duration and to a lesser extent the intensity of the exercise (322;398). The specific events initiating the immune response following exercise and exercise-induced muscle injury are not yet known (321).

The early findings of Bintliff and Walker (1960) indicated that chemotaxis of immune cells from the circulation is likely to be the primary mechanism by which immune cells proliferate and infiltrate the injured area (34). This finding, together with the knowledge that exercise-induced muscle damage results in the release of cytoplasmic proteins, cytokines and chemokines from the damaged muscle cell into the extracellular space (321;446), has led to the proposal of the wound hormone hypothesis. Possible wound hormones are peptide fragments, cytokines and growth factors (493). Furthermore, in a recent review article, Malm hypothesized that the indirect blood markers, such as creatine kinase and myoglobin, function as messenger molecules between the skeletal muscle and the immune system (328).

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Cytoplasmic peptide fragments

Peptides fragments, resulting from calpain-induced proteolytic cleavage of myofibrillar and cytoskeletal proteins, may be involved in the initiation of the inflammatory response following exercise-induced muscle damage. These peptides may provide the chemotactic stimulus for invading neutrophils (29;413).

Cytokines

Recent research in this area has focused on the participation of cytokines in the initiation, amplification and assimilation of the acute immune response to exercise-induced muscle damage (390;398;400;475). Cytokines are small polypeptides that are produced by a variety of cells including the vascular endothelium, tissue-resident macrophages, circulating macrophages, skeletal muscle and proliferating satellite cells (21;66;111;390;459;475)

Cytokines are represented by several different families, which include the interleukins (IL), tumor necrosis factors (TNF), interferons, growth factors, colony stimulating factors and cell adhesion molecules (459). Cytokines may be categorised as either pro- or anti-inflammatory based on their predominant action (398).

Immediately after a marathon, plasma levels of pro-inflammatory TNF-α and IL-1β increase twofold, levels of inflammatory responsive IL-6 increase 100 fold and levels of anti-inflammatory IL-10 increase 30 fold (389;398). An hour after the marathon there is a marked increase in the levels of cytokine inhibitors IL-1 receptor agonist (IL-1ra) and the soluble type receptors of TNF-

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α (sTNF-r1 and sTNF-r2) (389). Pro-inflammatory cytokines, IL-1β and TNF-α, are most likely released by resident macrophages at the site of the injury (111;459;493), whereas IL-6 is released from contracting skeletal muscle (390;398;475). The response of the various cytokines to strenuous exercise, for example a standard marathon, is summarised in Figure 1.3.2.

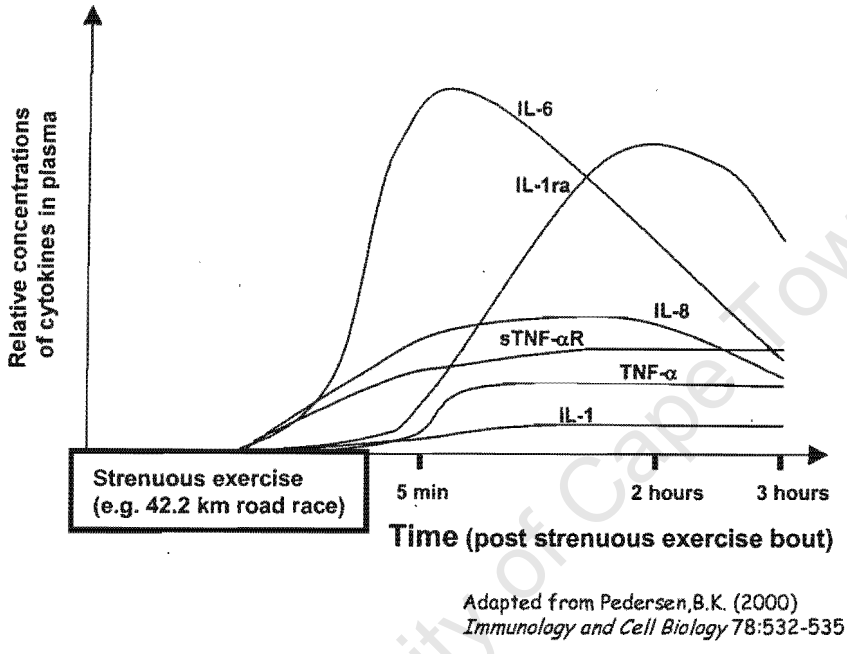

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Figure 1.3.2. Diagrammatic representation of the relative change in the plasma concentration (in arbitrary units) of the various cytokines following a bout of strenuous exercise. IL-6 = Interleukin-6, IL-1ra = Interleukin-1 receptor agonist, IL-8 = Interleukin-8, sTNF-αR = soluble tumor necrosis factor-α receptor, TNF-α = tumor necrosis factor-α, IL-1 = Interleukin- 1.

Growth factors

Studies involving crushed muscle extract have shown that several biologically active molecules are released from the damaged cells into the extracellular space (35;303;304;354). Although most of these molecules have been shown to attract and activate myogenic cells, their role in the attraction and activation

of inflammatory cells is not established (493;494). One such molecule is basic fibroblast growth factor (bFGF). The growth factor, bFGF, is present in muscle cells and fibroblasts and its release into the extracellular space occurs primarily through membrane lesions (493). Although bFGF is capable of attracting and activating myogenic cells it is not yet known whether it will have a similar effect on inflammatory cells (493).

The release of another growth factor, platelet-derived growth factor (PDGF), from damaged muscle cells, fibroblasts and resident macrophages into the extracellular space may initiate the early inflammatory response (493;494;530). The characteristically short half-life and low concentrations of PDGF in skeletal muscle may reduce its chemotactic function (493;494). However, a recent study suggested that PDGF was able to compensate for its low concentrations and short half-life by stimulating the synthesis and secretion of laminin, collagen IV and fibronectin via independent signalling pathways, all of which have a similar effect to PDGF on the regenerating muscle (4).

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Initiation by resident macrophages and fibroblasts

It is possible that the chemotactic signal responsible for attracting immune cells into the damaged area comes from the endothelial cells or non-muscle cells located in the muscle (494). This hypothesis was supported by the findings of a study by Robertson et al. (1993) in which the chemoattractant effect of crushed muscle extract on inflammatory cells was investigated (421). Crushed muscles removed 24 hours following injury were chemoattractive to

both neutrophils and macrophages whereas crushed muscle removed immediately post injury were not chemoattractive to either inflammatory cell (421). If the inflammatory cell invasion was triggered directly by substances released from the injured muscle then the crushed muscle removed immediately post injury would have shown the greatest chemoattraction. The crushed muscle removed 24 hours post injury, however, possessed the greatest chemoattractant effect, thus supporting the hypothesis that a substance, capable of attracting and activating inflammatory cells, is released by a third cell type (421).

Resident fibroblasts and macrophages are potentially suitable third party participants. It may be that wound hormones such as bFGF, PDGF, IL-1 and IL-6, which are known to be released from muscle following exercise-induced muscle injury, may activate the resident fibroblasts and macrophages to provide the signals necessary to initiate chemotaxis of additional inflammatory cells into the injured site (493). The close association of resident macrophages with muscle cell membranes throughout the muscle also make them prime candidates for initial sensors of muscle injury (493). Activated macrophages secrete numerous cytokines (transforming growth factor-α (TGF- α), transforming growth factor-β (TGF- β), tumor necrosis factor-α (TNF-α), IL-1α, IL-1β, IL-6 and PDGF, all of which are capable of inflammatory cell chemotaxis (111;459;493).

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Complement System

An additional mechanism by which inflammation may be initiated following exercise-induced muscle damage is via activation of the complement pathway (139;493). While certain products of the complement system may be involved in lysis of the muscle cell (174), other components of the activated complement system are powerful chemotactic and stimulating agents of macrophages (174). For example, C5b-9 attack complex is a component of the complement system found on the sarcolemma (174). The C5b-9 attack complex is activated in response to membrane damage and when activated is responsible for cell lysis (174). Activation of the complement pathway has been observed after different types of endurance exercise (64), however the precise affect of the products of this pathway in the process of exercise-induced muscle damage, repair and adaptation is unknown (321).

1.3.2.2. Identity and function of the infiltrating immune cells

Although numerous studies have reported large increases in leukocytes in circulating plasma (321-323;400;482) and the injured muscle (129;320) following damage inducing exercise, there is no clear consensus as to the identity of the inflammatory cells, the order of infiltration or even if an infiltration of inflammatory cells occurs (327).

Neutrophils

Neutrophils are speculated to be the first cells to infiltrate the damaged muscle fibres (411;457;493;494). Increases in circulating neutrophils (64;480;482) and accumulation of neutrophils in skeletal muscle

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(129;197;320) have been reported in humans immediately following and up to 5 days after eccentric exercise. Neutrophils are capable of invading host tissue and producing superoxide free radicals and cytotoxic enzymes (481). Neutrophils may contribute to the clearance of damaged ultrastructural components through their phagocytic activities (129;321). Neutrophils may also contribute to the increase in the ultrastructural damage in the days following eccentric exercise through the production of radical oxygen species (ROS) and cytotoxic enzymes (129;481;494).

Neutrophil chemotaxis

While certain researchers believe that neutrophils function as immunoregulatory cells that release cytokines (98), other researchers suggest that stress hormones mediate the release of cytokines (482) which subsequently induce a neutrophil response (198;480). Furthermore, peptide fragments resulting from the calpain-induced proteolytic cleavage of myofibrillar and cytoskeletal proteins, may also be associated with neutrophil chemotaxis (29;278;413;493). A study by Raj et al. (1998), reported a positive correlation between calpain-like protease activity and myeloperoxidase activity, which is indicative of neutrophil accumulation in the muscle (413). In addition to this, when the calpain-like protease activity was reduced by a specific inhibitor (E64c – a cell permeable cystein protease inhibitor), myeloperoxidase activity was also reduced. Based on these findings they suggested that neutrophil accumulation into skeletal muscle is at least partially dependent on the activation of cellular calpain-like protease activity (413).

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It has also been suggested that superoxide radicals may be important in neutrophil attraction and adherence to endothelium (196). One of the sources of superoxide radicals, particularly during inflammatory events, is the enzyme xanthine oxidase. Xanthine oxidase is localised in the vascular walls of skeletal muscle (195). A two-way interaction exists between xanthine oxidase and neutrophils. Firstly, xanthine oxidase increases the production of superoxide radicals, which in turn attract neutrophils. Secondly, on adhesion to endothelial cells, the activated neutrophils enhance the expression of xanthine oxidase (513). Hellsten et al. (1997) reported an increased expression of xanthine oxidase in association with secondary inflammatory processes following strenuous eccentric exercise in humans (196). The methods used in this study to detect changes in superoxide radicals were not sufficiently sensitive to detemine whether the increased expression of xanthine oxidase led to an increase in superoxide radicals and ultimately an increase in invading neutrophils (196). The authors suggested that the increase in xanthine oxidase might be induced by an increase of IL-6 in the plasma following eccentric exercise or by the adhesion of circulating leukocytes to the endothelium (196).

A number of studies investigating exercise-induced muscle damage have observed an inflammatory response without an infiltration of neutrophils (287;288;316;329;477). There are a number of possible explanations for this. Firstly, while peptide fragments resulting from calcium-activated proteolysis may be associated with neutrophil chemotaxis, the degree to which calcium-activated proteases contribute to the morphological changes observed in

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skeletal muscle following exercise-induced muscle damage is not known. It is possible that in the instances of exercise-induced muscle damage whereby these proteases do not play a prominent role, a neutrophil free inflammatory response occurs. Secondly, it was also observed that vascular trauma is necessary to trigger the sequentially organised inflammatory cascades that will lead to the appearance of neutrophils in damaged tissue (287). It is possible that the exercise models used in the above-mentioned studies do not induce sufficient vascular trauma necessary to trigger the adhesion molecules, cytokines and/or chemokines required for the recruitment and infiltration of neutrophils (287).

Macrophages

Most investigations have shown macrophages to be the predominant inflammatory cell type at all stages of inflammation following the first 12 hours post injury (82;321;457;493;494). It appears that resident macrophages and fibroblasts are the early responders or sensors of exercise-induced muscle injury due to their close association with, and proximity to, skeletal muscle fibres. Activated macrophages secrete cytokines such as transforming growth factor-alpha (TGF-α), transforming growth factor-beta (TGF-β), interluekin-1 alpha (IL-1α), interleukin-1beta (IL-1β), platelet derived growth factor (PDGF) and tumor necrosis factor-alpha (TNF-α), all of which are capable of attracting inflammatory cells (421). These cytokines accompanied by neutrophil invasion and fibroblast activation may provide the additional signals for the chemotaxis of inflammatory cells from the circulation to the site of damage (494).

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Functionally distinct subclasses of macrophages may play distinct roles in response to muscle injury. Two subpopulations of macrophages, $ED1^+$ and $ED2^+$, are present in the normal macrophage population in muscle (470;493;494). It is believed that $ED1^+$ cells are mainly involved in the removal of cellular debris, as they are highly concentrated in areas that contain necrotic fibres and their appearance in muscle coincides with increased rates of protein degradation (287). Although some studies have shown that inflammatory cells that invade muscle have the capability to induce further damage (82;196;287;288), a recent study by Tidball et al. (1999) showed conclusively that $ED1^+$ macrophages were not responsible for the muscle membrane injury that occurred during reloading of rat hind limb following a period of hind limb suspension (494).

$ED2^+$ macrophages are resident cells that do not invade or phagacytose necrotic fibres. The $ED2^+$ macrophages appear at a later stage in the inflammation process, when the tissue necrosis is complete and regeneration of the tissue begins (65;82;493). $ED2^+$ macrophages play a role in the activation and proliferation of satellite cells through the release of growth factors such as macrophage-derived growth factor (MDGF) and possibly basic fibroblast growth factor (bFGF) (174).

1.3.2.3. Summary

In the case of sublethal or minor exercise-induced muscle damage there is evidence to suggest that activation of satellite cells and regeneration of skeletal muscle occurs in the absence of necrosis and a subsequent immune

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response (100;175). In the case of more severe exercise-induced muscle damage that results in necrosis, the subsequent immune response is a very important part of the regeneration process.

1.3.3. Revascularisation

Revascularisation of the damaged area begins at the time of immune cell infiltration and most likely as a result of similar initiation factors, such as peptide fragments resulting from calcium-activated proteolysis or activation of the complement system (75;174). Revascularisation occurs by increasing the proliferation of endothelial cells in and around the area of damage (174). Numerous factors, which act to enhance endothelial cell proliferation, have been implicated in the process of revascularisation (75;174). Basic fibroblast growth factor (bFGF) is a powerful angiogenic agent that is released by infiltrating macrophages (261;293) and by endothelial cells themselves (259). In addition to this, heparinases, secreted by lymphocytes and macrophages, act on heparansulfateproteoglycans and glycosaminoglycans in the extracellular matrix to enhance the release of bFGF (174;259).

Research has shown that the gene expression of another angiogenic growth factor, vascular endothelial growth factor (VEGF), is regulated in human skeletal muscle in response to a single bout of dynamic exercise (182). Furthermore, byproducts from the breakdown of the sarcolemma, intracellular proteins and metabolites are also believed to stimulate angiogenesis (174).

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The process of revascularisation is an important event for successful muscle regeneration (75;174;176). Firstly, enhanced revascularisation of the damaged area decreases the extent of fibrosis in the damaged area (75;174), as prolonged ischaemia and low oxygen tension enhance the proliferation of fibroblasts (174). Secondly, enhanced revascularisation would result in an increased delivery of important immune cells to the damaged area, which would enhance the phagocytosis of necrotic tissue as well as the release of certain growth factors and cytokines necessary for the attraction and proliferation of satellite cells to the damaged area (174).

1.3.4. Satellite cells

1.3.4.1. Introduction

Skeletal muscle satellite cells were first described in frog muscle (336) and then in adult avian and mammalian muscle (13;37;438). Although, the exact origin of satellite cells is not known, it appears that during embryonic myogenesis, myoblasts fused to form myotubes. When these myotubes differentiate and mature into myofibres, a subpopulation of myoblasts remain undifferentiated and are not incorporated into the muscle but instead remain associated with the surface of the developing myofibre (439).

Satellite cells are located between the sarcolemma and the basement membrane of the muscle fibre (Figure 1.3.3.).

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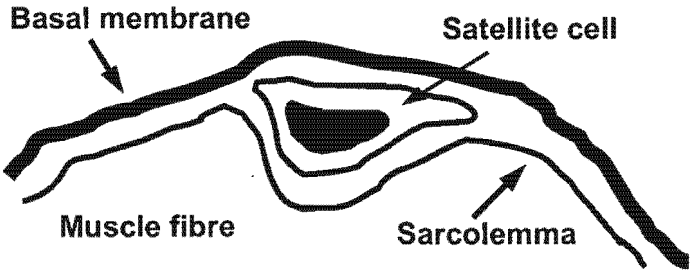

Figure 1.3.3. Diagram showing the location of the satellite cell between the basement membrane and the sarcolemma of the muscle fibre. SC = satellite cell. (Adapted from Chambers,R.L. and McDermott,J.C. Molecular basis of skeletal muscle regeneration. *Can J Appl Physiol.* 21(3) : 155-184, 1996)

The small cytoplasm of the satellite cell contains the usual organelles together with a single nucleus. It was previously believed that quiescent satellite cells did not express any specific skeletal muscle markers (92;177;442). Recent studies, however, have shown that besides m-cadherin (226), quiescent satellite cells express Myf5 (26) and myocyte nuclear factor (MNF)(157). It is hypothesized that there are subsets of precursor cells with phenotypic and functional differences and that the quiescent satellite cells expressing skeletal muscle markers are already committed to becoming muscle precursor cells even before being activated (539).

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1.3.3.2. Activation

Moss and Leblond (1970) were the first to demonstrate that satellite cells were responsible for the increase in the number of myonuclei that occurs with postnatal growth (363). In healthy, mature skeletal muscle, satellite cells are quiescent (in the G_0 phase of mitotic cell division, Figure 1.3.4.) (37;92;442;539). Satellite cells are activated to enter the G_1 phase of mitotic cell cycle approximately 30 to 48 hours following stimuli such as exercise,

traumatic injury, denervation, immobilisation and stretching (37;75;174) and approximately 16 hours following mitogen stimulation (37).

Although numerous agents, known as competence factors, have been implicated in the activation of satellite cells following injury, the precise molecular mechanisms involved are still under investigation (Table 1.2.1.)(37;75;174;442). An activated satellite cell is called a muscle precursor cell (mpc).

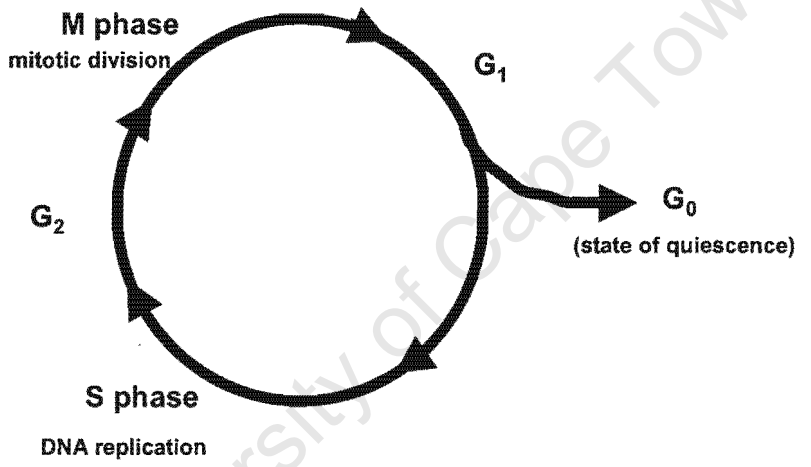

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Figure 1.3.4. Diagrammatic representation of the mitotic cell cycle. Under normal conditions satellite cells are in the G_0 phase (state of quiescence). Exercise is one of the many stimuli that activates the satellite cells to re-enter the cell cycle. G_0 = growth phase 0, G_1 = growth phase 1, G_2 = growth phase 2. S = synthesis, M = mitotic. (Adapted from Chambers,R.L. and McDermott,J.C. Molecular basis of skeletal muscle regeneration. *Can J Appl Physiol.* 21(3) : 155-184, 1996)

Bischoff's mitogen

In 1985, Richard Bischoff discovered that lightly crushed, adult rat muscle extract released a soluble factor that possessed the ability to activate satellite cells and initiate their entry into the cell cycle. This satellite cell mitogen stimulated both proliferation and differentiation of the myoblasts (35;36).

Recently Li et al. (2000) discovered two novel myogenic factors in crushed rat muscle extract (303) and a single novel myogenic factor in crushed human muscle extract (304). All three myogenic factors stimulated both proliferation and differentiation of satellite cells in culture and *in vivo* (303;304). Although the true identity of the myogenic factors is yet to be determined, their approximate molecular weights and heparin binding abilities are known (303;304).

Hepatocyte growth factor/scatter factor (HGF/SF)

A recent study providing evidence to support the role of HGF/SF in the activation of quiescent satellite cells, observed that mechanically stretched skeletal muscle satellite cells released hepatocyte growth factor/scatter factor (HGF/SF), which subsequently accelerated the entry of the satellite cells into the mitotic cell cycle (487). Furthermore, studies have shown that injection of recombinant hepatocyte growth factor/scatter factor (HGF/SF) into either cultured satellite cells or normal muscle, activated quiescent satellite cells to enter the cell cycle (7;354;486). In addition to this HGF/SF is present on intact myofibres (486) and the receptor for the HGF/SF ligand, c-Met proto-oncogene, is expressed in quiescent satellite cells in normal muscle (176;442). It has recently been suggested that Bischoff's mitogen is actually HGF/SF (175;486). Not only is HGF/SF a potent chemotactic agent (38) and satellite cell activator but also through the transcriptional inhibition of myogenic regulatory factors, it inhibits the differentiation of myoblasts (75;155). Miller et al. (2000) hypothesised that inhibition of differentiation by HGF/SF allowed the proliferating myoblasts to increase in number until an

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optimal cell density was attained, whereupon differentiation and fusion of myoblasts would occur (354). During regeneration HGF/SF expression appears to be proportional to the degree of injury, peaking 3 days post injury and then declining by 7 days post injury (486;507).

Infiltrating leukocytes

Jesse et al. (1998) suggest that infiltrating leukocytes may be involved in the activation of quiescent satellite cells on the basis that quiescent satellite cells express vascular cell adhesion molecule-1 (VCAM-1) and the infiltrating leukocytes express the co-receptor for this molecule, VLA-4. It is proposed that a cell-cell interaction initiates a genetic response within the satellite cells (233;442).

Macrophage derived growth factor

It is possible that other growth factors or substances released by infiltrating immune cells may be capable of initiating myogenesis. For example, in addition to phagocytosis of necrotic cell debris, macrophages secrete an undetermined soluble macrophage derived growth factor (MDGF), which may exert a specific mitogenic effect on satellite cells. Lescaudron et al. (1999) established a potentially important role for macrophages in the initiation of satellite cell activation, when they showed that in the absence of macrophage infiltration muscle regeneration did not occur (300).

1.3.4.3. Proliferation

Progression factors are responsible for the progress of activated satellite cells (muscle precursor cells) through the cell cycle up to the stage at which DNA is

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synthesized (S phase)(174). Numerous growth factors and cytokines assume the role of progression factors in the regeneration process (6;75;133;191). The growth factors and cytokines studied most extensively are basic fibroblast growth factor (bFGF), macrophage-derived growth factor (MDGF), platelet-derived growth factor (PDGF), insulin-like growth factor-I (IGF-I) and II (IGF-II), endothelium-derived growth factor (EDGF), transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α) and the interleukin-6 cytokines, interleukin-6 and leukaemia inhibiting factor (LIF). A summary of the growth factors and their permissive and/or inhibitory actions on satellite cells throughout the various stages of regeneration is shown in Table 1.3.1.

Additional factors

Recent research has indicated a role for hormones such as adrenocorticotrophic hormone (ACTH), glucocorticoids, testosterone and growth hormone (GH) in the regeneration process (reviewed in Grounds, 1991 (174)). Nitric oxide has also been implicated as a key factor in the regeneration process (10;191).

Satellite cells proliferate at least once before fusing to form myotubes (75;174;420). At this stage there are 3 different fates for the proliferated satellite cells. Firstly, the satellite cells could remain undifferentiated and thus restore the population of quiescent satellite cells. Secondly, the cells could fuse with the damaged fibres, thus adding a nucleus and assisting in the maintenance of the cytoplasm to nuclei ratio. Thirdly, several satellite cells

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could fuse to form myotubes that will eventually mature into myofibres and replace the necrotic muscle fibres (175;191).

Table 1.3.1. *A summary of the growth factors and their permissive (↑) or inhibitory (↓) actions on the satellite cell throughout the various stages of regeneration.*

Factor	Activation	Proliferation	Differentiation	Fusion	References
Bischoff's mitogen	↑	↑	↑		(35;36)
HGF/SF	↑	↑	↓		(486;487)
Li's mitogens	↑	↑	↑		(303;304)
MDGF	↑	↑	↓		(300)
bFGF		↑	↓	↓	(135)
PDGF		↑	↓	↓	(530)
IGF I & II		↑	↑	↑	(132;134)
EDGF		↑	↓		(249)
TGF-β		↓	↓	↓	{4744,355
TNF-α		↓↑	↓		(18)
LIF		↑			(18;279)
Interferon			↓	↓	(131)
IL-6		↑			(66)

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1.3.4.4. Differentiation

At some point during the G_1 phase of the cell cycle (Figure 1.2.4) the activated satellite cells begin to differentiate by express specific muscle genes (75;174;539). Myogenic regulatory proteins are specialised transcription factors that control the level of transcription of a given set of muscle genes (75). This step commits the muscle precursor cells to fusion and maturation into adult muscle fibres (75;174;442). The MyoD family of transcription factors are the first to be recognised as important regulators of muscle-specific gene expression (75). The members of this family are MyoD, myogenin, Myf5 and Mrf4. The expression of Myf5 and MyoD are required for the determination of myoblasts (251} and the expression of myogenin and Mrf4 regulate terminal differentiation and fusion of myotubes (442).

Cornelison and Wold (1997) performed a definitive study in which gene expression of c-Met (receptor for HGF/SF ligand), m-cadherin, Myf5, MyoD, myogenin and Mrf4 were assessed by RT-PCR of individual satellite cells following their activation in intact mouse muscle fibres (92). They found that c-Met was expressed in the satellite cells throughout the process, from quiescence to terminal differentiation (92). M-cadherin positive cells were present in only a small fraction of the quiescent satellite cells but at later time points following activation and proliferation, increasingly more of the cells expressed m-cadherin (92). The authors proposed that perhaps the fraction of satellite cells that expressed m-cadherin in the early stages were a functionally distinct subset that were specially programmed to differentiate rapidly upon activation (92). By 24 hours post activation 32% of the satellite

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cells were singularly positive for either MyoD or Myf5 and 35% of the satellite cells were positive for both MyoD and Myf5 (92). By 48 hours post activation myogenin and Mrf4 positive cells were noted (92). At this time point the majority of the satellite cells either expressed all four MRFs or a combination of myogenin/Myf5/MyoD. At 96 hours post activation the preferred expression state contained all four MRFs. It was also noted that, even at later time points following activation, cells that were positive for c-Met only were still present. The authors proposed that perhaps these cells were specifically programmed to maintain the progenitor pool of quiescent satellite cells instead of entering the cell cycle (92). This finding may shed some light on the mechanisms involved in sustaining the satellite cell population through multiple rounds of muscle regeneration. The significance of the different combinations of MRF, c-Met and m-cadherin expression is not yet understood. What is known is that the general pattern and timing of MRF expression is similar, no matter what type of damage or stimulus activates the process (92).

The expression of MyoD is up regulated within 12 hours of injury (455). An increased expression of MyoD and a decreased expression of Myf5 induce the myogenic precursor cells to withdraw from the cell cycle and begin the process of differentiation (258;539). While there is evidence supporting the role of MyoD in the regulation of myoblast exit from the cell cycle and entry into terminal differentiation and that an absence of MyoD will result in increased proliferation and delayed differentiation of activated satellite cells (184;394;539), there is also evidence suggesting that active satellite cells require the expression of MyoD in order to proliferate (351).

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The specific role of Myf5 has yet to be established. It seems that while an increased expression of Myf5 in quiescent satellite cells may hinder both cell proliferation and differentiation (311), the presence of Myf5 restricts the undifferentiated myoblasts to myogenesis (539). It has been suggested that the expression of Myf5 alone in activated satellite cells may define a stage during which renewal of the satellite cell population occurs (442). The expression patterns of the various myogenic regulatory factors during the different stages of the regeneration process are illustrated in Figure 1.3.5.

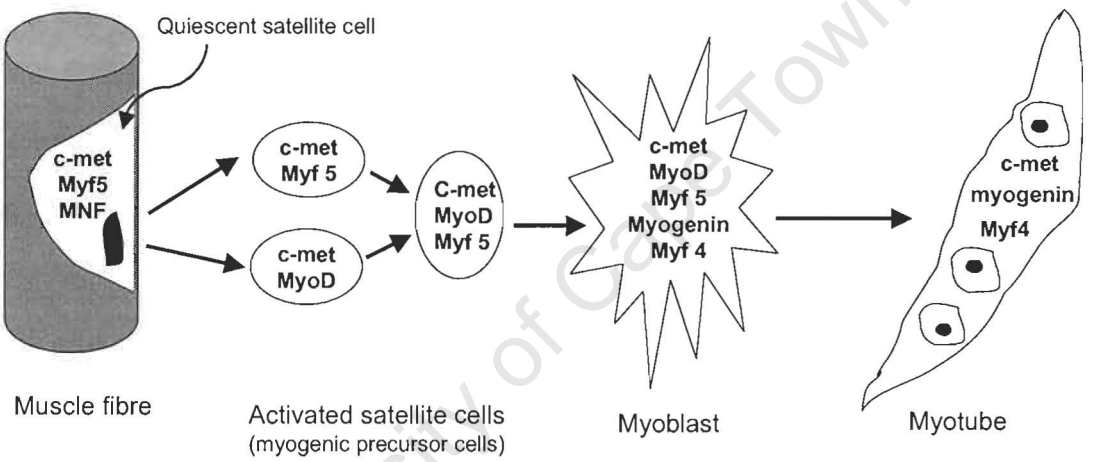

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Figure 1.3.5. Diagram illustrating the expression of myogenic regulatory factors (MRF) at the various stages of the regeneration process. MNF = myocyte nuclear factor. (Adapted from Cornelison and Wold (1997). *Dev Biol* 191:270-283).

Apart from the MyoD family of transcription factors, there are a host of other transcription factors involved in the regulation of muscle specific gene expression (refer to (75;539) for more details). Although the various roles of the muscle-specific transcription factors are well described in terms of developmental myogenesis, scientists are still uncovering the integral part

these factors play in the process of muscle repair and regeneration in mature skeletal muscle.

1.3.4.5. Fusion

The fusion of myoblasts and myotubes is a complex process involving the extracellular matrix, cell surface molecules, growth factors and elevated calcium and hydrogen ions in the extracellular space (174). For fusion of myoblasts and myotubes to be successful the cells must recognise each other, the electrostatic forces produced by the glycoproteins and glycolipids must be neutralised by elevated calcium and hydrogen ions in the extracellular space and specific membrane glycoproteins and glycolipids must be expressed (174).

While decreased amounts of hyaluronic acid and fibronectin may be necessary for fusion, increased collagen, N-acetylglycosamine and sialic acid tend to enhance fusion of myoblasts and myotubes (174). Although the expression of particular cell surface receptor molecules is evidently important there is little information regarding which receptors are integrally involved (174). Decreased availability of bFGF and PDGF and increased presence of IGF-II appear to be necessary for differentiation and fusion of myoblasts and myotubes (174). Furthermore, increased levels of prostaglandin E_1 initiate the fusion of embryonic chick myoblasts, *in vitro* (174;538).

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1.3.4.6. Maturation and innervation

The maturation process of the multinucleated myotubes is considered complete when the contractile filaments are assembled, the adult instead of embryonic isoform of various genes are expressed, the synapses and motor endplates are formed and the new myotube is connected to the appropriate motor neuron (174).

Part of the maturation process involves reinnervation of the newly formed myofibres (75;174). If the new myofibres are formed under the basement membrane of pre-existing muscle fibres then the old synaptic site is used to innervate the new myofibres (75;174). If a new synapse is to be formed, synaptic proteins such as agrin and gelasmin restrict the synapse-specific cell surface molecules, acetylcholine receptor (AchR) and neural cell adhesion molecule (N-CAM) to synaptic areas (53;232). On innervation of the muscle cell, myonuclei and golgi bodies close to the site of the new synapse are immobilised beneath the synapse by means of microtubules, actin and possibly even tropomyosin (174).

Prior to innervation, the fast muscle specific isoform is the default gene expression (122;479) but once innervated the fast and slow isoforms are differentially expressed within the muscle fibre (122).

If the basement membrane is intact complete regeneration may occur by 7 days post injury (222). If the injury is more severe it seems that connective tissue preferentially fills the area at about 7 days and by 21 days the new

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myofibres are still in the process of repairing the area. Under such conditions, the infiltration and formation of connective tissue may prevent complete regeneration of muscle in the long term (222).

1.3.5. Extracellular matrix

The extracellular matrix is composed of interstitial connective tissue (collagen types I, III, V and short chain collagen and fibronectin) and the basement membrane (collagen type IV, laminin, heparansulphate proteoglycans, glycoproteins, nidogen, enactin and fibronectin) (Figure 1.3.6.) (433). The components of the extracellular matrix have important regulatory and structural functions regarding the physiology of the muscle cell (174;433). Extracellular matrix molecules maintain quiescence of satellite cells, regulate movement, attachment, proliferation and fusion of muscle precursor cells and myoblasts and control innervation of muscle cells (174;433).

The connective tissue of the extracellular matrix is composed of structural proteins such as collagen and elastin and adhesive protein molecules such as fibronectin and laminin (255;384;433). These protein molecules are embedded in a medium called the ground substance. This ground substance consists primarily of water but it also contains organic molecules called proteoglycans and glycoaminoglycans (302).

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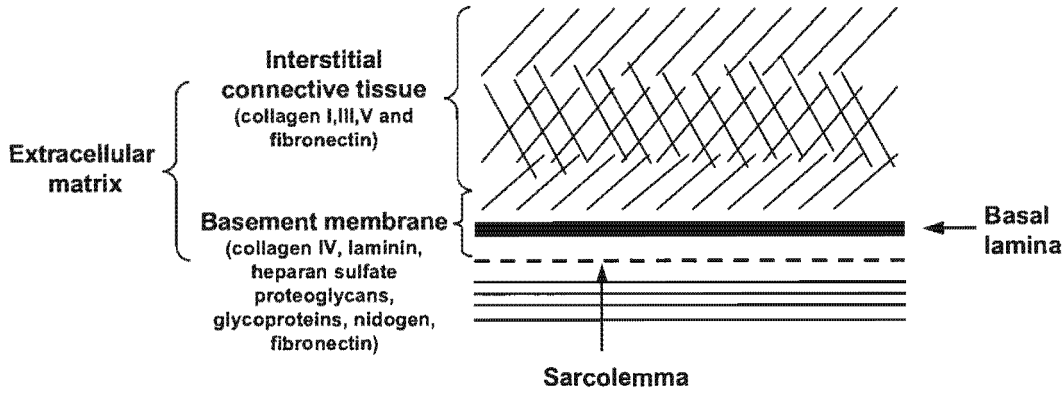

Figure 1.3.6. Diagrammatic representation of the components of the extracellular matrix surrounding an uninjured, mature muscle fibre. (Adapted from M.D. Grounds, 1991. *Path Res Pract.* 187:1-22.

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Physiologically important glycoaminoglycans are hyaluronic acid, heparin and heparan sulphate (302). The basement membrane consists of thin layers of specialised extracellular matrix that forms a supportive structure on which epithelial and endothelial cells grow (174;302;433).

The basement membrane of skeletal muscle forms a continual layer around the muscle fibre and so any changes that occur in the fibre, regarding size or shape during early development and later growth and adaptation, will require the remodelling of the basement membrane (507). The basement membrane acts as a transmission barrier in that force generated within the fibre is transmitted via the basement membrane to the surrounding tissue and extracellular signals must first pass through the membrane before entering the muscle cell (181). The basement membrane consists of 3 basic areas each with specific functions and characteristics (181). The myotendinous junction

occurs at the ends of the muscle fibre and it is the major site of force transmission from the interior of the muscle cell to the surrounding extracellular matrix. The neuromuscular junction is the site where the motor neuron attaches to the muscle fibre. The rest of the membrane is called the sarcolemmal basement membrane (181).

1.3.5.1. Components of the basement membrane

The family of proteins called laminins together with collagens (particularly collagen IV) constitute the majority of the basement membrane network. Interspersed throughout this network are proteoglycans, nidogens (linker molecules) and cell membrane receptors, integrins and dystroglycan complexes (174;181).

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Laminins

Laminins are the most abundant glycoprotein in the basement membrane and they are the major ligands for the cell surface receptors involved in the transmission of force from the cell's interior to the extracellular matix (174). Laminins are heterotrimers composed of 3 chains arranged in a crucifix-like shape (174). The individual laminin chains, alpha (α), beta (β) and gamma (γ) are distinct gene products (54). The five α, three β and three γ chains form various combinations to produce distinct laminin isoforms. Laminin –2 ($\alpha 2\beta 1\gamma 1$; also referred to as merosin) is the predominant isoform in the sarcolemmal basement membrane of mature muscle fibres (174). In vitro studies have identified both laminin-2 and laminin-1 isoforms to be important factors in myogenesis and muscle integrity. Muscle precursor cells will

preferentially attach, proliferate, migrate and differentiate into myotubes on these laminins compared to type I collagen or fibronectin (174). While laminin-1 reportedly sustains proliferation, migration and differentiation of myoblasts in vitro, laminin-2, although not essential for myogenesis (393), is important for myotube formation and stabilisation (465).

Integrins

Integrins are heterodimers consisting of α and β chains. Integrins are the major receptors for the laminin proteins and are the mechanical link between the cytoskeleton on the muscle fibre and the extracellular matrix. Integrins are also able to transduce signals regarding the proliferation or differentiation status of the cell (436). In vitro studies have revealed that the interactions of myoblasts with laminin-1 and laminin-2 are mediated by the β1 integrin series of receptors, in particular integrin α7β1, which is the predominant laminin binding integrin in adult skeletal muscle (510). A novel integrin α11β1 has recently been identified on human myotubes. This integrin appears to be involved in the binding of myoblasts to type I collagen (506).

Dystroglycans

The dystroglycan receptor is composed of 2 peptide chains, α and β. The dystroglycan receptors play a crucial role in the attachment of muscle fibres to the basement membrane in vivo (476). The α chain is highly glycosylated and is the extracellular component of the dystroglycan complex (DGC), whereas the β chain is the transmembrane component of the complex. An integral link is established between the cytoskeletal protein dystrophin and the

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extracellular matrix via the dystroglycan receptor complex (169). Although laminins do bind these receptors, other basement membrane proteins such as agrin and perlecan bind preferentially with the dystroglycan receptors (181).

Heparan sulphate proteoglycan

Heparan sulphate proteoglycan is a major component of the basement membrane. Presumably through its strong binding to growth factors such as bFGF, this proteoglycan indirectly inhibits proliferation of muscle precursor cells (247).

Fibronectin and Collagen

Fibronectin is a cell surface and extracellular matrix protein that is involved in cell-cell, cell-substrate adhesions, cell motility, binding of macromolecules and maintenance of the normal morphological phenotype of the muscle fibre (174). Studies have shown that while an up-regulation of fibronectin enhances myofibre formation a down-regulation of the protein enhances fusion and maturation of the myofibre (514). Collagen is essential for the differentiation and fusion of muscle precursor cells (174).

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1.3.5.2. Skeletal muscle regeneration and the extracellular matrix

It is important to note that the mode of repair is related to the severity of the muscle damage (75;191). Most studies investigating the process of muscle repair and regeneration have used extreme models of tissue damage such as muscle contusion, crush, strain (rupture) and ablation by means of toxic chemicals (75;174;176). In the case of these models where there is severe damage to the muscle tissue, extensive regeneration of the tissue must take

place. Although there is evidence to show that regeneration does occur in the absence of the basement membrane (62;434), the repair process is greatly enhanced if the basement membrane is intact (174;180;434).

The importance of the basement membrane in the regeneration and repair process is demonstrated by tissue culture experiments where attachment and proliferation of muscle precursor cells is significantly increased in cells grown on a reconstituted basement membrane compared with cells grown on a gelatin membrane (174). During the repair process the basement membrane acts as a scaffold upon which activated satellite cells (myoblasts) proliferate, migrate, differentiate and finally fuse to form myotubes (174;175). An intact basement membrane provides a favourable microenvironment in which new myofibres may regenerate and prevents the infiltration of fibroblasts and collagen fibres into the regenerating area (222).

1.3.5.3. Interaction of basement membrane, interstitium and myogenic cells

Most of the research investigating skeletal muscle regeneration and the factors involved in the process have investigated the influence of either the growth factors and cytokines or the basement membrane proteins on the regenerating muscle cells (75;174-176). The few studies that have investigated the integration/interaction between the growth factors and cytokines and the basement membrane proteins and the combined effect the interaction will have on the regenerating muscle cells will be discussed below.

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Changes in the relative proportions of extracellular components such as a down-regulation of heparan sulphate proteoglycan and an up regulation of laminin and fibronectin may contribute to the initiation of satellite cell replication (174;247). This interaction may occur via the release of bFGF from heparan sulphate proteoglycans, so enabling the growth factor to interact with the satellite cells (174;247).

A recent study revealed that an elegant relationship exists between the growth factor PDGF, the basement membrane proteins, laminin, collagen IV and fibronectin and regenerating muscle cells (4). PDGF stimulates proliferation and inhibits differentiation of regenerating muscle cells, as does fibronectin. Laminin and collagen IV promote chemotaxis and stimulate proliferation and differentiation of regenerating muscle cells. As a compensation for its short half-life, PDGF stimulates the synthesis and secretion of laminin, collagen IV and fibronectin via independent signalling pathways. As a result of this interaction the growth factor is able to amplify its effect on the regenerating muscle cells, be it to stimulate cell migration and proliferation or to inhibit differentiation. The basement membrane is modified according to the needs of the cell and its environment (4).

Similar interactions were investigated between MyoD and leukaemia inhibiting factor (LIF) and the basement membrane proteins (216;526). It was hypothesized that a lack of MyoD might effect the expression of certain extracellular matix proteins that are normally responsible for myoblast attachment, proliferation, migration and differentiation and as a result there

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would be delayed myotube formation and increased myoblast proliferation. There is recent evidence to show that this is not the case (216).

LIF has been shown to stimulate myoblast proliferation *in vitro* (18;526) and enhance myotube formation *in vivo* (503). It was thought that perhaps the effect LIF exerted on myoblasts and myotubes was mediated via LIF-induced changes in the extracellular matrix. Although LIF induced significant changes in the expression of fibronectin, tenascin-c, collagen IV and laminin during the regeneration process *in vitro* (526), no such changes have been noted *in vivo* (180;465;526).

1.3.6. Satellite cells and muscle fibre type

An increase in satellite cell density occurs in association with the proximity of capillaries, myonuclei and motorneuron junctions (191). Oxidative fibres usually have increased capillary and motorneuron density compared to glycolytic fibres and as a result oxidative fibres have a 5 to 6 times greater satellite cell content compared to glycolytic fibres (161;191;439). Growth characteristics, muscle fibre recruitment patterns and loading patterns also influence the number of satellite cells per fibre (439). Muscles with predominately oxidative fibres, such as the soleus muscle, are recruited with greater frequency than muscles with a high proportion of glycolytic fibres (439). Frequency of recruitment may increase the demands on the muscle and consequently the amount of damage and degeneration the muscle is

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exposed to (439). Therefore, a larger population of satellite cells is needed to meet the demands placed on the muscle (439).

