Skeletal muscle repair after micro-damage: effect of ice therapy on satellite cell activation.

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Thesis presented in partial fulfillment of the requirements for the degree of Master of Physiological Sciences at the University of Stellenbosch.

Prof. Kathy H. Myburgh and Dr. Carola U. Niesler

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I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Cryotherapy is one of the popular treatments used to alleviate muscle soreness, especially in the competitive sports arena. However, the therapeutic use of cryotherapy is unsubstantiated because of a lack of proper investigations in the literature, especially a hypothesised effect on muscle recovery. Thus, our aims were to characterise satellite cell (SC) activity in human subjects with delayed onset muscle soreness (DOMS) and to shed light on the effect of cryotherapy on SC activity. DOMS was induced in six male subjects (24 ± 3 years) by completion of a downhill-run (DHR) protocol (5 x 8 min bouts, 2 min rest between bouts) at 70 or 80% of their individual peak treadmill speed. Ice application was applied to only one leg per subject for 3 days: 30 min every 2 hours, 5 times per day. In total 5 muscle biopsies were obtained from each subject: 1 baseline and 4 post-DHR. Post-DHR biopsies: 1 from each leg on day 1 and 7 (1st group, n=3) and 1 from each leg on day 2 and 9 (2nd group, n=3). DOMS was successfully induced as indicated by significant increases in muscle soreness at days 1 and 2 post-DHR ($P < 0.01$), and creatine kinase activity at day 1 post-DHR ($P < 0.01$). No difference in muscle soreness was found between treated and untreated legs. SC quiescence and activation were characterised by their expression of the cell surface markers CD34 and CD56 respectively. No significant change in quiescent SC was observed in the untreated or treated legs over time. However, at day 1 post-DHR the number of quiescent SC was significantly lower in the untreated compared with the treated leg ($P < 0.05$). There was a significant increase in activated SC numbers at day 2 post-DHR in the untreated leg, which was sustained up to day 9 post-DHR ($P < 0.01$). However, no such increase was found in biopsies taken on days 1 and 7. Also, no

change was found in the treated leg, however a significant difference between the number of activated SC in untreated and treated legs on days 2 and 9 post-DHR ($P < 0.01$) was seen. No significant effect of DOMS or ice treatment was observed for the expression of the myogenic regulatory factors, MyoD and myogenin. C2C12 cell cultures induced to differentiate, however, did stain using these antibodies. This is the first study to report an effect of cryotherapy at the tissue level. In conclusion, this study highlights many unanswered questions on the SC response to DOMS at tissue level, and lays a good foundation for future studies.

Kreoterapie is een van die gewilde behandelings wat gebruik word om spierseerheid te verlig, veral in die kompeterende sport arena, maar die gebruik van kreoterapie is onbevestig as gevolg van ‘n gebrek aan voldoende ondersoeke in die literatuur, veral ‘n hipotese oor die effek op spier-herstel. Ons doelstellings was dus om satellietsel (SC) aktiwiteit te ondersoek in mens proefpersone met vertraagde aanvang spierseerheid (DOMS) en ook om lig te werp op die effek van kreoterapie op SC aktiwiteit. DOMS was in ses mans proefpersone (24 ± 3 jare) geïnduseer deur voltooïng van ‘n afdraend-hardloop (DHR) protokol (5 x 8 min rondtes, 2 min rus tussen rondtes) teen 70 of 80% van elkeen se individuele maksimum trapmeul-spoed. Ys was vir 3 dae op net een been per proefpersoon aangewend: 30 min elke 2 ure, 5 keer per dag. 5 spierbiopsies in totaal was van elke proefpersoon verkry: 1 basislyn en 4 post-DHR. Post-DHR biopsies: 1 van elke been op dae 1 en 7 (1ste groep, n=3) en 1 van elke been op dae 2 en 9 (2de groep, n=3). DOMS was suksesvol geïnduseer soos aangedui deur die betekenisvolle verhogings in spierseerheid op dae 1 en 2 post-HR ($P < 0.01$) en kreatien kinase aktiwiteit op dag 1 post-DHR ($P < 0.01$). Geen verskil in spierseerheid is gevind tussen die onbehandelde en behandelde bene nie. SC dormansie en aktivering was gekarakteriseer deur die onderskeidelike uitdrukking van die sel oppervlak merkers CD34 en CD56. Geen betekenisvolle verandering is in SC dormansie in die onbehandelde en behandelde bene waargeneem nie, maar op dag 1 post-DHR was die getal dormante SC betekenisvol laer in die onbehandelde been as in die behandelde been ($P < 0.05$). Daar was ‘n betekenisvolle verhoging in die getalle geaktiveerde SC op dag 2 post-DHR in die onbehandelde been wat volgehou was tot op dag 9 post-DHR ($P < 0.01$), maar so