Although, as a general rule, oxidative fibres within a muscle are associated with a larger satellite cell population compared to the glycolytic fibres, fibres of the same type in different muscles will not necessarily show the same pattern of distribution or satellite cell population size. The reasons for this are unknown (439).

In accordance with the different fibre types, it has been speculated that there are different satellite cell lineages that are capable of producing particular types of myotubes based on the myosin heavy chain isoform expression (174;428). Although there is evidence to suggest that satellite cells have a genetic predisposition to forming a particular fibre type (127), it is believed that innervation patterns and the cellular milieu at the time of regeneration are the ultimate determinants of the final phenotype of the cell (75;122).

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1.3.7 Satellite cells and exercise-induced training adaptations

Resistance and endurance training are capable of inducing damage to the skeletal muscle (refer to section 1.2). The extent of the exercise-induced muscle damage ranges from minor, sublethal membrane damage to focal necrosis of muscle fibres (175). It has been suggested that even in the absence of extensive damage and muscle fibre necrosis, macrophages infiltrate the area (470) and satellite cells are activated to proliferate (100).

1.3.7.1. *Hypertrophy and hyperplasia*

Resistance or strength training induces an increase in the size of the trained muscle. The increased muscle size can result from an increase in the cross sectional area of the muscle fibres due to increased protein synthesis (hypertrophy) or an increase in the number of muscle fibres that make up the muscle mass (hyperplasia) (507). Hyperplasia can occur either by the regenerating myotubes fusing with the hypertrophying muscle fibre or by the myotubes fusing with one another to form a new myofibre (175)

1.3.7.2. *Atrophy*

Muscle atrophy occurs after immobilisation, denervation or extreme malnutrition of skeletal muscle with a reduction in the number of myonuclei and the cross-sectional area of the muscle fibre (176;191). Following a period of immobilisation, satellite cells from adult muscle are able to recover and regenerate the atrophied muscle once weight bearing is resumed (191). In adolescents, however, a period of immobilisation may irreversibly disrupt the developmental progress and prevent the myofibres from incorporating new myonuclei, even after weight bearing has resumed (191).

In the initial stages of denervation the percentage of satellite cells increases (508). However, after a prolonged period of denervation (18 month period) the percentage of satellite cells decreases significantly (191). Viguie et al. (1997) hypothesize that prolonged denervation may result in satellite cells apoptosis (508). Prolonged denervation ultimately results in the inability of the muscle to

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regain its previous functional capacity even if the muscle is reinnervated (191).

The severe and rapid consequences of immobilisation and denervation on both the muscle fibre and the satellite cells highlight the importance of mechanical and neural stimuli to the functional homeostasis of the skeletal muscle.

1.3.7.3. Remodelling of the basement membrane following exercise

Although exercise-induced muscle damage may be considerably extensive in focal regions of the muscle, it is unlikely that the damage would be sufficiently severe to result in damage to the basement membrane. Exercise-induced muscle damage may however result in remodelling of the basement membrane. Previous research has shown that rats, which undergo habitual endurance training, have an increased content of type IV collagen (267). In addition to this an acute, strenuous bout of exercise rapidly elevated steady-state mRNA levels of type IV collagen (186). A recent study revealed that the pattern of changes seen in type IV collagen following acute, damage inducing exercise, differ between muscle groups with different fibre types and correlate with the severity of the muscle damage and the subsequent regeneration of the basement membrane (266).

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1.3.8. Satellite cells and aging

1.3.8.1. Number of satellite cells

The precise evolution of the satellite cell population during normal human aging is still controversial (492). In general, however, the percentage of satellite cells decreases as a function of age (191;439;492). At birth, human satellite cells account for 15% of all the muscle nuclei. By age 2, 6-10% of the total myonuclei are accounted for by satellite cells and in adults 4% of the myonuclei are represented by satellite cells (492). Studies have observed a range of different values (between 0.6 and 3.4%) for the percentage of satellite cells in skeletal muscle of older individuals (65 - 75 years of age) (418;423;492).

The reduction in the relative number of satellite cells may reflect an increase in the number of myonuclei, as the absolute number of satellite cells remains constant (439). Another explanation for the reduction in the relative number of satellite cells with age could be that as the number of myonuclei increase there is a concurrent decrease in the number of satellite cells (439). Studies quantifying the number of satellite cells in the soleus and EDL muscles of rats at different ages, demonstated that both explanations described above occurred depending on the muscle examined (439).

1.3.8.2. Proliferative capacity

Proliferation of the satellite cells is an integral part of skeletal muscle regeneration (174;176;191;417). It is well established that human diploid cells,

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including satellite cells, have a limited proliferative capacity that decreases with age (Figure 1.3.7.)(106;192;193;417). One of the mechanisms that may control the proliferative capacity of cells is the shortening of the telomeric region found at the end of all eukaryotic chromosomes (104-106;417). This region of the chromosome is comprised of non-coding TTAGGG repeats called telomeres (168;231;417). As a result of incomplete DNA replication, approximately 113 base pairs of telomeric DNA are lost with each *in vitro* cell replication (168;219;231). Studies have shown that a decrease in telomere length is associated with onset of replicative senescence, whereby the cell loses its ability to replicate (187;188;219;273).

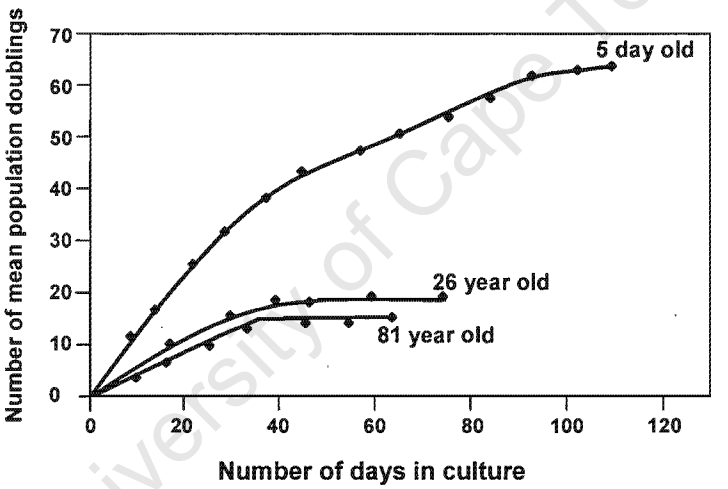

Figure 1.3.7. Graph showing the proliferative life span of human satellite cells isolated from donors of differing ages. The cell populations were serially passaged and cultured in Ham's F10 medium until the cells stopped dividing. At each passage the number of mean population doublings were determined by counting the number of cells. (Adapted from Thornell et al. (2003). *Scand J Med Sci Sport.* vol 13: 48-55)

Despite there being a decrease in the number of satellite cells and a limit to the proliferative capacity of cells with aging, these factors are not the rate limiting steps in the decreased regenerative capacity observed in aged

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skeletal muscle (175;191;492). The cellular environment in which the regeneration occurs appears to be a critical factor in the success of the regenerative response to injury (68;69;175;191). With increasing age there is a thickening of the basal lamina (460), increased fibrosis within the muscle (334), a reduced capillary density (86), decreased immune response (99), decreased serum levels of critical growth factors (499) and reduced ability for neuronal regeneration (68;69). All of these factors will impair the regenerative process of the muscle (191). In addition to this, it seems that thinner more fragile myotubes are formed in skeletal muscle of individuals who are over the age of 60 years (67;417).

1.3.9. Maintenance of the satellite cell population

Constant mechanical activity throughout adult life renders skeletal muscle prone to continual episodes of damage and degeneration. In early adulthood the number of satellite cells is maintained but the chronic demand for new myonuclei over one's lifespan eventually leads to a reduction in the number of satellite cells in aged muscles (162;460;539). In fast myofibres of mice there are ~300 myonuclei and 5-12 satellite cells per fibre and in the slow myofibres there are ~450 myonuclei and about 30 satellite cells per fibre (539). Approximately 1-2% of myonuclei from normal adult rats are replaced each week (437) and activated satellite cells undergo only one or two rounds of division before being incorporated into myofibres (178). The number of satellite cells per fibre, the turnover rate of myonuclei and the number of mitotic divisions prior to incorporation into myofibres, show that the satellite

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cell population are capable of self-renewal. Indeed, if this were not the case, the population of satellite cells would be rapidly exhausted instead of merely depleted (539).

Numerous mechanisms have been proposed to explain how the satellite cell population is maintained throughout an individual's lifetime. Moss and Leblond (1971) proposed asymmetrical division of the satellite cells, whereby one daughter cell enters the regeneration process and becomes incorporated into a myofibre and the other maintains the satellite cell pool (179). Zammit and Beauchamp (2001) suggest that symmetrical satellite cell division may occur and that the expression of the specific myogenic regulatory factors will determine whether or not the daughter cells enters the differentiation pathway or returns to a state of quiescence (539). In support of this theory, quiescent satellite cells express Myf5 but not MyoD (26) and the expression of MyoD appears to commit the cell to terminal differentiation. Furthermore in the absence of MyoD, activated satellite cells show increased proliferation and delayed differentiation (531). Also, the results of a study by Yoshida et al. (1998) showed that when C2C12 myogenic cultures were induced to differentiate, a population of undifferentiated MyoD negative cells persisted (536). On further investigation, these MyoD negative cells were capable of producing daughter cells that could either enter the differentiation pathway or replenish the satellite cell pool (536).

Another theory suggests that self-renewal of satellite cells may occur through dedifferentiation of committed muscle precursor cells and even myofibres

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(442), An alternative theory is that a specific subpopulation of satellite cells, or perhaps a separate population of multipotent stem cells, is responsible for the maintenance of the satellite cell population (539). Recent research has revealed the presence of multipotent stem cells in adult skeletal muscle. Although the true identity and the functional relevance of these cells in normal skeletal muscle remains to be determined, the presence of these cells adds a new dimension for research in this area. This theory, however, remains to be thoroughly investigated.

1.3.10. General morphology of the repair process

Hurme et al. (1991) used ultrastructural and immunohistochemical techniques to record the healing process that occurs in skeletal muscle following a contusion injury (222). Injuries involving contusions or ruptures of the muscle tissue completely disrupt the muscle fibres and their connective tissue sheaths. The damaged area is divided into four zones. The central zone is where the damaged fibres retract to create a gap between the muscle fibres. The ruptured gap is filled with hematoma, proliferating granulation tissue and later by connective tissue scar. The regeneration zone is where necrosis of myofibres takes place. The stumps of preserved, undamaged muscle fibres appear in the surviving zone. The fourth zone is the fibrillary cap covering the traumatised area (222;243).

Damage to the sarcolemma, which occurs with rupture of the muscle fibre, allows uncontrolled entry of extracellular constituents into the cytoplasm of the

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muscle cell. These constituents are invariably toxic to the muscle cell (228;239). Approximately 9 hours following the insult to the muscle, membrane vesicles accumulate in the undamaged portion of the muscle fibre and interdigitating membranes form at the interface of the damaged portion (174;243). In this way the damaged area is sealed off from the undamaged area (221), to prevent further damage and to contain the infiltration of immune cells. This new membrane separates the regenerating zone from the surviving zone (243).

By the second day after the injury, infiltrating macrophages have removed the necrotic muscle tissue (243) and fibroblasts have begun to form a connective tissue scar in the central zone (222;243). On the third day, activated myoblasts begin to aggregate within the basal lamina cylinders in the regeneration zone. By day 5, myoblasts in the regenerating zone have fused to form thin myotubes and the connective tissue in the central zone is denser. By day 7, the newly regenerating myotubes form multiple thin branches that extend out of the old basal lamina cylinders into the central zone. These branches gradually pierce through the scar of connective tissue. In the central zone, growth of the regenerating myotubes occurs in the absence of a basement membrane and chains of fused myoblasts act as guides (222). By day 14, the connective tissue scar of the central zone has further condensed and the regenerating myofibres have nearly crossed the gap of the central zone. If the basement membrane remains intact, it will act as a scaffold, and together with the undamaged muscle fibres it will help align the regenerating fibres (75;174;221;243). However, in the absence of an intact basement

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membrane, the uninjured fibres together with the surrounding connective tissue guide the parallel alignment of the regenerating myotubes (222). After 21 days the scar now consists of interlacing myofibres with little intervening connective tissue (222).

Active synthesis of sarcoplasmic components occurs in the growing myotubes (222). This is shown by the abundant mitochondria, ribosomal rosettes, the presence of sarcomeres and the active synthesis of structural myofibrillar proteins in the growing tip (222). In addition to this the nuclei move out to the periphery, indicative of myofibre maturity (222).

1.3.11. Summary

While some studies have shown that the regeneration process may proceed in the absence of damage, and therefore necrosis, of the skeletal muscle (10;100;327;329;517;537), the process of regeneration and repair of skeletal muscle typically follows a sequence of events including, infiltration of immune cells, phagocytosis of necrotic debris, revascularisation of the damaged area, activation of satellite cells, proliferation of myogenic precursor cells, fusion of myoblasts and finally maturation of myotubes.

In the presence of an intact basement membrane and undamaged muscle fibres, the deposition of scar tissue is greatly reduced and complete regeneration of skeletal muscle may occur within 7 days (75;221). However, if the muscle is more severely damaged, connective tissue is deposited in the

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ruptured gap of the central zone. The formation of the connective tissue scar is integral to the regeneration process as it bridges the ruptured gap and guides the growth of the regenerating myotubes across the central zone (222;243). The connective tissue scar is, however, vulnerable to rupture, particularly in the early stages of the repair process (243). While contraction of the regenerating muscle enhances alignment and extension of growing myotubes across the gap of the central zone by bringing the ruptured ends of the myofibres closer together (222), contraction or lengthening of the connective tissue scar within the first 10 days of the regeneration process, may rupture the scar (243). Repeated disruption of the scar results in a more compact connective tissue scar (222;243). If the connective tissue scar becomes too compact the regenerating myotubes will have difficulty penetrating it and this could potentially result in incomplete repair of the damaged area (243). Contrary to this, early mobilisation of an injured muscle appears to enhance the expression of specific integrin molecules. These integrin molecules are responsible for the attachment of cells to the extracellular matrix and transduction of signals, specifically regarding mechanical stress, between the extracellular matrix and the cells. These signals regulate the composition of the surrounding extracellular matrix and the cellular functions essential for muscle repair.

Although the damage induced by exercise is not likely to be as extensive as a contusion injury, the principles regarding the repair and regeneration process may be relevant to the endurance athlete who is constantly exposing his muscle to bouts of exercise-induced damage and repair. The connective

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tissue scar is at its weakest up to 10 days after the event that caused damage. Endurance athletes usually start training again within 10 days of an event that causes muscle damage (282). As a result, it is possible that they rupture the connective tissue scar within this period. If this happens repeatedly it will make the scar more compact and therefore more difficult for the regenerating myotubes to penetrate. This raises the question of whether incomplete repair of the exercise-induced muscle damage, which may accumulate over the years, results in chronic skeletal muscle damage. This will be discussed further in the experimental section of this thesis.

1.4. SKELETAL MUSCLE ADAPTATION

1.4.1. Introduction

Physical activity is essential for normal muscle development and maintenance (47). The absence of physical activity results in skeletal muscle atrophy and decreased functional capacity (3). Skeletal muscle has a remarkable capacity to adapt to the specific demands of the various physical stresses (47;165). Adaptation to these stressors usually results in enhanced functional capacity of the muscle (63;432).

Exercise induces both mechanical and metabolic disruptions of the cellular environment of skeletal muscle. These disruptions activate a host of second messengers and signalling pathways, which alter the cellular environment of the muscle fibre so enabling it to adapt to the mechanical and metabolic demands placed on it (47;63). Scientist are in the process of uncovering the

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multitude of cellular signals that stimulate exercise-induced adaptation in the muscle, most of which are not yet clearly identified or understood (for recent review articles see references (136;205;431)).

In addition to cytokines, growth factors and various catecholamines, reactive oxygen species also have a very important role in regulating intracellular skeletal muscle adaptation to exercise (337;339). Studies have shown the reduction/oxidation (REDOX) state of the cell to be influential in the activation of several key transcription factors and signalling molecules (9;285;339). Nitric oxide has also been implicated in the up-regulation of particular genes, which mediate changes in protein composition in response to mechanical stimuli (262;495).

While exercise is a potent stimulus for skeletal muscle adaptation, damage to the skeletal muscle that is caused through exercise, is a unique stimulus such that adaptive response induced by the stimulus protects the muscle from a subsequent bout of damage-inducing exercise.

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1.4.2. Exercise-induced muscle damage and the repeat bout effect

Exercise-induced damage to the muscle cell induces skeletal muscle adaptation and remodelling, such that the clinical symptoms associated with exercise-induced muscle damage (i.e. force decrement, myofibrillar ultrastructural disruption, delayed onset muscle soreness, immune response, increased serum creatine kinase) are greatly reduced following the second

bout a subsequent bout of damage inducing exercise (326;341;343;477). This phenomenon is known as the repeat bout effect (326;341;343;477).

The initial bout of damaging exercise does not have to cause appreciable muscle damage in order to provide a protective effect (381;382). However, the intensity of the subsequent bout of damage-inducing exercise must not exceed that of the initial bout if a protective effect is to be provided (378). The repeat bout effect appears to be muscle specific, in that only the specific muscle that was exercised in the initial bout will be protected during the subsequent bout of exercise (88). A number of recent studies have shown that prior concentric training appears to increase the muscle's susceptibility to exercise-induced muscle damage (164;405;527). As discussed previously (see page 48), this appears to be associated with the increase in muscle stiffness that is believed to result from concentric training.

It is not known whether the repeat bout effect reflects a decrease in the actual damage to the skeletal muscle, a decrease in the response of the muscle cell to the subsequent bout of damage-inducing exercise, or perhaps a combination of the two (343). In a recent review, McHugh (2003) discussed the scientific evidence for and against a number of theories that have been proposed to explain the repeat bout effect (343). These and other theories will be discussed below.

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1.4.2.1. *Neural theory*

During an eccentric action, fewer motor units are recruited per unit force produced compared to during a concentric contraction (120). The resultant high stress placed on a small number of activated muscle fibres is believed to be a major cause of the mechanical damage that occurs to the muscle fibre during an eccentric contraction (343).

The neural theory is based on the principle that there is an altered pattern of muscle fibre recruitment following the initial bout of exercise-induced muscle damage, such that the force is distributed over a greater number of muscle fibres so reducing the stress placed on the individual fibres (343;376). It is also thought that during the initial bout of exercise-induced muscle damage fast twitch fibres are preferentially recruited while during the second bout of exercise-induced muscle damage a greater proportion of slow twitch fibres, which are believed to be less susceptible to muscle damage, are recruited (518).

There are studies which show increased integrated electromyograph (iEMG) activity following eccentric strength training, which may be interpreted as there being a redistribution of the workload over a greater number of active muscle fibres (209;210;263). It is not clear, however, whether the increase iEMG resulted from the repeated bouts of eccentric training or from the initial bout of eccentric exercise. This is of particular concern, especially since an increased iEMG has not been seen following repeated bouts of damage inducing exercise in the hamstring or the tibialis anterior muscles (343;344;518).

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The fact that the initial bout of exercise need not induce a considerable amount of muscle damage in order to afford effective protection during the second bout of exercise (211) and that a protective effect following a repeated bout of damage-inducing exercise is seen prior to full recovery of the muscle following the initial bout of damage-inducing exercise (326) lends further support to the neural theory of the repeat bout effect (343).

On the other hand when the neural component was bypassed through electrical stimulation, a protective effect was still noted (380). While this does not exclude the involvement of a neural component in the repeat bout effect, it does introduce the possibility of additional components being involved in the process (341;343).

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1.4.2.2. Mechanical theory

The most extensive exercise-induced muscle damage occurs when the muscle is lengthened beyond the point at which there is no longer crossbridge interaction between the actin and myosin filaments (i.e. the descending limb of the length-tension curve; see section 1.2. for more details)(410). At this point on the length-tension curve the muscle is only able to produce passive force, the strength of which is primarily dependent upon the integrity of the intermediate filaments, titin, desmin, vimentin and synemin and the amount of intramuscular connective tissue (140;286;397;521).

The principle upon which the mechanical theory is based is that the initial bout of damaging exercise induces alterations in the mechanical properties of the

non-contractile elements of skeletal muscle will strengthen the muscle and protects it from damage during the subsequent bout of exercise (343). While some researchers believe that increased passive tension will afford greater protection following a repeat bout of damage-inducing exercise (286;414) others believe that decreased passive tension may afford this effect (340;376;377).

Intramuscular connective tissue

An increase in intramuscular connective tissue is believed to increase muscle stiffness and so provide protection during a repeat bout of damage-inducing exercise (286). Studies in which the intramuscular connective tissue was experimentally increased have shown a dramatically reduced strength decrement following a repeat bout of damaging exercise (286). It is thought that tissue repair following exercise-induced muscle damage is associated with an increased synthesis and deposition of intramuscular connective tissue, which will absorb and so dissipate the excessive strain on the myofibrils (341;343).

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Intermediate filaments

Apart from being responsible for the structural integrity of serial and parallel sarcomeres and providing a link for lateral transmission of force, the intermediate filaments are also responsible for the passive tension of the myofibril (397). The cytoskeletal muscle proteins are extremely vulnerable to damage resulting from eccentric exercise (521). Alterations in the properties of the cytoskeletal proteins could provide enhanced mechanical reinforcement

during a subsequent bout of damage-inducing exercise. There is no direct evidence to support this theory, however, Barash et al. (2002) recently observed an increase in the desmin content of rat skeletal muscle, 3-7 days after undergoing a damaging contraction (23).

The highly elastic intermediate filament, titin, is believed to be the major determinant of passive tension in the myofibril (498). A recent study investigating the adaptability of cardiac titin revealed that cardiac titin was a highly adjustable spring and through differential splicing of various elastic regions of the molecule and co-expression of the various cardiac titin isoforms, the passive stiffness of the cardiac muscle could be readily adjusted (172). Similar adaptability of skeletal muscle titin needs to be investigated, particularly with respect to changes in the passive tension of the myofibril and its involvement in the repeat bout effect following an initial bout of damage-inducing exercise.

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1.4.2.3. Cellular theory

Cellular adaptations that occur at the level of the muscle fibre, the myofibril and the sarcomere are used to support the cellular theory of the repeat bout effect. Some researchers believe that the initial bout of damage-inducing exercise eliminates the weak, stress susceptible fibres and sarcomeres, thus explaining the reduced muscle damage following the repeat bout of exercise (15;326). However, this theory does not explain the situation where a protective effect is observed after an initial bout of exercise that does not cause substantial muscle damage (341;343).

An additional cellular theory involves adaptation at the level of the proteolytic pathways within the cell. For effective repair of the exercise-induced muscle damage to occur, damaged proteins must be degraded and removed (see section 1.3). This proteolysis would require activation of various proteolytic pathways (388). One such pathway is the ATP-dependent ubiquintin pathway (388). A recent study revealed that this particular pathway was up regulated to a greater extent following the second bout of eccentric leg press (477). This may indicate a more efficient removal of damaged contractile and cytoskeletal proteins thus promoting regeneration and repair of the damaged tissue (477).

1.4.2.4. Additional sarcomeres

There is evidence to suggest that additional sarcomeres are added in series following eccentric muscle exercise (319). Furthermore, the rightward shift in the length-tension curve of the skeletal muscle following recovery from a damaging contraction has been attributed to an increase in the number of sarcomeres in series (43). Additional sarcomeres in series would reduce the individual sarcomeric strain thus preventing myofibrillar disruption (341;343). Although this is a possible explanation for the repeat bout effect, the time course of the adaptations and the initiating stimuli do not reconcile with the present evidence regarding the repeat bout effect (341). For instance the protective effect that has been noted prior to full recovery from the initial bout of damage inducing exercise cannot be as a result of additional sarcomeres as this adaptation could not occur in this time frame. Furthermore, this explanation is based on the fact that myofibrillar disruption is the initiating

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stimulus for this adaptation to occur, but very often a protective effect has been noted without substantial damage being induced by the initial bout of exercise (381;382).

1.4.2.5. Altered immune response

Although the immune response following exercise-induced muscle damage is an essential part of the regeneration, repair and adaptation process, it may also contribute to the secondary damage characteristic of exercise-induced muscle damage (see section 1.3). A blunted immune response has been noted following a repeat bout of damage-inducing exercise (403). The decreased muscle damage, inferred for the most part by indirect measures of muscle damage, during a subsequent bout of damaging exercise may attributed to a decreased immune response to the primary muscle damage (343;404).

A recent study revealed the importance of the inflammatory response to exercise-induced muscle damage and its role in the repeat bout effect (288). The authors hypothesized that the ED2+ macrophages, in addition to producing collagen themselves, may activate fibroblasts to proliferate and synthesize collagen. Increased intracellular collagen deposition may protect the muscle cell against future bouts of damage-inducing exercise, as discussed earlier.

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1.4.2.6. Alterations in the excitation-contraction coupling mechanism

Although the characteristic decrease in force production following a bout of damage-inducing exercise is most likely due to mechanical disruption of the myofibrils, disruption of the excitation contraction coupling mechanism may also contribute to the decreased force production (5). Studies have shown extensive ultrastructural damage to the sarcoplasmic reticulum and the t-tubules as well as impaired calcium release and sensitivity following exercise-induced myofibrillar disruption (22;56;520;534). An adaptation in the excitation contraction coupling mechanism may explain the reduced force decrements following a repeat bout of the exercise. Remodelling of the sarcolemma, sarcoplasmic reticulum and t-tubules following the initial bout of damage-inducing exercise may result in decrease influx of calcium and reduced cellular disruption and necrosis as well as a reduced efflux of cytoplasmic proteins such as creatine kinase (83).

1.4.2.7. Heat shock proteins (HSPs) and antioxidants

Heat shock proteins are important molecules involved in protecting the cell against oxidative and thermal stress (339). Cytoplasmic levels of HSPs increase in response to increased free radicals, oxidative stress, protein denaturation, aggregation and lysis and exercise (251;338;491). A recent study investigating HSPs and the repeat bout effect observed reduced basal levels of both HSP27 and HSP70 following the initial bout of exercise and a similar percentage response of each HSP to both bouts of exercise (490). The response of the HSPs is proportional to the magnitude of the stress. Since both bouts of exercise were the same it follows that the HSP should respond

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similarly after both bouts of exercise (490). High levels of HSPs have been shown to be toxic to the cell, thus in order to respond appropriately to the stressful stimuli without poisoning the cell, basal levels of HSP were reduced (490). HSP may also facilitate rapid recovery and remodelling following exercise-induced damage by acting as molecular chaperones and ensuring that newly synthesized proteins are folded and function correctly (339).

1.4.2.8. Altered gene expression

Considerable research has focused on the phenotypic nature of exercise-induced skeletal muscle adaptations (109;205;366;401). Modern technologies, such as microarrays, have provided new insight into the complex patterns of gene expressions necessary for the phenotypic skeletal muscle adaptations induced by exercise (63). Gene transcription may be activated within seconds of the initial contraction and/or up to hours following the cessation of the exercise bout (63). Numerous studies have demonstrated significant elevation in the mRNA concentration of metabolic, coordinatory and immuno-modulatory genes in the muscle sample of healthy human subjects following a single bout of exercise (for extensive reviews see references (63;136)).

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1.4.3. Summary

The process of skeletal muscle adaptation to exercise in general is extremely complex. Similarly, the mechanisms, either separately and/or simultaneously involved in the repeat bout effect are also complex. Before the precise mechanisms involved in the repeat bout effect can be established a

consensus needs to be reached regarding whether the repeat bout effect reflects a reduction in the actual myofibrillar damage, a reduction in the response of the muscle to the damaging stimulus, or a combination of both (343).

Exercise and exercise-induced muscle damage are potent stimuli for skeletal muscle adaptation. Furthermore, skeletal muscle is remarkably adept at responding to these stimuli and adapting accordingly. However, this capacity for adaptation appears to be a limited, resulting in maladaptation and malfunctioning of the skeletal muscle. This will be discussed in the following section.

1.5. "MALADAPTATION"

An erroneous belief is that the harder one trains the greater the degree of adaptation and the better one's performance. Although skeletal muscle has a remarkable capacity for adaptation (section 1.4), specific intrinsic and extrinsic conditions must prevail for successful, performance enhancing adaptations to occur. If these specific requirements are not met, maladaptations and decrements in performance may occur.

The aging process (intrinsic) and the overtraining syndrome (self-induced) are examples whereby the adaptive capacity of the body has been exceeded. In these two examples, there is an imbalance between catabolic and anabolic

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processes, resulting in maladaptation and as a consequence, a reduction in performance capacity.

1.5.1. The aging process

If the various cellular processes were infinitely perfect, with a limitless capacity for repair and adaptation, the cell would not age. The reality is all organisms, from single cells to highly evolved, multi-tissued organisms, age (523). In humans, the aging process results in structural and functional maladaptations in the entire body, particularly the skeletal muscle (71;339;523). Not only is there a reduction in the number and the cross sectional area of the muscle fibres with aging, but older muscle fibres are weaker per unit cross sectional area (150;151). This loss of muscle mass, strength, quality and function that occurs with aging is known as sarcopenia (71;339;523). The factors associated with age-related sarcopenia are discussed in more detail below.

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1.5.1.1. Inactivity

It is interesting to note that a reduction in the levels of physical activity with aging occur in all organisms, from fruitflies to roundworms to monkeys and humans (523). As much as inactivity may contribute to the sarcopenia of aging, the age-related decrease in muscle mass, strength and function may very well contribute to the age-related decline in physical activity (523). As such it is not known whether age-related physical activity is a cause or consequence of age-related sarcopenia. Older individuals who continue to

exercise on a regular basis seem to be able to delay certain aspects of age-related sarcopenia, however, they are not able to stem the relative decline in muscle function and mass (361;425;471). Therefore, although sarcopenia is accelerated by inactivity (425), inactivity is one of many factors which contribute to the loss of muscle mass and function that occurs with increasing age.

1.5.1.2. Skeletal muscle innervation and contractility

The decrease in muscle strength that occurs with age-related sarcopenia may result from maladaptations that occur in the innervation or contractility of the skeletal muscle. A noted consequence of the aging process is a loss of motor neurons and a decrease in the number of motor units, which is compensated for, to a certain extent, by an increase in the size of the motor unit (i.e. a particular nerve innervates a greater number of fibres)(318). Although some studies show a reduced capacity for reinnervation with increasing age (69;70) other studies have not confirmed this finding (246). Furthermore, the strength deficits associated with increasing age, appear to result from a decreased firing rate of the motor nerve during a maximal voluntary contraction (245), decreased motor unit recruitment during a sustained isometric contraction (33) and ultra structural abnormalities in the neuromuscular junction (318). The changes in the neuromuscular system, which occur with increasing age, may either be the cause or consequence of the loss and atrophy of muscle fibres. While the loss of muscle fibres occurs indiscriminately, there is a preferential atrophy of type II fibres (86). The selective atrophy of type II

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muscle fibres may also contribute to the decreased strength of aged muscle (523).

Aging also appears to affect the contractibility of the muscle fibre in that the both the release and the uptake of Ca^{2+} by the sarcoplasmic reticulum is impaired in old age (218;523). In addition to this, changes in the ion content (i.e. potassium and chloride) that determine the excitability of the cell are also affected with aging (497). These changes would certainly contribute to the loss of contractile function that occurs with aging.

1.5.1.7. Skeletal muscle regenerative capacity

Previous studies have shown that the regenerative capacity of old muscle is reduced compared to that of young muscle (67). The age-related reduction in the regenerative capacity may be related to the reduction in the number of satellite cells (418), the decreased proliferative capacity of satellite cells (106) and alterations in the cellular environment that impair regeneration (68), all of which occur as a result of the aging process.

All eukaryotic cells have a limited capacity for proliferation and on reaching this limit (Hayflick limit) they will no longer be able to divide (192;193). However, the proliferative capacity of the satellite cells does not appear to be the rate-limiting step in the aging process as a satellite cell from a 75 year old man was capable of doubling more than thirty times, *in vitro* (406).

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Whole muscle transplant studies have shown the age of the host, rather than the age of the donor to be the critical determinant of successful muscle regeneration (68;523). Stages of the regeneration processes that are dependent upon the cellular environment, for example differentiation and fusion of myoblasts, are more likely to be impaired and thus limit the regeneration process of aging muscle (406). The delayed and potentially incomplete regeneration that occurs with increasing age, may contribute to age-related sarcopenia.

1.5.1.7. Hormones, growth factors and enzymes

Numerous hormones, growth factors and enzymes regulate the development and function of muscle fibres (71;189;523). Circulating levels of anabolic hormones such as testosterone, growth hormone, dehydroepiandrosterone (DHEA), estrodiol and progesterone decrease with increasing age (284;360;523).

Very little is known about the role of growth factors, other than insulin-like growth factor-1 (IGF-1), in age-related sarcopenia (189;244). The decreased release of growth hormone from the anterior pituitary with aging results in a decreased release of IGF-1 by the liver (189;284;523). This results in decreased circulating levels of IGF-1 in older individuals (189;284). IGF-1 is an important mediator of protein synthesis, reinnervation and myoblast proliferation and differentiation (1). Studies have shown that the up-regulation of mechano growth factor (MGF), a specific isoform of IGF-1 that is sensitive

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to mechanical alterations or micro trauma in skeletal muscle, is decreased in older rats in response to mechanical overload (392).

Although circulating levels of most of the catabolic hormones and cytokines do not appear to be affected by age, circulating plasma levels of interleukin-6 (IL-6) and tumor necrosis factor (TNFα) increase with increasing age (50;128;399). The increased circulating levels of TNFα appear to be associated with insulin resistance (50), which may contribute to the decreased glycogenolysis and glycolysis that occurs with increased aging (396). The precise role of increased circulating levels of IL-6 are not yet fully understood, however it has been shown that older women with high levels of circulating IL-6 have a higher risk of being physically disabled (128).

The activity of certain enzymes involved in skeletal muscle metabolism changes with increasing age (reviewed in Carmeli et al. (2002) (71)). Studies have shown a decline in glycolysis and glycogenolysis and the related enzymes in aged human vastus lateralis muscle (396). In addition to this, enzymes associated with the citric acid cycle are also reduced, with the end result being an age-related reduction in respiration and oxidative capacity (71;488). To compensate for the reduction in respiration and oxidative capacity, aged rat muscle tends to increase gluconeogenesis (71;238). Furthermore mitochondrial respiratory chain function decreases considerably in humans between the ages of 17 to 90 years (41). The reduced capacity of aged muscle to produce energy may account for the impaired muscle function and decreased oxidative capacity noted in older individuals (71).

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1.5.1.7. Skeletal muscle protein turnover

As there is only a gradual decline in muscle mass with aging, any changes in protein synthesis should be matched with similar changes in protein breakdown. While there is evidence to show an age-related reduction in the synthesis of total muscle proteins (533), total myofibrillar proteins (525), myosin heavy chain proteins (190) and mitochondrial proteins (422), there is no real evidence to suggest a similar reduction in proteolysis (523). It is, however, clear that age-related sarcopenia is not mediated by a chronic increase in the rate of muscle proteolysis, but rather by a slow decrease in the rate of protein synthesis (25;189;190).

1.5.1.6. Oxidative damage to the skeletal muscle mitochondria

The by-products of metabolism, such as free radical oxygen, nitrogen and aldehyde species, cause extensive damage to DNA, proteins and lipids (339). Highly oxidative, post mitotic tissue such as the heart, brain and skeletal muscle are particularly susceptible to oxidative damage by free radicals.

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An increased production of free radicals in the resting muscles of rats (27) and an accumulation of oxidative damage to skeletal muscle mitochondria occurs with increasing age (290). The mitochondrial accumulation of free radicals may be associated with increased mitochondrial DNA deletion mutations, which together with the insufficient functioning of the electron transport chain will further enhance the generation of free radicals (71;339;346). The increased production of free radicals will not only result in

increased oxidative damage to structural components of the muscle cell but the shift in the redox state of the cell will lead to a decrease in the function of oxidised proteins, such as myosins, creatine kinase and some ATPases (339).

The antioxidant system of the cell, including amongst others, glutathione peroxidase, superoxide dismutase, catalase and vitamin A, C and E, appears to be overwhelmed and unable to sufficiently up-regulate in response to the increased production of free radicals with aging (236;339). The induction of heat shock proteins (HSPs), imperative for protection against free radical-mediated cellular and molecular damage, appears to be reduced in some cells of aged individuals (313;339). This further reduces the adaptive capacity of aged cells to stress and oxidative damage. Although most studies have indicated increased oxidative damage with aging, the full impact of this damage is yet to be elucidated.

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1.5.1.7. Gene expression

There appears to be a reduced expression of mRNA encoding proteins involved in mitochondrial electron transport and ATP synthesis and mRNA encoding enzymes involved in glucose and glycogen metabolism with increasing age (292;523). The reduced expression of these proteins and enzymes may be responsible for the reduced oxidative capacity of older muscle (292;524). There also appears to be a reduced expression of a calcineurin subunit in older muscle (523). Since calcineurin is involved in the nuclear translocation of certain transcription factors that are required for

muscle hypertrophy in response to overload, this is an important finding in terms of the age-related loss of muscle mass (444).

1.5.1.8. Exercise and aging

The increased demand for ATP during bouts of sustained muscle contractions is met by an increased rate of mitochondrial respiration (126;339). The increased oxidative phosphorylation dramatically increases the production of reactive oxygen and nitrogen species (339). It was recently found that at the same relative workload (% VO_{2max}), reactive oxygen species production was 77% higher in the muscles of old rats compared to young rats (235). Furthermore, the repair process following exercise-induced muscle damage involves the invasion of immune cells into the injured area and the phagocytosis of the necrotic muscle tissue by these cells involves the generation of free radicals (235).

Studies have shown that the degree of exercise-induced muscle damage is greater in older individuals compared to younger individuals (330). Interestingly, when the effect of a 9 weeks heavy-resistance strength training programme on the extent of skeletal muscle ultra structural damage was compared between young and old men and women, it was discovered that although there was no difference in the degree of muscle damage between the young and old men, a significantly greater degree of damage was induced in the older women compared to the young women (423;424). This finding might be explained by the protective effect of oestrogen. Younger women would have greater amounts of circulating oestrogen and therefore would

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benefit from the proposed protective effect of this hormone (250). It has also been noted that the ability to adapt to repeat bouts of muscle damage inducing exercise is reduced with increasing age (339).

Furthermore, strenuous, prolonged exercise may produce areas of ischaemia as well as the release of pro-inflammatory cytokines, both of which are capable of activating reactive oxygen species-generating enzymes (196). Although the antioxidant enzyme activity increases with increased aging, the increase in activity is not sufficient to counter-balance the dramatic increase in the production of free radicals (294).

Although exercise in older individuals is associated with certain risks it seems that the benefits of regular exercise training far out-weigh the risks. Studies have shown that aged muscle is capable of adapting to exercise in the same manner as younger muscle and so similar benefits such as increased oxidative capacity, increased antioxidant defence mechanisms and increased protein synthesis, all of which will enhance the structure and function of the aging body.

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1.5.1.9. Summary

There is an accumulation of random free radical damage to the DNA throughout an individual's lifetime. Although there are mechanisms in place to repair this damage, these mechanisms appear to be imperfect (523). The damaged DNA may result in the inactivation or altered expression of one or a number of genes. In a multinucleated muscle cell the effect of random genetic

mutations will be diluted and isolated to the surrounding cytoplasm. However, in a mononucleated motor nerve cell the impact of these genetic mutations will be far greater and are likely to lead to malfunctioning of the cell. Dysfunctional motor nerves are believed to be one of the major underlying mechanisms responsible for the sarcopenia associated with the aging process (523).

There appears to be an increase in the accumulation of cellular damage, both mechanical and metabolic in origin, with increasing age (523). The disrupted cell does not provide an environment that is conducive to successful and efficient muscle repair and regeneration. As a result of this, and other intrinsic factors, there is a decreased capacity for skeletal muscle repair and regeneration with increasing age. The increased accumulation of cellular damage together with the decreased capacity for repair results in skeletal muscle maladapations, which are expressed in the form of age-related skeletal muscle sarcopenia.

While aging is an innate process that occurs in every individual, the overtraining syndrome is an example of an extrinsic or self-induced form of maladaptation. The overtraining syndrome will be discussed in the next section.

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1.5.2. OVERTRAINING

1.5.2.1. Introduction

The concept of "adaptation energy" was first described in the General Adaptation Syndrome of Hans Selye (Figure 1.5.2.1.)(443). He observed that disruption of an organism's homeostasis by a stressful stimulus led to an initial, temporary decrease in "adaptation energy" (alarm phase) followed by a phase of adaptation. The duration of the adaptation phase depended on the amount of recovery between consecutive exposures to stressful stimuli. If the recovery was insufficient the organism entered an exhaustion phase. If this phase persisted the organism would ultimately die. Using a hard exercise training session as an example of a stressful stimulus, the alarm phase would be analogous to the temporary decrease in performance that occurs after a hard training session to which the body is unaccustomed. The adaptation phase can be likened to the enhanced performance that occurs after the development of training adaptations. The exhaustion phase can be likened to the impaired performance that occurs after excessive training with insufficient rest and recovery (52;385;443).

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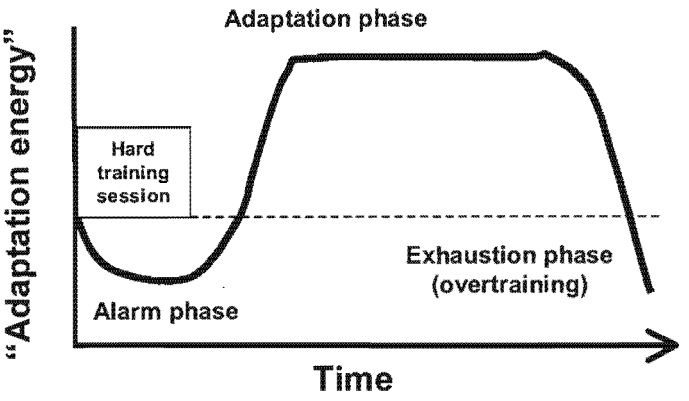

Figure 1.5.2.1. Diagrammatic representation of the General Adaptation Syndrome proposed by Hans Selye (1950).

Based on this theory, coaches and athletes regularly include a series of training sessions that progressively overload the system in order to optimise performance (training periodisation). If the hard training sessions are continued without adequate recovery, the athlete becomes exhausted and is unable to perform adequately (385). If the athlete is able to recover from this exhausted state within a couple days it is termed "overreaching". Overreaching is the first phase of overtraining (52;274;458). It is usually transient and the symptoms of muscular fatigue are easily reversed within a couple of days (up to 14 days), with appropriate rest and recovery (52;274;458;461). Previous studies have shown that if this state of overreaching is maintained for longer than 3 weeks, with intense, prolonged training sessions lasting approximately 3 hours per day, the individual's performance will continue to decrease and the overreaching phase will progress into overtraining (153;274;297)(Figure 1.5.2.2.). Once the athlete

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has reached this state of overtraining, a number of weeks and even months of rest may be required for the athlete to recover (458;461).

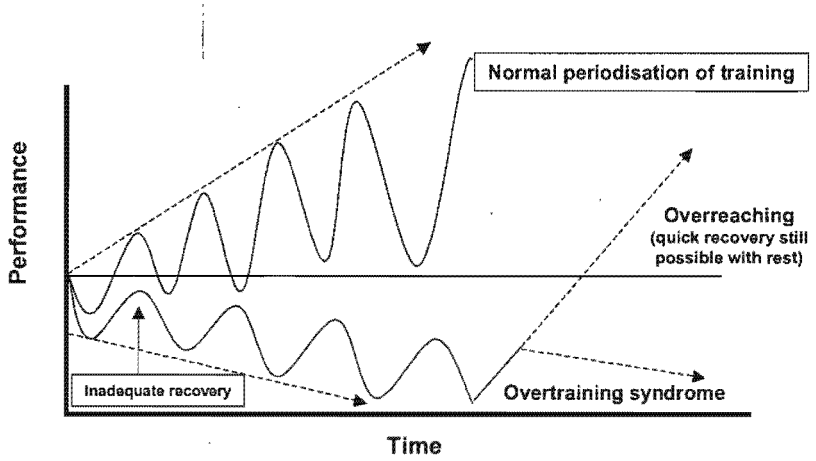

Figure 1.5.2.2. A diagram illustrating how normal periodisation of training with adequate recovery leads to improved performance whereas periodisation of training with inadequate recovery results in overreaching and ultimately overtraining in the athlete. (Adaptation of diagram in Budgett, R. *British Journal of Sports Medicine*, 1998; 32:107-110 (52)).

Many athletes' training programmes are based on the principle idea that the harder one trains the better one performs. This may occur in a previously untrained individual, as in the initial stages of this individual's training programme there is a positive relationship between training load and performance. As the training program progresses and the individual's fitness increases the relationship between increased training load and performance tends to plateau and large increases in training load elicit small improvements in performance. Often the individual interprets this as a sign of weakness and poor fitness, and instead of resting the individual trains even harder, forcing the body into a state of overreaching which progressively becomes a state of overtraining (385).

1.5.2.2. Prevalence of overtraining

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While there is very little published information on the prevalence of overreaching and overtraining in endurance athletes, a study by Morgan et al. (1987)(359) reported that 65% of elite runners experienced staleness (overtraining) at some point during their running careers (359). Anecdotal evidence suggests that there is a high prevalence of overtraining in almost all endurance sports, particularly endurance running, swimming, cycling, speed skating and rowing (385). Overtraining is not unique to elite athletes as there is anecdotal evidence suggesting that overtraining is common in sub-elite and recreational endurance runners, particularly if the athlete is highly driven to meet self-imposed performance expectations (375).

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1.5.2.3. Risk factors associated with overtraining

Increased training: volume versus intensity

An increased training load can be achieved by either increasing one's training volume or intensity. While some studies have shown training intensity to be the major contributing factor in the development of overtraining in athletes (49;93;200;201;257;295;298;385). Although no study has established a cause and effect relationship between training volume and risk of overtraining, most of the overuse syndromes have been associated with high training volumes (385). The fact that many athletes and coaches equate large training volumes with improved performance, together with the numerous anecdotal accounts of elite athletes performing large training volumes (375;385), possibly tips the scales towards increased training volume being the major contributing factor in the development of overtraining in athletes.

Monotony

It is believed that prolonged, intense, high volume training that lacks day-to-day variation in terms of both activity and rest is one of the factors responsible for the onset of overtraining symptoms (137;297;385). Proponents of this theory believe that training programmes with a high strain index (the product of load and monotony) lead to psychological stress in the athlete and this in turn results in physiological stress and consequent decreased performance (137;138). It has been shown in horses, that monotony of training at high training loads was as critical to the development of overtraining as the total training load (49). It is also believed that by doing the same intense training every day continual, excessive strain will be placed on particular parts of the musculoskeletal system, so increasing the chance of injury to these parts (138).

Recovery

It has been suggested that inadequate recovery is the most important factor contributing to the development of overtraining (153;385;458). Regular days of active rest consisting of low-intensity exercise or days of inactivity, interspersed throughout the training programme, are essential as they allow the body time to recover and adapt to the present stimulus (153). There is substantial evidence to show that continuous hard training without rest (active or inactive) will lead to the development of overreaching and overtraining (49;93;137;138;153;385). Furthermore the fact that the majority of athletes adopt various tapering techniques (allowing the body adequate time to recover from hard training by reducing training volume by as much as 90%

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prior to competition) indirectly supports the idea that adequate recovery is essential for optimal performance and the prevention of overtraining (212;385;448).

Non-training related stress factors

The athlete's dietary, social, economical, educational and occupational status are all factors, which if not optimal, could contribute to the development of overtraining in the athlete (51;138;297). Travelling across time zones to participate in competitions might also contribute to the risk of developing overtraining (297).

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1.5.2.4. Signs and symptoms associated with overtraining

The overtraining syndrome is associated with multiple signs and symptoms and Fry et al. (1991) published a comprehensive list of the major symptoms previously reported in the literature (Table 1.5.2.1) (153). Just as adaptation to training varies according to the individual so the presentation of signs and symptoms associated with overtraining and the degree to which they are presented varies with each individual athlete. A factor, which is common to all forms of overtraining, even with varying symptoms, is a decrement in sport specific performance (52;153;385;458).

1.5.2.5. Physiological response to overtraining

While the signs and symptoms associated with overtraining are extensively described, the physiological response to overtraining is variable and equivocal. Overtraining, per se, is an ill-defined state with no definitive

diagnostic physiological markers. Due to ethical considerations and the difficulties associated with inducing a true state of overtraining in athletes in a laboratory setting, most of the published overtraining data are based on findings from studies in which overreaching, and not overtraining, was induced (297;385). Furthermore, it is very difficult to distinguish whether the reported physiological effects of overtraining are as a result of the prolonged, hard training prior to the onset of overtraining or as a result of the overtraining syndrome per se.

Autonomic imbalance

It has been suggested that prolonged overtraining results in an imbalance of the autonomic nervous system resulting in either a sympathetic or parasympathetic-type overtraining syndrome (227;296;297;347). Even though there is little evidence to substantiate the autonomic imbalance theory, it is thought that overtrained power athletes, such as sprinters and power lifters, present with sympathetic type signs and symptoms of overtraining (Table 1.5.2.2.)(296;297). On the other hand, overtrained endurance athletes present with parasympathetic type signs and symptoms of overtraining (Table 1.5.2.2.)(296;297).

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Table 1.5.2.1. The most frequently reported symptoms of overtraining. (Fry et al. (1991), *Sports Medicine*, 12: 32-65)

Physiological performance	Psychological/information processing
▪ Decreased performance	▪ Feelings of depression
▪ Inability to meet previously attained performance standards	▪ General apathy
▪ Prolonged recovery	▪ Decreased self-esteem or worsening feelings of self
▪ Reduced tolerance of training load	▪ Emotional instability
▪ Decreased muscular strength	▪ Difficulty in concentrating at work or during training
▪ Decreased maximum work capacity	▪ Sensitive to environmental or emotional stress
▪ Loss of coordination	▪ Fear of competition
▪ Decreased efficiency and decreased amplitude of movement	▪ Changes in personality
▪ Reappearance of mistakes already corrected	▪ Decreased ability to narrow concentration
▪ Reduced capacity of differentiation and correcting technical faults	▪ Increased internal and external distractibility
▪ Increased difference between lying and standing heart rate	▪ Decreased capacity to deal with large amounts of information
▪ Abnormal T wave pattern in ECG	▪ Gives up when going gets tough
▪ Heart discomfort on slight exertion	**Immunological**
▪ Changes in blood pressure	▪ Increased susceptibility to, and severity of illnesses, colds and allergies
▪ Changes in heart rate at rest, exercise and recovery	▪ Flu-like illnesses
▪ Increased frequency of respiration	▪ Unconfirmed glandular fever
▪ Perfuse respiration	▪ Minor scratches heal slowly
▪ Decreased body fat	▪ Swelling of the lymph glands
▪ Increased oxygen consumption at submaximal workloads	▪ One-day colds
▪ Increased ventilation and heart rate at submaximal workloads	▪ Decreased functional activity of neutrophils
▪ Shift of lactate curve towards the x axis	▪ Decreased total lymphocyte counts
▪ Decreased evening post-workout weight	▪ Reduced response to mitogens
▪ Elevated basal metabolic rate	▪ Increased blood eosinophil count
▪ Chronic fatigue	▪ Decreased proportion of null (non-T, non-B) lymphocytes
▪ Insomnia with and without night sweats	▪ Bacterial infection
▪ Feels thirsty	▪ Reactivation of herpes viral infection
▪ Anorexia nervosa	▪ Significant variation in CD4:CD8 lymphocytes
▪ Loss of appetite	**Biochemical**
▪ Bulimia	▪ Negative nitrogen balance
▪ Amenorrhea or oligomenorrhea	▪ Hypothalamic dysfunction
▪ Headaches	▪ Flat glucose tolerance curves
▪ Nausea	▪ Depressed muscle glycogen concentration
▪ Increased aches and pains	▪ Decreased bone mineral content
▪ Gastrointestinal disturbances	▪ Delayed menarche
▪ Muscle soreness or tenderness	▪ Decreased hemoglobin
▪ Tendonostic complaints	▪ Decreased serum iron
▪ Periosteal complaints	▪ Decreased serum ferritin
▪ Muscle damage	▪ Lowered total iron-binding capacity (TIBC)
▪ Elevated C-reactive proteins	▪ Mineral depletion (Zn, Co, Al, Mn, Se, Cu, etc.)
▪ Rhabdomyolysis	▪ Increased urea concentrations
	▪ Elevated cortisol levels
	▪ Elevated ketosteroids in urine
	▪ Low free testosterone
	▪ Increased serum homone binding globulin
	▪ Decreased ratio of free testosterone to cortisol of more than 30%
	▪ Increased uric acid production

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Table 1.5.2.2. *Signs and symptoms associated with sympathetic and parasympathetic-type overtraining syndromes (Lehmann, M. et al. (1998); Overtraining in Sport, Chapter 2, pg 21; Kreider, R.B., Fry, A.C. and O"Toole, M.L. eds; Human Kinetics).*

Overtraining syndrome	
Sympathetic-type (power athletes)	**Parasympathetic-type (endurance athletes)**
▪ Impaired performance	▪ Impaired performance
▪ Lack of supercompensation	▪ Lack of supercompensation
▪ Restlessness, irritability	▪ Fatigue, depression, apathy
▪ Disturbed sleep	▪ Not sleep disturbed
▪ Weight loss	▪ Constant weight
▪ Increased resting heart rate	▪ Low resting heart rate
▪ Retarded recovery after exercise	▪ Suppressed heart rate-exercise profile
	▪ Suppressed glucose-exercise profile
	▪ Suppressed lactate-exercise profile
	▪ Suppressed neuromuscular excitability
	▪ Suppressed sympathetic intrinsic activity
	▪ Suppressed catecholamine sensitivity
	▪ Altered hypothalamo/pituitary, adrenal/gonadal function.

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It has also been suggested that the sympathetic-type overtraining is an early manifestation of the syndrome, resulting from increased psycho-emotional stress such as too many competitions and/or too many non-training related stress factors (347). According to this theory, the overtraining response then progresses into a parasympathetic-type overtraining syndrome (347).

Neuroendocrine response

It has been suggested that an imbalance in the neuroendocrine system (hypothalamus, hypothalamic-pituitary-adrenal (HPA) axis and hypothalamic-

pituitary-gonadal (HPG) axis) is one of the physiological reponses of the body to overtraining. While low basal catecholamine excretions (296;501) and elevated plasma levels of norepinephrine (200;295;298) have been observed in overtrained athletes, further studies have not confirmed these findings (194;500;502). The low basal catecholamine excretions have been interpreted to reflect a decreased intrinsic activity of the sympathetic nervous system (296;501). It is possible that the increased circulating catecholamines may down- regulate the intrinsic activity of the sympathetic nervous system by negative feedback mechanisms (296;297), however, this theory needs to be investigated further.

Hormonal response

The ratio of free testosterone to cortisol in the plasma should reflect the anabolic/catabolic balance of the tissue. Most studies have, however, not been able to show a convincing increase in basal cortisol levels or a decrease in free testosterone levels (for more details refer to Urhausen and Kindermann, 2002)(501).

Neuromuscular excitability

Neuromuscular excitability is defined as the minimal current pulse required to induce a single contraction of the fibres in the reference muscle. Reduced neuromuscular excitability, an increased amount of current is required to induce a single contraction of the muscle fibres, has been reported in overtrained athletes (297;299). A similar neuromuscular response has also been observed in stressed muscles following prolonged exercise (297). In this respect it is difficult to distinguish between the

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reduced neuromuscular excitability being due to the fatigue-inducing training preceding the onset of overtraining or to the state of overtraining itself.

Adrenocorticotropin hormone (ACTH) and adrenal sensitivity

Following a period of prolonged overtraining, in a group of long distance runners, Barron et al. (1985)(24) observed an 81% elevation in ACTH release in combination with a 25% decrease in adrenal cortisol release, following insulin-induced hypoglycemia (24). Further studies have shown slightly decreased basal and exercise-induced cortisol levels in overreached/overtrained athletes (152;200;201;257;295;298). Lehmann et al. (1998) believes this to be caused by a decreased sensitivity of the adrenal cortex to ACTH (297). This, however, remains to be confirmed.

1.5.2.6. Overtraining hypotheses

In an attempt to explain and understand the underlying pathophysiology of the overtrained state numerous hypotheses have been proposed. These will be discussed briefly.

Branched chain amino acids (BCAA) and tryptophan hypothesis

This hypothesis is based on the premise that prolonged, intense exercise depletes the carbohydrate energy stores resulting in a higher proportion of BCAA and lipids being utilised as a source of fuel (158;458). This results in an increase in serum free fatty acids (FFA) and a decrease in serum BCAA (158;268;458). The FFA competes with tryptophan for binding with albumin in the plasma, the result of this being an

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increase in unbound tryptophan (158;350). Tryptophan also competes with BCAA for entry into the brain via the blood brain barrier. The decreased serum BCAA concentration and the increased free tryptophan will favour the entry of tryptophan into the brain (158). In the brain tryptophan is converted into the neurotransmitter, serotonin (268). An increase in serotonin in certain areas of the brain has been shown to result in mood and behaviour changes similar to those reported in the overtrained athlete (268). A review article by Gastmann and Lehmann (1998) (158), concluded that while there is conclusive evidence from animal studies to support this hypothesis, the evidence from human studies in support of this hypothesis is contradictory (158).

Glutamine and immuno-suppression hypothesis

Glutamine is the most abundant amino acid and skeletal muscle is the major tissue involved in the *de novo* synthesis of glutamine (323;458). Glutamine is important for lymphocyte proliferation and macrophage function (72;323;458). Glutamine and alanine are important precursors of gluconeogenesis and the production of acute phase proteins in the liver (323;458). The glutamine hypothesis is based on the belief that due to the decreased availability of glutamine the immune system of an overtrained athlete is suppressed and as a result these athletes are more prone to upper respiratory tract infections (URTI). While decreased serum glutamine has been observed following prolonged, intense high volume training and in overtrained athletes (324;395;427;515), there is no clear evidence to support the hypothesis that decreased glutamine in overtrained athletes results in immuno-suppression (323;324). Furthermore, while minor changes in the immune profiles of overtrained

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athletes have been observed, the athletes are not clinically immune deficient (154;323). While there is evidence to show a decreased immunosupression with glutamine supplementation, following prolonged exercise (73) there is also evidence that is contrary to this (370;419). There is however, extensive evidence supporting a relationship between prolonged, intense, high volume training and competition and an increased incidence of URTI (72;323). A recent study showed that exhaustive exercise resulted in a prolonged (at least 24 hours) suppression of macrophage ability to present antigens to T-cells (74). This immune suppression together with a combination of all the other minor changes in the immune system that occur as a result of prolonged exhaustive exercise may ultimately compromise the body's resistance to infection and so result in the increased incidence of URTIs in the overtrained athlete (323).

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Glycogen hypothesis

The glycogen hypothesis is based on the fact that with a dramatic increase in training load and intensity, athletes are unable to maintain their caloric intake, in particular carbohydrate intake (449;458). While the decrease in muscle glycogen could be responsible for the fatigue and performance decrements experienced by overtrained athletes (93), it is yet to be established whether or not decreased muscle glycogen initiates the onset of the overtraining syndrome (449;450;461). A study by Snyder et al. (1995) showed that overreaching and potential overtraining was not prevented in cyclists even when their glucose intake and muscle glycogen was maintained (462). To date, no study has established a causal relationship between a reduction in muscle glycogen and either overreaching or overtraining (449).

All the hypotheses discussed previously (BCAA and tryptophan, glutamine and immune suppression and the glycogen hypothesis) are based on the consequences of being in an overtrained state. While the hypotheses explain possible reasons for various signs and symptoms of the overtrained state, they do not account for what might initiate the onset of the overtrained state. The cytokine hypothesis, however, is the most integrative of all the hypotheses (458).

Cytokine hypothesis

The basis for this hypothesis is that daily physical activity results in microtrauma to the musculoskeletal system and joints of the body (458). This microtrauma induces an appropriate inflammatory response, which in turn initiates healing of the traumatised tissue. The end result of this process is regeneration and consequent adaptation of the tissue (458). This scenario will only occur if there is sufficient time for the body to rest, regenerate and adapt to the environment to which it is being subjected. If however, there is insufficient time for complete recovery, benign microtrauma may develop into subclinical and perhaps eventually pathological trauma to the tissue (458). Unresolved and exacerbated micotrauma to the musculoskeletal system and joints induced by prolonged, intense, high volume training and racing, without sufficient recovery is the initiator and perpetuator of the overtrained state (458).

Smith (458) suggests that as a result of the exacerbated microtrauma, the local, acute inflammatory response becomes a systemic, chronic inflammatory response. Cytokines are released from the traumatised tissue, which activate circulating

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monocytes and attract them to the injured area. These infiltrating macrophages produce and release large quantities of proinflammatory cytokines, namely IL-6, IL-1β and TNFα. The proinflammatory cytokines are the messengers that coordinate the response of the body to the excessive training and racing and insufficient rest. This cytokine hypothesis provides a viable explanation for all aspects of the overtraining state (458).

While the majority of the proposed hypotheses explain the response of the body to overtraining or to an increased training load, the cytokine hypothesis appears to be the only hypothesis to suggest a mechanism of action that initiates and perpetrates the overtraining syndrome.

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1.5.2.7. Prevention and treatment of overtraining

It is generally accepted that adequate nutrition and sleep, optimal management of one's non-training related stress factors and a varied training regime incorporating cross training and regular rest days are the means by which overtraining may be prevented. The fact that, even after extensive research in the field of overtraining, there are no reliable tools or methods by which overtraining may be diagnosed (501) further complicates the prevention and treatment of this syndrome.

1.5.2.8. Summary

There is a fine line between training hard enough to elicit the required adaptations and training too hard and subsequently inducing a state of overreaching and overtraining. Increased training volume and intensity, monotonous training

programmes that do not incorporate adequate rest days and non-training related stress are all factors that could increase the athlete's risk of the developing overtraining. While numerous signs and symptoms are associated with the overtrained state, the universal indicator of overreaching and overtraining is a decrement in sport-specific performance. Although further studies are required to substantiate this, the general physiological response to overtraining is a decreased activity of the intrinsic sympathetic nervous system and decreased sensitivity of target organs to hormonal stimuli (297).

Most hypotheses of the pathophysiology of the overtraining syndrome are incomplete because they tend to explain limited aspects of the bodies physiological response to overtraining. The cytokine hypothesis, however, proposes both an initiator (unresolved microtrauma to the musculoskeletal system) and perpetrators (cytokines and the immune response to the unresolved microtrauma) responsible for the onset and progression of the overtraining syndrome (458).

Prolonged, high volume, intense training and competition place the body in a constant state of high stress, both physically and psychologically. If the body were to maintain this state without any rest or recovery it would eventually enter the exhaustion phase of the General Adaptation Syndrome as proposed by Seyle (1950) (443). Persistence of this state would result in irreparable damage to the body. The overtraining syndrome is primarily interpreted as a form of maladaptation by the body to high volume, prolonged, intense physical activity.

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1.6. AIMS AND OBJECTIVES

A review of the literature has established that prolonged, endurance exercise is capable of inducing skeletal muscle damage and temporary impairment of muscle function (as reviewed in section 1.2). In addition to this, exercise-induced muscle damage is a powerful stimulus for skeletal muscle adaptation. An integral part of the repair process, which follows exercise-induced muscle damage, is the remodelling process that ultimately enables the muscle to rebuild and adapt in the appropriate manner to the mechanical and metabolic demands place on it (see section 1.4).

Skeletal muscle possesses an extraordinary capacity for repair (12;75;174;191) and adaptation (20;136;165;207). However, there are limits to the capacity of skeletal muscle to repair and adapt to damaging stimuli. The skeletal muscle dysfunction associated with age-related sarcopenia (section 1.5.1) and the overtraining syndrome (section 1.5.2) provides evidence of this.

As mentioned in the introduction (section 1.1), anecdotal evidence regarding the training beliefs and practises of endurance athletes suggests that these athletes rarely allow their muscles sufficient time to recover completely before exposing them to further bouts of damage inducing exercise. In the case of a soft tissue injury, it is generally believed that exposure of the injury to a damaging stimulus in the early phase of the recovery process is harmful to the tissue (243;378). Contrary to this, previous studies have observed that an additional bout of damage inducing exercise on already damaged muscles does not exacerbate the extent of the muscle or impair

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the repair process (118;376;378). However, it must be noted that in these studies only indirect measures of skeletal muscle damage (i.e. maximal isometric force output, range of motion, limb circumference, muscle soreness and plasma creatine kinase) were used to assess the extent of muscle damage (118;376;378). None of the studies investigated the impact of repeated bouts of exercise on the extent of disruption of the skeletal muscle at the structural and ultra structural level. Furthermore, while the acute effects of repeated bouts of exercise-induced muscle damage may prove to be benign, the chronic effect of damaging already damaged muscles is not known (378). Since endurance athletes are constantly exposing their already damaged muscles to further damaging bouts of exercise, this is a potential area whereby the finite regenerative capacity of skeletal muscle may be exceeded.

In a recent study it was observed that daily bouts of damage-inducing exercise significantly increased satellite cell activation and proliferation in the soleus muscle of rats (456). As discussed in the review of the literature, satellite cells are responsible for the repair and regeneration of skeletal muscle following injury (see section 1.3.4 for more details). Although the proliferative capacity of satellite cells decreases with increasing age, the finite proliferative capacity of satellite cells is not believed to be a limiting factor during normal aging of an individual (see section 1.3.8.2 for details). However, if excessive regenerative demands are placed on the satellite cells in the form of repeated bouts of damaging exercise, particularly on already damaged muscles, as is the case with most endurance athletes, it is possible that the finite proliferative capacity of the satellite cells may become exhausted (175;191;492).

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Transplantation of a whole muscle graft from a young donor into an aged host was not as successful as the transplantation of a muscle graft from an aged donor into a young host (68;523). Research into the regenerative capacity of aging skeletal muscle highlights the importance of the state of the cellular environment in the success or efficacy of the regeneration process. Although purely speculative at this stage, it may be argued that the cellular environment of skeletal muscle that is in a constant state of disrepair as a result of the already damaged muscles being exposed to further damaging bouts of exercise, is not conducive to effective and successful regeneration.

Most competitive endurance athletes train 6-7 days a week and often resume training within 24–48 hours after completing a race, despite previous studies reporting that there are still signs of regeneration present in the muscle up to 12 weeks after a 42.2 km road race (517). As a result it is quite possible that the damage induced by their prolonged endurance training is incompletely or ineffectively repaired. Furthermore, many endurance athletes train and race in this manner for several years incurring repeated muscle damage that requires repair and regeneration, and as such it is possible that the finite capacity of skeletal muscle to repair and adapt to damaging stimuli might be exhausted. This raises the question: what are the long-term, cumulative effects of repeated bouts of endurance training and racing in combination with potentially ineffective or incomplete repair of the muscle, on the morphology and function of skeletal muscle?

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While the results of several case studies and a case series study suggest an association between long-term high volume endurance training and racing and chronic skeletal muscle damage and dysfunction (108;426;452;468), no study has systematically examined the impact of repeated bouts of muscle damage and repair, over a number of years, on the structure and function of skeletal muscle. Accordingly, the aim of this thesis was to investigate the impact of repeated bouts of muscle damage and repair, over a number of years, on the structure and function of the skeletal muscle of a group of endurance athletes with acquired training intolerance.

These athletes are a suitable model for examining the potential detrimental effects of long-term high volume endurance training and racing as they have a history of high volume endurance training and racing. In addition to this, we postulate that these athletes have exceeded the finite capacity of their skeletal muscle to repair and adapt to exercise-induced muscle damaging stimuli, as their skeletal muscle is no longer able to adapt to training stimuli to which they were previously accustomed. The athletes are characterised by maladaptations such as a precipitous decline in running performance that is not due solely to aging and skeletal muscle symptoms including excessive delayed onset muscle soreness after exercise, stiffness, tenderness and skeletal muscle cramps, occur whenever they attempt any form of endurance training.

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The original objectives of this thesis were to compare:

1. The functional characteristics of the skeletal muscle of athletes with acquired training intolerance to asymptomatic control athletes.
2. The structural and ultra structural characteristics of the skeletal muscle of athletes with acquired training intolerance to asymptomatic control athletes.

The final objectives of this thesis, which evolved out of the findings of the above-mentioned objectives, were to:

3. Compare the regenerative history, as measured by the telomere length of the skeletal muscle cells of the vastus lateralis muscle, of athletes with acquired training intolerance to asymptomatic control athletes.
4. Investigate possible reasons for the pathologically shortened telomeres of three of the athletes with acquired training intolerance.

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CHAPTER 2

DESCRIPTIVE, PHYSIOLOGICAL AND TRAINING CHARACTERISTICS OF ENDURANCE ATHLETES WITH ACQUIRED TRAINING INTOLERANCE

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2.1. INTRODUCTION

Several case studies have alluded to the possible association between chronic skeletal muscle damage and exercise-associated chronic fatigue and long-term high volume endurance training and racing (108). There are, however, to our knowledge, no studies that have systematically investigated the impact of repeated bouts of damage inducing endurance exercise, over a number of years, on skeletal muscle function.

Furthermore, there are no studies that have compared the endurance training volume of athletes with acquired training intolerance and exercise-associated chronic fatigue with similarly aged asymptomatic endurance athletes. The aim, therefore, of this chapter is to compare the descriptive, physiological and training characteristics of athletes with acquired training intolerance to those of control athletes that have been matched for age and years of high volume endurance training.

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2.2. METHODS

2.2.1. Subjects

This study was approved by the Research and Ethics committee of the Faculty of Health Sciences at the University of Cape Town. Eighteen endurance-trained athletes (13 runners, 2 cyclists, a rower, a squash player and a triathlete) were recruited for the study. These subjects had an extensive history of high volume endurance training and competition, decreased physical performance during

exercise and a clinical profile that was dominated by skeletal muscle symptoms including excessive delayed onset muscle soreness after exercise, stiffness, tenderness and skeletal muscle cramps. Subjects were recruited from the Sports Medicine Clinic at the Sports Science Institute of South Africa. The most commonly reported "symptom" that led these athletes to seek medical advice was acquired training intolerance, whereby the athletes were unable to tolerate endurance training loads to which they were previously accustomed. Any attempt to do so would result in chronic, excessive exercise related fatigue. A sports physician examined the athletes prior to their participation in the trial to exclude Chronic Fatigue Syndrome (199) or a related diseased state as possible diagnoses for the athletes' symptoms. In addition to this all dystrophies or classic myopathies were excluded as possible causes for the reported symptoms of these athletes. Furthermore, the physical symptoms of these athletes were not consistent with classical overtraining, as in most cases the athletes had significantly reduced their training loads and extensive rest periods did not alleviate the symptoms, as would be expected with overtraining (458).

Seventeen control athletes (16 runners and a triathlete), without symptoms or signs of chronic fatigue or exercise intolerance, were recruited from local running clubs (CON group). The control athletes were matched with the acquired training intolerant (ATI) athletes for age and years of endurance training, prior to the onset of the above-mentioned symptoms. A sports physician also examined these athletes prior to their participation in the trial.

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2.2.2. Endurance training history

Informed consent was received from all the athletes prior to testing (Appendix 1). On the day of testing, all subjects completed a retrospective training (Appendix 2) and racing questionnaire (Appendix 3). The endurance runners and the triathletes recalled their endurance training history from the age at which they started running greater than 40 km per week. The endurance cyclists recalled their endurance training history from age at which they cycled greater than 150 km per week. The squash player and the rower recalled their endurance training history from the age at which they spent greater than 4 hours per week training for their specific sport. All subjects recalled the age at which they started high volume endurance training (HVETage), the number of years of high volume endurance training (HVETyrs) and their endurance training volume (days.wk^{-1}, hours.wk^{-1} and km.wk^{-1}) during this period. ATI athletes recalled their endurance training volume both prior to (pre) and following (post) the onset of symptoms of acquired training intolerance.

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2.2.3. Descriptive and physiological analyses

Percent body fat was assessed as the sum of four skinfolds (biceps, triceps, subscapular, suprailliac) using the procedure described by Durnin and Womersley (1974)(115). Stature and body mass were also measured. Maximal oxygen uptake was determined after an incremental treadmill test to exhaustion as previously described (445)(see Appendix xi for details of the method). The maximal voluntary isometric force output of the right knee extensor muscles of each subject was measured using a Kin-Com isokinetic dynamometer (Chattanooga Group Inc., USA) as described previously (469)(see Appendix xii for details of the method).

Additional information regarding the medical and dietary history, psychological profile (Beck Inventory) and previous running injuries, overtraining episodes and biomechanical problems of the individual ATI athletes have been presented and discussed in the MD thesis of Alan St Clair Gibson (466).

2.3. STATISTICS

Descriptive statistics are expressed as mean ± S.D. A Levene's test for homogeneity of variance indicated a significant difference in the variance between the ATI and CON groups for the number of days (days.wk^{-1}) and hours (hours.wk^{-1}) per week of endurance training. Consequently, a Mann-Whitney test for non-parametric data was used to detect any significant difference between the two groups for the number of days (days.wk^{-1}) and hours (hours.wk^{-1}) per week of endurance training. An independent t-test was used to determine if there were any significant differences ($P < 0.05$) in the remainder of the descriptive, physiological and endurance training history data of the ATI and CON subjects. A dependent t-test was used to determine differences between the pre and post endurance training volumes of the ATI athletes.

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2.4. RESULTS

The individual symptoms of fatigue and muscle soreness and the physical and emotional incidences that preceded and/or precipitated the onset of this condition in these athletes are varied and complex. In light of this, we felt it best to present the

data in tabular form. In this way each individual's descriptive, physiological, training, morphological and molecular characteristics may be followed throughout the thesis. Table 2.1. and 2.2. show the descriptive and physiological characteristics of the individual ATI and CON athletes respectively. The means, standard deviations and minimum and maximum values for the descriptive and physiological characteristics of both groups are summarised in Table 2.3.

As shown in Table 2.1., the number of years with ATI symptoms, recorded retrospectively by each ATI athlete, varied quite considerably within the group. Five ATI athletes reported being symptomatic for 1 year, two ATI athletes reported being symptomatic for 2 years, two ATI athletes reported being symptomatic for 3 years, three ATI athletes reported being symptomatic for 4 years, two ATI athletes reported being symptomatic for 7 years, three ATI athletes reported being symptomatic for 8 years and one ATI athlete reported being symptomatic for 10 years. On average, the ATI athlete was symptomatic for 4 ± 3 years.

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Table 2.1. Descriptive and physiological characteristics of the athletes with acquired training intolerance (ATI).

Subject	Gender	Years with ATI	Age (yrs)	Stature (cm)	Body mass (kg)	% Body fat	VO_{2max} ($ml.kg^{-1}.min^{-1}$)	MVC (N)
ATI 1	M	4	27	177.6	76.7	13.0	56.2	496
ATI 2	F	8	36	168.0	54.2	19.9	59.7	321
ATI 3	F	7	44	161.0	54.5	31.3	38.8	346
ATI 4	M	3	46	166.2	76.0	26.5	42.2	469
ATI 5	M	1	39	177.0	79.1	18.1	59.2	555
ATI 6	M	1	52	187.0	85.0	27.2	47.4	624
ATI 7	M	8	48	179.5	103.3	30.7	31.1	603
ATI 8	M	4	48	167.0	81.0	21.5	48.3	629
ATI 9	M	3	42	176.0	73.0	13.3	55.9	577
ATI 10	M	1	34	184.0	80.5	20.7	55.6	818
ATI 11	F	1	32	153.0	51.0	25.6	49.5	370
ATI 12	M	10	43	185.0	92.0	19.2	39.4	640
ATI 13	F	2	57	165.0	54.5	24.3	52.9	270
ATI 14	M	2	43	177.0	79.5	26.1	44.3	643
ATI 15	M	7	33	184.0	110.0	24.2	47.2	843
ATI 16	M	4	43	171.0	66.5	16.6	47.1	739
ATI 17	M	8	49	185.0	88.5	19.7	55.6	588
ATI 18	M	1	32	172.0	67.5	14.6	68.1	740

MVC = maximal voluntary contraction.

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Table 2.2. Descriptive and physiological characteristics of the control (CON) athletes.

Subject	Gender	Age (yrs)	Stature (cm)	Body mass (kg)	% Body fat	VO_{2max} ($ml.kg^{-1}.min^{-1}$)	MVC (N)
CON 1	F	50	160.0	52	24.4	45.7	504
CON 2	F	31	165.0	62	23.2	52.8	372
CON 3	M	56	176.5	75	18.3	61.0	448
CON 4	F	35	175.5	77	27.1	41.6	519
CON 5	M	27	186.0	85	9.2	73.1	731
CON 6	M	44	175.5	76	20.7	57.6	702
CON 7	M	35	176.0	74	13.8	56.7	607
CON 8	M	34	159.8	67	21.3	58.5	483
CON 9	F	25	163.7	57	26.6	49.3	469
CON 10	F	27	165.8	58	22.3	56.2	456
CON 11	M	48	176.0	73	15.0	56.9	616
CON 12	M	49	179.5	76	21.0	54.4	520
CON 13	M	55	190.0	91	23.3	41.9	571
CON 14	M	45	169.6	78	18.0	45.2	681
CON 15	M	24	171.0	71	9.1	73.1	775
CON 16	F	53	164.5	49	25.0	54.3	377
CON 17	M	32	165.0	73	8.4	70.3	825

MVC = maximal voluntary contraction.

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Table 2.3. Summary of the descriptive and physiological characteristics of the acquired training intolerant (ATI) and control (CON) athletes.

	ATI (n = 18)	CON (n = 17)	P value
Gender	14 (M) 4 (F)	10 (M) 7 (F)	0.3
Age (yrs)	42 ± 8 (27 ~ 57)	39 ± 11 (24 ~ 56)	0.5
Stature (cm)	174.2 ± 9.5 (153.0 ~ 187.0)	171.7 ± 8.7 (159.8 ~ 190.0)	0.4
Body mass (kg)	76 ± 17 (51 ~ 110)	70 ± 11 (49 ~ 91)	0.2
% Body fat	21.8 ± 5.5 (13.0 ~ 31.3)	19.3 ± 6.0 (8.4 ~ 27.1)	0.2
$VO_{2\ max}$ ($ml\ O_2.kg^{-1}.min^{-1}$)	49.9 ± 9.0 (31.1 ~ 68.1)	55.8 ± 9.7 (41.9 ~ 73.1)	0.07
MVC (N)	571 ± 166 (270 ~ 843)	568 ± 136 (372 ~ 825)	1.0

With the exception of gender, all values are means ± SD. The minimum and maximum and n values are indicated in parenthesis. MVC = maximal voluntary contraction

There was no significant difference between the two groups for gender. The ATI and CON athletes were well matched for age, stature, body mass and % body fat. There was no significant difference in the peak oxygen consumption ($VO_{2\ max}$) and maximal voluntary contraction (MVC) between the ATI and CON athletes (Table 2.3.).

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Table 2.4. and 2.5. show the endurance training history of the individual ATI and CON athletes. In the case of the ATI athletes, the training volume both before (Pre) and after (Post) the onset of the ATI symptoms is shown (Table 2.4.). The endurance training history for both the ATI and CON athletes is summarised in Table 2.6.
The CON and ATI athletes were well matched for the age at which they started high volume endurance training (P = 0.92) and the number of years of high volume endurance training (P = 0.70), as there was no significant difference between the two groups for these variables (Table 2.6). Despite this, however, prior to the onset of symptoms, the ATI athletes trained a significantly greater number of days (ATI 6 ± 1 vs CON 5 ± 1 days.wk^{-1}; P = 0.004), kilometres (ATI 83 ± 32 vs CON 50 ± 25 km.wk^{-1}; P = 0.001) and hours (ATI 9 ± 5 vs CON 5 ± 2 hours.wk^{-1}; P = 0.004) per week than the CON athletes (Table 2.6).

Compared to their training volume prior to the onset of symptoms, the ATI athletes significantly reduced the number of days (ATI pre 6 ± 1 vs ATI post 4 ± 3 days.wk^{-1}; P = 0.002), kilometres (ATI pre 87 ± 29 vs ATI post 32 ± 34 km.wk^{-1}; P = 0.001) and hours (ATI pre 9 ± 5 vs ATI post 3 ± 3 hours.wk^{-1}; P = 0.0003) per week of endurance training following the onset of their symptoms (Table 2.6).

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Table 2.4. Endurance training history of ATI athletes before (Pre) and after (Post) the onset of symptoms.

				Training volume					
				days.wk^{-1}		hours.wk^{-1}		km.wk^{-1}	
Subject	**Sport**	**HVETage**	**HVETyrs**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
ATI 1	Triathlete	13	10	5	2	5.3	0	80	0
ATI 2	Runner	18	12	7	5	9.5	4.3	130	50
ATI 3	Squash	21	15	6	0	24	0	NA	NA
ATI 4	Runner	35	10	6	4	7.5	4	90	40
ATI 5	Runner	32	7	6	4	5	4.5	60	50
ATI 6	Runner	44	7	5	5	3.8	4.1	45	45
ATI 7	Runner	40	3	4	0	3.3	0	50	0
ATI 8	Runner	40	4	6	6	6.7	3	80	30
ATI 9	Cyclist	26	8	7	3	17	0	NA	NA
ATI 10	Rower	27	7	7	6	15	8	NA	NA
ATI 11	Runner	20	10	6	5	6.4	3.1	70	25
ATI 12	Runner	28	7	6	1	5	1.7	60	10
ATI 13	Runner	39	18	6	0	5.4	0	65	0
ATI 14	Runner	21	21	5	0	5.8	0	70	0
ATI 15	Runner	18	9	7	6	12	3	160	30
ATI 16	Cyclist	23	20	7	7	8.6	2.3	NA	NA
ATI 17	Runner	27	25	6	6	7.4	8.5	105	100
ATI 18	Runner	15	13	6	6	6.7	6.7	100	100

Triathlete = running data are shown. Squash player = number of days and hours per week of squash training are shown. Cyclists = number of days and hours per week of endurance cycling are shown. Rower = the number of days and hours per week spent rowing are shown.
HVETage - age in years at which the athlete began high volume endurance training.
HVETyrs - number of years the subject has participated in of high volume endurance training.
NA = not applicable

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Table 2.5. Endurance training history of the CON athletes.

				Training volume		
Subject	Sport	HVETage	HVETyrs	days.wk^{-1}	hours.wk^{-1}	km.wk^{-1}
CON 1	Runner	38	14	6	2	15
CON 2	Runner	25	7	4	4	40
CON 3	Runner	47	10	6	5	50
CON 4	Runner	20	15	3	3	22
CON 5	Runner	19	8	4	6	60
CON 6	Runner	33	10	4	3	30
CON 7	Runner	17	13	4	6	60
CON 8	Runner	12	22	7	13	130
CON 9	Runner	18	7	6	5	45
CON 10	Runner	23	4	6	5	50
CON 11	Runner	30	18	4	4.5	45
CON 12	Runner	39	10	4	5	50
CON 13	Runner	33	22	4	4	40
CON 14	Runner	35	10	4	5	50
CON 15	Runner	14	10	6	6	60
CON 16	Runner	33	20	3	4	40
CON 17	Triathlete	18	14	6	6	70

Triathlete = running data are shown. HVETage = age in years at which the athlete began high volume endurance training. HVETyrs = number of years of high volume endurance training.

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Table 2.6. Comparison of the endurance training history between the CON and ATI athletes, before (ATI pre) and after (ATI post) the onset of symptoms.

	CON (n = 17)	ATI pre (n = 18)	ATI post (n = 18)
HVETage (yrs)	27 ± 10 (12 ~ 47)	27 ± 9 (13 ~ 44)	
HVETyrs (yrs)	13 ± 5 (4 ~ 22)	12 ± 6 (3 ~ 25)	
Training (days.wk $^{-1}$)	5 ± 1 (3 ~ 7)	6 ± 1 * (4 ~ 7)	4 ± 3 † (0 ~ 7)
Training (km.wk $^{-1}$)	50 ± 25 (15 ~ 130)	83 ± 32 ** (45 ~ 160)	34 ± 34 †† (0 ~ 100)
Training (hours.wk $^{-1}$)	5 ± 2 # (2 ~ 13)	9 ± 5 *** (3 ~24)	3 ± 3 ††† (0 ~ 9)

All values are means ± SD. The minimum and maximum values are in parenthesis. There was no data for the number of km.wk^{-1} of endurance training for the squash player, rower and 2 cyclists in the ATI group, therefore, only the data of 14 ATI athletes were analysed when determining differences for this variable between the two groups. HVETage = age in years at which the athlete began high volume endurance training. HVETyrs = number of years of high volume endurance training.

* P = 0.002; 5 ± 1 (CON) vs 6 ± 1 (ATIpre) days.wk $^{-1}$
** P = 0.003; 50 ± 25 (CON) vs 83 ± 32 (ATIpre) km.wk $^{-1}$
*** P = 0.02; 5 ± 2 (CON) vs 9 ± 5 (ATIpre) hours.wk $^{-1}$
† P = 0.0003; 6 ± 1 (ATI pre) vs 4 ± 3 (ATI post) days.wk $^{-1}$
†† P = 0.0002; 83 ± 32 (ATI pre) vs 34 ± 34 (ATI post) km.wk $^{-1}$
††† P = 0.001; 9 ± 5 (ATI pre) vs 3 ± 3 (ATI post) hours.wk $^{-1}$
\# P = 0.02; 5 ± 2 (CON) vs 3 ± 3 (ATI post) hours.wk $^{-1}$

The exercise intolerance of the ATI athletes was such that, following the onset of symptoms, the ATI athletes trained significantly fewer number of hours per week than that of the CON athletes (ATIpost 3 ± 3 vs CON 5 ± 2 hours.wk^{-1}; P = 0.02). There was no significant difference in the number of days (ATIpost 4 ± 3 vs CON 5 ± 1days.wk^{-1}; P = 0.11) and kilometres (ATIpost 34 ± 34 vs

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CON 50 ± 25 km.wk^{-1}; P = 0.14) trained per week by the control and ATI athletes, after the onset of symptoms (Table 2.6).

2.5. DISCUSSION

The aim of this chapter was to systematically compare the descriptive, physiological and training characteristics of athletes with acquired training intolerance (ATI) to those of asymptomatic control athletes (CON), who were matched for chronological age and years of endurance training. There was no significant difference in the descriptive characteristics of the ATI and CON athletes as they were well matched for age, stature, body mass and percentage body fat. Despite the ATI athletes' inability to tolerate endurance training loads to which they were previously accustomed and to maintain their expected levels of racing performance, their maximal oxygen uptake and maximal force output was not reduced compared to that of the asymptomatic control athletes (CON).

No conclusions may be drawn regarding the effect of the ATI athletes' present state of health and fitness on their short term, maximal performance capacity, as we do not have any data for these particular performance parameters prior to the onset of symptoms in these athletes. However, previous case studies investigating the physiological characteristics of individual athletes with similar symptoms to those described by the ATI athletes have shown that although the individual's peak oxygen consumption is significantly reduced from previous measurements obtained prior to the onset of the symptoms, the

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value is within the normal range as would be expected of similarly aged and activity matched endurance athletes (426). In addition to this, objective physiological data from individuals diagnosed with chronic fatigue syndrome show very little reduction in muscle strength and peak aerobic power (447).