‘n verhoging was nie in biopsies wat op dae 1 en 7 geneem is gevind nie. Daar is ook geen verandering in die behandelde been gevind nie, maar ‘n betekenisvolle verskil in die getal geaktiveerde SC is tussen die onbehandelde en behandelde bene op dae 2 en 9 post-DHR gevind($P < 0.01$). Geen betekenisvolle effek van DOMS en ys-aanwending vir die uitdrukking van die miogeniese (myogenic) regulatoriese faktore, MyoD en myogenin, is waargeneem nie. C2C12 sel kulture wat geïnduseer is om te differensieer het wel gekleur vir hierdie antiliggame. Dit is die eerste studie wat ‘n effek van kreoterapie op weefselvlak rapporteer. Ten slotte, hierdie studie beklemtoon baie onbeantwoorde vrae oor die SC respons op DOMS op weefselvlak en dit lê ‘n goeie grondslag neer vir toekomstige studies.

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CHAPTER 1: INTRODUCTION

Apart from the mechanical movements that are requirements for activities of daily life, skeletal muscle may be challenged by several stressors, including exercise, mechanical overloading and direct trauma. These challenges can cause damage to skeletal muscle ranging from mild (micro-damage) to severe (e.g. a frank muscle tear). Delayed onset muscle soreness (DOMS) is a form of micro-damage that typically manifests itself within one to two days following a session of unaccustomed exercise. Skeletal muscle is very adaptable and is capable of responding to the above-mentioned stimuli via remodelling, regeneration and repair. For example, upon overloading, as seen in weight training, skeletal muscle will enlarge by hypertrophy (Dangott *et al.*, 2000) which is considered a form of remodelling, whereas upon direct muscle damage it will respond via the regeneration or repair pathways (Hurme & Kalimo, 1992).

Although skeletal muscle fibres themselves are terminally differentiated, skeletal muscle regeneration is possible due to a special cell population, namely satellite cells, which is capable of promoting and facilitating the synthesis of new muscle proteins using a recurrence of the developmental pathways. Hypertrophy and muscle repair can progress without activation of satellite cells, but via myonuclear upregulation of relevant proteins' transcription and translation. Although the terms regeneration and repair are sometimes used interchangeably, some authors imply that the regeneration process requires the activation of the original embryonic pathways of muscle generation (Musaro *et al.*, 2004). In adults, stem

cells are known to be able to differentiate along embryonic pathways (Bailey *et al.*, 2001). Satellite cells are a stem cell-like population located between the sarcolemma and basal lamina of myofibres.

1.1 Skeletal muscle

1.1.1 Basic structure

Skeletal muscle is composed of many elongated, cylindrical, multinucleated muscle cells known as myofibres. These fibres are arranged parallel to each other and are bound together by three layers of connective tissue: the epimysium, perimysium and the endomysium. The epimysium surrounds a whole muscle (see Figure 1.1). Within the muscle the myofibres are grouped into bundles (fasciculi) by the perimysium in which the muscle's blood and nerve supplies are situated. Within the fasciculi each myofibre is surrounded by the endomysium which in turn is attached to the fibre cell membrane (sarcolemma) and conveys some of the contractile force to the tendons (McComas, 1996).

The sarcolemma encases the muscle cell cytoplasm (sarcoplasm) that contains the muscle nuclei (myonuclei), mitochondria, energy substrates and the myofibrils. The sarcolemma forms a boundary between myonuclei (within a myofibre) and satellite cells (outside the myofibre) making it possible to distinguish between a myonucleus and the nucleus of a satellite cell (Mauro, 1961). Satellite cells are sequestered between the sarcolemma and basal lamina of their associated myofibres (Mauro, 1961). In addition to being a boundary between satellite cells and other cells outside of the myofibre (also aiding in identification of satellite