It is also possible that the VO_{2max} test and the maximal voluntary isometric contraction test, although valid measures of maximal oxygen uptake and strength in a healthy population are not valid measures of the competitive racing performance in the athletes with acquired training intolerance. A submaximal intensity exercise test that challenges the ATI athletes' ability to resist fatigue over a longer period of time may be a more appropriate test to detect functional changes in perfomance. An alternative conclusion is that the skeletal muscle of the ATI athletes is capable of performing short-term, maximal contractions and that the impairment in muscle function only occurs after sustained contractions.

Thirdly, although the ATI and CON athletes were well matched for the age and the number of years of high volume endurance training, prior to the onset of training intolerance the ATI athletes trained a significantly greater number of hours, days and kilometres a week compared to the CON athletes. The ATI athletes, however, significantly reduced their training volume following the onset of training intolerance.

Isolated case studies and a case series suggest that high volume endurance training over a number of years may impact negatively on one's training and

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racing performance. For example, St Clair Gibson et al. (1998), described a 28-year-old international male runner who experienced a sudden decline in running performance and an inability to tolerate high training loads after 7 years of high volume endurance training and racing (468).

Sjöström et al. (1987) presented a case study of a previously well-trained (10000 km.year $^{-1}$), 46-year-old man, who ran a distance of 3529 km in 7 weeks. Following the race the subject's running speed continuously decreased (452).

A recent case study of a 64-year-old long distance runner, with 37 years of running experience, showed an accelerated decrement in the individual's running performance compared to what would be expected for his age. Although a reduced training load may explain this decrement in performance, it is possible that the high volume of training and racing might be responsible for the accelerated decline in running performance (281).

Rowbottom et al. (1998) reported a decrement in performance capacity of a previously elite ultra-endurance male cyclist who developed chronic fatigue (426). The case further supports a possible association between ultra-endurance activity, chronic fatigue and decreased performance capacity. The case series study by Derman et al. (1997) also suggests an association between high volume endurance training over a number of years and acquired training intolerance and impaired racing performance (108).

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In conclusion, despite being unable to tolerate endurance training loads to which they were previously accustomed and to attain expected endurance racing performance, the maximal oxygen consumption and force output of the ATI athletes was not impaired in comparison to asymptomatic control athletes who were matched for age and years of high volume endurance training. Furthermore, while the ATI and CON athletes had a similar number of years of endurance training experience, the volume of endurance training undertaken by the ATI athletes, prior to the onset of symptoms, was significantly greater than that of the asymptomatic control athletes.

Case studies suggest a possible association between a history of high volume endurance training, exercise-associated chronic fatigue and skeletal muscle pathology (468). Furthermore, a study by Kuipers et al. (1989), noted a gradual increase in skeletal muscle degeneration as the endurance training distance of the athletes increased (276). Therefore, as a result of these findings we investigated the structural and ultra structural characteristics of the skeletal muscle of the ATI athletes and the asymptomatic control athletes, in the next study.

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CHAPTER 3

MORPHOLOGICAL CHARACTERISTICS OF THE SKELETAL MUSCLE OF ENDURANCE ATHLETES WITH ACQUIRED TRAINING INTOLERANCE

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3.1. INTRODUCTION

The previous sections have shown that it is well established that prolonged, exhaustive endurance exercise is capable of inducing skeletal muscle damage and temporary impairment of muscle function (reviewed in section 1.2). Although skeletal muscle has a remarkable capacity for repair and adaptation (see section 1.3 and 1.4), research on exercise-induced muscle damage (203;281;283;452;453;468;517) aging (section 1.5.1) and the overtraining syndrome (section 1.5.2) suggest that this capacity may be limited. In the previous chapter, analysis of the physiological data showed that the maximal strength and endurance capacity of the ATI athletes was not impaired compared to that of the control athletes. Analysis of the training history, however, revealed that the ATI athletes trained a significantly greater number of hours, days and kilometres per week compared to the control athletes (Chapter 2).

Case studies of chronically fatigued athletes with impaired exercise performance and exercise training intolerance suggest a possible association between a history of high volume training, exercise-associated chronic fatigue and skeletal muscle pathology (108;452;468). Furthermore, a study by Kuipers et al. (1989) investigated the changes in skeletal muscle ultra structure of previously untrained individuals who undertook an 18-month endurance training programme with the end goal of completing a standard marathon (42.2 km) (276). While no signs of pathology were noted in the original biopsy samples, a gradual increase in the prevalence of ultra

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structural disturbances was noted as the training distance increased. It was also noted that the increased ultra structural disturbances were more likely to be related to the increased training distance, rather than the increased number of races the subjects participated in during the 18-month training period (276). The results of this study suggest that endurance training, particularly with respect to the training distance, is associated with an increased accumulation of skeletal muscle structural and ultra structural disturbances.

Numerous researchers investigating the acute effects of endurance exercise on muscle structure and ultra structure have commented on the fact that "*there is a lack of data regarding the effects of endurance training for longer than 6 months*" (203) and "*that the effects of long term tissue damage and repair and how it impacts on muscle adaptation/maladaptation are unknown*" (517). Researchers have also questioned the "reversibility" of the muscle damage induced by long distance running and how acute and chronic damage effects muscle function in the long term (453). Despite this, however, no one has systematically investigated the impact of repeated bouts of muscle damage and repair over a number of years on the structure and ultra structure of skeletal muscle.

Accordingly, the aim of this study was to compare the presence of structural and ultra structural disruptions in the skeletal muscle of endurance athletes who presented with acquired training intolerance to asymptomatic endurance athletes who were matched for age and years of endurance training.

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3.2. METHODS

3.2.1. Subjects

This study was approved by the Research and Ethics committee of the Faculty of Health Sciences at the University of Cape Town. The same subjects as described in Chapter 2 participated in this study (see Chapter 2.2. for more details).

3.2.2. Light and electron microscopic analyses of the skeletal muscle

All subjects were instructed to refrain from any strenuous or unaccustomed exercise for at least 72 hours prior to testing. A muscle biopsy of the vastus lateralis was obtained from each subject using the percutaneous needle biopsy technique of Bergstrom (30), as modified by Evans et al (123)(detailed methods in Chapter 8: Appendix). A portion of the muscle sample was mounted on a piece of cork with embedding medium (Tissue-Tek, Miles Laboratories Inc. Naperville Illinois, USA), frozen in liquid nitrogen-cooled isopentane, and stored at -20°C for future light microscopic analysis.

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Various histological stains, haemotoxylin and eosin, gomori trichorome, succinate dehydrogenase and NADH-tetrazolium reductase stains (Chapter 8: Appendix), were used to assess the presence of internal nuclei, variation in muscle fibre size, necrosis and inflammation and subsarcolemmal aggregations of mitochondria. All are typical markers of skeletal muscle structural pathology (114).

The remaining portion of the muscle sample was placed in a chilled (4°C) fixative (3% gluteraldehyde in a 0.1M Cacodylate buffer; pH 7.2) until further electron microscopic analysis. After the initial fixation, the tissue samples were postfixed in osmium tetroxide, dehydrated in graded baths of ethyl alcohol and embedded in an epoxy resin (Spurr's). Ultrathin sections of the tissue blocks were cut and subsequently stained with uranly acetate and lead citrate. The sections were mounted on 200 square copper grids and viewed through an electron microscope (detailed methods in Chapter 8: Appendix). The presence of Z disc streaming, focal deletions of myofibrils, atrophic muscle fibres, enlarged mitochondria (mitochondia extends over the length of 2 sarcomeres), subsarcolemmal mitochondria and lipid and glycogen accumulations, all of which are markers of skeletal muscle ultra structural pathology (114), were then determined. Due to technical difficulties no electron microscopic data was obtained for ATI 16 and CON 3.

The fibre type composition of the muscle sections was determined by staining for myosin ATPase activity at pH 9.4, 4.6 and 4.3 (44) (Chapter 8: Appendix). Based on the myosin ATPase activity, the muscle fibres were classified as type I, type IIA, IIB and IIC fibres, according to the nomenclature of Brooke et al. (1970)(44). Images of the stained sections were captured using an interactive graphic digitiser (Carl Zeiss "Axioplan" 2 MOT). Approximately 10 to 15 images were captured and ± 400 fibres were counted per subject. Each fibre type (I, IIA, IIB and IIC) was expressed as a percentage of the total number of fibres counted.

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A pathologist, blinded to the identity and physical condition of the subject, analysed the muscle samples. A score of "0" indicated an apparently normal muscle sample from a physically active, healthy individual, with no visible signs of pathology. A "1+" to a "3+" score indicated an increasingly greater presence of the various markers of skeletal muscle structural (internal nuclei, fibre size variations, necrosis and inflammation and subsrcolemmal aggregations of mitochondria) and ultra structural pathology (Z disc streaming, focal deletions of myofibrils, atrophic fibres, enlarged mitochondria, subsarcolemmal aggregations of mitochondria and aggregations of lipid and glycogen deposits within the muscle fibres). Actual examples of the varying degrees ("0" to "3+") of skeletal muscle structural and ultra structural pathology are shown in Figure 3.1. and 3.2., respectively. A cumulative total pathology score was calculated for each individual as well as for the group.

To ensure repeatability the same pathologist analysed and scored the light microscopy samples a total of three times until good agreement, defined by Kappa statistics (97;362;451), was obtained between successive analyses.

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3.3. STATISTICS

Descriptive statistics are expressed as mean ± S.D. A Levene's test for homogeneity of variances indicated a significant difference in the variance between the ATI and CON groups for the total structural (light microscopy) and ultra structural (electron microscopy) pathology scores. Consequently, a Mann-Whitney test for non-parametric data was used to detect any significant

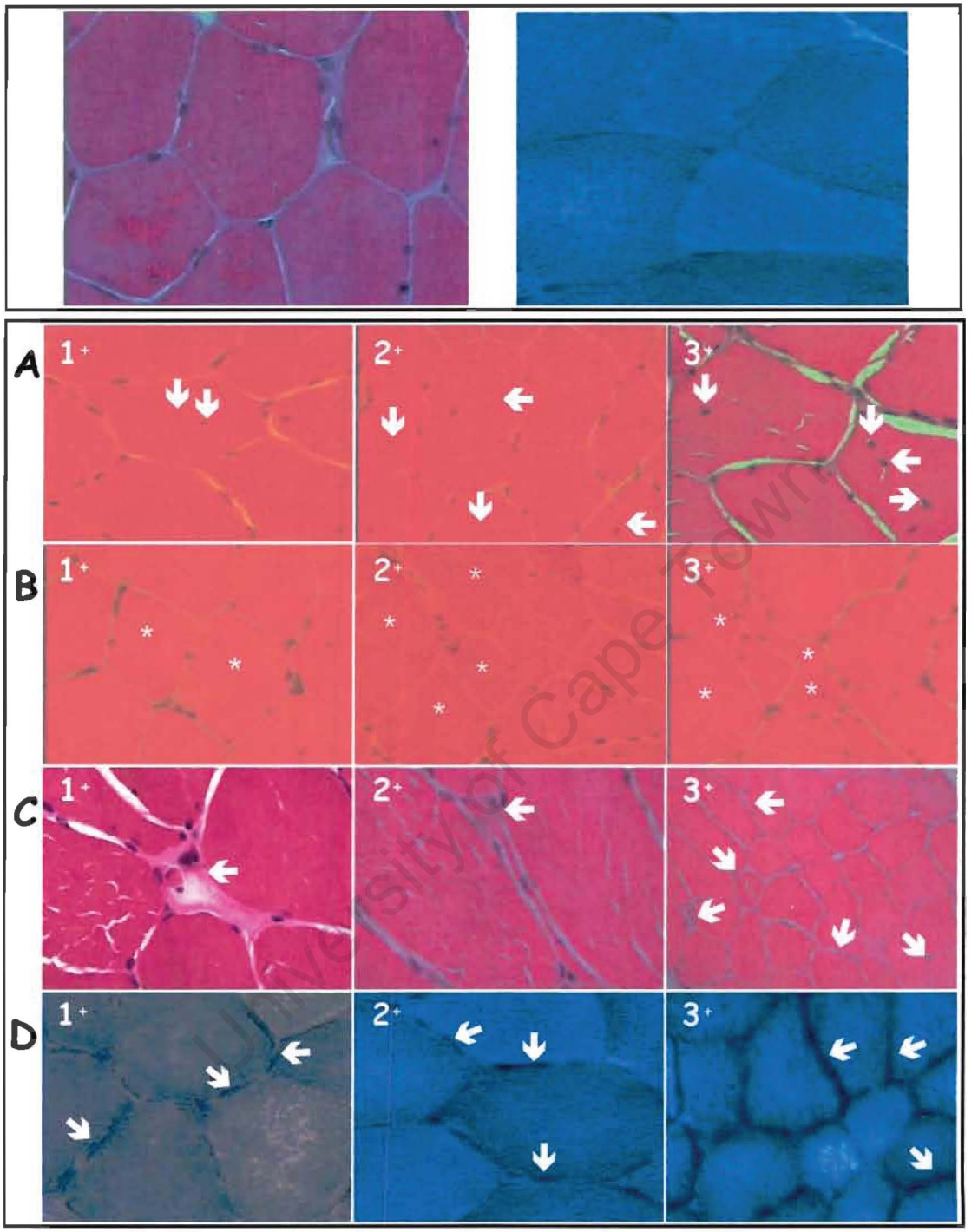

Figure 3.1. Top panel: Light micrographs of cross sections of skeletal muscle from control subjects stained with H&E (left) and NADH (right). Bottom panel: Light micrographs illustrating the degrees (1+, 2+, 3+) of internal nuclei (A), fibre size variation (B), necrosis/inflammation (C) and aggregations of subsarcolemmal mitochondria (D). A, B and C are H&E stains, while D is an NADH stain. Arrows indicate internal nuclei (A), necrotic fibres (C) and subsarcolemmal aggregations of mitochondria (D). Asterisks indicate varying sized muscle fibres (B). For visual purposes particular areas of interest have been enlarged in some of the cases.

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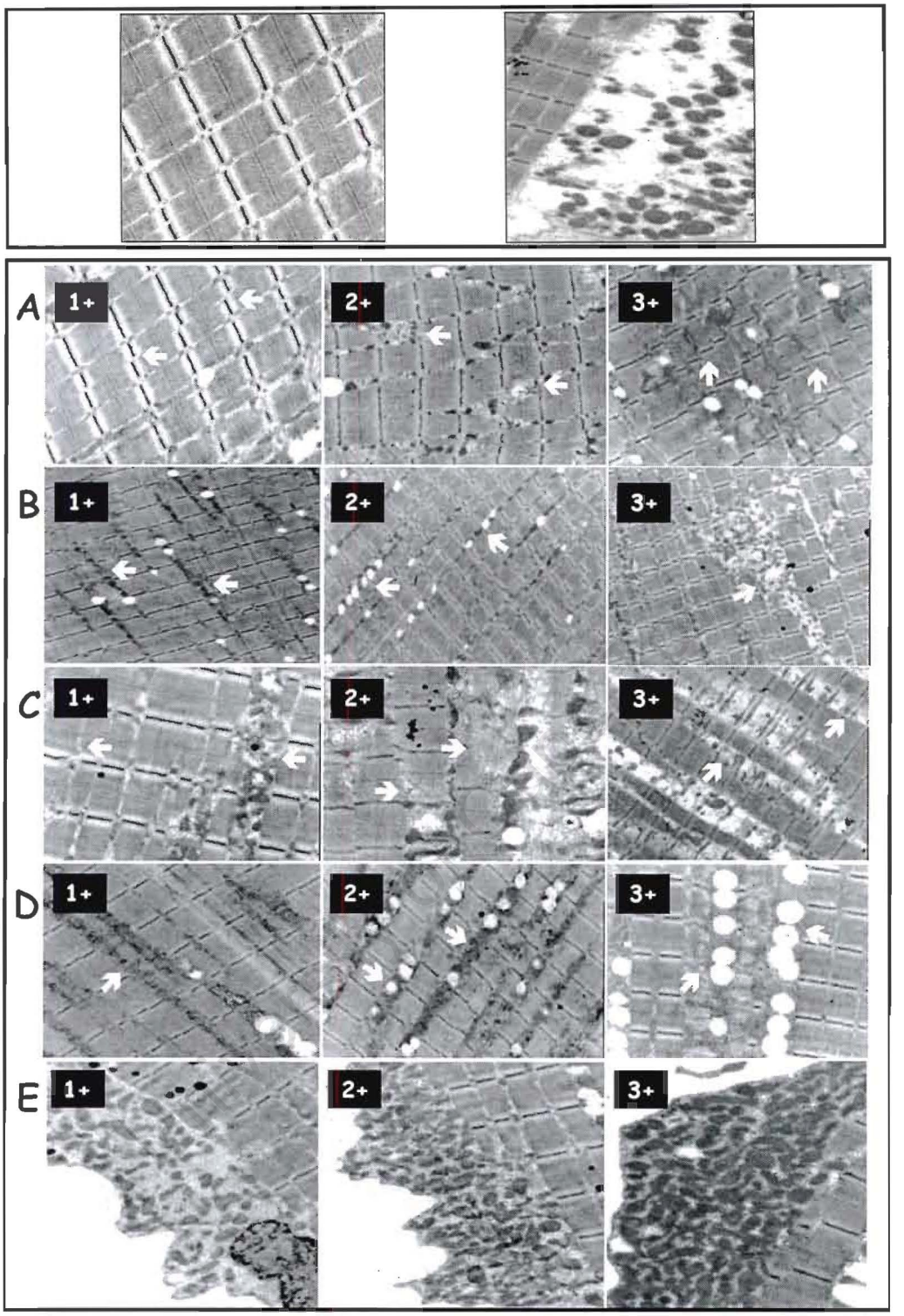

Figure 3.2. Top panel: Electron micrographs of longitudinal sections of skeletal muscle from control athletes showing well aligned Z discs (left) and normal aggregations of subsarcolemmal mitochondria (right). Bottom panel: Electron micrographs illustrating the degrees (1+, 2+, 3+) of Z disc streaming (A), focal deletions of myofibrils (B), atrophic fibres (C), lipid and glycogen accumulations (D) and aggregation of subsarcolemmal (SSL) mitochondria (E). Arrows indicate particular areas of interest.

differences in the total structural and ultra structural pathology scores. An independent t-test was used to detect significant differences in the percentage of type I muscle fibres present in the muscle samples of the ATI and CON subjects.

A Kappa statistic (κ)(97;362;451) was calculated to ascertain the level of agreement between the successive scores of the pathologist. An acceptable Kappa score (κ = 0.8) was obtained between the second and third observation for all the categories, with the exception of the category that rated the degree of accumulation of subsarcolemmal mitochondria. This category had a Kappa score of (κ = 0.22), indicating only a fair agreement between repeated observations.

The structural pathology scores, determined on the third occasion by the pathologist, and the ultra structural pathology scores, were divided into two groups with a score of "0" indicating no pathology and scores of "1+", "2+" or "3+" indicating the presence of pathology. A Fisher's Exact two-tailed Chi squared test was used to determine if there was a significant difference ($P < 0.05$) in the proportion of athletes from the ATI and CON groups who presented with symptomatic skeletal muscle structural and ultra structural pathology. The odds ratio (OR) within a 95% confidence interval was also calculated in order to determine whether either group was more likely to present with skeletal muscle structural and ultra structural pathology.

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3.4. RESULTS

3.4.1. Light microscopy

The degree to which the various markers of skeletal muscle structural disturbances, as determined by light microscopy, are present in the muscle samples of the individual ATI and CON athletes is shown in Table 3.1. and 3.2. , respectively. No specific changes indicative of a myopathy, neuropathy or muscular dystrophy were noted in the skeletal muscle of any of the athletes.

The ATI athletes, who had as their predominant sporting activity a non-weight bearing and low impact sport (ATI 16, cyclist; ATI 15, canoeist; ATI 10, rower), appeared to have a reduced presence of skeletal muscle structural disturbances. This, however, is not the case with ATI 9, as despite being an endurance cyclist, his muscle showed a high prevalence of size variation (3+), necrosis and inflammation (3+) and subsarcolemmal aggregations of mitochondria (3+). In addition to the high volume endurance cycling undertaken by this individual, he also followed a rigorous resistance-training programme. As resistance training is known to induce muscle damage, the resistance-training programme may either be an additional or alternative factor (472) implicated in the increased presence of skeletal muscle structural disturbances in this individual.

The percentage of type I fibres in the athletes of the ATI group ranged from 19% (ATI 12) to 91% (ATI 2)(Table 3.1). The percentage of type I fibres in the

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CON athletes ranged from 37% (CON 14) to 75% (CON 17)(Table 3.2). There was, however, no significant difference in the percentage type I fibres between the ATI and CON group (56 ± 18, ATI vs 53 ±11, CON; P = 0.56), as determined by an independent t-test.

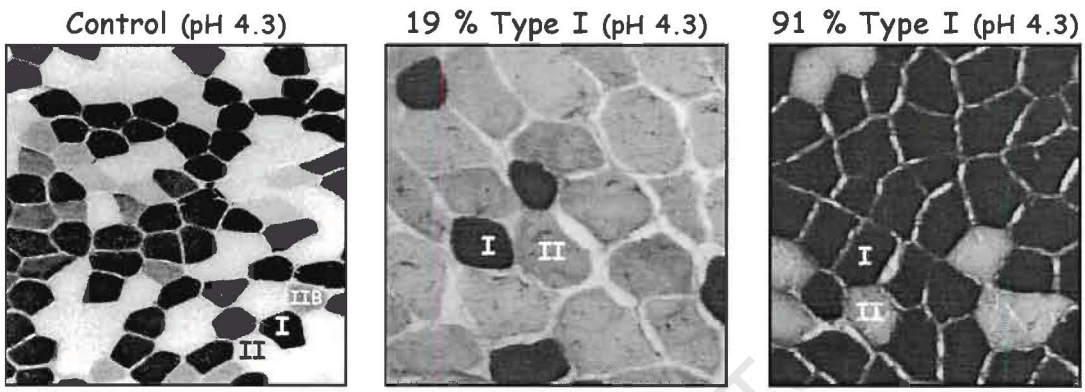

Figure 3.3. Light micrographs of skeletal muscle showing differences in the percentage distribution of type I and II muscle fibres (Myosin ATPase stain, pH 4.3; original magnification X 40).

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Table 3.1. Structural disruptions, as determined by light microscopy, in the skeletal muscle of athletes with acquired training intolerance (ATI).

Subject	% Type I fibres	Size variation	Internal nuclei	Necrosis/ inflammation	SSL Mitochondria	Total score
ATI 1	53	1+	2+	0	2+	**5**
ATI 2	91	3+	3+	0	3+	**9**
ATI 3	56	2+	1+	0	2+	**5**
ATI 4	66	1+	0	0	2+	**3**
ATI 5	45	0	0	0	2+	**2**
ATI 6	52	2+	0	0	2+	**4**
ATI 7	41	3+	2+	2+	1+	**8**
ATI 8	42	3+	2+	0	1+	**6**
ATI 9	59	3+	1+	3+	3+	**10**
ATI 10	83	1+	0	0	2+	**3**
ATI 11	76	1+	0	0	3+	**4**
ATI 12	19	2+	0	0	1+	**3**
ATI 13	67	2+	1+	0	2+	**5**
ATI 14	39	2+	1+	1+	1+	**5**
ATI 15	53	1+	1+	0	2+	**4**
ATI 16	43	1+	0	0	2+	**3**
ATI17	70	0	0	0	2+	**2**
ATI 18	54	0	0	0	1+	**1**
Average	**56**	**2**	**1**	**0.3**	**2**	**5**

SSL = subsarcolemmal aggregations of mitochondria.

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Table 3.2. Structural disruptions, as determined by light microscopy, in the skeletal muscle of control (CON) athletes.

Subject	% Type I fibres	Size variation	Internal nuclei	Necrosis/ inflammation	SSL Mitochondria	Total score
CON 1	71	1+	0	0	3+	**4**
CON 2	64	1+	0	0	2+	**3**
CON 3	50	0	0	0	2+	**2**
CON 4	41	0	0	0	1+	**1**
CON 5	49	1+	0	0	2+	**3**
CON 6	49	1+	0	0	2+	**3**
CON 7	60	0	0	0	2+	**2**
CON 8	42	0	0	0	2+	**2**
CON 9	58	1+	1+	0	2+	**4**
CON 10	59	0	0	0	2+	**2**
CON 11	55	0	0	0	2+	**2**
CON 12	40	2+	1+	1+	1+	**5**
CON 13	54	0	0	0	2+	**2**
CON 14	37	0	0	0	1+	**1**
CON 15	48	0	0	0	3+	**3**
CON 16	52	0	0	0	3+	**3**
CON 17	75	0	0	0	3+	**3**
Average	**53**	**0.4**	**0.1**	**0.06**	**2**	**3**

SSL = subsarcolemmal aggregations of mitochondria.

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The mean total score for the degree of skeletal muscle structural pathology present in the ATI athletes (5 ± 2) was significantly greater than that of the CON athletes (3 ± 1; P = 0.008).

3.4.1.1. Internal nuclei

The haematoxylin and eosin (H&E) and the gomori-trichrome stains enabled the pathologist to determine the presence of internal nuclei in the muscle fibres of each sample. The presence of internal nuclei may be indicative of various neuromuscular disorders as well as muscle degeneration/regeneration (32;114;159;532). The presence of internal nuclei within more than 3% of the muscle fibres is generally considered to be abnormal (114).

According to the Fishers Exact two-tailed Chi squared test a significantly greater proportion of ATI athletes compared to CON athletes, presented with internal nuclei (9 ATI vs 2 CON; P = 0.03; Figure 3.4). Furthermore the ATI athletes were significantly more likely to present with internal nuclei (OR = 7.5; 95% CI: 1.3 to 42.8) compared to the control athletes.

3.4.1.2. Fibre size variation

The H&E and gomori-trichrome stains also enabled the pathologist to assess the prevalence of fibre size variation in each muscle sample. The myosin ATPase stain and the NADH-tetrazolium reductase stain enabled the pathologist to determine if there was atrophy or hypertrophy of a specific fibre type. In a normal muscle sample the fibres should all be similarly sized (Figure 3.5.B). Increased muscle fibre size or muscle fibre hypertrophy is characteristic of skeletal muscle adaptation to increased workload (refer to Chapter 1.3.7. for further details) (63;507). A decrease in the size of the muscle fibres is known as muscle fibre atrophy. Certain skeletal muscle

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myopathies and dystrophies are known to cause diffuse and random atrophy of the muscle fibres (114), whereas denervation or immobilisation of the muscle normally results in selective atrophy of a particular fibre type or atrophy of a group of closely associated fibres (11;114;387). It is generally believed that the presence of a certain degree of variation in the size of the muscle fibres is abnormal (114).

A significantly greater proportion of ATI athletes presented with fibre size variation (15 ATI vs 6 CON; P = 0.006; Figure 3.5) compared to the CON athletes. Furthermore the ATI athletes were significantly more likely to present with fibre size variation (OR = 9.2; 95% CI: 1.9 to 44.9) compared to the control athletes. The muscle fibre atrophy present in both groups of athletes was random and diffuse and was not limited to a particular fibre type or group of fibres. Although this implies the presence of myopathy, denervation type pathology may be excluded.

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3.4.1.3. Necrosis and inflammation

The H&E and gomori-trichrome stains enabled the pathologist to assess the prevalence of necrosis and inflammation in the biopsy sample of the muscle. While the presence of necrosis and inflammation in the muscle sample may be indicative of certain inflammatory myopathies or muscular dystrophy, it is also indicative of muscle fibres in the process of degeneration/regeneration. As discussed in the review of the literature prolonged, eccentric or unaccustomed exercise is capable of inducing both structural and ultrastructural damage to the muscle fibres (Chapter 1.2). Severely damaged

muscle fibres become necrotic and are eventually phagocytosed by infiltrating neutrophils and macrophages.

There was no significant difference in the proportion of ATI and CON athletes who presented with necrosis/inflammation (P = 0.6; OR = 3.2; 95% CI: 0.3 to 34.3; Figure 3.6).

3.4.1.4. Subsarcolemmal mitochondria

The NADH tetrazolium reductase stain and the succinate dehydrogenase (SDH) stain were used to assess the degree of subsarcolemmal mitochondrial aggregation as well as the presence of any abnormalities in the distribution of the mitochondria within the cytoplasm (114). In a normal muscle fibre the mitochondria are evenly dispersed throughout the cytoplasm. In the case of various myopathies, dystrophies or metabolic disturbances abnormalities in the number, size, structure and distribution of mitochondria within the cytoplasm may occur (114). Alternatively it is well established that endurance exercise training induces changes in the number, size, structure and distribution of mitochondria in the cytoplasm (2;39;77;206;242;256;489;496;540).

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There was no significant difference in the proportion of ATI or CON athletes who presented with subsarcolemmal aggregations of mitochondria (P = 0.7; OR = 0.6; 95% CI: 0.1 to 2.8; Figure 3.7).

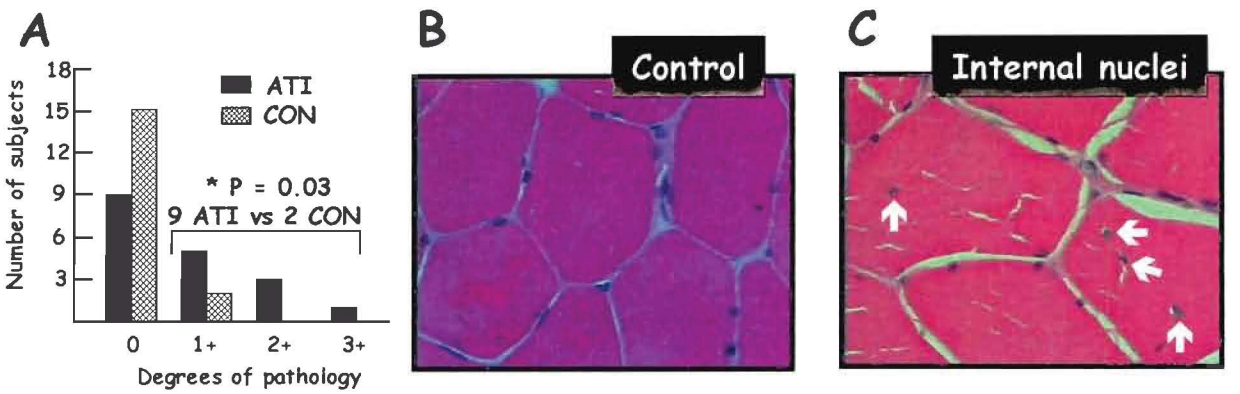

Figure 3.4. Internal nuclei. Panel A - graphic representation of the proportion of ATI (solid bars) and CON (hatched bars) athletes presenting with varying degrees of internal nuclei. Panel B - cross section of a muscle sample from the vastus lateralis muscle of a control athlete. Panel C - cross section of a muscle sample from the vastus lateralis of an ATI athlete showing extensive internal nuclei as indicated by the white arrows (Haemotoxylin and eosin stain; original magnification, x40).

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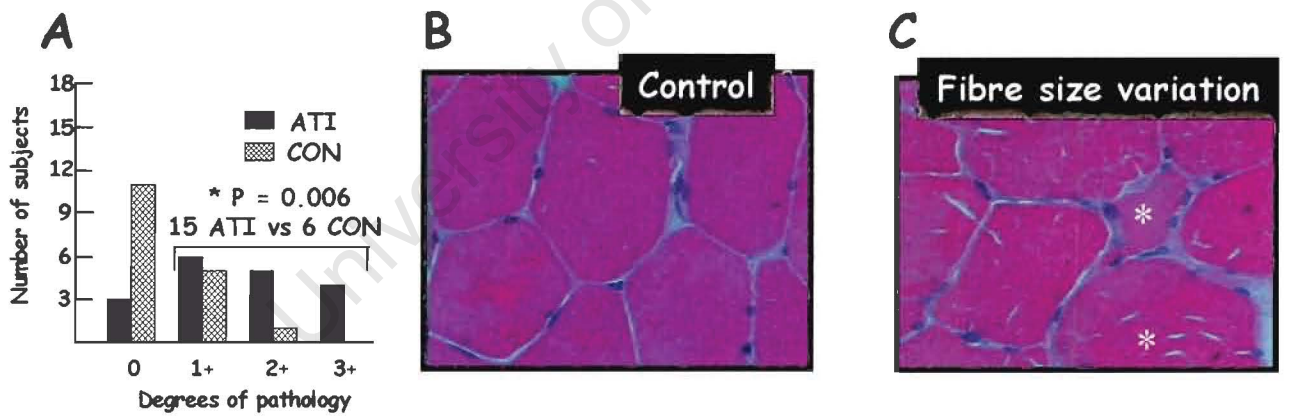

Figure 3.5. Variation in muscle fibre size. Panel A - graphic representation of the proportion of ATI (solid bars) and CON (hatched bars) athletes presenting with varying degrees of variation in the size of their muscle fibres. Panel B - cross section of a muscle sample from the vastus lateralis muscle of a control athlete. Panel C - cross section of a muscle sample from the vastus lateralis of an ATI athlete showing a variation in the size of the muscle fibres (Haemotoxylin and eosin stain; original magnification, x40).

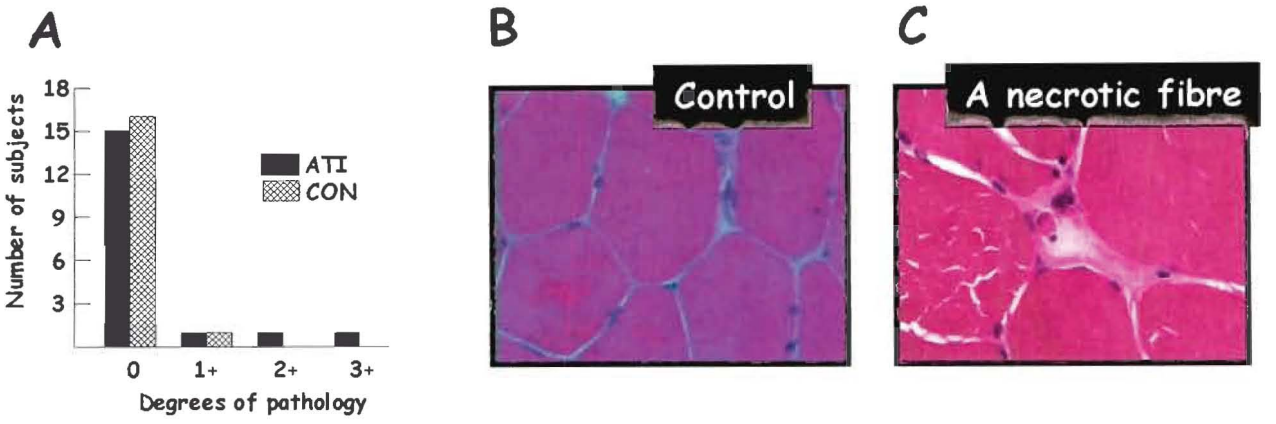

Figure 3.6. Necrosis and inflammation. Panel A - graphic representation of the proportion of ATI (solid bars) and CON (hatched bars) athletes presenting with signs of necrosis and inflammation. Panel B - cross section of a muscle sample from the vastus lateralis muscle of a control athlete. Panel C - cross section of a muscle sample from the vastus lateralis of an ATI athlete showing necrosis and inflammation in the muscle fibres (Haemotoxylin and eosin stain; original magnification, x400).

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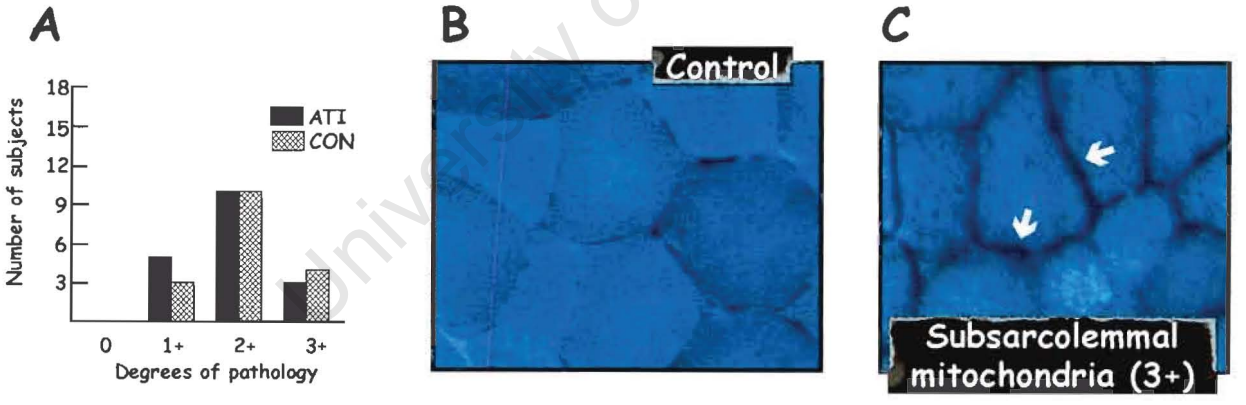

Figure 3.7. Subsarcolemmal aggregations of mitochondria. Panel A - graphic representation of the proportion of ATI (solid bars) and CON (hatched bars) athletes presenting with subsarcolemmal aggregations of mitochondria. Panel B - cross section of a muscle sample from the vastus lateralis muscle of a control athlete. Panel C - cross section of a muscle sample from the vastus lateralis of an ATI athlete showing necrosis and inflammation in the muscle fibres (NADH – tetrazolium reductase stain; original magnification, x400).

3.4.2. Electron microscopy

The various degrees of skeletal muscle ultra structural changes, as determined by electron microscopy, present in the ATI and CON athletes are shown in Table 3.3. and 3.4., respectively. The total score for ultra structural pathology in the ATI group (8 ± 5) was significantly greater than that of the CON group (4 ± 3; P = 0.01).

Table 3.3. Ultra structural changes, as determined by electron microscopy, in the skeletal muscle of the ATI athletes.

Subjects	Z disc	Focal	Atrophic	Mitochondria	SSL	Lipid	Glycogen	Total score
ATI 1	0	3+	0	E	2+	3+	3+	**11**
ATI 2	3+	3+	2+	E	2+	0	0	**10**
ATI 3	3+	3+	2+	N	2+	3+	3+	**16**
ATI 4	0	3+	0	E	1+	3+	3+	**10**
ATI 5	0	3+	3+	E	3+	0	3+	**12**
ATI 6	1+	3+	0	E	1+	3+	3+	**11**
ATI 7	0	1+	0	N	1+	3+	3+	**8**
ATI 8	3+	3+	0	N	1+	3+	3+	**13**
ATI 9	3+	3+	0	N	3+	3+	3+	**15**
ATI 10	1+	3+	0	E	3+	3+	3+	**13**
ATI 11	0	0	0	N	2+	0	0	**2**
ATI 12	0	2+	1+	N	0	0	2+	**5**
ATI 13	0	1+	1+	E	1+	0	0	**3**
ATI 14	0	1+	1+	E	0	0	0	**2**
ATI 15	0	1+	2+	N	3+	0	0	**6**
ATI 17	0	0	0	N	0	0	0	**0**
ATI 18	0	1+	2+	E	2+	0	0	**5**
Average	**1**	**2**	**1**		**2**	**1**	**2**	**8**

Z disc = Z disc streaming, Focal = focal deletions of myofibres, Atrophic = atrophic muscle fibres, Mitochondria = size of mitochondria, E = enlarged mitochondria, N = normal mitochondria, SSL = subsarcolemmal aggregations of mitochondria, Lipid = lipid deposits and Glycogen = glycogen deposits.

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Table 3.4. Ultra structural changes, as determined by electron microscopy, in the skeletal muscle of the CON athletes.

Subjects	Z disc	Focal	Atrophic	Mitochondria	SSL	Lipid	Glycogen	Total score
CON 1	0	3+	1+	E	3+	0	2+	**9**
CON 2	0	0	1+	N	0	0	0	**1**
CON 4	0	3+	1+	E	2+	0	0	**6**
CON 5	0	2+	2+	E	0	0	0	**4**
CON 6	0	2+	2+	E	1+	0	0	**5**
CON 7	0	1+	0	E	3+	0	0	**4**
CON 8	0	0	0	N	2+	0	0	**2**
CON 9	0	0	0	N	1+	0	0	**1**
CON 10	0	3+	1+	E	3+	0	0	**7**
CON 11	0	2+	1+	E	3+	0	0	**6**
CON 12	0	0	0	N	1+	0	0	**1**
CON 13	0	0	0	N	1+	0	0	**1**
CON 14	0	3+	0	E	3+	0	0	**6**
CON 15	0	1+	1+	N	1+	0	0	**3**
CON 16	0	3+	1+	E	3+	0	0	**7**
CON 17	0	0	0	N	2+	0	0	**2**
Average	**0**	**1**	**1**		**2**	**0**	**0.1**	**4**

Z disc = Z disc streaming, Focal = focal deletions of myofibres, Atrophic = atrophic muscle fibres, Mitochondria = size of mitochondria, E = enlarged mitochondria, N = normal mitochondria, SSL = subsarcolemmal aggregations of mitochondria, Lipid = lipid deposits and Glycogen = glycogen deposits.

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3.4.2.1. Z disc streaming

Although a certain degree of Z disc streaming may be present in normal muscle (352;472), extensive Z disc streaming may be indicative of either exercise-induced ultra structural muscle damage as well as non-specific myopathies (see section 1.2.3.1).

A significantly greater proportion of ATI athletes presented with Z disc streaming compared to the CON athletes (6 ATI vs 0 CON; OR = 17.2; 95% CI: 0.9 to 334.3; P = 0.02; Figure 3.8).

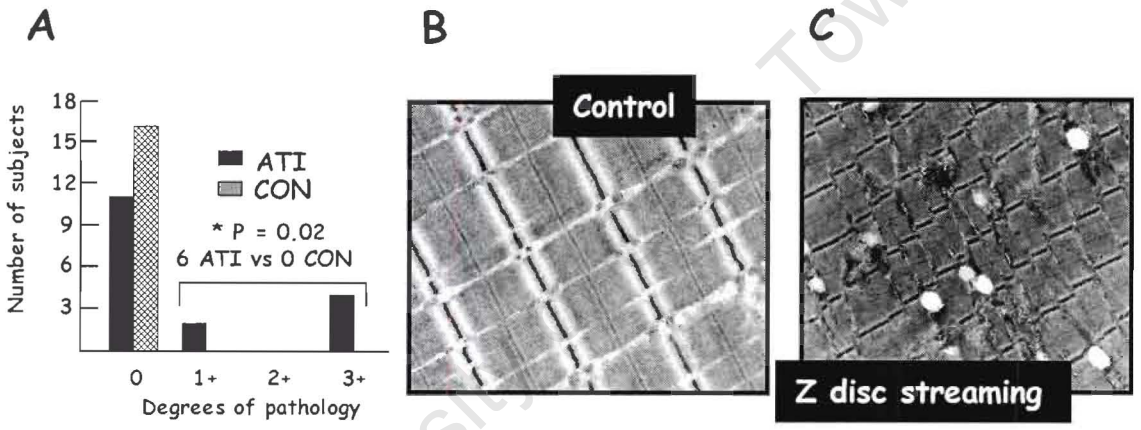

Figure 3.8. Z disc streaming. Panel A - graphic representation of the proportion of ATI (solid bars) and CON (hatched bars) athletes presenting with Z disc streaming. Panel B - longitudinal electron micrograph section of a muscle sample from the vastus lateralis muscle of a control athlete. Panel C - longitudinal electron micrograph section of a muscle sample from the vastus lateralis of an ATI athlete showing extensive Z disc streaming (original magnification, x4500).

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3.4.2.2. *Focal deletions of myofibrils*

While focal deletions in isolated fibres is a normal occurrence in healthy muscle, extensive loss of myofibrils in several muscle fibres is considered abnormal.