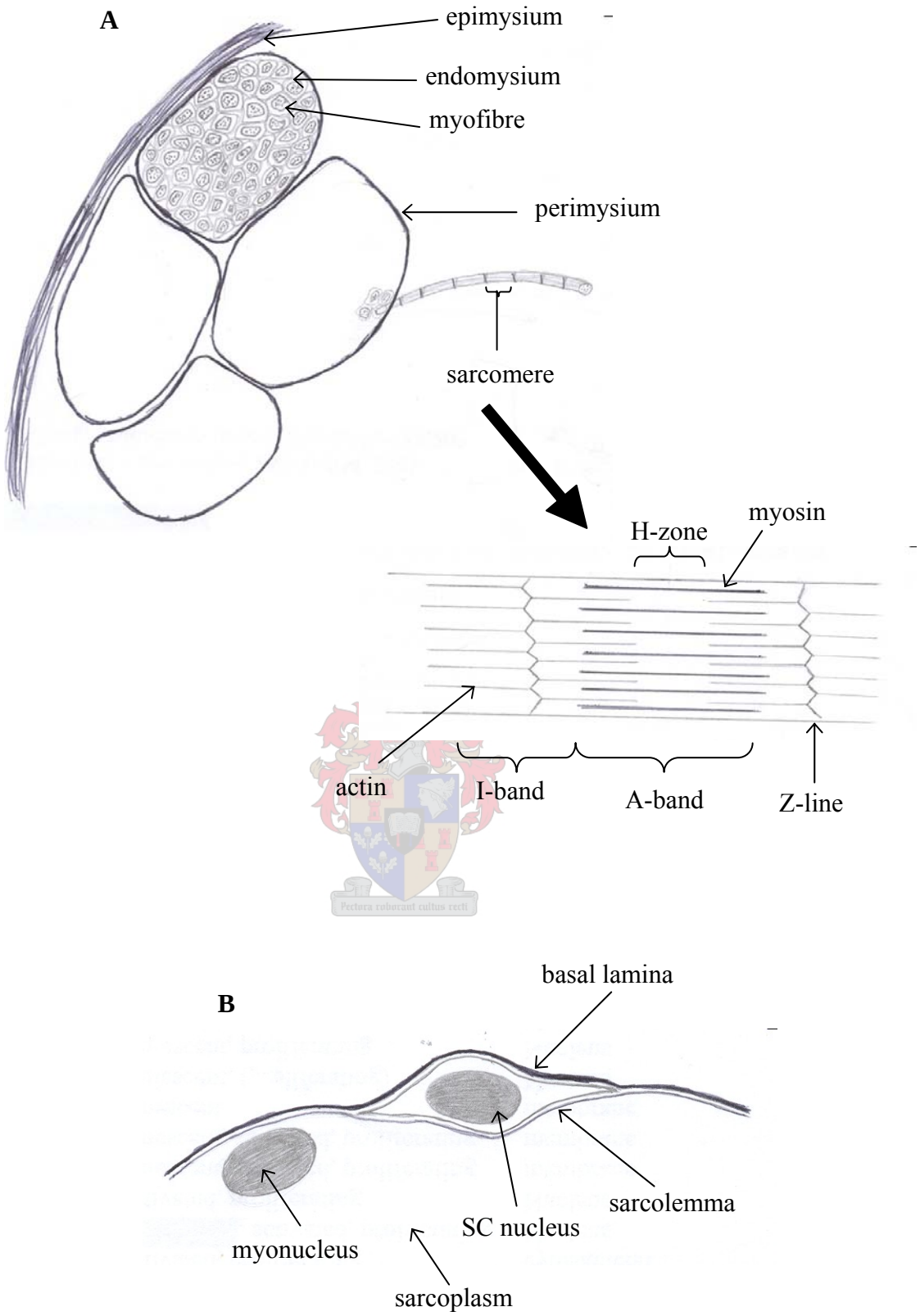


Figure 1.1 Basic structure of skeletal muscle. A cross-section of skeletal muscle, showing the gross components and the structure of a sarcomere (A). A satellite cell sequestered between the basal lamina and the sarcolemma of its associated myofibre (B).

cells) (Cooper *et al.*, 1999), the basal lamina also plays a role in keeping the satellite cells in contact with their associated myofibres (Bischoff, 1990).

Contraction of skeletal muscle is the responsibility of the myofibrils which consist mainly of the contractile proteins (myofiliments) myosin and actin. These proteins are arranged into light and dark bands, consequently giving myofibres their striated appearance. Myosin is associated with the dark bands (A-band) and actin with the light bands (I-band). During relaxation, there is a region where the myosin and actin do not overlap, i.e. the H-zone. This bands' arrangement can be demonstrated in the smallest contractile unit of skeletal muscle, the sarcomere (refer to Figure 1.1). Many of these sarcomeres are arranged end-to-end (ends indicated by the Z-line) to constitute a myofibril (Ganong, 1997b). Unaccustomed exercise may cause disarrangement of this banded structure, e.g. Z-line streaming or even Z-line disruption, both of which are associated with muscle micro-damage (Friden *et al.*, 1981; Friden & Lieber, 1992).

Calcium is required for muscle contraction and is stored in a tubular system, i.e. sarcoplasmic reticulum, that runs along the myofibres and also surrounds individual myofibrils (McComas, 1996). Muscle damage will cause disturbance to the sarcoplasmic reticulum, leading to an imbalance in calcium homeostasis (reviewed by Byrd, 1992). Consequently an increase in intracellular calcium will activate certain enzymes (calpains) that may contribute to further muscle damage (reviewed in Belcastro *et al.*, 1998).

1.1.2 Damage

The response to severe acute muscle damage is similar irrespective of the cause and can be divided into several stages. First, the disruption of the intracellular and/or extracellular compartments of the affected area occurs (Tidball, 1995). Calcium homeostasis within the severely damaged fibres is disrupted and autolysis of the damaged fibre components follows (Armstrong, 1990; Tidball, 1995). In addition to myofibre damage, the connective tissue, nerves and blood vessels may also be disrupted. If vascular disruption is present, platelets form a plug to close any damaged vessel. This disruption phase causes several proteins, including enzymes and other factors, to be released into the circulation. As reviewed by Tidball (1995), neutrophils are the first inflammatory cells to invade the injury site 1-6 hours following a severe insult. Hereafter (9-12 hours after injury) monocyte and macrophage invasion accompanies the neutrophil invasion. 12 hours after injury the neutrophil number starts to decrease with an increase of macrophages, which remain the predominant cell type (Tidball, 1995). The macrophages not only remove the debris, but also secrete several factors and cytokines that might be important in the muscle repair and regeneration processes. The recovery phase from severe damage includes re-vascularisation (if any vascular damage occurred), as well as the proliferation and differentiation of satellite cells (and possibly other stem cells e.g. bone marrow-derived stem cells (Ferrari *et al.*, 1998; LaBarge & Blau, 2002).

Several of the factors released during the course of muscle damage have been identified as important factors in the activation of satellite cells, their proliferation

as well as their migration from undamaged to the damaged area (Tidball, 1995; Vierck *et al.*, 2000). These factors include hepatocyte growth factor (HGF), IL-6 (interleukin 6), IL-1 (interleukin 1), leukaemia inhibitory factor (LIF), insulin, insulin-like growth factor (IGF-I), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) (Arsic *et al.*, 2004; Germani *et al.*, 2003; Vierck *et al.*, 2000). Some of these factors will be discussed in more detail later (see "Regulation of satellite cells").

The amount of inflammation caused by muscle trauma will depend on the severity of damage as well as the degree of vascularisation. Muscle damage can be divided into macro-trauma (which includes crush injuries and moderate to severe sprains or strains) and micro-trauma that occurs with acute and chronic overuse syndromes (Merrick, 2002). Delayed onset muscle soreness (DOMS) has been shown to be associated with muscle micro-damage (Friden *et al.*, 1983) and is experienced by most individuals at least once in their lifetime. Thus it is relevant to study DOMS as a model of muscle damage in humans. This model is not as extreme as some animal models of severe muscle injury, thus the processes of regeneration and repair may differ. For example, the extent of, or even presence of embryonic or foetal contractile protein expression may differ, or the extent of the inflammatory response.

1.1.3 Delayed onset muscle soreness (DOMS)

1.1.3.1 What is DOMS?

Most of us have experienced the tender, aching and stiff feeling after going to the gym following a long absence, or after an unaccustomed bout of exercise, especially hiking. Strangely this soreness only really becomes evident about 1-2 days after the exercise bout, and usually continues for another 1-2 days. This is what is known as DOMS. DOMS occurs predominantly after unaccustomed eccentric (lengthening contraction) exercise (Byrnes *et al.*, 1985a; Cleak & Eston, 1992b).

The clinical symptoms of DOMS include muscle soreness, muscle stiffness, swelling, decreased strength, tenderness on palpation (Cleak & Eston, 1992b; Eston *et al.*, 1994; Eston *et al.*, 1996b; Yackzan *et al.*, 1984) and decreased active range of movement in the joints of the affected muscles (Yackzan *et al.*, 1984). Although symptoms of DOMS may become evident within 8-12 hours after the exercise bout (Nosaka & Kuramata, 1991), in general these symptoms peak a day or two days (24-48 hours) post-exercise whereafter they resolve within a day or two (Pizza *et al.*, 1995; Thompson *et al.*, 1999). In extreme cases however, DOMS may peak only at five days and require a further five days to resolve (Manfredi *et al.*, 1991). These differences in peak and duration times of DOMS infer that the type and extent of exercise plays a role in the severity of DOMS. It has been reported that the intensity, rather than the duration, of an exercise is the determining factor in inducing DOMS (Tiidus & Ianuzzo, 1983). Interestingly,

type II fibres have been reported to be more susceptible to the micro-damage associated with DOMS (Friden *et al.*, 1983).

In most studies, plasma or serum creatine kinase (CK) activity is used as a measure of muscle damage (Schwane *et al.*, 1983; Tiidus & Ianuzzo, 1983). Although used as an indicator of muscle damage, CK activity is not a predictor of the extent of muscle damage, but merely the presence of some damage. Data have shown no linear statistical correlation between CK activity and the extent of muscle damage (Manfredi *et al.*, 1991), nor between CK activity and muscle soreness (Nosaka & Kuramata, 1991). This is also confirmed by data showing that muscle tenderness is not associated with serum CK activity (Eston *et al.*, 1994). Although CK activity is the general blood parameter measured to indicate muscle damage in DOMS studies, other studies have measured proteins such as lactate dehydrogenase (LDH) (Schwane *et al.*, 1983), malondialdehyde (MDA - a free radical production indicator) (Eston *et al.*, 1996a) and myoglobin (Byrnes *et al.*, 1985b).