There was no significant difference between the ATI and CON groups for the proportion of athletes who presented with focal deletions of myofibrils (16 ATI vs 10 CON; OR = 4.8; 95% CI: 0.8 to 28.6; P = 0.11; Figure 3.9).

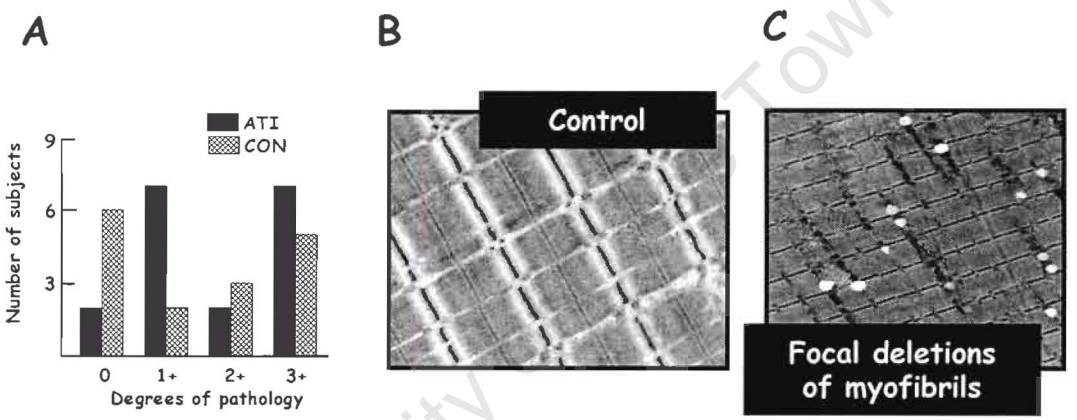

Figure 3.9. Focal deletions of myofibrils. Panel A - graphic representation of the proportion of ATI (solid bars) and CON (hatched bars) athletes presenting with focal deletions of myofibrils. Panel B - longitudinal electron micrograph section of a muscle sample from the vastus lateralis muscle of a control athlete. Panel C - longitudinal electron micrograph section of a muscle sample from the vastus lateralis of an ATI athlete showing extensive focal deletions of myofibrils (original magnification, x4500).

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3.4.2.3. Atrophic fibres

There was no significant difference between the ATI and CON groups for the proportion of athletes who presented with atrophic muscle fibres (8 ATI vs 9 CON; OR = 0.6; 95% CI: 0.2 to 2.4; P = 0.7; Figure 3.10).

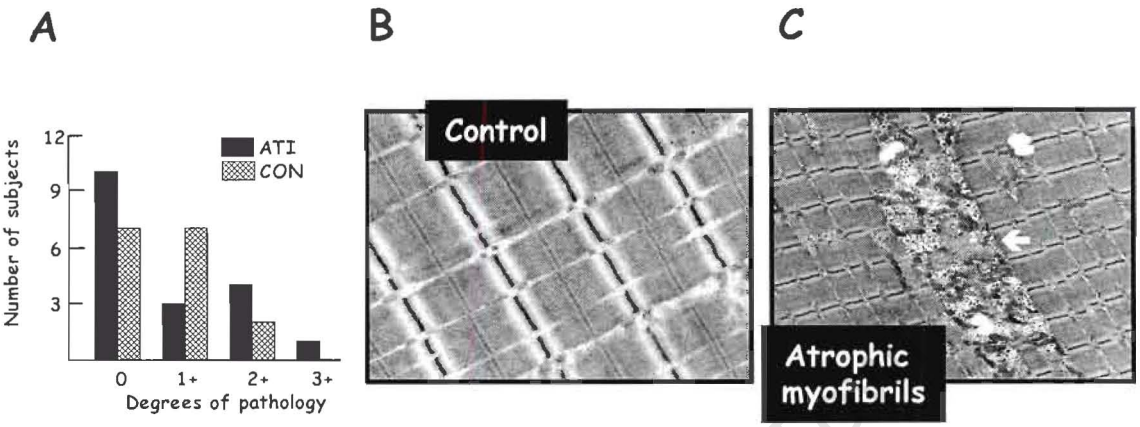

Figure 3.10. Atrophic myofibrils. Panel A - graphic representation of the proportion of ATI (solid bars) and CON (hatched bars) athletes presenting with atrophic myofibrils. Panel B – longitudinal electron micrograph section of a muscle sample from the vastus lateralis muscle of a control athlete. Panel C - longitudinal electron micrograph section of a muscle sample from the vastus lateralis of an ATI athlete showing atrophic myofibrils (original magnification, x4500).

3.4.2.4. Mitochondrial abnormalities

While the presence of enlarged mitochondria may be indicative of certain pathologies (114), it is also a well-described characteristic of endurance training (2;206).

There was no significant difference in the proportion of ATI or control athletes who presented with enlarged mitochondria (P = 0.74; OR = 1.3; 95% CI: 0.3 to 5.0; Figure 3.11) or subsarcolemmal aggregations of mitochondria (P = 1.0; OR = 0.8; 95% CI: 0.2 to 4.3; Figure 3.12).

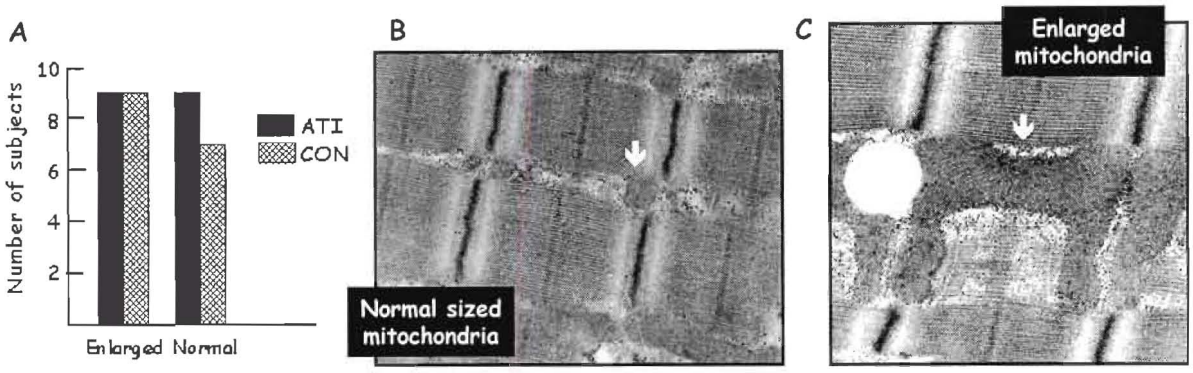

Figure 3.11. Enlarged mitochondria. Panel A - graphic representation of the proportion of ATI (solid bars) and CON (hatched bars) athletes presenting with enlarged mitochondria. Panel B – longitudinal electron micrograph section of a muscle sample from the vastus lateralis muscle of a control athlete. Panel C - longitudinal electron micrograph section of a muscle sample from the vastus lateralis of an ATI athlete showing enlarged mitochondria (original magnification, x4500).

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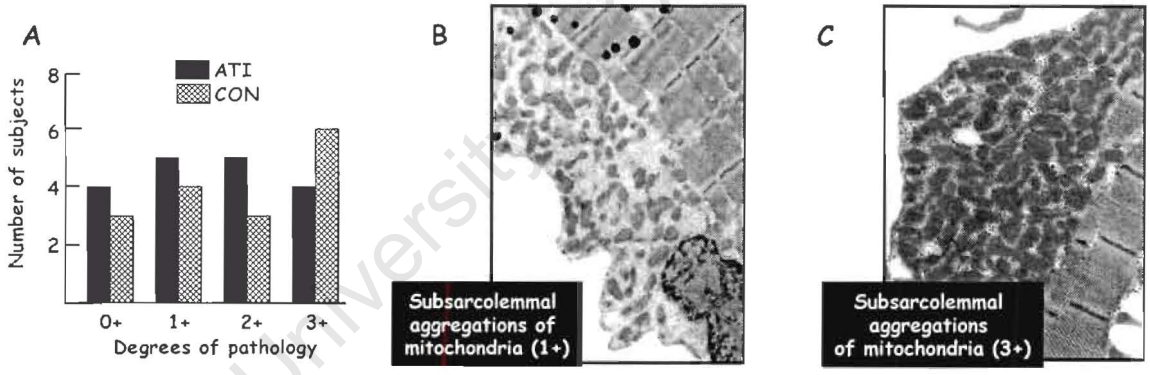

Figure 3.12. Subsarcolemmal aggregations of mitochondria. Panel A - graphic representation of the proportion of ATI (solid bars) and CON (hatched bars) athletes presenting with subsarcolemmal aggregations of mitochondria. Panel B – longitudinal electron micrograph section of a muscle sample from the vastus lateralis muscle of a control athlete. Panel C - longitudinal electron micrograph section of a muscle sample from the vastus lateralis of an ATI athlete showing extensive subsarcolemmal aggregations of mitochondria (original magnification, x10 000).

3.4.2.5. Accumulation of lipid and glycogen droplets

A significantly increased proportion of ATI athletes presented with lipid (8 ATI vs 0 CON; OR = 35.0; 95% CI: 1.8 to 670.2; Figure 3.13) and glycogen (10 ATI vs 1 CON; OR = 20.0; 95% CI: 2.2 to 185.0; Figure 3.14) deposits in the skeletal muscle compared to the CON group (P = 0.001). While an increase in lipid and glycogen deposits, particularly surrounding the mitochondria, is a well described adaptation to endurance training (19;173;272;289), there was extensive accumulation of intramuscular glycogen and lipid in the muscle of the ATI athletes.

3.5. DISCUSSION

The aim of this chapter was to determine whether or not the acquired training intolerance of the ATI athletes was associated with an increased presence of chronic skeletal muscle structural and ultra structural pathology. While there were no signs of a specific myopathy or dystrophy, a significantly greater proportion of ATI athletes presented with internal nuclei, fibre size variation, Z disc streaming and lipid and glycogen deposits, compared to the CON athletes. All of these characteristics reflect a state of either muscle damage or repair or a general alteration in the homeostasis of the muscle cell.

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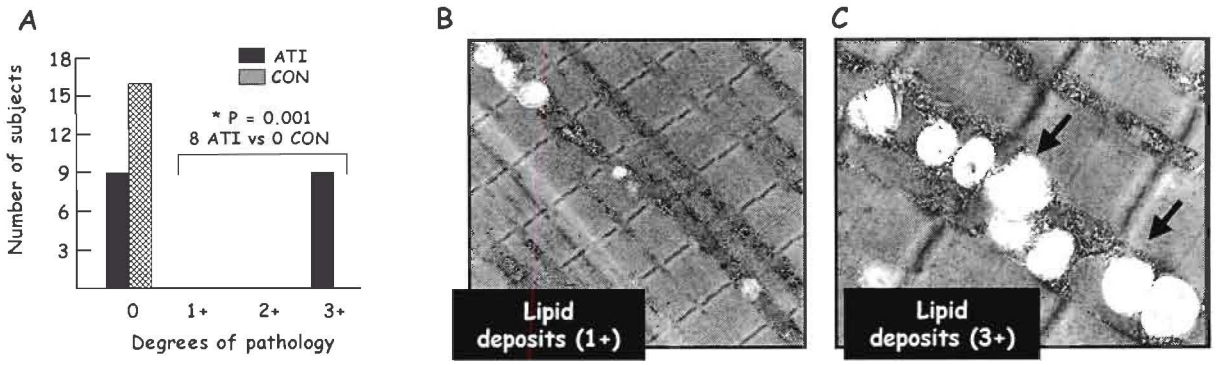

Figure 3.13. Lipid deposits. Panel A - graphic representation of the proportion of ATI (solid bars) and CON (hatched bars) athletes presenting with deposits of lipid droplets. Panel B – longitudinal electron micrograph section of a muscle sample from the vastus lateralis muscle of a control athlete. Panel C - longitudinal electron micrograph section of a muscle sample from the vastus lateralis of an ATI athlete showing extensive accumulation of lipid droplets (original magnification, x10 000).

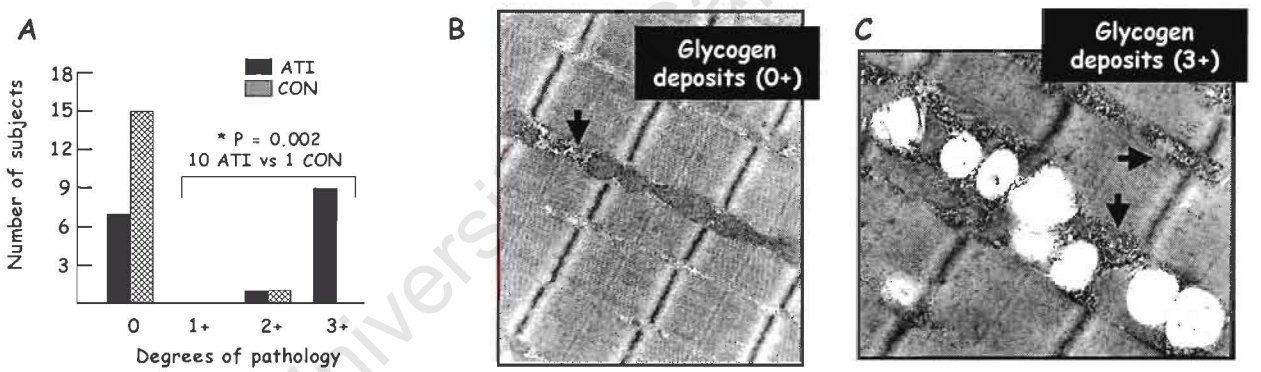

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Figure 3.14. Glycogen deposits. Panel A - graphic representation of the proportion of ATI (solid bars) and CON (hatched bars) athletes presenting with glycogen deposits. Panel B – longitudinal electron micrograph section of a muscle sample from the vastus lateralis muscle of a control athlete. Panel C - longitudinal electron micrograph section of a muscle sample from the vastus lateralis of an ATI athlete showing extensive deposits of glycogen (original magnification, x10 000).

Myonuclei are usually located around the periphery of the muscle cell. The presence of internal nuclei is usually indicative of skeletal muscle degeneration/regeneration. A number of studies have shown an increased presence of internal nuclei to be both an acute (197;203;275;452;517) and chronic (276;453) consequence of prolonged, exhaustive endurance exercise. The increased prevalence of internal nuclei in the ATI muscle samples is most likely a reflection of the perpetual state of damage and repair the muscle has been subjected to as a result of the many years of prolonged, exhaustive endurance training and racing.

Although a certain degree of variation in the size of the muscle fibres is expected, the muscle fibres from a normal, healthy muscle sample are usually of a similar size. Variations in muscle fibre size may be indicative of physiological (hypertrophy) or pathological (atrophy) changes occurring in the muscle (114). In response to increased activity muscle fibres increase in size (hypertrophy or hyperplasia)(63;507) and in response to denervation, immobilisation, myopathy and muscular dystrophy, muscle fibres decrease in size (atrophy) (11;114;387).

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A number of studies investigating the effect of prolonged, exhaustive endurance exercise on the skeletal muscle morphology have noted an increased variation in the size of the muscle fibres as both an acute (197;452;517) and chronic (276;453) response to endurance training and racing. The increased prevalence of muscle fibre size variation in the ATI athletes reflected both physiological as well as pathological changes, as there

were signs of muscle fibre hypertrophy as well as atrophy present in the muscle samples.

Only 4 out of all 35 athletes (3 ATI athletes and 1 CON athlete) presented with signs of necrosis and inflammation. In the absence of specific inflammatory myopathies, signs of necrosis and inflammation are normally only present in the acute phase following exercise-induced muscle damage (197;203;265;275;276;452). There are studies that have not shown any evidence of muscle fibre necrosis or inflammation following exercise-induced muscle damage (329;517), even though extensive muscle damage was noted (517).

Electron microscopic analyses revealed that an increased proportion of ATI athletes presented with Z disc streaming compared to the CON athletes. The majority of studies investigating the effect of prolonged exhaustive endurance exercise on the ultra structure of the muscle have reported both acute (197;203;275;517) and chronic (276) alterations to the Z discs. These include Z disc streaming, smearing, broadening, disruption and dissolution. The Z disc appears to be the structure that is most susceptible to exercise-induced muscle damage (5;12;15;147-149;241;281;308;369). While none of the control athletes showed any signs of extensive Z disc streaming, the increased prevalence of Z disc streaming in the muscle of the ATI athletes is perhaps further evidence of the chronic state of exercise-induced muscle damage and disrepair in the skeletal muscle of the acquired training intolerant athletes.

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Although a similar proportion of ATI and CON athletes presented with atrophic muscle fibres, focal deletions of myofibrils and atrophic fibres were present in the muscles of both groups of athletes. Most of the studies investigating the acute effects of prolonged, exhaustive endurance exercise on skeletal muscle ultra structure have noted the increased presence of focal deletions of myofibrils and atrophic fibres (197;203;275;517). In terms of the chronic effects of prolonged, exhaustive endurance exercise on skeletal muscle ultra structure, previous studies have observed an increased presence of atrophic fibres (276;452;453) and focal deletions of myofibrils (203).

Previous studies investigating the structure, function and distribution of mitochondria in athletes with exercise-associated chronic fatigue, have reported enlarged mitochondria with dense matrices containing broad cristae, extensive subsarcolemmal aggregates of these mitochondria and crystalline inclusions within the mitochondria (468). Studies investigating the acute effects of prolonged, exhaustive endurance exercise on mitochondrial structure have noted alterations such as mitochondrial swelling, crystalline inclusions within the mitochondria, loss of mitochondrial matrices and dissolution of the cristae (197;203;275;517). Chronic alterations to the structure of mitochondria as a result of prolonged, exhaustive endurance training include an increase in the volume density of mitochondria (203), pleomorphic mitochondria, increased electron density and paracrystalline inclusions in the mitochondria (166). No such mitochondrial alterations were noted in the muscle samples of the athletes in the present study.

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Endurance training increases the size and distribution of mitochondria within the muscle cell (2;206). Studies have shown a greater increase in the volume density of subsarcolemmal mitochondria compared to interfibrillar mitochondria with endurance training (39;77;203). Both the ATI and CON athletes were experienced endurance athletes. This may explain why there was a similar prevalence of subsarcolemmal mitochondria was noted in both groups.

Both groups also showed a similar presence of enlarged, abnormally shaped mitochondria. There is evidence to suggest that once mitochondrial volume density reaches a certain threshold point the mitochondria join together to form a reticulum or network (2;47). As mentioned earlier, no signs of specific myopathies or dystrophies were noted so in all likelihood the presence of enlarged mitochondria may be a normal adaptive response to endurance training in both groups of athletes. This study was not designed to assess the function of the mitochondria, so it is not possible to reach a conclusion regarding the functional state of the enlarged mitochondria.

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Glycogen and lipid droplets accumulate in normal muscle in varying amounts depending upon the fibre type (121;213;522) and the dietary (170;204;252) and training status (107;207;335) of the individual. Excessive accumulations of glycogen and lipid may also be indicative of a diseased state or a myopathy (114). While the increased accumulation of glycogen and lipid droplets in the ATI muscle may be indicative of a mitochondrial deficiency or non-specific

muscle pathology, it may also reflect the decreased activity levels, and therefore, decreased utilisation of endogenous fuel stores, in these endurance trained athletes.

In conclusion, the significantly increased proportion of ATI athletes who presented with skeletal muscle structural and ultra structural disruptions, compared to the CON athletes, establishes an association between increased skeletal muscle disruptions and acquired training intolerance in endurance athletes with a history of high volume training and racing. Further studies are required to determine the nature of this association and the possible mechanisms involved.

We suggest that although exercise-induced muscle damage is a precursor to muscle adaptation and skeletal muscle has a remarkable ability to repair this damage and to adapt accordingly (reviewed in section 1.4), there may however, be a limit to the regenerative capacity and adaptability of muscle (203;231;274;283;339;453;458;517;523). Further muscle damage beyond this limit may result in irreversible, irreparable chronic muscle damage (467). This chronic muscle damage may underlie both the decreased performance capacity as well as the exercise intolerance experienced by the ATI athletes. However, the extent of any causal relationships between these variables requires further investigation.

The increased degree of disruption in the skeletal muscle of the athletes with acquired training intolerance provides evidence that there is incomplete repair

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in the skeletal muscle of these athletes. The presence of residual muscle damage may be as a result of the athletes not allowing the muscles sufficient time to recover completely from a bout of damage-inducing exercise. An alternative reason for the residual muscle damage may be that the finite regenerative capacity of the satellite cells has been exhausted and the skeletal muscle damage can no longer be repaired. With this in mind, the regenerative history of the skeletal muscle and its associated satellite cells was investigated.

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CHAPTER 4

REGENERATIVE HISTORY OF THE SKELETAL MUSCLE OF ENDURANCE ATHLETES WITH ACQUIRED TRAINING INTOLERANCE

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4.1. INTRODUCTION

Skeletal muscle cells are highly differentiated, post-mitotic cells. As a result of this, muscle cells themselves are unable to repair or replace damaged or necrotic muscle fibres. In the event of damage to the muscle cell, quiescent satellite cells, situated between the sarcolemmal and the basement membrane (336), are activated to proliferate by cell division (100;101;440;441;456). A portion of the proliferated daughter satellite cells remains undifferentiated and in this form restores the satellite cell population (4;417). The remaining daughter satellite cells either fuse with the damaged muscle cell or form new myotubes to replace the necrotic muscle cells (see Chapter 1.3.9. for more details) (4;492).

Human diploid cells, including muscle satellite cells, have a limited replicative capacity (105;192;193;417). When isolated satellite cells are first placed in culture they proliferate rapidly. Over time this rate of proliferation declines until the cell is no longer able to proliferate. This stage is known as proliferative or replicative senescence (231;417). One of the mechanisms that regulate the proliferative capacity of human somatic cells is the loss of telomeric DNA (231;417). It has been shown, *in vitro*, that approximately 113 bp of telomeric DNA are lost with each division of a human muscle satellite cell (105;417).

Telomeres are sections of non-coding DNA that occur at the ends of all eukaryotic chromosomes (Figure 4.1) (104;168;219;231). If a chromosome were likened to a shoelace then the telomere would be the section at the tip of

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the shoelace that is encased in the protective plastic covering. The molecular structure of telomeres is highly conserved and consists of numerous tandem repeats of the DNA sequence $(TTAGGG)_n$, (168;219;231;417). Human telomeres can be up to 20 kb in length (231). Telomeres act to stabilise the ends of chromosomes and to protect the genetic information of the chromosomes from degradation and aberrant recombination (105;219;231;417).

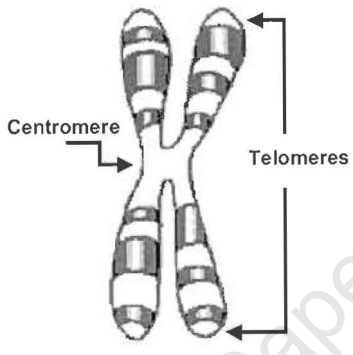

Figure 4.1. A diagrammatic representation of a human chromosome in metaphase. The telomeres and the centromere are labelled.

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The process by which linear DNA replicates is such that the DNA polymerase is unable to copy the 3' terminal segment of each DNA strand. This "end replication problem" results in the loss of 50 – 200 base pairs of telomeric DNA per cell division (219;231;260;417). The presence of the non-coding telomeres at the ends of the chromosomes prevents the loss of important genetic information with every cell division. Therefore, accepting that with every cell division telomeric DNA is lost, measuring the length of the telomeres provides an indirect, yet reliable indication of the regenerative history of the cell (417).

There is evidence to show that there is a reduction in the mean telomere length as the cell ages (105). As mentioned previously, the reduction in telomere length with every cell division is one of the mechanisms responsible for satellite cell senescence (219). Therefore, not only is the length of the telomere indicative of the regenerative history of the cell but it is also an indication of the replicative potential or regenerative capacity of the cell (417).

In determining the telomere length of human cells, total genomic DNA is digested with a restriction enzyme that removes the terminal fragment of each chromosome. The digested DNA is resolved on an agarose gel by electrophoresis. The terminal restriction fragments (TRFs) are composed of telomeric $(TTAGGG)_n$ repeat sequences and a subtelomeric fragment of non-TTAGGG DNA. The TRFs are detected by hybridisation to a ^{32}P-labeled telomeric oligonucleotide probe and a typical smear pattern is generated on the gel. An autoradiogram is made of the probed gel and the lengths of the TRFs are determined by densitometric analysis of the autoradiogram. The size of the terminal restriction fragments (TRFs) reflects the length of the telomeres in the cell population (106). A summary of the process by which terminal restriction fragments are generated from the total genomic DNA and an example of a typical autoradiogram with the TRF smears is presented in Figure 4.2.

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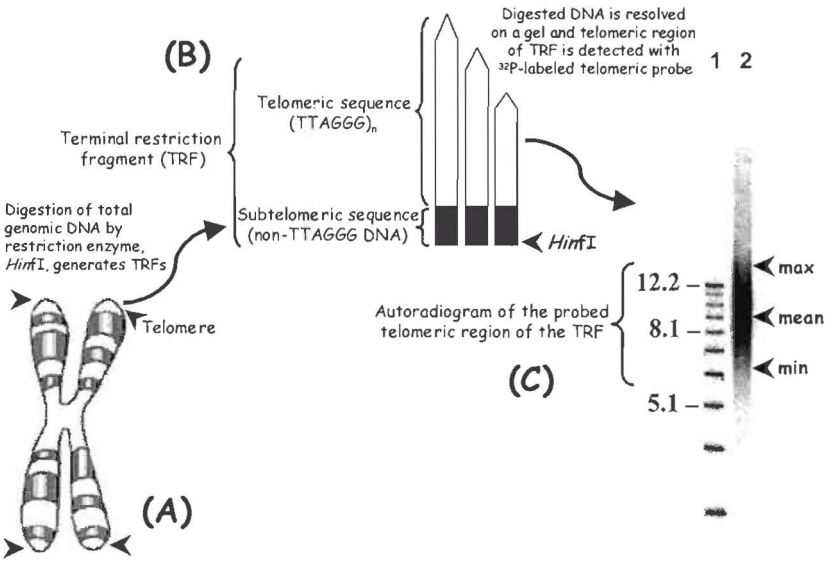

Figure 4.2. (A) A human chromosome in metaphase with the telomeric region marked with arrowheads. (B) Terminal restriction fragments (TRFs) are generated by digestion of the total genomic DNA with restriction enzyme, *Hinf*I. The arrowhead marks the site at which the restriction enzyme generates the TRF. (C) A representative autoradiogram showing the maximum (max), mean and minimum (min) lengths of an individual's TRF (lane 2). Molecular weight standards are indicated in kbp (lane 1).

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The population of cells in a sample of muscle tissue will be at different stages of their replicative lifespan, from terminally differentiated post-mitotic muscle cells to undifferentiated, pre-mitotic satellite cells. As a result, varying lengths of terminal restriction fragments will be observed. In a sample of muscle tissue, the mean length of the TRFs (meanTRF) is representative of the telomere lengths of both the satellite cells and the post-mitotic muscle cells (418). While the meanTRF is usually a good marker of the cell turnover of actively dividing mitotic tissue, it is not usually sufficiently sensitive to detect the small loss of telomeric DNA that occurs in post-mitotic tissue such as skeletal muscle (104;106). In post-mitotic muscle tissue, the maximum length of the TRFs (maxTRF) corresponds to the length of the telomeres on the

chromosomes within the post-mitotic myonuclei. The myonuclei that were included in the muscle fibre early on during development would only have undergone a few cell divisions and therefore would have considerably longer telomeres compared to those from satellite cells that have recently been included into the muscle fibre (104). The minimum length of the TRFs (minTRF) represents the length of the telomere from chromosomes of satellite cells that have undergone the most number of cell divisions (104;106). The minimum length of the terminal restriction fragments (minTRF) is the most sensitive indicator of the low rate of telomere shortening that occurs in normal skeletal muscle tissue (104;106).

The physiological data described in Chapter 2 revealed that the short term, maximal muscle function of the athletes with acquired training intolerance (ATI) is not impaired compared to that of the asymptomatic control athletes (Chapter 2). In addition to this it has also been shown that although the ATI and CON athletes were well matched for age and years of high volume endurance training, prior to the onset of symptoms, the ATI athletes trained a significantly greater number of hours, days and kilometres per week compared to the CON athletes (Chapter 2).

The results of Chapter 3 revealed that although there was some degree of skeletal muscle pathology in the muscle of the CON athletes, a significantly greater proportion of the ATI athletes presented with an even greater degree of skeletal muscle disruptions. The nature of these disruptions was not

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specific to a particular myopathy or dystrophy but resembled that of chronic exercise-induced muscle damage (Chapter 3).

Research has shown that prolonged, exhaustive endurance exercise induces skeletal muscle damage (reviewed in section 1.2). Repair of this muscle damage would be carried out by the satellite cells (reviewed in section 1.3). Extensive endurance training over a number of years will therefore involve numerous cycles of damage and repair and hence an increased proliferation of satellite cells (168;417). Accordingly, the aim of this section was to compare the presence of pathology and the regenerative history, as indicated by the minimum, maximum and means length of the terminal restriction fragments (TRFs), in the skeletal muscle of athletes with acquired training intolerance and asymptomatic control athletes.

4.2. METHODS

4.2.1. Subjects

This study was approved by the Research and Ethics committee of the Faculty of Health Sciences at the University of Cape Town. 13 ATI (tATI) and 13 CON (tCON) subjects were randomly selected from the original group of ATI and CON athletes. Two additional subjects (ATI 20 and CON 18), who met with the inclusion criteria described in Chapter 2, were recruited for this study. All the descriptive, physiological and training characteristics were collected as previously described in Chapter 2, section 2.1. A history of the

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endurance training of ATI 19 was not obtained. Furthermore the endurance training history of ATI 20 and CON 18 was incomplete.

4.2.2. Skeletal muscle morphology

A muscle biopsy of the vastus lateralis was obtained from each subject using the percutaneous needle biopsy technique of Bergstrom (30), as modified by Evans et al (123). Light (Chapter 3.2.2.) and electron microscopic (Chapter 3.2.3.) analyses were carried out as previous described in Chapter 3. Due to technical difficulties no electron microscopy data could be obtained from the muscle sample of subjects, ATI 16 and CON 3.

4.2.3. Determination of telomere lengths

A portion of the muscle sample was rapidly frozen in liquid nitrogen and stored at – 80 ^{o} C for subsequent DNA extraction and determination of the mean (meanTRF), minimum (minTRF) and maximum (maxTRF) lengths of the terminal restriction fragments (TRFs) as previously described (106) (Figure 4.2.). Total genomic DNA was extracted from at least 10 mg of skeletal muscle from the vastus lateralis. The muscle sample was ground to a powder in liquid nitrogen and digested overnight at 55 ^{o}C with gentle agitation in 650 µl proteinase K digestion buffer (10 mM Tris-Cl, pH 8.0; 100 mM EDTA,pH 8.0; 100 mM NaCl; 1% Triton X-100) containing 20 units.ml^{-1} Proteinase K. The digest was extracted twice with 1 volume of 25:24:1 (vol:vol:vol) phenol:chloroform:isoamyl alcohol. The DNA was precipitated with 1 volume of a 1:4 (vol:vol) 7.5 M ammonium acetate and 100% ethanol mixture, washed with 70% ethanol, resuspended in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM

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EDTA, pH 8.0) and stored at 4°C. The intact genomic DNA was digested for 4 hours at 37°C with the restriction enzyme, *Hinf* I. This digestion step produced terminal restriction fragments (TRFs) of DNA, which consist of telomeric $(TTAGGG)_n$ repeat sequences of varying lengths and a subtelomeric fragment of non-TTAGGG DNA of uniform length (See Figure 4.2) (8). Since the TRF lengths from a tissue range in size, mean, minimum and maximum TRF lengths were determined by Southern blot analysis using a ^{32}P-$(TTAGGG)_4$ probe as described by Renault et al. (418). Undigested DNA samples were also resolved on an agarose gel to verify the absence of DNA degradation (see Figure 4.2).

On completion of the Southern blot analysis, the gels were exposured to X-ray film (BioMax, Kodak, EIS, Massy, France) with a BioMax transcreen (Kodak, EIS, Massy, France). The signal responses on the autoradiograms were analysed by a computer-assisted system using NIH Image 1.62, which generates densitometric data from one-dimensional gels, and ProFit software, which analyses densitometric profiles. The mean TRF (L) was calculated by integrating the signal intensity above background over the entire TRF distribution as a function of TRF length using the formula: $L = \Sigma(OD_i.L_i)/\Sigma(OD_i)$, where OD_i and L_i are the signal intensity and the TRF length (the value of the corresponding standard molecular weight marker), respectively, at position " i " on the gel image (188;505). To determine the minimum value of the TRF in a homogeneous way for all samples, the densitometric profile of the TRF length was integrated over the distance of migration and the minimum TRF value corresponds to 95% of this integration (i.e. the minimum

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signal intensity at the lower side of the peak signal intensity; Figure 4.2) (104). Similarly, the maximum TRF value corresponds to 5% of the integration (i.e. the maximum signal intensity on the upper side of the peak signal intensity; Figure 4.2) (104). The maximum, mean and minimum TRF values (in kbp) for each sample were determined in triplicate on three independent gels.

Due to technical difficulties, the minimum value of the terminal restriction fragment for CON 14 was not determined.

4.3. STATISTICS

With the exception of gender, descriptive, physiological and training values are expressed as mean ± S.D. An independent t-test was used to determine if there were any significant differences ($P < 0.05$) in the descriptive, physiological and training data between the two groups. An independent t-test was also used to determine if there was a significant difference in the percentage type I muscle fibres. A Mann-Whitney test for non-parametric data was used to detect any significant difference between the two groups for the total structural and ultra structural pathology score.

A Fisher's Exact two-tailed Chi squared test was used to determine if there was a significant difference ($P < 0.05$) in the gender ratio of tATI and tCON athletes and in the proportion of tATI and tCON athletes who presented with structural and ultra structural skeletal muscle pathology. The odds ratio (OR) within a 95% confidence interval (CI) was also calculated so as to determine

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whether either group was more likely to present with skeletal muscle pathology.

A Levene's test for homogeneity of variances indicated a significant difference in the variance between the two groups for maximum, minimum and mean terminal restriction fragments. Therefore, to reduce the risk of a type II error, which might occur after analysing the data using nonparametric statistics, the data were logarithmically transformed so as to reduce the variance (202). Telomeres shorten with increasing age, therefore the data were analysed with an analysis of covariance, with age as the covariate, to determine if a significant difference ($P < 0.05$) existed between the mean, minimum and maximum values of the telomere restriction fragments for the tATI and tCON groups.

4.4. RESULTS

4.4.1. Descriptive and physiological characteristics

The individual descriptive and physiological characteristics of the tATI and tCON athletes are presented in Table 4.1. and 4.2. and summarised in Table 4.3.

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Table 4.1. Descriptive and physiological characteristics of tATI athletes.

Subject	Gender	Age (yrs)	Years with ATI	Stature (cm)	Body mass (kg)	% Body fat	VO_{2max} ($ml.kg^{-1}.min^{-1}$)	MVC (N)
ATI 1	M	27	4	177.6	76.7	13.0	56.2	496
ATI 2	F	36	8	168.0	54.2	19.9	59.7	321
ATI 3	F	44	7	161.0	54.5	31.3	38.8	346
ATI 4	M	46	3	166.2	76.0	26.5	42.2	469
ATI 5	M	39	1	177.0	79.1	18.1	59.2	555
ATI 6	M	52	1	187.0	85.0	27.2	47.4	624
ATI 7	M	48	8	179.5	103.3	30.7	31.1	603
ATI 13	F	57	2	165.0	54.5	24.3	52.9	270
ATI 14	M	43	2	177.0	79.5	26.1	44.3	643
ATI 15	M	33	7	184.0	110.0	24.2	47.2	843
ATI 16	M	43	4	171.0	66.5	16.6	47.1	739
ATI 19	M	38	3	176.0	72.0	21.2	49.3	467
ATI 20	M	22	ND	172.5	58.5	14.3	58.8	480

MVC = maximal voluntary contraction. ND = not determined

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Table 4.2. Descriptive and physiological characteristics of tCON athletes.

Subject	Gender	Age (yrs)	Stature (cm)	Body mass (kg)	% Body fat	VO_{2max} ($ml.kg^{-1}.min^{-1}$)	MVC (N)
CON 3	M	56	176.5	75.0	18.3	61.0	448
CON 4	F	35	175.5	76.5	27.1	41.6	519
CON 7	M	35	176.0	73.5	13.8	56.7	607
CON 8	M	34	159.8	67.0	21.3	58.5	483
CON 9	F	25	163.7	57.0	26.6	49.3	469
CON 10	F	27	165.8	58.0	22.3	56.2	456
CON 11	M	48	176.0	73.0	15.0	56.9	616
CON 12	M	49	179.5	75.5	21.0	54.4	520
CON 13	M	55	190.0	91.0	23.3	41.9	571
CON 14	M	45	169.6	77.5	18.0	45.2	681
CON 15	M	24	171.0	70.9	9.1	73.1	775
CON 16	F	53	164.5	49.0	25.0	54.3	377
CON 18	M	58	166.0	75.0	26.1	30.5	440

MVC = maximal voluntary contraction

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Table 4.3. Summary of the descriptive and physiological characteristics of the tATI and tCON athletes.

	tCON (n = 13)	tATI (n = 13)	P value
Gender	9 (M) 4 (F)	10 (M) 3 (F)	1.00
Age (yrs)	42 ± 12 (24 ~ 58)	41 ± 10 (22 ~ 57)	0.77
Stature (cm)	172 ± 8 (160 ~ 190)	174 ± 8 (161 ~ 187)	0.55
Body mass (kg)	71 ± 11 (49 ~ 91)	75 ± 18 (54 ~ 110)	0.50
% Body fat	21 ± 5 (9 ~ 27)	23 ± 6 (13 ~ 31)	0.37
$VO_{2\,max}$ ($ml.kg^{-1}.\,min^{-1}$)	52 ± 11 (31 ~ 73)	49 ± 9 (31 ~ 60)	0.37
MVC (N)	536 ± 111 (377 ~ 775)	527 ± 165 (270 ~ 843)	0.88

With the exception of gender, values are means ± S.D. Minimum and maximum values are indicated in parentheses. MVC = maximal voluntary contraction.

There was no significant difference in age, stature, body mass, or percent body fat between the tATI and tCON groups. There was also no significant difference in the maximal oxygen consumption (VO_{2max}) or the maximal isometric voluntary force output (MVC) between the two groups.

4.4.2. Endurance training history

The endurance training history of the tATI athletes, both prior to and after the onset of symptoms, and the tCON athletes are presented in Table 4.4. and

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4.5. Table 4.6. presents a summary of the endurance training data for both groups.

Table 4.4. Endurance training history of the tATI athletes before (Pre) and after (Post) the onset of symptoms.

				Training volume					
				days.wk^{-1}		hours.wk^{-1}		km.wk^{-1}	
Subject	**Sport**	**HVETage**	**HVETyrs**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
ATI 1	Triathlete	13	17	5	2	5	0	80	0
ATI 2	Runner	18	12	7	5	10	4	130	50
ATI 3	Squash	21	15	6	0	24	0	NA	NA
ATI 4	Runner	35	10	6	4	8	4	90	40
ATI 5	Runner	32	7	6	4	5	5	60	50
ATI 6	Runner	44	7	5	5	4	4	45	45
ATI 7	Runner	40	3	4	0	3	0	50	0
ATI 13	Runner	39	18	6	0	5	0	65	0
ATI 14	Runner	21	21	5	0	6	0	70	0
ATI 15	Runner	18	9	7	6	12	3	160	30
ATI 16	Cyclist	23	20	7	7	9	2	NA	NA
ATI 19	Runner	ND	ND	ND	ND	ND	ND	ND	ND
ATI 20	Runner	18	4	ND	ND	ND	ND	ND	ND

Triathlete = running data are shown for all variables. Squash player = number of days and hours per week of squash training are shown. Cyclist = number of days, hours and kilometres per week of endurance cycling are shown. HVETage = age in years at which started high volume endurance training. HVETyrs = years of high volume endurance training. NA = not applicable. ND = Not determined

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Table 4.5. Endurance training history of tCON athletes.

				Training volume		
Subject	**Sport**	**HVETage**	**HVETyrs**	**days.wk^{-1}**	**hours.wk^{-1}**	**km.wk^{-1}**
CON 3	Runner	47	10	6	5	50
CON 4	Runner	20	15	3	3	22
CON 7	Runner	17	13	4	6	60
CON 8	Runner	12	22	7	13	130
CON 9	Runner	18	7	6	5	45
CON 10	Runner	23	4	6	5	50
CON 11	Runner	30	18	4	5	45
CON 12	Runner	39	10	4	5	50
CON 13	Runner	33	22	4	4	40
CON 14	Runner	35	10	4	5	50
CON 15	Runner	14	10	6	6	60
CON 16	Runner	33	20	3	4	40
CON 18	Cyclist	ND	ND	ND	ND	ND

HVETage = age in years at which started high volume endurance training. HVETyrs = years of high volume endurance training. ND = Not determined.

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Table 4.6. Summary of the endurance training history of the tATI (pre and post) and tCON athletes.

	tCON (n = 12)	tATIpre (n = 11)	tATIpost (n = 11)
HVETage (yrs)	27 ± 11 (12 ~ 47)	27 ± 11 (13 ~ 44)	
HVETyears (yrs)	13 ± 6 (4 ~ 22)	12 ± 6 (3 ~ 21)	
Training (days.wk $^{-1}$)	5 ± 1 (3 ~ 7)	6 ± 1 # (4 ~ 7)	3 ± 3 † (0 ~ 7)
Training (km.wk $^{-1}$)	54 ± 26 (22 ~ 130)	86 ± 35 ## * (45 ~ 160)	24 ± 23 †† (0 ~ 50)
Training (hours.wk $^{-1}$)	5 ± 3 ** (3 ~ 13)	8 ± 6 (3 ~24)	2 ± 2 ††† (0 ~ 4.5)

All data are means ± standard deviations (SD). Minimum and maximum values are in parentheses. HVETage = age, in years, at which started high volume endurance training. HVETyrs = years of high volume endurance training. Only data from 11 tATI subjects were analysed when determining differences in the number of days.wk^{-1} and hours.wk^{-1} of endurance training between the two groups. There were no data for the number of km.wk^{-1} of endurance training for the squash player (ATI 3), the cyclist (ATI 16), ATI 19 and ATI 20, therefore, only data from 9 tATI subjects were analysed when determining differences in this variable between the two groups.

\# P = 0.04; tATIpre 6 ± 1 vs tCON 5 ± 1 days.wk $^{-1}$
\## P = 0.04; tATIpre 86 ± 35 vs tCON 54 ± 26 km.wk $^{-1}$
* P = 0.01; tATIpost 24 ± 23 vs tCON 54 ± 26 km.wk $^{-1}$
** P = 0.002; tATIpost 2 ± 2 vs tCON 5 ± 3 hours.wk $^{-1}$
† P = 0.01; tATIpre 6 ± 1 vs tATIpost 3 ± 3 days.wk $^{-1}$
†† P = 0.001; tATIpre 86 ± 35 vs tATIpost 24 ± 23 km.wk $^{-1}$
††† P = 0.003; tATIpre 8 ± 6 vs tATIpost 2 ± 2 hours.wk $^{-1}$

Following the onset of symptoms the tATI athletes significantly reduced the number of days (6 ± 1 vs 3 ± 3 days.wk^{-1}; P = 0.01), kilometres (86 ± 35 vs 24

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± 23 km.wk^{-1}; P = 0.001) and hours (8 ± 6 vs 2 ± 2 hours.wk^{-1}; P = 0.003) of endurance training per week. In addition to this, tATIpost kilometres and hours of endurance training per week were also significantly less than that of the tCON athletes (24 ± 23 vs 54 ± 26 km.wk^{-1}; tATIpost vs tCON; P = 0.01 and 2 ± 2 vs 5 ± 3 hours.wk^{-1}; tATIpost vs tCON P = 0.002).

The tATI and tCON athletes were well matched for age at which they started high volume endurance training (HVETage) and the years of high volume endurance training (HVETyrs). Although the tATI athletes trained a significantly greater number of days (6 ± 1 vs 5 ± 1 days.wk^{-1}; P = 0.04) and kilometres per week than the tCON athletes (86 ± 35 vs 54 ± 26 km.wk^{-1}; P = 0.04), there was no significant difference in the number of hours spent training per week between the two groups.

4.4.3. Light microscopy

The presence of various structural disruptions in the skeletal muscle of each individual tATI and tCON athlete are presented in Table 4.7. and 4.8. There was no significant difference in the proportion of type I muscle fibres between the tATI (52 ± 16) and the tCON (50 ± 8) groups (P = 0.58).

The tATI athletes had a significantly higher total score for the degree of structural pathology present in their muscle compared to that of the tCON athletes (4 ± 2 vs 2 ± 1; P = 0.02).

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Table 4.7. Structural disruptions, as determined by light microscopy, in the skeletal muscle of tATI athlete.

Subject	% Type I fibres	Size variation	Internal nuclei	Necrosis/ inflammation	SSL mitochondria	Total score
ATI 1	53	1+	2+	0	2+	**5**
ATI 2	91	3+	3+	0	3+	**9**
ATI 3	56	2+	1+	0	2+	**5**
ATI 4	66	1+	0	0	2+	**3**
ATI 5	51	0	0	0	2+	**2**
ATI 6	52	2+	0	0	2+	**4**
ATI 7	41	3+	2+	2+	1+	**8**
ATI 13	67	2+	1+	0	2+	**5**
ATI 14	39	2+	1+	1+	1+	**5**
ATI 15	53	1+	1+	0	2+	**4**
ATI 16	43	1+	0	0	2+	**3**
ATI 19	31	0	0	0	1+	**1**
ATI 20	39	0	0	0	1+	**1**
Average	**52.5**	**1.4**	**0.9**	**0.2**	**1.8**	**4.2**

SSL = Subsarcolemmal aggregations of mitochondria.

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Table 4.8. Structural disruptions, as determined by light microscopy, in the skeletal muscle of tCON athletes.

Subject	% Type I fibres	Size variation	Internal nuclei	Necrosis/ inflammation	SSL mitochondria	Total score
CON 3	50	0	0	0	2+	**2**
CON 4	41	0	0	0	1+	**1**
CON 7	60	0	0	0	2+	**2**
CON 8	42	0	0	0	2+	**2**
CON 9	58	1+	1+	0	2+	**4**
CON 10	59	0	0	0	2+	**2**
CON 11	55	0	0	0	2+	**2**
CON 12	40	2+	1+	1+	1+	**5**
CON 13	54	0	0	0	2+	**2**
CON 14	37	0	0	0	1+	**1**
CON 15	48	0	0	0	3+	**3**
CON 16	52	0	0	0	3+	**3**
CON 18	51	1+	0	0	0	**1**
Average	**49.8**	**0.3**	**0.2**	**0.1**	**1.8**	**2.3**

SSL = Subsarcolemmal aggregations of mitochondria.

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4.4.3.1. Internal nuclei

There was no significant difference in the proportion of tATI and tCON athletes who presented with internal nuclei (7 vs 2; P = 0.1; OR = 6.4; 95% CI: 1.0 to 41.2; Figure 4.3.)

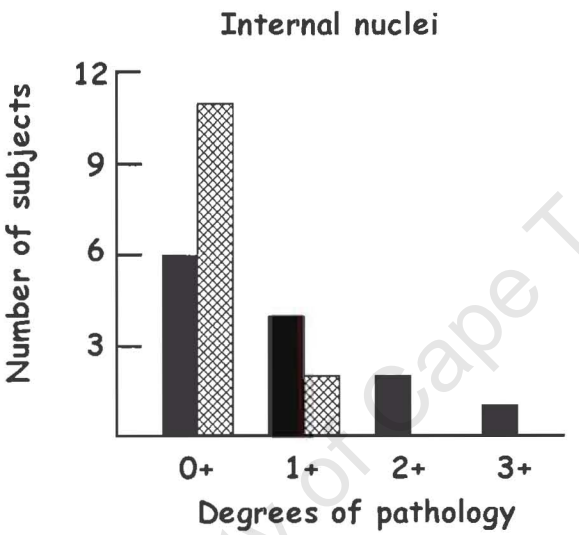

Figure 4.3. The proportion of tATI (solid bars) and tCON (hatched bars) athletes who presented with internal nuclei.

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4.4.3.2. Fibre size variation

A significantly greater proportion of tATI athletes presented with an abnormal degree of variation in the size of their muscle fibres compared to the tCON athletes (9 vs 2; P = 0.02; Figure 4.4). Furthermore the tATI athletes were significantly more likely to present with fibre size variation, compared to the tCON athletes (OR = 11.1; 95% CI: 1.8 to 68.9).

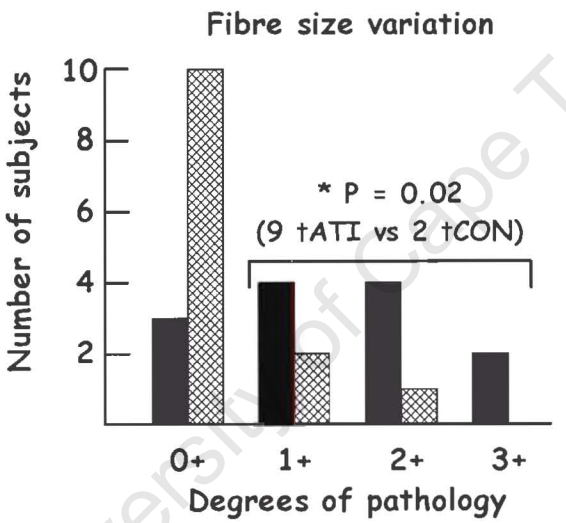

Figure 4.4. The proportion of tATI (solid bars) and tCON (hatched bars) athletes who presented with abnormal variation in muscle fibre size.

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4.4.3.3. Necrosis and inflammation

The proportion of tATI and tCON athletes who presented with skeletal muscle fibre necrosis and inflammation was minimal (2 vs 1; P = 1.0; OR = 2.2; 95% CI: 0.2 to 27.6; Figure 4.5).

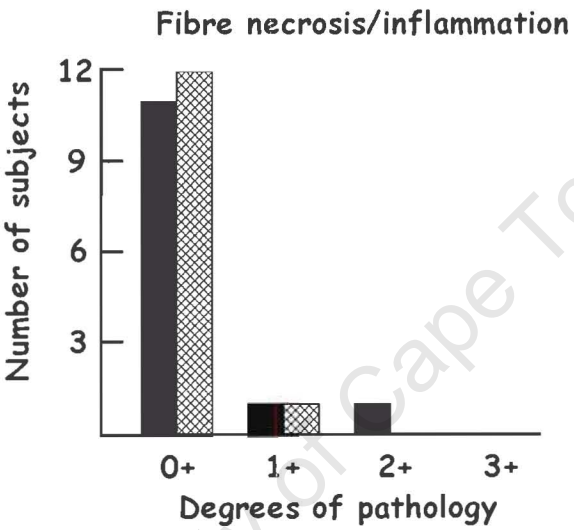

Figure 4.5. The proportion of tATI (solid bars) and tCON (hatched bars) athletes who presented with signs of necrosis and inflammation.

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4.4.3.4. Subsarcolemmal aggregations of mitochondria

A similar proportion of tATI and tCON athletes presented with subsarcolemmal aggregations of mitochondria (9 vs 9; P = 1.0; OR = 3.2; 95% CI: 0.1 to 87.2; Figure 4.6.).

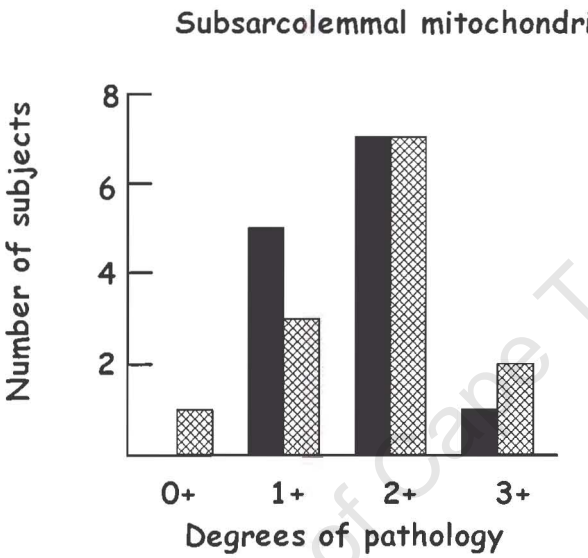

Figure 4.6. The proportion of tATI (solid bars) and tCON (hatched bars) athletes who presented with subsarcolemmal aggregations of mitochondria.

Apart from the there being no significant difference in the proportion of tATI and tCON athletes presenting with internal nuclei, the results of the light microscopy in this subset of control (tCON) and acquired training intolerant (tATI) athletes are similar to that of the original CON and ATI groups. In this regard it can be said that this subset of athletes is representative of the original group of athletes.

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4.4.4. Electron microscopy

The presence of various ultra structural disruptions in the skeletal muscle of each individual tATI and tCON athlete are presented in Table 4.9. and 4.10.

4.4.4.1. Z disc streaming

Z disc streaming was noted in five of the muscle of 5 tATI athletes, whereas no Z disc streaming was observed in any of the muscle samples from the tCON athletes (P = 0.04; OR = 18.3; 95% CI: 0.9 to 381.0; Figure 4.7).

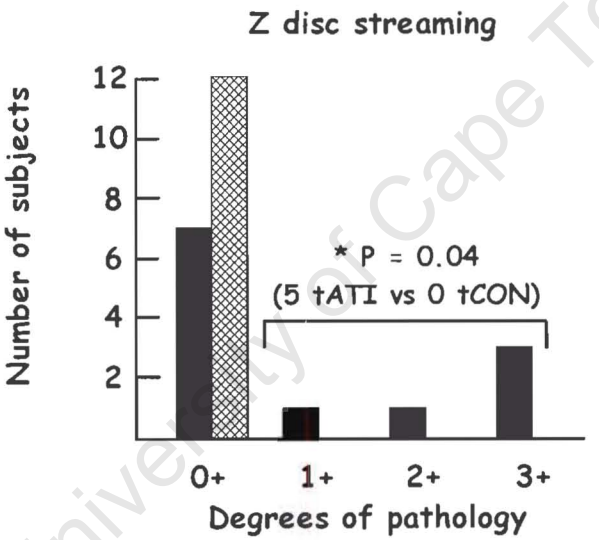

Figure 4.7. The proportion of tATI (solid bars) and tCON (hatched bars) athletes who presented with Z disc streaming.

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Table 4.9. Ultrastructural changes, as determined by electron microscopy, in the skeletal muscle of the tATI athletes.

Subjects	Z disc	Focal	Atrophic	Mitochondria	SSL	Lipid	Glycogen	Total score
ATI 1	0	3+	0	Enlarged	2+	3+	3+	**11**
ATI 2	3+	3+	2+	Enlarged	2+	0	0	**10**
ATI 3	3+	3+	2+	Normal	2+	3+	3+	**16**
ATI 4	0	3+	0	Enlarged	1+	3+	3+	**10**
ATI 5	0	3+	3+	Enlarged	3+	0	3+	**12**
ATI 6	1+	3+	0	Enlarged	1+	3+	3+	**11**
ATI 7	0	1+	0	Normal	1+	3+	3+	**8**
ATI 13	0	1+	1+	Enlarged	1+	0	0	**3**
ATI 14	0	1+	1+	Enlarged	0	0	0	**2**
ATI 15	0	1+	2+	Normal	3+	0	0	**6**
ATI 16	ND	ND	ND	ND	ND	ND	ND	**ND**
ATI 19	2+	3+	0	Enlarged	0	0	3+	**8**
ATI 20	3+	1+	0	Normal	0	0	0	**4**
Average	**1**	**2.2**	**0.9**		**1.3**	**1.3**	**1.8**	**8.4**

Z disc = Z disc streaming, Focal = focal deletions of myofibres, Atrophic = atrophic muscle fibres, Mitochondria = size of mitochondria, SSL = subsarcolemmal aggregations of mitochondria, Lipid = lipid deposits and Glycogen = glycogen deposits.
ND = Not determined.

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Table 4.10. Ultrastructural changes, as determined by electron microscopy, in the skeletal muscle of *tCON* athletes.

Subjects	Z disc	Focal	Atrophic	Mitochondria	SSL	Lipid	Glycogen	Total score
CON 3	ND	ND	ND	ND	ND	ND	ND	**ND**
CON 4	0	3+	1+	Enlarged	2+	0	0	**6**
CON 7	0	1+	0	Enlarged	3+	0	0	**4**
CON 8	0	0	0	Normal	2+	0	0	**2**
CON 9	0	0	0	Normal	1+	0	0	**1**
CON 10	0	3+	1+	Enlarged	3+	0	0	**7**
CON 11	0	2+	1+	Enlarged	3+	0	0	**6**
CON 12	0	0	0	Normal	1+	0	0	**1**
CON 13	0	0	0	Normal	1+	0	0	**1**
CON 14	0	3+	0	Enlarged	3+	0	0	**6**
CON 15	0	1+	1+	Normal	1+	0	0	**3**
CON 16	0	3+	1+	Enlarged	3+	0	0	**7**
CON 18	0	1+	0	Normal	2+	0	0	**4**
Average	**0**	**1.4**	**0.4**		**2.1**	**0**	**0**	**4**

Z disc = Z disc streaming, Focal = focal deletions of myofibres, Atrophic = atrophic muscle fibres, Mitochondria = size of mitochondria, SSL = subsarcolemmal aggregations of mitochondria, Lipid = lipid deposits and Glycogen = glycogen deposits.
ND = Not determined. ND = Not determined.

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4.4.4.2. Focal deletions of myofibrils

There was no significant difference in the proportion of tATI athletes and tCON athletes who presented with focal deletions of myofibrils (12 vs 8; P = 0.09; OR = 13.2; 95% CI: 0.6 to 279.4; Figure 4.8).

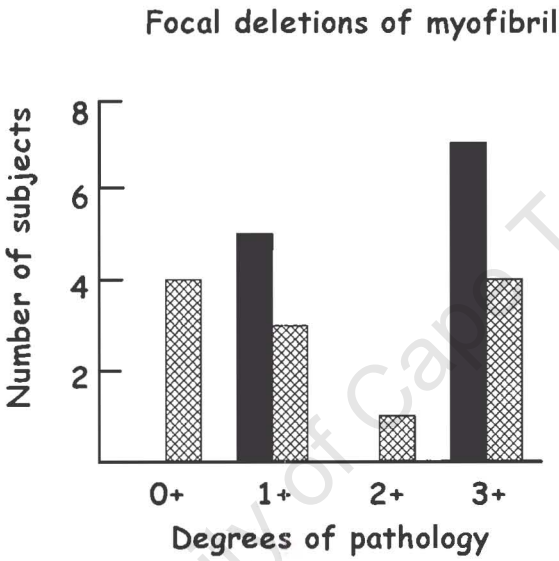

Figure 4.8. The proportion of tATI (solid bars) and tCON (hatched bars) athletes who presented with focal deletions of myofibrils.

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4.4.4.3. Atrophic fibres

There was no significant difference between the proportion of tATI and tCON athletes who presented with atrophic fibres (6 vs 5; P = 1.0; OR = 1.4; 95% CI: 0.3 to 7.0; Figure 4.9).

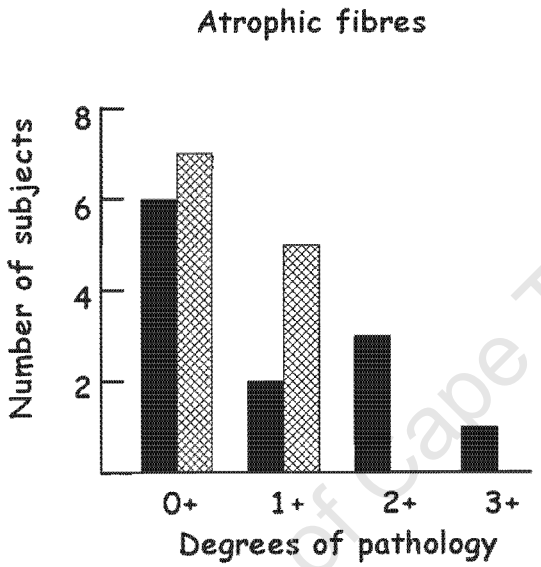

Figure 4.9. The proportion of tATI (solid bars) and tCON (hatched bars) athletes who presented with atrophic muscle fibres.

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4.4.4.4. Mitochondrial abnormalities

A similar proportion of tATI and tCON athletes presented with enlarged mitochondria (8 tATI vs 6 tCON; P = 0.7; OR = 2.0; 95% CI: 0.4 to 10.4; Figure 4.10A) and aggregations of subsarcolemmal mitochondria (9 vs 12; P = 0.2; OR = 0.1; 95% CI: 0.005 to 2.4; Figure 4.10B).

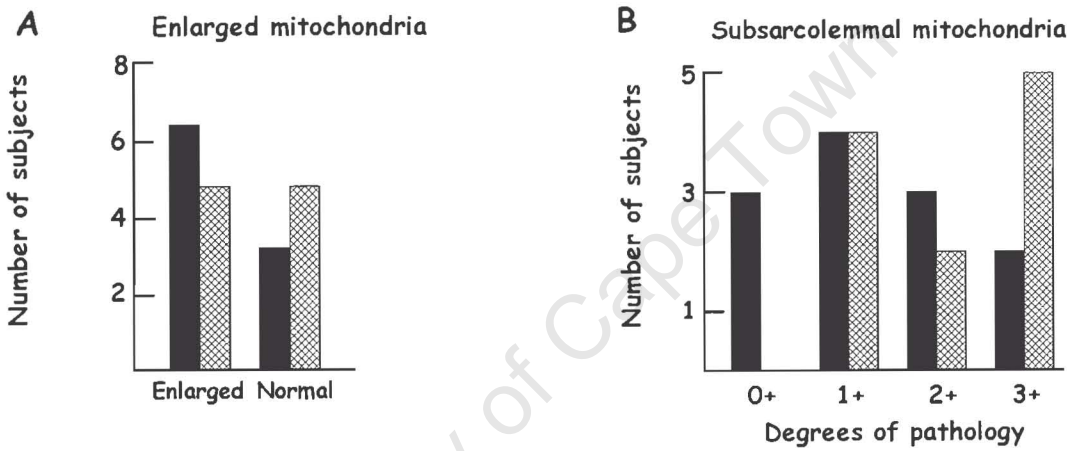

Figure 4.10. The proportion of tATI (solid bars) and tCON (hatched bars) athletes who presented with enlarged (A) and subsarcolemmal aggregations of mitochondria (B).

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4.4.4.5. Lipid and glycogen deposits

A significantly greater proportion of the tATI athletes presented with both lipid (5 tATI vs 0 tCON; P = 0.04; OR: 18.3; 95% C.I. 0.9 to 381.0; Figure 4.11A) and glycogen (7 tATI vs 0 tCON; P = 0.005; Figure 4.11B) deposits in the muscle of the tATI athletes. In addition to this the tATI athletes were more likely to present with an accumulation of glycogen deposits compared to the tCON athletes (OR: 31.4; 95% C.I. 1.6 to 708.4).

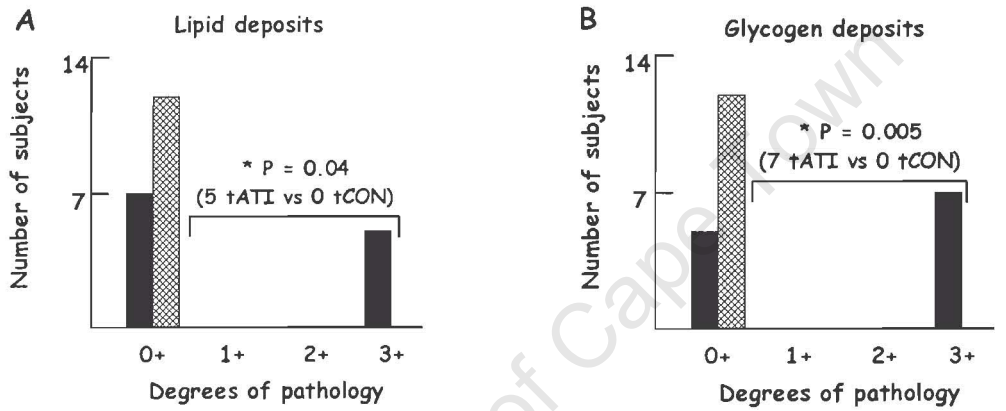

Figure 4.11. The proportion of tATI (solid bars) and tCON (hatched bars) athletes who presented with lipid and glycogen deposits.

The tATI athletes had a significantly higher total score for the degree of ultra structural disturbances present in their muscle compared to that of the tCON athletes (8 ± 4 vs 4 ± 2; P = 0.004).

The results of the electron microscopy in this subset of ATI and CON athletes are similar to that of the original group. In this regard it can be said that this subset of athletes is representative of the original group of athletes.

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4.4.5. Telomere lengths

A typical autoradiogram used to determine mean (meanTRF), minimum (minTRF) and maximum (maxTRF) lengths of the terminal restriction fragments (TRFs) is shown in Figure 4.12. As mentioned in the methods, prior to digestion with the restriction enzyme, *Hin*fI, the integrity of the extracted DNA was checked on an agarose gel. The results of this precautionary step showed that there was no degradation of the DNA (Figure 4.12 B). The meanTRF, minTRF and maxTRF of each individual tATI and tCON athlete are presented in Table 4.11. and 4.12. A summary of the gender ratio, age, mean (meanTRF), minimum (minTRF) and maximum (maxTRF) lengths of the telomere restriction fragments (TRFs) between the tATI and tCON groups is presented in Table 4.13.

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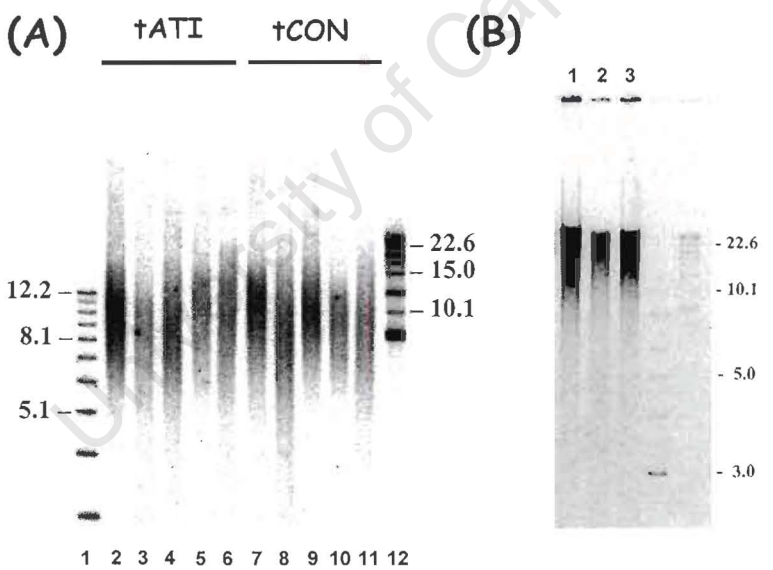

Figure 4.12. (A) A representative autoradiogram showing the size distribution of telomere restriction fragments (TRFs) from the vastus lateralis muscle of tATI (lanes 2 - 6) and tCON (lanes 7 - 11) athletes. Molecular weight standards are indicated in kbp (lane 1 and 12). (B) Representative undigested DNA samples (lanes 1-3) resolved on an agarose gel verifying the absence of DNA degradation (visualisation on ultraviolet Geldoc 2000, BioRad).

Table 4.11. The gender, age and mean (meanTRF), minimum (minTRF) and maximum (maxTRF) length of the terminal restriction fragments (TRFs) for each tATI athlete.

Subject	Gender	Age (yrs)	meanTRF (kbp)	minTRF (kbp)	maxTRF (kbp)
ATI 1	M	27	9.4	5.0	17.5
ATI 2	F	36	3.8	1.2	10.0
ATI 3	F	44	9.8	5.3	19.4
ATI 4	M	46	8.1	4.0	15.6
ATI 5	M	39	2.1	0.7	5.0
ATI 6	M	52	9.5	4.9	18.6
ATI 7	M	48	3.2	1.0	8.2
ATI 13	F	57	8.6	4.8	14.6
ATI 14	M	43	10.2	5.8	18.2
ATI 15	M	33	8.7	4.8	14.8
ATI 16	M	43	9.3	4.7	18.3
ATI 19	M	38	8.6	4.2	17.0
ATI 20	M	22	10.5	5.1	20.3

meanTRF = mean length of the telomere restriction fragments. minTRF = minimum length of the telomere restriction fragments. maxTRF = maximum length of the telomere restriction fragments.

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Table 4.12. The gender, age and mean (meanTRF), minimum (minTRF) and maximum (maxTRF) length of the terminal restriction fragments (TRF) for each tCON athlete.

Subject	Gender	Age (yrs)	meanTRF (kbp)	minTRF (kbp)	maxTRF (kbp)
CON 3	M	56	10.2	5.8	18.2
CON 4	F	35	9.2	5.4	15.8
CON 7	M	35	9.2	4.7	16.3
CON 8	M	34	10.2	5.4	17.5
CON 9	F	25	12.0	6.6	21.4
CON 10	F	27	9.9	5.7	15.4
CON 11	M	48	10.4	5.9	17.3
CON 12	M	49	8.1	4.1	14.9
CON 13	M	55	8.9	4.9	16.0
CON 14	M	45	7.8	ND	15.8
CON 15	M	24	9.7	5.1	16.4
CON 16	F	53	9.1	5.3	15.4
CON 18	M	58	9.6	5.6	16.0

meanTRF = mean length of the telomere restriction fragments. minTRF = minimum length of the telomere restriction fragments. maxTRF = maximum length of the telomere restriction fragments. ND = Not determined.

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Table 4.13. Summary of the gender, age and mean (meanTRF), minimum (minTRF) and maximum (maxTRF) lengths of the terminal restriction fragments (TRF) for both the tATI and the tCON groups.

	tCON (n = 13)	tATI (n = 13)	P value
Gender	9 (M) 4 (F)	10 (M) 3 (F)	1.00
Age (yrs)	42 ± 12 (24 ~ 58)	41 ± 10 (22 ~ 57)	0.69
meanTRF (kbp)	9.6 ± 1.1 (7.8 ~ 12.0)	7.8 ± 2.8 (2.1 ~ 10.5)	0.06
minTRF (kbp)	5.4 ± 0.6 (4.1 ~ 6.6)	4.0 ± 1.8 * (0.7 ~ 5.8)	0.04
maxTRF (kbp)	16.7 ± 1.7 (14.9 ~ 21.4)	15.2 ± 4.7 (5.0 ~ 20.3)	0.21

With the exception of gender, all data are means ± S.D. Minimum and maximum values as well as n values are indicated in parentheses. meanTRF = mean length of the telomere restriction fragments. minTRF = minimum length of the telomere restriction fragments. maxTRF = maximum length of the telomere restriction fragments. The minTRF value for CON 14 could not be determined, therefore data from 12 tCON subjects were analysed when determining differences in this variable between the two groups.

* tCON minTRF, 5.4 ± 0.6 kbp vs tATI minTRF, 4.0 ± 1.8 kbp; P = 0.04

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There was no difference in the age (P = 0.69) and gender (P = 1.0) distribution between the two groups. While there was no significant difference in maxTRF (16.7 ± 1.7 kbp, tCON vs 15.2 ± 4.7 kbp, tATI; P = 0.21) and meanTRF (9.6 ± 1.1 kbp, tCON vs 7.8 ± 2.8 kbp, tATI; P = 0.06) between the two groups, minTRF of the tATI group (4.0 ± 1.8 kbp) was significantly shorter than that of the tCON group (5.4 ± 0.6 kbp; P = 0.04; Table 4.13. and Figure

4.13). Careful inspection of the telomere lengths of the tATI group revealed that 3 of the tATI athletes (ATI 2, ATI 5 and ATI 7) appeared to have significantly smaller mean, minimum and maximum terminal restriction fragment values compared to the rest of the tATI group (Table 4.11). All three of the athletes met with the inclusion criteria outlined in the methods described in Chapter 2 and as a result were not excluded from the tATI group in the present chapter. It is important to note, however, that when ATI 2, ATI 5 and ATI 7 were excluded from the tATI group, the minimum terminal restriction fragment value for the tATI group (4.9 ± 0.5 kbp) was still significantly smaller than that of the tCON group (5.4 ± 0.6 kbp; P = 0.043). These three athletes (ATI 2, ATI 5 and ATI 7) will be discussed in greater detail in Chapter 5.

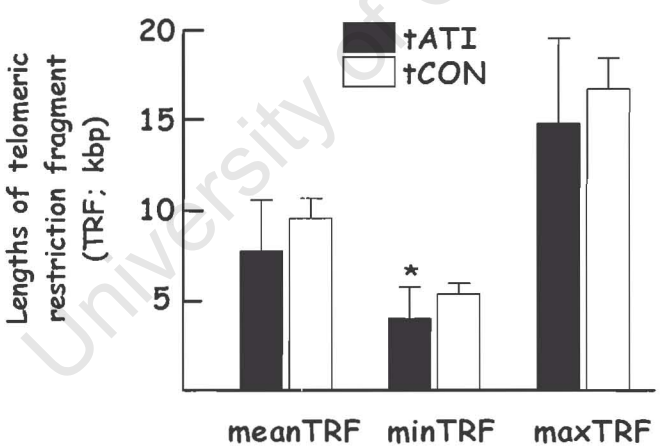

Figure 4.13. Graphic representation of the meanTRF, minTRF (*P = 0.043) and maxTRF between the tATI (n = 13) and tCON (n = 13) athletes.

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4.5. DISCUSSION

Throughout an individual's lifespan the cells in the body are constantly turning over and being replaced. With each cell division the length of the telomeres is reduced (417). The rate of telomere shortening as a consequence of normal *in vivo* aging of skeletal muscle is approximately 13 base pairs per year (106;417). Although the mean telomere length (meanTRF) is a good marker of cell turnover in mitotic tissue, it is not sensitive enough to measure the small loss of telomeric DNA in post-mitotic tissue such as skeletal muscle (104;106). Previous studies have shown the minimum terminal restriction fragment (minTRF) value, predominantly reflective of the length of the satellite cells in skeletal muscle tissue, to be the most sensitive marker of cell turnover in healthy skeletal muscle (104;106).

The most important finding of this chapter was that the athletes with acquired training intolerance had significantly shorter minimum (minTRF) telomere lengths compared to the control athletes, implying an increased proliferation or turnover of satellite cells in the skeletal muscle of these athletes (104).

Satellite cells are responsible for the repair of skeletal muscle damage (reviewed in section 1.3). If the muscle were continually exposed to damaging stimuli, be it pathological or physiological, an increased proliferation or turnover of satellite cells would occur. The telomeres of satellite cells obtained from dystrophic muscle, an example of a pathological stimulus associated with extensive skeletal muscle degeneration/regeneration, show a loss of 187

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base pairs per year. This is a 14-fold greater turnover of satellite cells per year compared with that of healthy skeletal muscle (13 base pairs per year) (104). Prolonged, endurance exercise is a well-established physiological stimulus capable of inducing muscle damage (12;16;117;146;197;203;276;283;452;453;517). The resultant skeletal muscle damage would require the activation and proliferation of satellite cells for the repair/remodelling process (280;416;429;485;507).

Results from this study revealed that an increased proportion of tATI athletes presented with chronic skeletal muscle structural (fibre size variation) and ultra structural (Z disc streaming and accumulation of lipid and glycogen deposits) pathology, compared to the control (tCON) athletes. This suggests that the increased presence of skeletal muscle pathology in the muscle of the tATI athletes may be associated with an increased proliferation and turnover of satellite cells and hence a significant reduction in minimum length of the terminal restriction fragments in these athletes.

While the tATI and tCON athletes were well matched for years of high volume endurance training, the tATI athletes trained a significantly greater number of days and kilometres per week compared to the control athletes. It is tempting to speculate that the significantly increased training volume of the tATI athletes resulted in an increased presence of structural and ultra structural skeletal muscle disruptions. This in turn leads to an increased turnover of satellite cells required to repair the exercise-induced muscle damage with the resultant consequence being a significant reduction in telomere length.

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It is interesting to note that the average minimum telomere length of the control athletes in this study (5.4 ± 0.6 kbp) was shorter than previously reported minimum values for vastus lateralis muscle (6.6 ± 0.7 kbp) sampled from healthy, presumably sedentary, individuals of a similar average age (41.7 ± 13.8 years) (104). So although the control athletes did not present with the symptoms of acquired training intolerance and exercise-associated chronic fatigue, there is preliminary evidence of an increased proliferation or turnover of satellite cells in their quadriceps muscle compared to healthy, sedentary individuals. This requires further investigation.

Although the tATI athletes had significantly shortened telomeres their maximal strength (MVC) and oxygen consumption/endurance capacity (VO_{2max}) was not impaired compared to the tCON athletes. Purely speculative reasons for this finding might be the difference in the level of motivation required to perform the task, as well as the potential of either of the tasks to cause muscle damage. This may suggest that short-term, maximal exercise is not associated with telomere length, whereas long-term, submaximal exercise that requires greater motivation and has an increased potential to cause damage may be regulated by or associated with telomere length.

In conclusion, athletes with acquired training intolerance presented with significantly smaller minimum TRF values compared to control athletes who were matched for age and years of endurance training. These findings suggest that there is an increased proliferation or turnover of satellite cells in the muscle of athletes with acquired training intolerance, possibly as a result

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of the increased muscle damage induced by years of high volume endurance training and racing.

Furthermore, it was noted that 3 of the tATI group had significantly shorter mean, minimum and maximum TRF values compared to the rest of the tATI group. It is thought that additional or alternative mechanisms to an increased proliferation or turnover of satellite cells may be implicated in these athletes. These three cases will be investigated in greater detail in the Chapter 5.

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CHAPTER 5

PATHOLOGICALLY SHORT TELOMERES IN ENDURANCE ATHLETES WITH ACQUIRED TRAINING INTOLERANCE

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5.1. INTRODUCTION

Adult skeletal muscle is a relatively stable tissue with a low turnover of muscle nuclei over the lifespan of a healthy individual (106;418;492). In the event of muscle damage (229;416;439;485), disease (104;110;386) or merely a change in the homeostasis of the muscle cell (36;100), resident satellite cells are activated. The satellite cells proliferate and either repair or replace damaged muscle fibres. While there is a decrease in the proliferative capacity of satellite cells with normal, healthy aging, it is not believed to be the limiting factor in the aging process (175;191;492;523). However, if the regenerative demands of the muscle on the satellite cells throughout the individual's lifetime are excessive, the finite proliferative capacity of the satellite cells could possibly be exhausted (175;191;492). Muscular diseases and metabolic abnormalities place increased or excessive regenerative demands on the satellite cells of the skeletal muscle (104;175). Similarly, repeated bouts of exhaustive exercise over an individual's lifetime may also place excessive regenerative demands on the skeletal muscle satellite cells and eventually impair their ability to regenerate and repair muscle damage (175;191;492). In a review article by Hawke and Garry (2001) (191), the authors pose this very question: does "*....repeated exhaustive and /or resistance training exercise programs have a negative impact on the long-term satellite cell content. If satellite cells have a limited proliferative capacity (~ 60 doublings) does a lifetime of intense exercise have a negative influence on their ability to regenerate skeletal muscle as aging progresses?"* (191). In addition to this, Miranda Grounds (1998) (175) suggested that "*if it is possible for the replicative potential of satellite cells to be exhausted by*

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repeated cycles of regeneration in diseases such as muscular dystrophy, a similar situation might theoretically arise after repeated bouts of extreme exercise over a lifetime" (175). Furthermore, in a recent review article, Thornell et al. (2003) (492) state that: *" while the number and quality of satellite cells and hence regenerative capacity are not a limiting factor during healthy ageing…..this would become a limiting factor if they were to be over solicited during the lifetime of an individual."* (492).

As previously mentioned, measurement of telomere length is an indirect yet reliable means of assessing the regenerative history as well as the proliferative capacity of skeletal muscle and it's associated satellite cells (Chapter 4.1). The population of cells in a sample of muscle tissue will be at different stages of their replicative lifespan, and hence, varying lengths of terminal restriction fragments will be observed. In a sample of muscle tissue, the mean length of the TRFs (meanTRF) is representative of the telomere lengths of both the satellite cells and the post-mitotic muscle cells (418). In post-mitotic muscle tissue, the maximum length of the TRFs (maxTRF) corresponds to the length of the telomeres on the chromosomes within the post-mitotic myonuclei. The myonuclei that were included in the muscle fibre early on during development would only have undergone a few cell divisions and therefore would have considerably longer telomeres compared to those from satellite cells that have recently been included into the muscle fibre (104). The minimum length of the TRFs (minTRF) represents the length of the telomere from chromosomes of satellite cells that have undergone the most number of cell divisions (104;106). The minimum length of the terminal restriction fragments (minTRF) is the

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most sensitive indicator of the low rate of telomere shortening that occurs in normal skeletal muscle tissue (104;106).

In the previous chapter, endurance athletes with acquired training intolerance (ATI) presented with significantly smaller minimum terminal restriction fragment (TRF) values compared to asymptomatic endurance athletes (Chapter 4). It was postulated that the significant decrease in the minimum TRF value of the ATI athletes most likely reflected an increased proliferation or turnover of satellite cells in the vastus lateralis muscle. Furthermore, it was noted that three of the tATI athletes (ATI 2, ATI 5 and ATI 7) presented with extremely short mean, minimum and maximum telomeric restriction fragment values compared to the rest of the tATI group. It is hypothesized that additional or alternative mechanisms might be responsible for the pathological shortening of the telomeres in these three athletes. Accordingly, the aim of the present chapter was to carry out an in-depth investigation into the descriptive, physiological and training characteristics, the morphological status of the skeletal muscle and the medical history of these three tATI athletes in an attempt to ascertain possible mechanisms responsible for the dramatic shortening of their minimum, maximum and mean TRF lengths.

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5.2. METHODS

5.2.1. Subjects

This study was approved by the Research and Ethics committee of the Faculty of Health Sciences at the University of Cape Town. The thirteen endurance athletes

with acquired training intolerance (tATI athletes) were investigated in this chapter (for more subject details see Chapter 4.2).

5.2.2. Determination of telomere length

The telomere lengths of the muscle samples were determined as previously described in Chapter 4.2.3.

5.2.3. Descriptive, physiological and training characteristics

The descriptive, physiological and endurance training characteristics of the athletes with acquired training intolerance were collected as described in Chapter 2.2.1 and 2.2.3.

5.2.4. Morphological characteristics of the skeletal muscle

The morphological characteristics of the skeletal muscle of the athletes with acquired training intolerance were assessed as described in Chapter 3.2.2.

5.2.5. Medical history

All subjects completed a retrospective questionnaire regarding their medical history both before and after the onset of symptoms associated with acquired training intolerance (Appendix IV).

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5.3. RESULTS

5.3.1. Telomere length

In the previous chapter it was observed that the athletes with acquired training intolerance had significantly shorter minimum terminal restriction fragments (minTRF) compared to the asymptomatic control athletes (Chapter 4.4.5). Closer inspection of the data in Table 14, revealed that three of the tATI athletes (ATI 2, ATI 5 and ATI 7; tATIshort) had significantly shorter mean (3.0 ± 0.9 kbp, tATIshort vs 9.3 ± 0.8 kbp, tATI; P = 0.00), minimum (1.0 ± 0.3 kbp, tATIshort vs 4.9 ± 0.5 kbp, tATI; P = 0.00) and maximum (7.7 ± 2.5 kbp, tATIshort vs 17.4 ± 1.9 kbp, tATI; P = 0.0001) TRFs compared to the rest of the athletes with acquired training intolerance (tATI; Table 5.1; Figure 5.1).

In order to prevent the loss of telomeric DNA from the agarose gel, a shorter gel migration time was used to determine the telomere lengths of ATI 2, ATI 5 and ATI 7 (0.7% Agarose gel, migration at 80 volts for 14 hours). In addition to this, the undigested DNA of these three samples was resolved on an agarose gel in order to verify the absence of DNA degradation (Figure 5.1).

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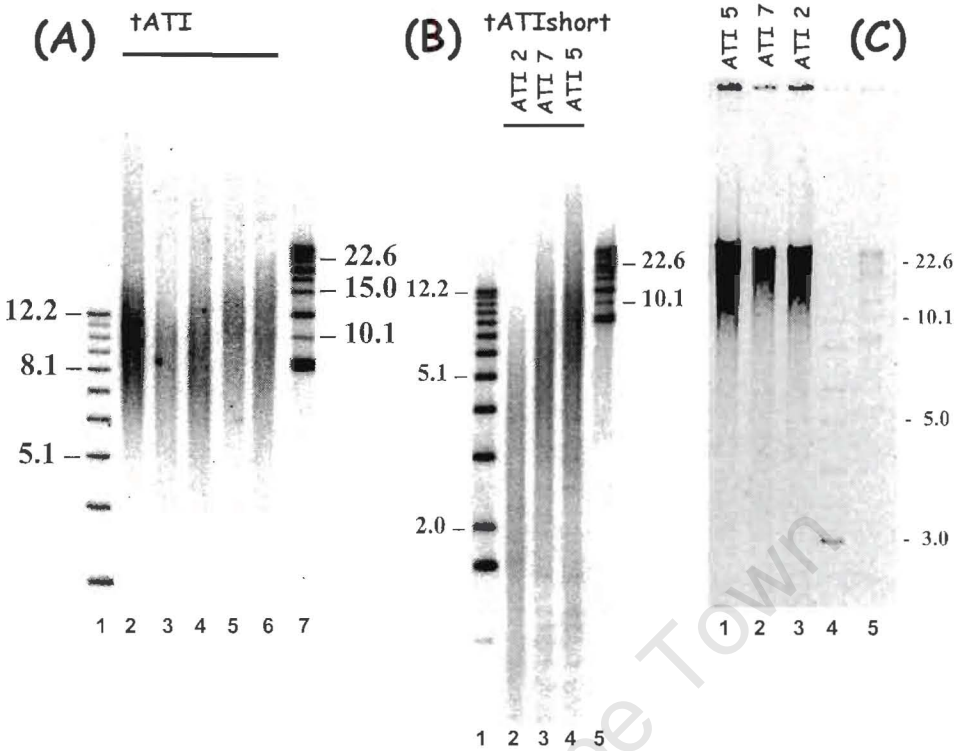

Figure 5.1. (A) A representative autoradiogram showing the size distribution of the terminal restriction fragments (TRFs) from the vastus lateralis muscle of tATI athletes (lanes 2-6). Molecular weight standards are indicated in kbp (lanes 1 and 7). (B) An autoradiogram showing the size of the terminal restriction fragments of the three tATIshort athletes, ATI 5, ATI 7 and ATI 2 (lanes 2-4). Molecular weight standards are indicated in kbp (lanes 1 and 5). (C) A 0.7% agarose gel (migration at 80 volts for 14 hours) showing the absence of DNA degradation in the samples of the three tATIshort athletes, ATI 5, ATI 7 and ATI 2 (lanes 1-3). Molecular weight standards are indicated in kbp (lanes 4 and 5; ultraviolet visualisation, GelDoc 2000, BioRad).