1.1.3.2 Pain associated with DOMS

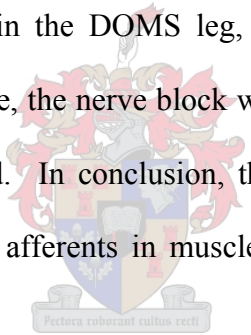
Several theories postulating the pathophysiology of pain associated with the syndrome of DOMS suggest that connective tissue damage, inflammation and sarcomere damage may all play a role. Alternatively, physiological conditions such as lactic acid accumulation and muscle spasms may cause pain. However, lactic acid has been shown not to be the culprit and data on muscle spasms are inconclusive. Although muscle damage and inflammation are both associated

with DOMS, a cause-effect relationship has not been established (reviewed in Cleak & Eston, 1992a). Recent data implicate the role of muscle mechanoreceptors in generating pain during DOMS (Weerakkody *et al.*, 2001; Weerakkody *et al.*, 2003).

In their 2001 study (Weerakkody *et al.*, 2001), the authors induced DOMS in one leg of human subjects using a protocol consisting of walking backwards on an inclined (13^{o}) moving treadmill. Subjects had to step backward using the toe-to-heel movement which caused eccentric contraction in the *triceps surae* (DOMS leg). The other leg was placed flat alongside which did not cause eccentric contraction in the *triceps surae*. Some subjects received isotonic saline injections before and after the exercise bout. The rationale being that if the nociceptors (pain receptors) were sensitised during DOMS, these injections could cause an increase in pain in the DOMS leg. However, no significant difference in pain experienced was found between the exercised and non-exercise leg, thus excluding a role for nociceptors in pain during DOMS. In another group of subjects, vibration (20 and 80 Hz) was applied to the muscle. This caused an increase in pain in the exercised leg with more pain experienced at 80 Hz. These authors suggest that their data indicate the involvement of primary muscle spindle endings in pain during DOMS. To control for possible skin sensations, an anaesthetic cream was applied to three subjects, showing that skin sensation did not play a role in perceived pain sensation. Some subjects also participated in a nerve block experiment. Blocking the sciatic nerve was achieved by placing a wooden bar under the exercised thigh (distal to the ischial tuberosity) with the

subjects leaning towards the bar with their full body weight. Pain sensation significantly decreased with this nerve compression block, whereas latency for hot and cold sensation was unchanged. In summary, these authors concluded that muscle mechanoreceptors, including muscle spindles, are involved in generating pain during DOMS.

In 2003, this research group conducted a similar study (Weerakkody *et al.*, 2003) with some modifications. The same one-legged backward walking protocol was used to induce DOMS in one leg. They included the vibration test in the saline and nerve block groups, as well as including vibration and saline injection during a nerve block. Vibration in the DOMS leg, with added saline, increased the perceived pain. Furthermore, the nerve block was capable of reducing pain, even when vibration was applied. In conclusion, these authors speculate that large-diameter mechanoreceptive afferents in muscle are involved in generating pain during DOMS.



1.1.3.3 Possible DOMS treatments

Treatments for the soreness and stiffness associated with DOMS that are currently used include massage (Lightfoot *et al.*, 1997; Smith *et al.*, 1994b; Tiidus & Shoemaker, 1995), passive stretching (Lund *et al.*, 1998), pulsed ultrasound (Hasson *et al.*, 1990), cryotherapy (Paddon-Jones & Quigley, 1997), anti-inflammatory and analgesic drugs (Francis & Hoobler, 1987; Hasson *et al.*, 1993; Lecomte *et al.*, 1998; Tokmakidis *et al.*, 2003; Trappe *et al.*, 2001), hyperbaric oxygen therapy (Mekjavic *et al.*, 2000; Staples *et al.*, 1999), vitamin C

supplementation (Childs *et al.*, 2001; Kaminski & Boal, 1992; Thompson *et al.*, 2001), a fabric with electromagnetic shielding properties (*Farabloc*) (Zhang *et al.*, 2000), exercise (Carpinelli & Gutin, 1991; Jamurtas *et al.*, 2000) and homoeopathic medicine (Vickers *et al.*, 1997). Ingestion of non-steroidal anti-inflammatory drugs is a popular treatment used for DOMS (Donnelly *et al.*, 1990; Hasson *et al.*, 1993; Semark *et al.*, 1999). However, data regarding these non-steroidal anti-inflammatory drugs are conflicting. In one study ibuprofen was not effective in alleviating muscle soreness or increasing muscle strength in people suffering from DOMS (Donnelly *et al.*, 1990), whereas another study found that ibuprofen can indeed decrease muscle soreness (Tokmakidis *et al.*, 2003). The latter study however also found ibuprofen to be ineffective in improving muscle strength after a DOMS-inducing exercise bout. In the study by Donelly *et al*. it was found that the serum CK activities and urea concentrations were higher in the ibuprofen-treated group than the placebo group. This infers that, apart from being ineffective in enhancing muscle recovery after a DOMS-inducing exercise, ibuprofen may also exacerbate muscle damage.

Studies have shown smaller changes in muscle soreness and serum proteins (e.g. CK, myoglobin) when a second bout of exercise follows the first (Byrnes *et al.*, 1985b; Donnelly *et al.*, 1990; Eston *et al.*, 1996a). This suggests that the first bout of exercise has a prophylactic effect on the second, decreasing muscle soreness and serum protein levels (Byrnes *et al.*, 1985b; Donnelly *et al.*, 1990; Eston *et al.*, 1996a). This is known as the second bout effect and the effectiveness of the first bout seems to depend on the type of exercise, e.g. the protective effect

of isometric exercise only lasts about 3 weeks (Triffletti *et al.*, 1988) whereas the prophylactic effect of an eccentric exercise bout lasts up to 6 weeks (Byrnes *et al.*, 1985b). Newham and his colleagues (Newham *et al.*, 1987) showed that the recovery rate of strength and force-frequency parameters of skeletal muscle also improved with the second and third bout of exercise. In this study, where the bouts were performed two weeks apart, plasma CK activity did not significantly change from baseline values after the second and third bouts. When an eccentric bout of exercise is repeated 48 hours after the first, the time-course and severity of muscle soreness, serum CK activity and muscle strength is similar in both the first and second bout (Smith *et al.*, 1994a).

The use of cryotherapy dates back to the ancient Greeks and Romans. Cryotherapy is a collective term for all cold treatments, including ice massage, ice packs, cold packs, gel packs, and ice-water baths (Knight, 1995). There is much controversy about whether it works to reduce muscle soreness or not.

1.1.3.4 Cryotherapy

Cryotherapy causes vasoconstriction, decreases microvascular permeability as well as oedema (Knight, 1995). The effect of cryotherapy on blood vessel diameter is variable. Initially cryotherapy will cause vasoconstriction, but after long exposure (after 15 – 40 minutes of cold exposure) to cold temperatures vasodilation will occur (cold-induced vasodilation) (Knight, 1995; Mac Auley, 2001). It is believed that this vasodilatation is a reflex to protect the muscle against cold damage. Cryotherapy's effect on the vasculature also seems

dependent on whether it is applied as a therapeutic intervention or not. Without any injury a cold application will decrease blood flow (Curl *et al.*, 1997; Karunakara *et al.*, 1999), but when applied to muscle after contusion injury no vasoconstriction occurs (Curl *et al.*, 1997) Cryotherapy is also believed to decrease inflammation (Knight, 1995) as well as secondary injury (Merrick *et al.*, 1999). In addition to cryotherapy decreasing microvascular permeability, and thus oedema, data also show that cryotherapy decreases interaction between leukocytes and endothelial cells that may contribute further to the decrement in oedema after cryotherapy (Deal *et al*., 2002).

The literature on the use of cryotherapy as a therapeutic intervention is inadequate (MacAuley, 2001; Mac Auley, 2001). It is also difficult to compare results of different studies due to the wide range of cryotherapy protocols used. The cooling effect of cryotherapy depends on the type of cold application (e.g. ice bag, or ice massage) (Zemke *et al*., 1998), duration of application (MacAuley, 2001; Mac Auley, 2001), and adipose tissue (Myrer *et al*., 2001). The size of the muscle group is also a contributing factor, because the deeper tissues, and thus a larger muscle, will need more time to cool down (Knight, 1995; Myrer *et al*., 2001; Zemke *et al*., 1998).

1.2 Skeletal muscle regeneration

Satellite cells, unlike their neighbouring myofibres, are capable of entering the mitotic cycle, an important factor for both muscle growth and regeneration. Satellite cell progeny fuse with existing myofibres, become true myonuclei (Moss

& Leblond, 1971) and thereby facilitate muscle growth and repair. Whether or not the satellite cells are highly involved in muscle hypertrophy in adults is still controversial.

1.2.1 Muscle growth and hypertrophy

The proliferation and fusion of satellite cells are responsible for myonuclei accretion during muscle growth. In the study by Moss and Leblond (1971), growing rats (14-17 days) were injected with a radioisotope to investigate muscle sections (radioautography) at 1, 18, 24, 48, and 72 hours after injection. Their results confirmed that myonuclei do not proliferate, but satellite cells are capable of DNA synthesis.

In a study by Kadi and Thornell it was shown that short-term (10 week) strength training can induce an increase in myonuclei (70%), as well as satellite cell (46%) number in human (female) *trapezius* muscles, in addition to increasing fibre size (Kadi & Thornell, 2000). It was suggested that the acquisition of myonuclei is important to assist hypertrophy after the strength-training program. A significant positive relationship between the proportion of satellite cells/mm^2 and the number of myonuclei/mm^2 ($r=0.5$) was found, however no distinction between quiescent and activated satellite cells was made in this study. Thus, it is unclear whether a 10-week strength training program increases the quiescent satellite cell pool in addition to increasing the activated satellite cells. An increase in satellite cell number (quiescent and activated) may be advantageous during training to 'prepare' for possible future muscle injuries, i.e. to allow for a better regenerative

response. Alternatively, the training may have caused unacknowledged micro-damage with subsequent satellite cell proliferation. Finally, it may be possible that the satellite cell pool has been enhanced by circulating stem cells (Ferrari *et al*., 1998; LaBarge & Blau, 2002). The following section explains the concept of stem cells in more detail.