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Table 5.1. The gender, age and mean (meanTRF), minimum (minTRF) and maximum (maxTRF) lengths of the telomeric restriction fragments (TRF) for the tCON, tATI and tATIshort groups.

	tATI (n = 10)	tATIshort (n = 3)	ATI 2	ATI 5	ATI 7
Gender	8 (M) 2 (F)	2 (M) 1 (F)	F	M	M
Age (yrs)	41 ± 11 (22 ~ 57)	41± 6 (36 ~ 48)	36	39	48
meanTRF (kbp)	9.3 ± 0.8 * (8.1 ~ 10.2)	3.0 ± 0.9 (2.1 ~ 3.8)	3.8	2.1	3.2
minTRF (kbp)	4.9 ± 0.5 ** (4.0 ~ 5.8)	1.0 ± 0.3 (0.7 ~ 1.2)	1.2	0.7	1.0
maxTRF (kbp)	17.4 ± 1.9 *** (15 ~ 19.4)	7.7 ± 2.5 (5.0 ~ 10.0)	10.0	5.0	8.2

tATIshort = athletes with acquired training intolerance who presented with pathologically short telomeres. tATI = athletes with acquired training intolerance who did not present with pathologically short telomeres. With the exception of gender, data for tATI and tATIshort are means ± S.D. Minimum and maximum values as well as n values are indicated in parentheses. The actual values for the three tATIshort athletes are provided. meanTRF = mean length of the terminal restriction fragments. minTRF = minimum length of the terminal restriction fragments. maxTRF = maximum length of the terminal restriction fragments.

* tATI 9.3 ± 0.8 vs tATIshort 3.0 ± 0.9 meanTRF; P = 0.000

** tATI 4.9 ± 0.5 vs tATIshort 1.0 ± 0.3 minTRF; P = 0.000

*** tATI 17.4 ± 1.9 vs tATIshort 7.7 ± 2.5 maxTRF; P = 0.0001

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5.3.2. Descriptive characteristics

ATI 2 is a previously elite female endurance runner. Although at 36 years of age, ATI 2 is younger than the average tATI athlete (41 ± 11 years), she was not the youngest

athlete in the group. The body mass of ATI 2 was the lowest in the group (54.2 kg) and according to normative data from the American College of Sports Medicine (ACSM) has low percentage body fat (19%) for her age (325). Even though the percentage body fat of ATI 2 was relatively low for her age, it did not reflect a state of anorexia (see section 5.3.6). The most likely reason for this is that at the time of the study ATI 2 was rehabilitated and her eating disorder was not as severe as in the past. ATI 2 reported experiencing symptoms associated with acquired training intolerance for the past 8 years, which together with ATI 7 is the longest reported period of acquired training intolerance in the whole group (Table 5.2).

ATI 5 is a 39-year-old male endurance runner. According to ACSM normative data (325) as well as in comparison to the other tATI athletes, ATI 5 is in good physical condition for his age (Table 5.2.). ATI 5 reported experiencing acquired training intolerance symptoms for about a year before being tested in our laboratory. This is a shorter time period of acquired training intolerance symptoms compared with the average tATI athlete (3 ± 2 years) and the other two tATIshort athletes.

ATI 7 is a 48-year-old male endurance runner. Although he is older than the average tATI athlete (41 ± 11years) he is not the oldest tATI athlete (Table 5.2). ATI 7 reported experiencing the symptoms associated with acquired training intolerance for a period of 8 years. Both the body mass (103.3 kg) and percentage body fat (30.7 %) of ATI 7 was higher than that of the average tATI athlete (22.4 ± 6.0 %). According to normative data from the American College of Sports Medicine, ATI 7 is in poor physical condition for his age (325).

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5.3.3. Physiological characteristics

The maximal oxygen consumption of ATI 2 (59.7 $ml.kg^{-1}.min^{-1}$) was higher than the average value for the rest of the tATI group (48.4 ± 6.2 $ml.kg^{-1}.min^{-1}$). According to ACSM normative data ATI 2 had superior endurance capacity for her age (325). While ATI 2 had a lower maximal voluntary contraction (321 N) compared to the average for the tATI group (538 ± 175 N), her value was not the lowest (Table 5.2).

According to normative data from the American College of Sports Medicine and in comparison with the rest of the tATI athletes (48.4 ± 6.2 $ml.kg^{-1}.min^{-1}$), ATI 5's maximal oxygen consumption (59.2 $ml.kg^{-1}.min^{-1}$) is superior for his age (325). ATI 5 has a greater maximal voluntary contraction (555 N) than the average tATI athlete (538 ± 175 N).

ATI 7 had the lowest maximal oxygen consumption (31.1 $ml.kg^{-1}.min^{-1}$) in the group of tATI athletes (48.4 ± 6.2 $ml.kg^{-1}.min^{-1}$) and according to ACSM normative data he is in poor physiological condition (325). However, ATI 7's maximal voluntary isometric force output (603 N) was higher than the average tATI athlete (538 ± 175 N).

The descriptive and physiological characteristics of the three tATIshort athletes (ATI 2, ATI 5 and ATI 7) compared to those of the tATI athletes are summarized in Table 5.2.

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Table 5.2. Descriptive and physiological characteristics of the three tATIshort athletes (ATI 2, ATI 5, ATI 7) and the tATI athletes.

	tATI (n = 10)	**ATI 2**	**ATI 5**	**ATI 7**
Years with ATI	3 ± 2 (1 ~ 7)	8	1	8
Gender	8 (M) 2 (F)	F	M	M
Age (yrs)	41 ± 11 (22 ~ 57)	36	39	48
Stature (cm)	173.7 ± 8.3 (161 ~ 187)	168	177	179.5
Body mass (kg)	73.3 ± 16.7 (54.5 ~ 110)	54.2	79.1	103.3
% Body fat	22.4 ± 6.0 (13 ~ 31.3)	19.9	18.1	30.7
VO_{2max} ($ml.kg^{-1}.min^{-1}$)	48.4 ± 6.2 (38.8 ~ 58.8)	59.7	59.2	31.1
MVC (N)	538 ± 175 (270 ~ 843)	321	555	603

tATIshort = endurance athletes with acquired training intolerance who presented with pathologically short telomeres. tATI = endurance athletes with acquired training intolerance who did not presented with pathologically short telomeres Values for the tATI group are means ± S.D. with the minimum and maximum values in parentheses. MVC = maximal voluntary contraction.

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5.3.4. Training history

While the age at which ATI 2 started high volume endurance training (HVETage; 18 years) is less than the average HVETage of other tATI athletes (26 ± 11), she was not the youngest committed endurance athlete in the group. ATI 2 was a highly competitive, elite endurance runner. For 12 years, prior to the onset of symptoms, ATI 2 followed a rigorous training schedule. ATI 2 trained a greater number of days

(7 days), km (130 km) and hours (9.5 hrs) per week compared to the average tATI athlete (6 ± 1 days.wk^{-1}, 85 ± 40 km.wk^{-1}, 9 ± 7 hrs.wk^{-1}). Following the onset of acquired training intolerance symptoms, ATI 2 continued to train 5 days.wk^{-1}, covering 50 km.wk^{-1}.

At the age of 32, ATI 5 began high volume endurance training. After 6 years of optimal running performance his performance declined drastically and he began to experience the symptoms associated with acquired training intolerance. The training volume of ATI 5 prior to the onset of ATI symptoms was very similar to that of the average tATI athlete (Table 5.3). ATI 5 trained 6 days.wk^{-1}, covered a training distance of 60 km.wk^{-1} and spent 5 hrs.wk^{-1} doing high volume endurance training. Although ATI 5 reduced the number of days (4 days.wk^{-1}), km (50 km.wk^{-1}) and hours (4.5 hr.wk^{-1}) of training per week following the onset of acquired training intolerance symptoms, his training volume was much greater than the post-symptoms training volume of the average tATI athlete (3 ± 3 days.wk^{-1}, 19 ± 22 km.wk^{-1}, 2 ± 2 hrs.wk^{-1}).

ATI 7 began high volume endurance training much later than the average tATI athlete (40 yrs vs 26 ± 11 yrs). For 3 years prior to the onset of symptoms, ATI 7 trained the least number of days.wk^{-1} compared to the other tATI athletes (4 vs 6 ± 1 days.wk^{-1}) and covered less training distance than the average tATI athlete (50 vs 85 ± 40 km.wk^{-1}). Following the onset of symptoms ATI 7 stopped training completely.

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The endurance training history of the three tATIshort athletes and the tATI athletes are presented in Table 5.3.

Table 5.3. Endurance training history of the three tATIshort athletes (ATI 2, ATI 5 and ATI 7) and the tATI athletes.

	TATI (n = 10)	**ATI 2**	**ATI 5**	**ATI 7**
HVETage (yrs) (tATI n = 9)	27 ± 11 (13 ~ 44)	18	32	40
HVETyrs (yrs) (tATI n = 9)	15 ± 5 (7 ~ 21)	12	7	3
Pre ATI: Training (days.wk $^{-1}$) (tATI n = 8)	6 ± 1 (5 ~ 7)	7	6	4
Post ATI: Training (days.wk $^{-1}$) (tATI n = 8)	3 ± 3 (0 ~ 7)	5	4	0
Pre ATI: Training (km.wk $^{-1}$) (tATI n = 6)	85 ± 40 (45 ~ 160)	130	60	50
Post ATI: Training (km.wk $^{-1}$) (tATI n = 6)	19 ± 22 (0 ~ 45)	50	50	0
Pre ATI: Training (hr.wk $^{-1}$) (tATI n = 8)	9 ± 7 (4 ~ 24)	9.5	5	3.3
Post ATI: Training (hr.wk $^{-1}$) (tATI n = 8)	2 ± 2 (0 ~ 4)	4.3	4.5	0

tATIshort = endurance athletes with acquired training intolerance who presented with pathologically short telomeres. tATI = endurance athletes with acquired training intolerance who did not presented with pathologically short telomeres Values for the tATI group are means ± S.D. with the minimum and maximum values in parentheses. HVETage = age in years at which started high volume endurance training. HVETyrs = years of high volume endurance training.

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5.3.5. Light and electron microscopy

ATI 2 had an extremely high percent of type I muscle fibres (91%; Figure 3.3. panel A). In terms of skeletal muscle structural pathology ATI 2 presented with a high degree of internal nuclei (3+; Figure 3.1A), muscle fibre size variation (3+; Figure 3.1B) and subsarcolemmal aggregations of mitochondria (3+; Figure 3.3 panel C). ATI 2 had the highest total score for structural pathology (9) in the whole tATI group. In accordance with the majority of the tATI athletes, there was no sign of necrosis/inflammation in her muscle sample (Table 5.4).

With regards to the ultra structural pathology present in her muscle, ATI 2 presented with a high degree of Z disc streaming (3+; Figure 3.2A) and focal deletions of myofibrils (3+; Figure 3.2B), and a slightly lesser degree of atrophic fibres (2+; Figure 3.2C). ATI 2's continued effort to train, even with ATI symptoms probably explains why in contrast to the majority of the other tATI athletes she did not present with increased lipid and glycogen deposits in the muscle (Table 5.5). ATI 2 also presented with enlarged mitochondria, most likely as a result of her history of high volume endurance training (Figure 3.3, panel B).

ATI 5 had an average percent of type I muscle fibres (51%). Apart from a 2+ aggregation of subsarcolemmal mitochondria, ATI 5 did not presented with any other signs of skeletal muscle structural pathology (Table 5.4 and Figure 3.3 panel C). ATI 5 did, however, present with a great deal of ultra structural pathology (total ultra structural pathology score = 16, Table 5.5) Although ATI 5 only presented with 1+ Z disc streaming (Figure 3.2A), electron microscopic analysis of his muscle sample

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revealed an extreme degree of focal deletions of myofibrils (3+; Figure 5.2A) as well as muscle fibre atrophy (3+, Figure 3.2C). Enlarged mitochondria as well as subsarcolemmal aggregations of mitochondria were also noted in the muscle sample of ATI 5 (Figure 5.2B and C). In addition to this, extensive accumulations of lipid (3+) and glycogen (3+) droplets were also noted (Figure 3.2D). Since ATI 5 still maintains a relatively demanding training schedule the accumulated lipid and glycogen stores are most likely not as a result of inactivity and consequent decreased utilization of endogenous fuel stores.

Although ATI 7 had quite a low percentage of type I muscle fibres (41%), he did not have the lowest percent observed as ATI 14 had 39% type I fibres. ATI 7 had extensive skeletal muscle structural pathology (3+ size variation, 2+ internal nuclei, 2+ necrosis/inflammation; Figure 5.3). The presence of necrosis and inflammation is indicative of acute skeletal muscle pathology.

In contrast to the extensive pathology present at the structural level in the muscle sample of ATI 7, there were only very minor signs of pathology present at the ultra structural level (Table 5.5). There was no sign of Z disc streaming or atrophic fibres and there were only minor indications of focal deletions of myofibrils (1+; Figure 3.2B) in a muscle sample from ATI 7 (Table 5.5). In contrast to the majority of the tATI athletes, ATI 7's mitochondria were not enlarged nor were they extensively aggregated in the region of the subsarcolemma (1+). There were, however, extensive accumulations of lipid (3+; Figure 5.3) and glycogen (3+; Figure 5.3) droplets present, which may be as a result of ATI 7's present state of inactivity.

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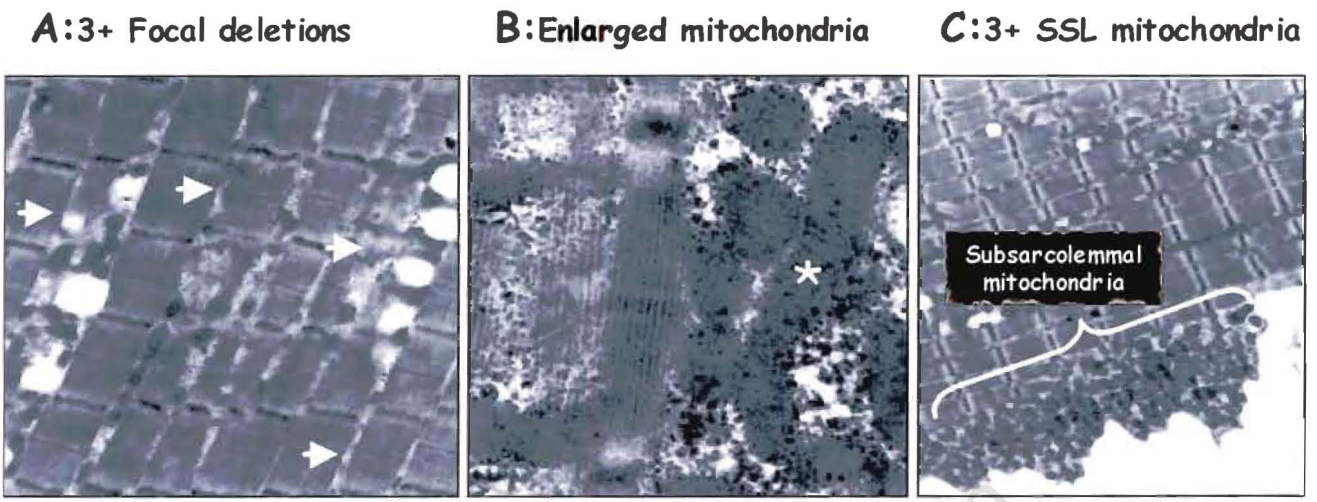

Figure 5.2. Electron micrographs illustrating the extent of focal deletions of myofibrils, indicated by arrowheads (A, X 10 000), enlarged mitochondria, indicated by asterisk (B, X 30 000) and subsarcolemmal aggregations of mitochondria (C, X 4500) in the muscle sample of ATI 5.

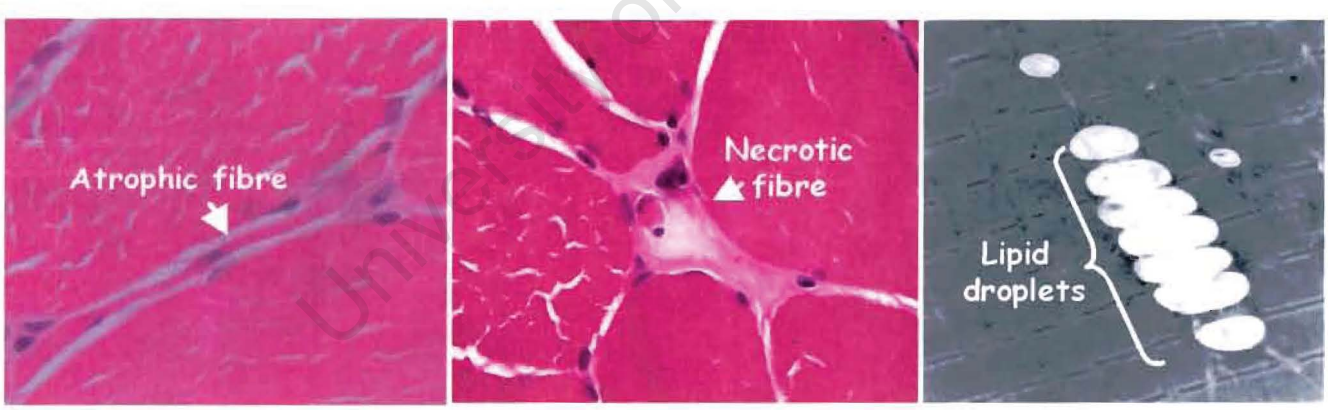

Figure 5.3. Light and electron micrographs illustrating the extent of muscle fibre size variation (A, X 40), necrosis and inflammation (B, X 40) and lipid droplet aggregations (C, X 4500) in the muscle sample of ATI 7.

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Table 5.4. The presence skeletal muscle structural pathology, as determined by light microscopy, in the three tATIshort athletes (ATI 2, ATI 5, ATI 7) and the tATI athletes.

Subject	% Type I fibres	Size variation	Internal nuclei	Necrosis/ inflammation	SSL Mitochondria	Total score
ATI 2	91	3+	3+	0	3+	**9**
ATI 5	51	0	0	0	2+	**2**
ATI 7	41	3+	2+	2+	1+	**8**
ATI 1	53	1+	2+	0	2+	**5**
ATI 3	56	2+	1+	0	2+	**5**
ATI 4	66	1+	0	0	2+	**3**
ATI 6	52	2+	0	0	2+	**4**
ATI 13	67	2+	1+	0	2+	**5**
ATI 14	39	2+	1+	1+	1+	**5**
ATI 15	53	1+	1+	0	2+	**4**
ATI 16	43	0	0	0	1+	**1**
ATI 19	31	0	0	0	1+	**1**
ATI 20	39	0	0	0	1+	**1**

tATIshort = endurance athletes with acquired training intolerance who presented with pathologically short telomeres. tATI = endurance athletes with acquired training intolerance who did not presented with pathologically short telomeres. SSL = subsarcolemmal aggregations of mitochondria.

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Table 5.5. The presence skeletal muscle ultra structural pathology, as determined by electron microscopy, in the three tATIshort athletes (ATI 2, ATI 5, ATI 7) and the tATI athletes.

Subjects	Z disc	Focal	Atrophic	Mitochondria	SSL	Lipid	Glycogen	Total score
ATI 2	3+	3+	2+	Enlarged	2+	0	0	**10**
ATI 5	1+	3+	3+	Enlarged	3+	3+	3+	**16**
ATI 7	0	1+	0	Normal	1+	3+	3+	**8**
ATI 1	0	3+	0	Enlarged	2+	3+	3+	**11**
ATI 3	3+	3+	2+	Normal	2+	3+	3+	**16**
ATI 4	0	3+	0	Enlarged	1+	3+	3+	**10**
ATI 6	1+	3+	0	Enlarged	1+	3+	3+	**11**
ATI 13	0	1+	1+	Enlarged	1+	0	0	**3**
ATI 14	0	1+	1+	Enlarged	0	0	0	**2**
ATI 15	0	1+	2+	Normal	3+	0	0	**6**
ATI 16	3+	1+	0	Normal	0	0	0	**4**
ATI 19	2+	3+	0	Enlarged	0	0	3+	**8**
ATI 20	3+	1+	0	Normal	0	0	0	**4**

tATIshort = endurance athletes with acquired training intolerance who presented with pathologically short telomeres. tATI = endurance athletes with acquired training intolerance who did not presented with pathologically short telomeres. Z disc = Z disc streaming, Focal = focal deletions of myofibres, Atrophic = atrophic muscle fibres, Mitochondria = size of mitochondria, SSL = subsarcolemmal aggregations of mitochondria, Lipid = lipid deposits and Glycogen = glycogen deposits.ND = Not determined.

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5.3.6. Medical history

The results of the medical history questionnaire revealed that in her late adolescence and early twenties ATI 2 was hospitalized on nine separate occasions as a result of her suffering from the eating disorder, anorexia nervosa. ATI 2 was diagnosed with anorexia nervosa for most of her 12 year running career. Furthermore, at the age of 46, ATI 7 was diagnosed as having adult onset, type II diabetes. He is presently being treated with Diamicron® (Gliclazide) and his diabetes is reportedly well controlled. None of the other tATI athletes, including the third tATIshort athlete, ATI 5, presented with any notable medical conditions.

5.4. DISCUSSION

As previously discussed (Chapter 4.1), in a sample of skeletal muscle, the maximum value of the telomeric restriction fragment (maxTRF) predominately reflects the telomere length of the post-mitotic myonuclei. The DNA of the majority of the post-mitotic myonuclei was incorporated early on in the individual's life, prior to the satellite cells having undergone numerous replicative cycles. In addition to this, once the DNA has been incorporated into the muscle cell it no longer replicates, thus halting any further loss of telomeric DNA in the myofibre. Therefore, the telomeric DNA of the post-mitotic myonuclei would be longer than the telomeric DNA present in any of the other cells in the muscle sample (Chapter 4.1) (104;417). The minimum value of the telomeric restriction fragment (minTRF) primarily reflects the telomere lengths of the satellite cell DNA as well as the DNA that has recently

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been incorporated into post-mitotic muscle cells. This DNA would have undergone numerous replicative cycles and as a result a relatively greater amount of telomeric DNA would have been lost (104;417).

Healthy, adult skeletal muscle is a stable tissue with a low turnover of post-mitotic myonuclei and satellite cells over an individual's lifetime (62;104;492). This is reflected in the stability of the maximum value of the telomeric restriction fragment and the relatively small decrease in the minimum value of the telomeric restriction fragment (13 base pairs per year), *in vivo*, over an individual's lifetime (106). Despite this, however, results of the present chapter revealed that within the group of endurance athletes with acquired training intolerance, there were three athletes in particular (tATIshort), who presented with significantly reduced mean, minimum and maximum telomere lengths compared to the remainder of the group (tATI).

As mentioned above, there is a small, but significant, loss of approximately 13 base pairs of telomeric DNA per year, as reflected by the reduction in the minimum value of the telomeric restriction fragment, in healthy skeletal muscle (104;417). In the skeletal muscle from an individual with Duchenne's muscular dystrophy there is a loss of approximately 187 base pairs of telomeric DNA, as indicated by the reduction in the minimum telomeric restriction fragment, per year (104). This profound increase in the loss of telomeric DNA reflects the extensive proliferation of satellite cells involved in the continual process of regeneration that is associated with skeletal muscle dystrophy (104;417).

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A significantly smaller minimum telomeric restriction fragment value, in the presence of stable or unchanged maximum and mean telomeric restriction fragment values, as was the case in the previous chapter (Chapter 4), primarily reflects an increased proliferation of satellite cells. The minimum value of the terminal restriction fragments observed for the three tATIshort athletes (Table 5.1) were even shorter than the values reported for individuals with Duchenne's muscular dystrophy (5.31 ± 0.93 kbp for a group of Duchenne's muscular dystrophy patients whose average age was 8 years) (104). This result may reflect the presence of extensive proliferation of satellite cells in the skeletal muscle of these three athletes, similar to that which would occur in dystrophied muscle.

An in depth investigation of the descriptive, physiological and endurance training characteristics of the three tATIshort athletes, failed to reveal any commonalities between the three subjects that might explain the presence of the shortened telomeres. All three tATIshort athletes did however present with extensive skeletal muscle pathology. Even though the skeletal muscle pathology was not specific to a particular skeletal muscle myopathy or dystrophy, nor was it expressed in a similar pattern in the three athletes, it is speculated that the increased presence of skeletal muscle pathology in these athletes would require an increased proliferation of satellite cells involved in the repair process. Therefore, the significantly reduced minimum TRF value, predominantly indicative of skeletal muscle satellite cell telomere length, might very well be associated with the increased presence of skeletal muscle pathology in the muscle of the three athletes.

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While it is possible that the high volume endurance training and racing history of the three tATIshort athletes is a likely cause of the increased skeletal muscle pathology, the medical conditions of ATI 2 and ATI 7 may also be implicated in the increased muscle pathology and the enhanced proliferation or turnover of satellite cells.

Severe anorexia nervosa is associated with metabolic abnormalities and profound skeletal muscle pathology that is characterized by selective atrophy of type II muscle fibres (348;349). The selective atrophy of type II muscle fibres associated with severe anorexia nervosa could account for the extremely high percent of type I muscle fibres (91%) observed in the muscle sample of ATI 2. Furthermore, muscles are a rich source of amino acids that in normal healthy individuals are very rarely utilized. In order to meet the energy demands of her high volume endurance training and racing, it is possible that in its malnourished state ATI 2's body was forced to utilize the amino acid stores of the skeletal muscle. The muscle would therefore be in a constant state of catabolism and degeneration and repair of the degenerated muscle would result in an increased proliferation of satellite cells and a subsequent decrement in the minimum telomere length.

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It is interesting to speculate that perhaps as a result of her malnourished state, ATI 2 deprived her muscles of the necessary "building blocks" for successful and complete repair of any muscle damage that may have been induced by her extensive endurance training and racing schedule. The damaged muscles would continue to send out distress signals and repair

would be attempted but would not be completed. A continuous cycle of damage and incomplete repair would perpetuate the turnover of satellite cells, resulting in a dramatically reduced minimum telomere length. This hypothesis deserves further investigation, particularly with the high incidence of anorexia nervosa and other eating disorders observed in female endurance athletes (217;478)

Diabetes mellitus is characterised by a relative lack of, or insensitivity to insulin, resulting in high blood glucose and cellular starvation (42;45). Amongst other things, diabetes is associated with muscular weakness and wasting (42;90;331). Research has shown that even acute diabetes is a potent stimulus for muscle protein degradation via the ubiquitin-proteasome proteolytic pathway (408;409). ATI 7 reported experiencing symptoms associated with acquired training intolerance for 8 years prior to this trial. He was diagnosed with diabetes 2 years before the trial. It is interesting to speculate that although ATI 7 reported his diabetes being well controlled under present medication, it is possible that his diabetes was undiagnosed, and therefore untreated, for a number of years. The increased protein degradation and skeletal muscle pathology associated with diabetes, particularly uncontrolled diabetes, could contribute to the increased proliferation and turnover of satellite cells involved in the repair process of this individual's skeletal muscle.

The skeletal muscle pathology associated with both anorexia nervosa and diabetes may provide an alternative or additional explanation for the

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increased turnover of satellite cells and the significantly reduced minimum telomeric restriction fragment value. The skeletal muscle pathology does not, however, account for the significantly reduced maximum telomeric restriction fragment values observed in the skeletal muscle of the three tATIshort athletes.

As mentioned previously, there is a very low turnover of post-mitotic myonuclei in healthy, adult skeletal muscle, such that a decrease in the maximum telomere length is rarely observed throughout an individual's lifetime (104;492). In contrast to this, the three tATIshort athletes presented with significantly reduced maximum telomeric restriction fragment values. The fact that both the minimum and the maximum values of the telomeric restriction fragments were profoundly reduced in the three tATIshort athletes indicates an increased loss of telomeric DNA not only in the satellite cells but also in the myonuclei. This finding suggests that additional or alternative mechanisms, besides extensive satellite cell proliferation, are responsible for the pathological shortening of the telomeres in the skeletal muscle of these athletes.

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There are a number of possible reasons that might explain the dramatically reduced maximum telomere lengths in the three tATIshort athletes. For example, a study investigating telomere length in the leucocytes of twins observed that 78% of the interindividual differences in the mean telomere length was genetically detemined (454). Furthermore, researchers have also observed individual differences in the structure of the telomeric t loop, which

might impact on the degree of telomeric degradation or synthesis that occurs in the end region of the telomeres (40;219). An additional, and perhaps more likely mechanism in the case of the three tATIshort athletes, is oxidative damage, specifically to the telomeric DNA of the post-mitotic myonuclei (231;511;512).

There are a number of factors to support this hypothesis. Firstly, it has been observed that telomeres are susceptible to oxidative damage, with the GGG triplets of the telomeric repeats particularly prone to attack by free radicals (231;435;511). Secondly, the post-mitotic skeletal muscle cells are also susceptible to the accumulation of oxidative damage (412). Thirdly, high volume endurance training and racing is associated with an increased production of damaging reactive oxygen and nitrogen species, which are byproducts of exercise-induced increase in metabolism (234;415;504). Fourthly, diabetes and the subsequent hyperglycemia are associated with increased oxidative stress in skeletal muscle (42;185;237;254). Finally, the malnutrition associated with anorexia nervosa may result in a sub-optimal intake of vitamins and minerals, which are essential for the maintenance of the antioxidant defense system (353). The compromised antioxidant defense system would further contribute to an increased oxidative state and an increased incidence of oxidative damage to the telomeres in the muscle tissue of these athletes (253;353;364;463).

The shortest mean (meanTRF), minimum (minTRF) and maximum (maxTRF) terminal restriction fragments of the tATIshort athletes were observed in ATI

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5, who did not present with a specific medical condition. It is possible that ATI 5 has an undiagnosed medical condition or compromised antioxidant defense system, both of which may contribute to the dramatic reduction in telomere length. Since there was no underlying medical condition present in ATI 5, that could possibly have exacerbated the shortening of the this individual's telomeres, ATI 5 may be an example of the detrimental effects of high volume endurance training and racing, over a number of years, on skeletal muscle and its resident satellite cells.

Apart from the findings of the previous chapter (Chapter 4), there is presently no scientific evidence to associate high volume endurance training and racing over a number of years, anorexia nervosa or diabetes with accelerated telomere shortening. There is, however, evidence to show that all three factors are associated with skeletal muscle pathology (90;197;230;331;348;349;517), an increased oxidative state (42;185;234;415;504) and/or a compromised antioxidant defense system (253;353;364;463). It is possible that the skeletal muscle pathology, increased oxidative state and/or compromised antioxidant defense system associated with excessive exercise alone, in the case of ATI 5, and in combination with conditions such as anorexia (ATI 2) and diabetes (ATI 7) may place excessive regenerative demands on the skeletal muscle of endurance athletes with acquired training intolerance.

In conclusion, there is evidence of pathologically short minimum and maximum telomeric restriction fragment values in endurance athletes with

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acquired training intolerance. It is possible that high volume endurance training alone and in conjunction with a diseased state could be implicated in the excessive turnover of satellite cells and post-mitotic myonuclei in these athletes.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

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In addition to inducing adaptations that are beneficial to the health and well being of the individual, exercise may also cause considerable damage, particularly to the skeletal muscle system. Under normal circumstances, skeletal muscle is capable of repairing this damage and adapting to the demands placed on it (reviewed in section 1.3 and 1.4). However, based on research investigating the aging process (see section 1.5.1) and the overtraining syndrome (section 1.5.2) there is a limit to the regenerative capacity of skeletal muscle. Exhaustion of the finite regenerative capacity of skeletal muscle manifests in maladaptations of both skeletal muscle function (138;458) and morphology (25;224;523). Anecdotal evidence regarding the training and racing practises of endurance athletes together with evidence from case studies suggests that the repeated bouts of muscle damage and repair, induced by endurance training and racing over a number of years, may exceed the limits of the repair and adaptation process of skeletal muscle.

There are no studies that have systematically examined the impact of repeated bouts of muscle damage and repair over a number of years on the structure and function of skeletal muscle. The specific objectives of this thesis, therefore, were to systematically examine the i) functional and ii) morphological characteristics and the iii) regenerative history of the skeletal muscle of endurance athletes who, after a number of years of high volume training and racing, were unable to tolerate training volumes to which they were previously accustomed (ATI group). The findings were then compared to those of control athletes who were matched for age and years of endurance

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training, but who did not have symptoms of acquired training intolerance (CON group).

i) Skeletal muscle function

The athletes with acquired training intolerance were well matched with the control athletes for age, gender, height, weight and % body fat. Furthermore, there was no significant difference in the peak oxygen consumption ($VO_{2\,max}$) and maximal voluntary contraction (MVC) between the two groups. Therefore, despite the inability of the athletes with acquired training intolerance to endure training loads to which they were previously accustomed and to maintain expected levels of racing performance, they were still able to perform short-term, maximal exercise.

A general complaint among the athletes with acquired training intolerance was the inability of their muscles to resist fatigue during exercise and to recover appropriately from an exercise bout. The VO_{2max} test and the MVC test, although valid measures of the muscle's maximal oxygen uptake and force output respectively, do not allow one to draw any conclusions regarding the ability of the muscle to resist fatigue or to recover from a potentially damage-inducing bout of exercise. With hindsight, an exercise test that challenged the athletes' ability to resist fatigue over a longer period of time, at a more submaximal level of exercise intensity, may have been a more appropriate means by which to assess the functional capacity of the athletes with acquired training intolerance.

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The ATI and CON athletes were well matched for the age at which they started high volume endurance training as well as for the number of years of high volume endurance training. However, prior to the onset of the acquired training intolerance, the athletes with acquired training intolerance trained a significantly greater number of hours, days and kilometres a week, compared to the control athletes. Although we do not have evidence to substantiate this, it is tempting to speculate that the increased training volume and subsequent reduced recovery time between training sessions of the ATI athletes not only increased the amount of muscle damage but also impaired the repair process.

ii) Skeletal muscle morphology

Although there was structural and ultra structural damage present in some of the muscle samples of the asymptomatic control athletes, a significantly greater proportion of athletes with acquired training intolerance presented with structural (internal nuclei and variation in muscle fibre size) and ultra structural (Z disc streaming and excessive accumulations of lipid and glycogen) pathology compared to the control athletes. Furthermore, the athletes with acquired training intolerance presented with a significantly greater degree of pathology compared to the control athletes. These results establish an association between increased skeletal muscle disruptions and the inability to tolerate endurance training loads. The extent of this association and whether any causality exists requires further investigation.

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iii) Regenerative history of the skeletal muscle

The results of Chapter 3 revealed that a greater proportion of athletes with acquired training intolerance presented with evidence of residual structural and ultra structural skeletal muscle damage, compared to control athletes. The presence of residual muscle damage implied the presence of incomplete repair or increased muscle damage, or a combination of the two. Satellite cells associated with the muscle would be activated to proliferate and participate in the repair process (280).

Telomeres are sections of non-coding DNA that occur at the ends of all eukaryotic chromosomes (104;219;231). Replication of linear DNA results in the loss of 50 – 200 base pairs of telomeric DNA per cell division (231;260;417). Therefore, measurement of the telomere length is an indirect marker of the regenerative history of the cell (417). Accordingly, the regenerative history, as indicated by the length of the telomeres, of the skeletal muscle of athletes with acquired training intolerance and asymptomatic control athletes were examined.

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The athletes with acquired training intolerance presented with significantly shorter minimum telomere lengths compared to control athletes. These findings suggest an increased proliferation of satellite cells in the muscle of athletes with acquired training intolerance. It was speculated that the increased presence of skeletal muscle pathology in these athletes, possibly as a result of the years of high volume endurance training and racing, induced an increased proliferation of satellite cells involved in the repair process.

Closer inspection of the telomere lengths of the group of athletes with acquired training intolerance revealed that three of the athletes had significantly shorter mean, maximum and minimum telomere lengths. While one of the athletes suffered from anorexia nervosa and another was diagnosed with type II diabetes, the third athlete was free of any such medical complication or disorder.

While an excessive proliferation of satellite cells could account for the dramatically reduced minimum telomere length, the dramatically shortened maximum telomere lengths of these three athletes implies the contribution of alternative or additional mechanisms in the shortening of their telomeres. The most likely mechanism responsible for the dramatic shortening of the maximum telomere length in these athletes, was postulated to be oxidative damage, specifically to the telomeric DNA of the post-mitotic myonuclei (231;511;512).

There is presently no scientific evidence to associate high volume endurance training and racing over a number of years, anorexia nervosa or diabetes with accelerated telomere shortening. There is, however, evidence to show that all three factors are associated with skeletal muscle pathology (90;197;230;331;348;349;517), an increased oxidative state (42;185;234;415;504) and/or a compromised antioxidant defence system (253;353;364;463). It is possible that the skeletal muscle pathology, increased oxidative state and/or compromised antioxidant defence system associated with excessive exercise alone, and in combination with conditions such as

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anorexia and diabetes may place excessive regenerative demands on the skeletal muscle of endurance athletes with acquired training intolerance.

In conclusion, we suggest that although skeletal muscle has a remarkable capacity to repair and adapt to muscle damage induced by prolonged endurance exercise, there is a limit to this capacity for repair and adaptation. The inability of athletes with acquired training intolerance to endure training loads to which they were previously accustomed suggests that these athletes have exhausted the finite capacity of their muscles to repair and adapt to exercise-induced muscle damage. The aim of this thesis was to examine the function, morphology and regenerative history of the skeletal muscle of athletes with acquired training intolerance. While the short term, maximal muscle function of these athletes was not affected; an increased proportion of athletes with acquired training intolerance presented with a greater degree of skeletal muscle pathology compared to asymptomatic control athletes. Furthermore, the regenerative history of the skeletal muscle of athletes with acquired training intolerance was far more extensive than that of asymptomatic control athletes.

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It is postulated that the high volume endurance training and racing, over a number of years, induces skeletal muscle damage that requires repair by satellite cells associated with the muscle fibres. The rigorous training and racing regime of most endurance athletes rarely incorporates sufficient time for the muscles to recover completely from previous bouts of muscle damage. As a result, these athletes often train and race on already damaged muscles.

In addition to increasing the risk of incurring even greater skeletal muscle damage, this practise may hinder or exhaust the repair process. The increased degree of skeletal muscle disruptions in the muscle of the athletes with acquired training intolerance is evidence of there being either increased damage or decreased repair, or a combination of both, in the skeletal muscle of these athletes. The increased presence of residual muscle damage may lead to an increased proliferation of satellite cells involved in the repair process. The significantly decreased minimum telomere length in the skeletal muscle of these athletes provided evidence for this. Research has shown that once the telomeres reach a certain length the cell will no longer divide. It is therefore postulated that excessive regenerative demands are placed on the satellite cells as a result of the muscle damage induced by years of high volume endurance training and racing. The increased proliferation of satellite cells leads to a reduction in the length of the telomeres to a point at which the cells will no longer divide and participate in the repair process. This will result in there being incomplete repair and a further increase in the residual muscle damage.

It is interesting to speculate that the acquired training intolerance might be a protective mechanism, against further damage in the skeletal muscles. Alternatively, the acquired training intolerance may be a manifestation of the muscle's inability to function appropriately as a result of the increased residual muscle damage.

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The impact of repeated bouts of muscle damage and repair, over a number of years, on skeletal muscle function, morphology and regenerative capacity requires further investigation so that sound training and racing advice may be given to endurance athletes which will enable them to enhance their performance and minimise their risk of depleting the regenerative resources of their skeletal muscle.

Future research

The results of this thesis have inspired a plethora of interesting and exciting research questions that require further investigation. For instance, based on the cytokine hypothesis of overtraining (see section 1.5.2), proposed by Smith (458), it would be very interesting to examine the plasma levels of circulating cytokines, interleukin-6 in particular, in athletes with acquired training intolerance and compare it with asymptomatic control athletes. It would also be useful to determine whether the circulating levels of cytokines are in any way related to the increased degree of skeletal muscle pathology in these athletes.

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Furthermore, it would be interesting to compare the regenerative capacity of the skeletal muscle of athletes with acquired training intolerance to asymptomatic control athletes who were matched for age, volume and years of endurance training. For this experiment muscle damage would be induced in a controlled manner in the laboratory and the regeneration process would be monitored.

It would also be very interesting to assess the oxidative and antioxidative status of the skeletal muscle of athletes with acquired training intolerance, compared to that of asymptomatic control athletes. This would provide us with a better understanding of the role of oxidative damage in the shortening of the telomeres, particularly in post mitotic skeletal muscle cells.

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INFORMED CONSENT

I, the undersigned, have been fully informed about the potential risks associated with participation in this trial. I also understand that the following tests/measurements will be carried out as part of this trial :

- Routine medical examinations
- Routine haematological investigation, including viral screening
- Lifestyle and sporting history questionnaire
- Performance testing
- Anthropometric measurements
- Isokinetic muscle strength testing
- Skeletal muscle biopsy

The University of Cape Town and the investigators are not liable for any harm or damage suffered by me during the course of the trial or arising from the trial.

I understand that I am free to withdrawal from the trial at any time, without prejudice. All of the investigation that is collected during the course of the investigation will be treated

.

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with the strictest confidentiality and will only be used for scientific research purposes. Names and personal particulars will not be released under any circumstances. I will be free to ask any questions about the procedures and results of the study.

Subject:_____

Signature:_____

Date:_____

Investigator:_____

Signature:_____

Witness:_____

Signature:_____

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RUNNING HISTORY

1. At what age did you start running > 40 km/week? _____ yrs
2. For how many years did you train before you noticed problems starting which led you to volunteer for this trial? _____ yrs
3. How many years has it been since you were last able to run at your perceived optimal capacity?_____ yrs
4. How many days a week did you train prior to your performance deterioration? _____ days
5. How many days a week do you train at present? _____ days
6. What was your average weekly training distance prior to your performance deterioration? _____ km
7. What is your weekly training distance at present? _____ km
8. What was your average training speed prior to your performance deterioration? _____ km/hr
9. What is your average training speed at present _____ km/hr
10. What was your best 5 km time trial time prior to your performance deterioration? _____ min Year:_____
11. What is your best 5 km time trial result in the month prior to starting the trial? _____ min

12. PRIOR TO YOUR PERFORMANCE DETERIORATION, how many time a week did you train specifically for:

Speed (Fartlek/Sprints)	_____/week
Endurance (Long runs)	_____/week
Strength (Gymnasium)	_____/week
Flexibility (Stretching)	_____/week

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MEDICAL CONDITIONS

PRIOR TO DETERIORATION IN PERFORMANCE

18. Did you suffer from any medical problems (eg asthma, high blood pressure, cancer).

Y N

19. If yes please name the problems and the dates they occurred and how they were treated.

ILLNESS DATE TREATMENT

AT THE TIME AND DIRECTLY RELATED TO DETERIORATION IN PERFORMANCE

20. Did you suffer any medical problems (eg asthma, high blood pressure, cancer). Y N

21. If yes please name the date it occurred and how it was treated

ILLNESS DATE TREATMENT

AFTER DETERIORATION IN PERFORMANCE

22. Did you suffer any medical problems (eg asthma, high blood pressure, cancer). Y N

23. If yes please name the dates they occurred and how they were treated

ILLNESS DATE TREATMENT

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SURGICAL CONDITIONS

PRIOR TO DETERIORATION IN PERFORMANCE

18. Did you suffer from any surgical problems (eg gall bladder operations, trauma, orthopaedic operations). Y N

24. If yes please name the problems and the dates they occurred and how they were treated.

INJURY DATE TREATMENT

AT THE TIME AND DIRECTLY RELATED TO DETERIORATION IN PERFORMANCE

25. Did you suffer from any surgical problems (eg gall bladder operations, trauma, orthopaedic operations). Y N

26. If yes please name the date it occurred and how it was treated

INJURY DATE TREATMENT

AFTER DETERIORATION IN PERFORMANCE

27. Did you suffer any from any surgical problems (eg gall bladder operations, trauma, orthopaedic operations). Y N

28. If yes please name the dates they occurred and how they were treated

INJURY DATE TREATMENT

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DRUG HISTORY

PRIOR TO DETERIORATION IN PERFORMANCE

18. Did you ever take any type of medication or drugs (Prozac, anti-hypertensives, anabolic steroids. Y N

39. If yes please name the problems and the dates they occurred and how they were treated.

MEDICATION DATE TREATMENT

AT THE TIME AND DIRECTLY RELATED TO DETERIORATION IN PERFORMANCE

40. Did you take any type of medication or drugs (Prozac, anti-hypertensives, anabolic steroids). Y N

41. If yes please name the date it occurred and how it was treated

MEDICATION DATE TREATMENT

AFTER DETERIORATION IN PERFORMANCE

42. Did you ever take any type of medication or drugs (Prozac, anti-hypertensives, anabolic steroids) Y N

43. If yes please name the dates they occurred and how they were treated

MEDICATION DATE TREATMENT

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DIETARY HISTORY

PRIOR TO DETERIORATION IN PERFORMANCE

18. Did you ever change your diet dramatically or decrease or increase your food intake (eg Pritikin diet). Y N

44. If yes please name the problems and the dates they occurred and how they were treated.

DIET DATE TREATMENT

AT THE TIME AND DIRECTLY RELATED TO DETERIORATION IN PERFORMANCE

45. Did you ever change your diet dramatically or decrease or increase your food intake (eg Pritikin diet). Y N

46. If yes please name the date it occurred and how it was treated

DIET DATE TREATMENT

AFTER DETERIORATION IN PERFORMANCE

47. Did you ever change your diet dramatically or decrease or increase your food intake (eg Pritikin diet). Y N

48. If yes please name the dates they occurred and how they were treated

DIET DATE TREATMENT

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MAXIMAL OXYGEN CONSUMPTION (VO_{2max})

Maximal oxygen consumption (VO_{2max}) was determined using a continuous, incremental running protocol on a motor-driven treadmill (Quinton Instruments, Seattle, WA, U.S.A.). Subjects began running on a horizontal treadmill at 10 $km.hr^{-1}$. The speed was increased by 0.5 $km.hr^{-1}$ every 30 seconds thereafter. Oxygen consumption was measured continuously (Oxygen Alpha, Jaeger/Mijnhardt, Gronnengen, The Netherlands). The test continued until the subject was unable to maintain the pace of the treadmill. VO_{2max} was defined as the highest oxygen consumption recorded during the test over a 30-second period.