1.2.2 Cell biology and stem cells

Stem cells are present in mammals during the development stage, as well as during post-natal and adult life (Figure 1.2). In fact, each one of us started as a totipotent stem cell, i.e. the zygote. For a cell to be recognised as a stem cell it first has to fulfil specific criteria. Stem cells are capable of self-renewal (by proliferation) and they are capable of differentiating into more than one type of tissue (multilineage differentiation) (Jankowski *et al.*, 2002). Cells of the zygote and the early blastocyst are totipotent, meaning that they are capable of producing a complete organism. Pluripotential cells are capable of forming the three germ layers, but cannot give rise to a complete adult organism (extra embryonic and embryonic tissue). Multipotential stem cells cannot develop into all three embryonic germ layers, but are capable of multilineage differentiation (Jankowski *et al.*, 2002; Lodish *et al.*, 2004), whereas an unipotent stem cell is capable of self-renewal and producing only one cell type (Lodish *et al.*, 2004). Adult stem cells are stem cells obtained from the different tissues in an organism post-natally. Refer to Figure 1.2 for a schematic presentation of the different stem cell types, as well as Table 1.1 for definitions of different stem cells.

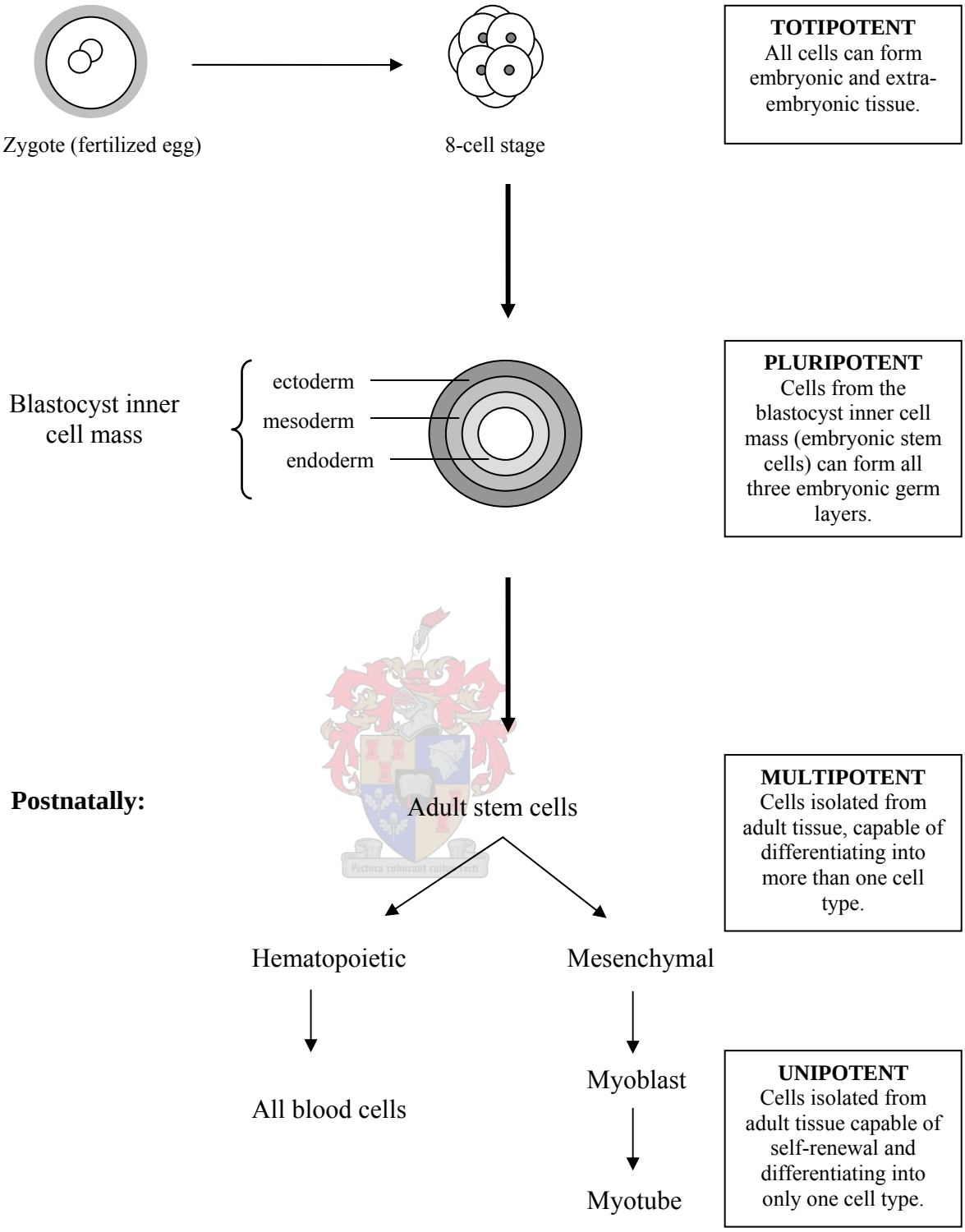
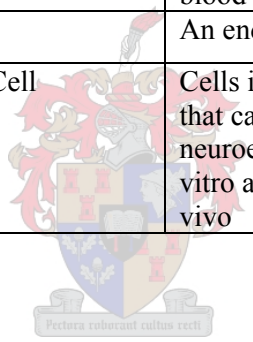