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MAXIMAL VOLUNTARY CONTRACTION (MVC)

The maximal voluntary contraction of the right knee extensor muscles of each subject was measured using a Kin-Com isokinetic dynamometer (Chattanooga Group Inc., USA). Subjects were secured to the dynamometer via shoulder and waist strapping. The axis of rotation of the dynamometer was visually aligned with the lateral femoral epicondyle, with the lower leg attached to the lever arm slightly above the level of the lateral malleolus. The knee was positioned at an angle of 60° of flexion, with the reference point being full knee extension. Prior to performing a maximal voluntary isometric contraction, the subjects performed four sub-maximal familiarization trials. Subjects were verbally encouraged throughout all trials to exert maximal effort. The highest of four MVC trials was used for subsequent analysis.

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0.1M sodium barbitone	5ml
0.18M calcium chloride	2.5ml
Distilled water	17.5ml
ATP (disodium salt)	0.075g

Adjust pH with NaOH. Use a relatively concentrated NaOH initially but once get closer to desired pH use very dilute. Solution will go milky as it nears pH 9.4. (**Place solution in oven at 37 degrees centigrade while cutting sections)

METHOD

1. Cut 10u cryostat section for the pH 9.4 ATPase and 20u for the pH 4.3 and pH 4.6 ATPase. Air-dry sections for at least 10 mins.

2. Pre-incubate sections at the specific buffers (ph 9.4, 4.3, 4.6) for 15 min.

3. Discard buffers and incubate all sections at pH 9.4 for 5 min.

4. Incubate all sections in the pre-warmed ATP solution at 37^0C for the appropriate times:

pH 9.4	7 mins
pH 4.6	17 mins
pH 4.3	32 mins

5. Wash well in 0.09M calcium chloride
   (stock $CaCl_2$ diluted 1:1 with distilled water)

6. Place in 2% Cobalt Chloride for:

pH 9.4	3 mins
pH 4.3 and 4.6	6 mins

7. Wash well in 0.01M Sodium Barbitone
8. (stock sodium barbitone diluted 1:10 with distilled water)

9. Rinse in distilled water

10. Develop the colour in dilute Ammonium Sulphide in the fume cupboard.
    (1% for pH 9.4 and 7% for pH 4.6 and 4.3)

11. Wash slides in running tap water for about 10 mins to get rid of excess ammonium sulphide

12. Dehydrate in various alcohol solutions, clear and mount in synthetic resin (DPX mounting stuff) and cover with coverslips.

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NADH

(Nicotinamide Adenine Dehydrogenase)

Solutions

0.2M Tris Buffer (ph 7.4)	100ml
Tris hydroxymethyl amino methane	0.606 g
Distilled water	58 ml
0.1M HCL	42 ml

Substrate

0.2M Tris Buffer (ph 7.4)	10 ml
NBT (Tetrazolium salt)	0.01 g
NADH (Reduced phosphopyridene nucleotide)	0.008 g

Method

1. Cut sections at 7 μm and air dry.
2. Incubate in the above substrate at 37°C for 30 – 45 minutes.
3. Rinse in distilled water.
4. Mount in glycerine jelly.

Results

NADH will stain blue. The darker the blue hue the greater the oxidative potential of the cell.

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SUCCINATE DEHYDROGENASE

Reagents

Solution A: Stock succinate solution (0.6M)

Sodium succinate	4.05 g
Distilled water	20 ml
1M HCL	0.13 ml

Adjust to ph 7 and make total volume of 25 ml. Store at –4°C.

Solution B: Tetrazolium solution (NBT)

Nitroblue tetrazolium (4mg/ml)	5 ml
0.2M tris buffer, ph 7.4	5 ml
Distilled water	7 ml

Final incubating solution

Add 1 ml of solution A to 9 ml solution B just before use.

Bakers Formal Calcium

0.1 M Calcium acetate	8.8 g / 500ml
Formaldehyde	20 ml

Weigh out 8.8 g Calcium acetate, add 400 ml distilled water and mix well. When dissolved add the formaldehyde and finally make up to volume of 500 ml.

Method

1. Cut 15μm sections and allow to air dry.
2. Incubate sections in final incubating solution at 37°C for about 45 minutes.
3. Drain slides and place in Baker's formal calcium at room temperature for 15 minutes.
4. Wash well in distilled water.
5. Mount in glycerine jelly.

Results

Mitochondria will stain up purple.

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HAEMOTOXYLIN AND EOSIN

Mayers haemotoxylin

Haemotoxylin	4 g
Sodium Iodate	0.4 g
Potassium alum (alum.potassium.sulfate)	100 g
Distilled water	2 L
Chloral hydrate	100 g
Citric acid	2 g

1. Dissolve the haemotoxylin, Na iodate and pot.alum in distilled water overnight.
2. Add chloral hydrate and citric acid the next day.
3. Then boil the solution for 5 min in a well-ventilated room. It is ready for use once cool.

Eosin

Stock solution: 1% aqueous eosin
1% aqueous phloxine

Mix 1 part phloxine to 2 parts eosin (500 ml: 1000 ml).

Working solution: 150 ml stock solution
150 ml tap water

Method

1. Stain in haemotoxylin for 10 min.
2. Briefly wash in tap water to remove haemotoxylin.
3. Differentiate (3 dips) in 1% acid alcohol (99 ml 70% Alcohol + 1 ml conc HCL).
4. Blue sections in running tap water for 8 minutes.
5. Stain in eosin for 10 mins.
6. Wash sections briefly in tap water (just to wash excess eosin off slides and rack)
7. Dehydrate slides in alcohol: 1 minute in 70%, 90% and absolute alcohol.
8. Clear sections in xylol (3 dishes; one dip in each dish).

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Electron microscopy: Processing schedule for small bocks

(using the Lynx automatic processor)

Solutions:

1. Cacodylate buffer

Solution A:

Sodium cacodylate [$Na(Ch_3)2AsO_2.3H2O$]	8.56 g	12.84 g	21.4 g	42.8 g
Distilled water (sabax water)	200 ml	300 ml	500 ml	1000 ml

Solution B: 0.2M HCl

Concentrated HCl	10 ml
Distilled water (sabax water)	603 ml

0.2M working solution: pH 7.2 (200ml)

Solution A	50 ml	125 ml
Solution B	4.2 ml	10.5 ml
Distilled water (sabax water)	up to 200 ml	500 ml

0.1M working solution:

0.2M working solution: pH 7.2 (200ml)	100 ml	
Distilled water (sabax water)	100 ml	

2. 3% Gluteraldehyde in 0.1M Cacodylate buffer

For 500 ml of 3% Gluteraldehyde:

25% Gluteraldehyde		60 ml	120 ml
0.2M Cacodylate buffer	up to	250 ml	500 ml
Distilled water (sabax water)	up to	500 ml	1000 ml

Aliquot into 4ml amounts and store in 4 °C

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Precautions:

Sodium Cacodylate

Toxic by inhalation or if swallowed
Keep locked up and when using do not eat, drink or smoke
If you feel unwell seek medical advice
After contact with skin, wash off with plenty of water
Work under a fume hood or well-ventilated area
Wear gloves

25% Gluteraldehyde (EM grade)

Harmful if swallowed
Irritant and harmful vapour
Avoid contact with eyes, skin and respiratory system-work under a fume hood or well-ventilated area
Store at 4 °C.

Osmium tetroxide

Very toxic by inhalation and if swallowed
Causes burns
Avoid contact with skin and eyes
Work under fume hood only and wear gloves at all times

Uranyl acetate

Very toxic by inhalation and if swallowed
Danger of cumulative effects
When using do not eat, drink or smoke
In case of an accident or if you feel unwell, seek medical advice immediately
Work under fume hood or well-ventilated area
Wear gloves

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ELECTRON MICROSCOPY PROCESSING SCHEDULE PROGRAM 1

(Total time: 5 hours, 30 mins)

1	Gluteraldehyde/cacodylate buffer	5 mins	4 °C
2	Cacodylate buffer-squeeze bottle	5 mins	4 °C
3	2% Osmium tetroxide	1 hour	4 °C
	(mix 4:2 with 0.2M Cacodylate)		
4	Cacodylate buffer-squeeze bottle	5 mins	4 °C
5	Uranyl acetate	30 mins	22 °C
6	70% Alcohol	5 mins	22 °C
7	85% Alcohol	10 mins	22 °C
8	95% Alcohol	10 mins	22 °C
9	Absolute alcohol	10 mins	22 °C
10	Absolute alcohol	10 mins	22 °C
11	Absolute alcohol	10 mins	22 °C
12	50:50 Absolute alcohol/Spurrs	45 mins	22 °C
13	Spurrs resin	10 mins	50 °C
14	Spurrs resin	1 hour	50 °C
15	Spurrs resin	1 hour	50 °C

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LEAD STAIN FOR ELECTRON MICROSCOPY

Solutions

2.66 g Lead nitrate

3.52 g Trisodium citrate

Add both of the above to 60 ml distilled water and place on the mixer for several hours. Add 16 ml of 10N NaOH (10N NaOH: 4 g in 100 ml distilled water) to the solution. The solution should be clear. Make up to 100 ml with distilled water. Filter and stand in fridge overnight. The solution lasts about 4 to 6 weeks.

Just before use, centrifuge at 3000 rpm for 5 min and remove supernatant for use. This ensures that all other precipitants are removed from the working solution.

Staining method

Place section in saturated uranyl acetate for 10 minutes.

Rinse with distilled water.

Place in lead citrate for 6 minutes

Rinse with distilled water.

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DNA EXTRACTION FROM MUSCLE SAMPLE

(Decary, S. et al. 2000)

MUSCLE SAMPLE STORAGE

- Place freeze fresh muscle sample into cryotube and freeze immediately in liquid nitrogen
- Store at –80 °C

DNA EXTRACTION

Isolation of genomic DNA from muscle samples

Reagents

- Proteinase K digestion buffer - 100 mM NaCl
  - 10 mM tris HCl, pH 8
  - 100 mM EDTA, pH 8
  - 1% Triton X-100
- Proteinase K - final concentration in buffer 20 U/ml
  - our specific activity is 30U/mg
  - ∴need 0.67mg Proteinase K per ml of buffer
- Liquid N_2
- Phenol / chloroform / isoamyl alcohol (25:24:1 vol/vol/vol)
  - For 25 ml tot vol in sterile bottle: 12.5 ml TE-saturated phenol
    12 ml chloroform
    0.5 ml iso-amyl alcohol
- Chloroform / isoamyl alcohol (24:1 vol/vol)
  - For 12 ml tot vol in sterile bottle: 12 ml chloroform
    0.5 ml iso-amyl alcohol
- 7.5 M Ammonium acetate / 100% ethanol (1:4 vol/vol)
  - For 10 ml total vol in sterile bottle: 2 ml 7.5 M ammonium acetate
    8 ml 100% alcohol
- 70% Ethanol
- TE buffer - 10 mM Tris-HCl
  - 1mM EDTA pH 8

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Washing DNA

22. Add 0.5ml 70 % ethanol
23. Invert to wash
24. Spin at 13 000RPM for 2 min (room temperature)
25. Repeat 22 – 24
26. Dry DNA (approximately 30 min) under light

Storage of DNA

27. Add 100µl TE buffer
28. If DNA does not completely dissolve, incubate on bench top
29. Store at 4 °C

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DETERMINATION OF TELOMERE LENGTH

(Allsopp, R. 1992, Decary, S et al. 1996, Decary, S. et al. 1997, Decary, S. et al. 2000, Renault, V. et al. 2002)

RESTRICTION DIGESTION OF TOTAL GENOMIC DNA

To generate TRF's containing the TTAGGG tandem-repeat sequence and a subtelomeric fragment of non-TTAGGG DNA.

Reagents

- *Hinf1* (10U/μl)
- Buffer H (10x)
- dH_2O
- Extracted genomic DNA

Procedure

1. Set heating block to 37 °C
2. Spin all components
3. Prepare cocktail

Component	[Stock]	[Rxn]	1 rxn (μl)
Buffer H	10x	1x	2
dH_20	-	-	6
Hinf1	10U	20U	2
Total volume			10

4. Pipette 10 μl cocktail into each reaction eppendorf
5. Pipette 10 μl DNA into respective eppendorf
6. Spin eppendorf
7. Incubate for 4 hours at 37 °C

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GEL ELECTROPHORESIS

Reagents

- Digested DNA
- Agarose
- 1x TAE buffer
- ^{32}P-DNA ladder (1kb and HMW)

Procedure

1. 30 ml of a 0.7% agarose gel (0.21g agarose + 30 ml 1x TAE)
2. Run each sample on three separate gels
3. Load 3 μg *(1 - 5 μg)* of digested DNA and a ^{32}P-DNA ladder (1kb and HMW)
4. Load 3 μg of undigested DNA sample (to verify the absence of DNA degradation.)
5. Run for 1400 Vh (or 600-700 Vh for a 0.5% agarose gel)

DRYING, DENATURING AND NEUTRALISING GEL

To avoid loss of high-molecular-weight DNA

Reagents

- 0.5 M NaOH / 1.5 M NaCl
- 0.5 M Tris, pH 8 / 1.5 M NaCl

Procedure

1. Dry gel under vacuum at 60 °C for 45 – 60 minutes (to dry gel)
2. Soak gel in 0.5 M NaOH / 1.5 M NaCl for 10 min (to denature gel)
3. Soak gel in 0.5 M Tris, pH 8 / 1.5 M NaCl for 10 min (to neutralise gel)

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HYBRIDISATION OF OLIGONUCLEOTIDE PROBES (Southern Blotting)

The TRF's are detected by hybridisation to a ^{32}P-$(TTAGGG)_4$ probe

Reagents

- 5 x standard saline / citrate and 3 x standard saline / citrate
- ^{32}P-end-labelled $(CCCTAA)_3$

Procedure

1. Incubate gel in 5x standard saline / citrate (SSC) at 37 °C with ^{32}P-end-labelled $(CCCTAA)_3$ for 8-12 hours
2. Wash gel 3 times in 3 X SSC at 48 °C (10 min each)

AUTORADIOGRAPHY

Procedure

1. Expose gel to preflashed Kodak XAR film for 1-2 days (*varies from 96 – 120 hours*)
2. Each of the three gels are exposed several times so that all of the samples have equal intensities within the linear response range of the X-ray film.

(Value for each sample is the mean of these 3) (Decary 2000)

SIGNAL RESPONSE ANALYSIS

- Analysed using NIH image 1.62 and ProFit software.
  1. The mean and minimal value of TRF length (in Kbp) is determined 3 times for each sample on 3 independent gels
  2. The mean TRF is calculated by integrating the signal intensity above background over the entire TRF distribution as a function of TRF length using the formula:

     $$L = \Sigma(OD_i \times L_i) / \Sigma(OD_i)$$

     where OD_i and L_i are the sinal intensity and TRF length respectively at position *i* on the gel image
  3. The min TRF corresponds to the value at which 95% of the densitrometric profile had been integrated according to the distance of migration

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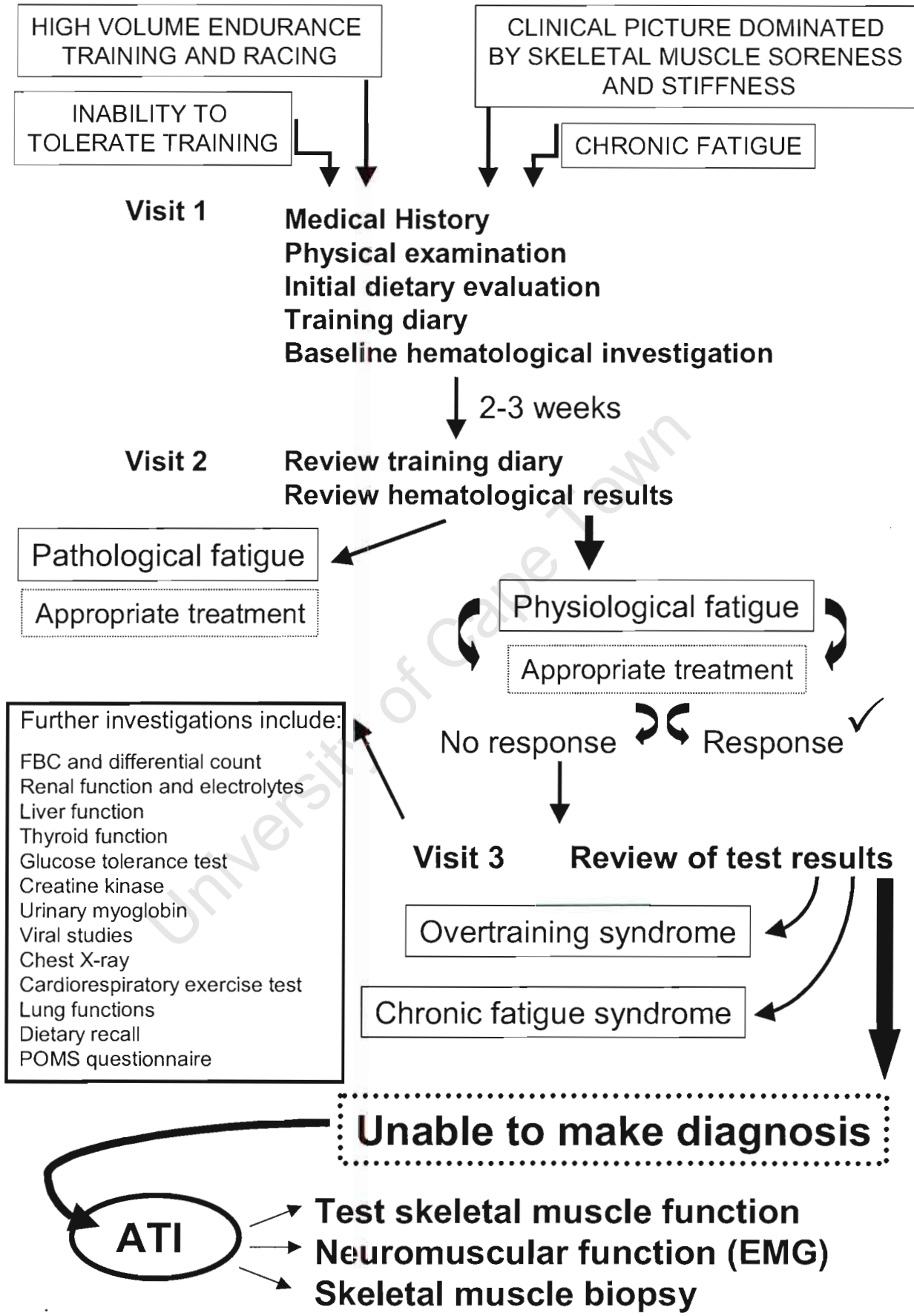

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Athletes with Exercise-Associated Fatigue Have Abnormally Short Muscle DNA Telomeres

MALCOLM COLLINS[1], VALÉRIE RENAULT[2], LIESL A. GROBLER[1], ALAN ST CLAIR GIBSON[1], MICHAEL I. LAMBERT[1], E. WAYNE DERMAN[1], GILLIAN S. BUTLER-BROWNE[2], TIMOTHY D. NOAKES[1], and VINCENT MOULY[2]

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ABSTRACT

COLLINS, M., V. RENAULT, L. A. GROBLER, A. ST CLAIR GIBSON, M. I. LAMBERT, E. W. DERMAN, G. S. BUTLER-BROWNE, T. D. NOAKES, and V. MOULY. Athletes with Exercise-Associated Fatigue Have Abnormally Short Muscle DNA Telomeres. *Med. Sci. Sports Exerc.*, Vol. 35, No. 9, pp. 1524–1528, 2003. **Introduction/Purpose:** Although the beneficial health effects of regular moderate exercise are well established, there is substantial evidence that the heavy training and racing carried out by endurance athletes can cause skeletal muscle damage. This damage is repaired by satellite cells that can undergo a finite number of cell divisions. In this study, we have compared a marker of skeletal muscle regeneration of athletes with exercise-associated chronic fatigue, a condition labeled the "fatigued athlete myopathic syndrome" (FAMS), with healthy asymptomatic age- and mileage-matched control endurance athletes. **Methods:** Muscle biopsies of the vastus lateralis were obtained from 13 patients diagnosed with FAMS and from 13 healthy control subjects. DNA was extracted from the muscle samples and their telomeric restriction fragment (TRF) or telomere lengths were measured by Southern blot analysis. **Results:** All 13 symptomatic athletes reported a progressive decline in athletic performance, decreased ability to tolerate high mileage training, and excessive muscular fatigue during exercise. The minimum value of TRF lengths (4.0 ± 1.8 kb) measured on the DNA from vastus lateralis biopsies from these athletes were significantly shorter than those from 13 age- and mileage-matched control athletes (5.4 ± 0.6 kb, $P < 0.05$). Three of the FAMS patients had extremely short telomeres (1.0 ± 0.3 kb). The minimum TRF lengths of the remaining 10 symptomatic athletes (4.9 ± 0.5 kb, $P < 0.05$) were also significantly shorter that those of the control athletes. **Conclusion:** These findings suggest that skeletal muscle from symptomatic athletes with FAMS show extensive regeneration which most probably results from more frequent bouts of satellite cell proliferation in response to recurrent training- and racing-induced muscle injury. **Key Words:** MUSCLE DAMAGE, EXERCISE, PATHOLOGY, INJURY, CHRONIC FATIGUE

Prolonged, demanding, weight-bearing exercise has been reported to cause both acute and more chronic muscle damage (reviewed in 12). Although adult skeletal muscles are made up of highly differentiated, elongated multinucleated postmitotic cells, they also contain a small population of quiescent mononucleated satellite cells that are located between the sarcolemma and the basement membrane (13). These cells are responsible for both muscle growth and repair. After skeletal muscle damage, the satellite cells proliferate and then fuse to repair or replace the damaged fibers. Some of these mononucleated cells will return to quiescence to restore the population of satellite cells (3). With age, there is a progressive reduction in both the proliferative capacity and the total number of satellite cells (15,16). The proliferative capacity of the satellite cells is limited, in part, by the loss of telomeric sequences, which are specialized DNA-protein structures at the end of the chromosomes (1). During each somatic cell division, a small piece of telomeric DNA is lost. Once the telomeric DNA becomes too short a DNA damage signal is initiated causing the cell to become senescent and stop dividing, thus limiting the regenerative capacity of the cell (6). Since the proliferating satellite cells will lose a small piece of telomeric DNA during each replicative cycle, the newly incorporated DNA in the repaired fiber will have shorter telomeres (5). Thus, the number of cycles of satellite cell proliferation as well as their remaining regenerative capacity can be determined indirectly by measuring the telomeric length or, more specifically, the minimum telomeric restriction fragment (TRF) lengths of DNA from skeletal muscle biopsies (5,16).

Athletes suffering from exercise-related chronic fatigue are ideally suited for studying any potential long-term detrimental effects of high-volume endurance training. These athletes have a long history of very high training loads and

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participation in competitions. They demonstrated a precipitous decline in performance which is not related to ordinary aging, they present with complaints of chronic fatigue dominated by skeletal muscle symptoms including excessive delayed-onset-muscle-soreness, muscle stiffness, weakness, and tenderness as well as a failure to adapt to training. Typically they have sought the help of many clinicians without success. Clinical characteristics and recognized skeletal muscle pathological features, including those of the muscular dystrophies and mitochondrial myopathies, are also excluded in these athletes. The athletes who fulfill these criteria have previously been described as suffering from the "fatigued athlete myopathic syndrome" (FAMS) (7,20,21).

In this study, we have compared the lengths of the TRF in DNA isolated from biopsies of vastus lateralis muscles sampled from endurance athletes suffering from FAMS with those from control athletes, matched for age and historical training volume. We postulate that the skeletal muscle symptoms of the athletes suffering from FAMS might be caused by extensive muscle regeneration.

METHODS

Subjects. Approval for this study was obtained from the Research and Ethics Committee of the Faculty of Health Sciences, University of Cape Town. Thirteen athletes diagnosed with FAMS and 13 healthy age-matched athletes were recruited for this study over a 3-yr period (7). The FAMS patients were recruited from the Sports Medicine Clinic at the Sports Science Institute of South Africa, Cape Town, South Africa. All the FAMS athletes had a history of high-volume training over several years, a history of chronic or excessive exercise-related fatigue, decreased physical performance during exercise and a clinical profile which was dominated by skeletal muscle symptoms including excessive delayed onset muscle soreness after exercise, stiffness, tenderness and skeletal muscle cramps (7). Control subjects (CON), matched for age and training volume and displaying none of the FAMS symptoms were recruited from local running clubs and the Sports Science Institute of South Africa. Athletes in the FAMS group were matched for the level of training before the onset of symptoms with individuals in the control group. Both the FAMS and control subjects were examined by a physician to exclude the presence of identifiable organic disease that would explain the athlete's symptoms.

Before participation in the trial, all subjects completed informed consent forms as well as extensive questionnaires detailing their sporting and training history, current training and racing performance, and medical status. In addition, symptomatic athletes also described their medical, injury, training and running histories both before, and after the onset of symptoms.

Exercise tolerance and skeletal muscle function. Each subject's maximal oxygen uptake ($\dot{V}O_{2peak}$) was determined during a treadmill test of increasing intensity according to a previously described method (18). Maximum voluntary contraction (MVC) of the knee extensors was measured with a Kin-Com isokinetic dynamometer (Chattanooga Group, Inc. Chattanooga, TN) as previously described (19).

Skeletal muscle biopsy and fiber typing. A percutaneous needle muscle biopsy sample was obtained from the vastus lateralis muscle of all subjects. Muscle fiber type proportions were determined using the myofibrillar ATPase method (8).

Telomere length analysis. A portion of the sample was rapidly frozen in liquid nitrogen and stored at −80°C for subsequent DNA extraction and TRF length determination as previously described (4,5,16). For total genomic DNA extraction, at least 10 mg of the muscle biopsy was ground to a powder in liquid nitrogen and digested overnight at 55°C with gentle agitation in 650 μL of proteinase K digestion buffer (10 mM Tris-Cl, pH 8.0; 100 mM EDTA, pH 8.0; 100 mM NaCl; 1% Triton X-100) containing 20 units·mL^{-1} proteinase K. The digest was extracted twice with 1 volume of 25:24:1 (vol:vol:vol) phenol/chloroform/isoamyl alcohol and the DNA precipitated with 1 volume of a 1:4 (vol:vol) 7.5 M ammonium acetate and 100% ethanol mixture, washed with 70% ethanol, resuspended in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0) and stored at 4°C. The extracted DNA samples were resolved on agarose gels to verify the absence of any DNA degradation during the biopsy, storage or extraction processes (Fig. 1, top left panel). The intact genomic DNA was digested for 4 h at 37°C with the restriction enzyme, *Hinf* I, to generate a smear of DNA fragments containing TRF with different lengths of the TTAGGG repeat sequence and a subtelomeric fragment of non-TTAGGG DNA with a constant length (2). Mean and minimum TRF lengths were determined by Southern blot analysis as described by Decary et al. (5) and Renault et al. (16). Briefly, 3 μg of the digested DNA, together with a high molecular weight and 1 kb ^{32}P-labeled DNA ladders, were resolved by electrophoresis on 0.7% agarose gels. The gels were dried, denatured, then neutralized and the TRF were detected directly in the dried treated gels by hybridization to a ^{32}P-labeled $(TTAGGG)_4$ probe, followed by exposed to x-ray film (BioMax, Kodak, EIS, Massy, France) with a BioMax transcreen (Kodak, EIS, Massy, France). Because the TRF lengths from a tissue range in size, mean and minimum TRF lengths were determined as previously described (10,22). The signal responses were analyzed by a computer-assisted system using NIH Image 1.62 (densitometric data of one-dimensional gels) and ProFit (densitometric profils analysis) software. The mean and minimum value of telomere length (in kilobase pairs) was determined three times for each sample on three independent gels. The mean telomere length (L) was calculated by integrating the signal intensity above background over the entire TRF distribution as a function of TRF length using the formula: $L = \Sigma(OD_i \cdot L_i)/\Sigma(OD_i)$, where OD_i and L_i are the signal intensity and TRF length, respectively, at position i on the gel image (10,22). To determine the minimum value of telomere length in a homogenous way for all samples, the densitometric profile of the TRF length was

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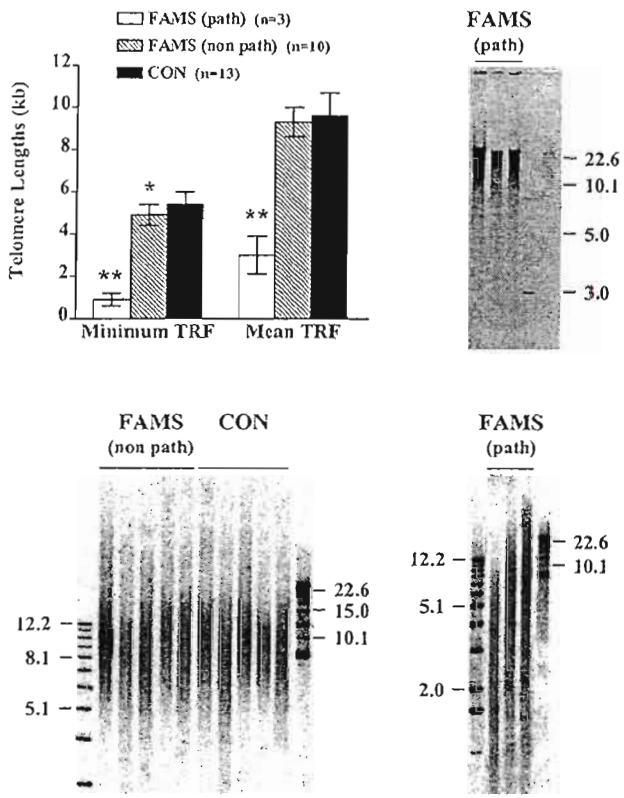

FIGURE 1—Left top panel: minimum and mean telomere lengths of DNA extracted from the vastus lateralis muscle of control (CON), FAMS athletes with pathologically short telomeres (FAMS path), and athletes whose telomeres are not pathologically short (FAMS nonpath). Data are represented as mean ± SD. * ($P < 0.05$) and ** ($P < 0.001$) indicate a significant difference between FAMS and controls. Right top panel: total genomic DNA was extracted from vastus lateralis biopsies. Undigested DNA from the FAMS athletes with pathological short telomeres (FAMS path) and all the other FAMS and control subjects (data not shown) were resolved on a 0.7% agarose gel for 14 h at 80 V. The gels were visualized under UV light (GelDoc 2000, BioRad). Left bottom panel: representative autoradiogram showing the size distribution of telomere lengths (TRF) from the vastus lateralis muscle of control (CON) and FAMS athletes with no pathological shortening of their telomeres (FAMS nonpath). Total genomic DNA was extracted from vastus lateralis biopsies, digested and resolved on agarose gels for 17 h, and digested and resolved on agarose gels for 17 h at 100 V. Molecular weight standards are indicated in kilobase pairs. Right bottom panel: representative autoradiogram showing the size distribution of telomere lengths (TRF) from the thigh muscle of the FAMS athletes with pathological short telomeres (FAMS path). Total genomic DNA was extracted from vastus lateralis biopsies, digested and resolved on agarose gels for 14 h, and digested and resolved on agarose gels for 14 h at 80 V. Molecular weight standards are indicated in kilobase pairs.

integrated over the distance of migration. The minimum telomere length corresponds to 95% of this integration.

Statistical analysis. Data were analyzed using the STATISTICA version 5.5 (StatSoft Inc., Tulsa, OK) statistical program. Where applicable, data were presented as means ± standard deviations (SD). Pearson's chi-square analysis was used to analyze differences between the genders of the FAMS and CON groups. A one-way analysis of variance was used to determine any significant differences between the subject characteristics, the training history, maximum voluntary contraction, $\dot{V}O_{2peak}$, fiber type proportions and the telomere lengths of the FAMS and CON groups. A dependent t-test was used to determine any significant differences between the training history of the FAMS group before and after the onset of FAMS. Statistical significance was accepted when $P < 0.05$.

RESULTS

Subject characteristics and training history. The FAMS group comprised 10 male and 3 female athletes. Included were 11 distance runners, a triathlete and an international squash player who had trained at a high level. Sporting achievement ranged from club to international level. The control group consisted of nine male and four female healthy asymptomatic athletes comprising 11 distance runners, a triathlete, and an endurance cyclist.

Subjects in each group were individually matched for gender, age, height and weight and for $\dot{V}O_{2peak}$ and quadriceps isometric MVC (Table 1). Both groups also had a similar proportion of Type I skeletal muscle fibers. With the exception of a single FAMS athlete whose skeletal muscles comprised 91% Type I fibers, the range of Type I fiber type proportions was similar in the FAMS (31–67%) and CON (37–60%) groups.

The FAMS athletes showed a significant decrease in the training load ($km \cdot h^{-1}$, $km \cdot wk^{-1}$, and $d \cdot wk^{-1}$) and in their 5-km personal best times once they developed symptoms (Table 2).

Both the FAMS and CON athletes, who were endurance runners, ran more than 40 km continuously during training and/or racing at similar ages (FAMS: 27.6 ± 10.6 yr (N = 11); CON: 24.5 ± 9.0 yr (N = 10)) and continued to do so for a similar numbers of years (FAMS: 11.8 ± 5.6 yr (N = 11); CON: 14.2 ± 6.9 yr (N = 9)). The current training volume of the CON athletes was very similar to the training volume of the FAMS athletes before the onset of their symptoms (Table 3) but was more than the current training of the FAMS group after the onset of symptoms (Table 2).

Telomere lengths. The minimum TRF lengths in the vastus lateralis muscle of the FAMS athletes (FAMS all) were significantly shorter than those of the CON subjects (P = 0.017) (Table 4). Even when corrected for age, the minimum TRF lengths remained significantly shorter in the FAMS athletes (data not shown). There was no significant difference in the mean (P = 0.052) TRF lengths of the FAMS and CON athletes (Table 4).

Three of the FAMS athletes (Fig. 1 and FAMS path in Table 4) had significantly shorter mean and minimum TRF lengths than the remaining 10 FAMS subjects (FAMS nonpath in Table 4). When the data of these three subjects were excluded, the minimum TRF lengths of the remaining 10 FAMS athletes were still significantly shorter than those of the CON group (P = 0.043) (Table 4).

DISCUSSION

The novel finding of this study was that the minimum TRF lengths of DNA from the vastus lateralis muscle of

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TABLE 1. General physiological characteristics of the FAMS and control (CON) subjects.

	FAMS	CON
Gender (male/female)	10/3	9/4
Age (yr)	42.0 ± 10.2 (13) (22–59)	41.2 ± 11.5 (13) (24–58)
Height (cm)	174.1 ± 8.2 (11) (161.0–187.0)	173.0 ± 9.1 (13) (159.8–190.0)
Weight (kg)	74.8 ± 18.5 (12) (54.2–110.0)	70.6 ± 10.8 (13) (49.0–91.0)
$\dot{V}O_{2peak}$ (mL $O_2 \cdot kg^{-1} \cdot min^{-1}$)	48.3 ± 8.7 (13) (30.5–74.7)	52.4 ± 10.9 (13) (31.1–59.7)
MVC (N)	527 ± 165 (13) (270–843)	532 ± 103 (13) (377–735)
Type I fibres (%)	52.0 ± 15.8 (13) (31–91)	49.6 ± 7.7 (13) (37–60)

Except for gender, values are expressed as means ± SD, with *N* in parentheses. The range is also given in parentheses. MVC, maximum voluntary contraction.

symptomatic athletes (4.0 ± 1.8 kb) were significantly shorter than those of the CON subjects (5.4 ± 0.6 kb). Decary et al. (5) have reported that the minimum TRF length of the vastus lateralis muscle decreases by about 13 bp per year from birth to 86 yr of age. When corrected for age, the minimum TRF lengths remained significantly shorter in the FAMS athletes, implying that, at least in the thigh muscles, muscle regeneration was occurring at a faster rate than in the control subjects. This suggests, that in an attempt to repair repeated bouts of exercise-induced muscle damage, the satellite cells of the FAMS patients have undergone more frequent rounds of replication, resulting in extensive regeneration of those muscles that are involved in repeated eccentric muscle contractions during running.

Indeed, histological studies of samples from these same FAMS patients have shown a significantly greater incidence of fiber size variation and the presence of internal nuclei than in controls. Ragged-red fibers, or any of the other histological signs of muscular dystrophies or mitochondrial myopathies, were not present in any of the samples. Because the presence of internal nuclei is a histological marker of muscle regeneration, the greater number of pathological changes in the FAMS patients is another indication that the damaged muscle in these patients may have reflected an accelerated aging process due to extensive regeneration (23,24).

Decary et al. (4) reported that the muscles of patients with Duchenne muscular dystrophy and limb girdle muscular dystrophy 2C have a 14-fold accelerated rate of telomeric DNA loss of 187 $bp \cdot yr^{-1}$. Although not to the same extent, these findings are similar to those we report in athletes with FAMS.

The minimum TRF lengths of the control endurance athletes were also slightly shorter than previously reported values for a sedentary population (5). This suggests that, although the control endurance athletes did not present with any of the FAMS symptoms, they nevertheless also showed evidence for extensive regeneration in their thigh muscles.

Close inspection of the maximal, mean and minimum TRF lengths of the FAMS athletes showed that 3 of the 13 athletes had significantly shorter telomeres. The mean and minimum TRF lengths of these three athletes were significantly shorter than those of the control group. Because skeletal muscle is a postmitotic tissue, mean and minimum TRF lengths should remain constant throughout its life (5). However, both mean and minimal TRF lengths in these three athletes were significantly shorter than the remaining 10 FAMS or control athletes; an alternative or additional mechanism, other than an accelerated satellite cell replication, could be implicated in telomere shortening. Because of its high guanine and cytosine content, which is a major target of reactive oxygen species (17), telomeric DNA may be more susceptible to oxidative damage. Telomeric sequences are also susceptible to breakages (11). During exercise, there is an increase in metabolism and the production of reactive oxygen species, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals. Poulsen et al. (14) have shown that 30 d of extreme endurance exercise caused a significant increase in DNA modification. Exercise would only be expected to increase the rate of oxidative damage to DNA and other cellular structures if the capacity of the antioxidant defense mechanisms is exceeded. Dufaux et al. (9) have demonstrated a large interindividual variation in the oxidative stress experienced by moderately trained athletes after a 2.5-h run. It is therefore tempting to speculate that certain athletes are more susceptible to exercise-induced oxidative damage to their telomeres. However, one of these athletes had previously been diagnosed with anorexia, while a second had Type II diabetes. There is no evidence that either of these conditions is associated with accelerated telomere shortening in skeletal muscle. However, the effects of these conditions on premature telomere shortening remains to be investigated.

Finally, although the FAMS athletes had short telomeres their $\dot{V}O_{2peak}$ and MVC were no different to the controls, suggesting that their short-duration muscle function was not affected in contrast to endurance activities.

In conclusion, the telomere lengths of DNA isolated from muscle biopsies from endurance athletes presenting with FAMS was significantly shorter than those of control athletes who did not have symptoms. These findings suggest

TABLE 2. Training history and 5-km personal best times of the FAMS subjects who were endurance runners prior to (Pre FAMS) and after (Post FAMS) the onset of the FAMS symptoms

	Pre FAMS	Post FAMS
Training speed ($km \cdot h^{-1}$)	12.6 ± 0.8 (5)	10.7 ± 0.7 (5)*
Training distance ($km \cdot wk^{-1}$)	68.7 ± 25.6 (9)	23.9 ± 23.4 (9)*
Training frequency ($d \cdot wk^{-1}$)	5.5 ± 1.3 (12)	3.1 ± 2.6 (12)*
Best 5-km time (min)	18.8 ± 2.2 (6)	23.9 ± 4.7 (6)*

Values are means ± SD, with n in parentheses; * denotes $P < 0.05$.

TABLE 3. Current training history of the CON subjects and of the FAMS subjects prior to (Pre FAMS) the onset of symptoms.

	CON	Pre FAMS
Training speed ($km \cdot h^{-1}$)	12.4 ± 1.2 (9)	12.8 ± 1.1 (10)
Training volume ($km \cdot wk^{-1}$)	52.0 ± 12.7 (10)	67.8 ± 24.3 (10)
Training volume ($km \cdot yr^{-1}$)	2629 ± 656 (9)	3526 ± 1261 (10)
Training frequency ($d \cdot wk^{-1}$)	4.6 ± 1.0 (9)	5.5 ± 1.3 (12)

Values are means ± SD, with *N* in parentheses.

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TABLE 4. Minimum (Min) and mean (Mean) telomeric restriction fragment (TRF) lengths of the vastus lateralis muscle of FAMS and control (CON) athletes.

	CON	FAMS (All)	FAMS (Path)	FAMS (Nonpath)
N	13	13	3	10
Mean TRF (kb)	9.6 ± 1.1 (7.8–12.0)	7.8 ± 2.8 (2.1–10.5)	3.0 ± 0.9** (2.1–3.8)	9.3 ± 0.7 (8.1–10.5)
Min TRF (kb)	5.4 ± 0.6 (4.1–6.6)	4.0 ± 1.8* (0.7–5.8)	0.9 ± 0.3** (0.7–1.2)	4.9 ± 0.5* (4.0–5.8)

Values are means ± SD; The range is given in parentheses. The * ($P < 0.05$) or ** ($P < 0.001$) indicates a difference between FAMS and corresponding CON. FAMS (all), telomere lengths of all the FAMS athletes; FAMS (path), FAMS athletes with pathologically short telomeres; FAMS (nonpath), FAMS athletes whose telomeres are not pathologically short.

that skeletal muscle from FAMS athletes shows extensive regeneration, which likely results from more frequent cycles of satellite cell proliferation induced by repeated bouts of muscle damage in these symptomatic athletes.

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