Figure 1.2. Stem cell potency during development.

Table 1.1 Different stem cell definitions. This table was taken from a journal article (Niesler, 2004) with the permission of the author, Dr. Carola U. Niesler.

STEM CELL AND PROGENITOR CELL DEFINITIONS	
Adult Stem Cell	An unspecialised cell, derived from a postnatal animal, capable of self renewing **AND** generating specialized mature cells
Hematopoietic Stem Cell	A stem cell, which can proliferate and differentiate into mature blood cells.
Mesenchymal Stem Cell	A stem cell, which can proliferate and differentiate into mesenchymal tissues (such as bone, cartilage, muscle)
Mesodermal Progenitor Cell	An unspecialised cell capable of yielding mesodermal tissue (such as muscle). Progenitor cells are not capable of self-renewal.
Hemangioblast	Earliest mesodermal precursor of both blood and vascular endothelial
Angioblast	An endothelial cell progenitor cell.
Multipotent Adult Progenitor Cell	Cells isolated from postnatal bone marrow that can differentiate into mesodermal, neuroectodermal and endodermal cells in vitro and into all embryonic lineages in vivo



Skeletal muscle contains a special cell population situated only in skeletal muscle, namely the satellite cells. These cells are mononucleated (Bailey *et al.*, 2001; Hughes & Blau, 1990) and located between the basal lamina and basement membrane (one of the layers of the sarcolemma (McComas, 1996)) of each skeletal muscle fibre (Bailey *et al.*, 2001) (Figure 1.1) Under normal conditions satellite cells are mitotically quiescent (Seale & Rudnicki, 2000), but are capable of maintaining or increasing their pool by proliferation (Hawke & Garry, 2001). Numerous stimuli, e.g. stretch, muscle damage, and exercise are capable of activating satellite cells in humans (Crameri *et al.*, 2004; Kadi *et al.*, 2004b), as

assessed by immunohistochemistry, and in animal models (Darr & Schultz, 1987), as assessed by autoradiography.

Although satellite cells have functional characteristics of stem cells, authors disagree on whether they are truly adult stem cells or whether they are progenitor cells (see Table 1.1 for definition). Satellite cells will therefore be referred to as stem cell-like cells, rather than stem cells.

1.2.3 Satellite cells

1.2.3.1 Cell cycle

In normal adult skeletal muscle, satellite cells reside in the G_0 phase, i.e. they are mitotically quiescent. Under appropriate conditions these cells become activated, start to proliferate and differentiate into myoblasts. After proliferation the myoblasts can fuse with each other or with an existing myofibre. During the different phases of the cell cycle satellite cells express distinct proteins that help to identify in which phase these cells exist. These cycle markers include CD34 (expressed by most quiescent satellite cells) (Beauchamp *et al.*, 2000), CD56 (expressed predominantly by activated satellite cells) (Malm *et al.*, 2000), MyoD (expressed by proliferating satellite cells) (Cooper *et al.*, 1999), and myogenin (expressed by differentiating satellite cells) (Rantanen *et al.*, 1995). For this whole process of satellite cell activation, proliferation, and differentiation, many growth factors and cytokines are needed.

1.2.3.2 Model systems

Cell cultures are a good model for studying different aspects of muscle regeneration and through them much can be learned about satellite cells' responses to stimuli during the activation, proliferation and differentiation processes. However, application of this information to the regeneration process *in vivo* is difficult and must be approached with caution. In some circumstances the effects *in vivo* contradict what has been observed *in vitro*.

Different cell lines are often used to investigate the response of muscle to damage or stress *in vitro*, for example C2C12 cells are specifically used to investigate satellite cells. It is also possible to study satellite cells in single myofibre cultures (Bischoff, 1990; Li *et al.*, 2000). Bischoff (Bischoff, 1986) designed a protocol to study satellite cells *in vitro*. This protocol entailed the isolation of muscle fibres with their associated satellite cells still intact. In cell culture, the proliferating satellite cells do not detach from their associated muscle fibre, but their numbers increase to cover the entire myofibre. This culture method of intact fibres is a better simulation of the *in vivo* state of satellite cells compared to cultures of satellite cells in the absence of mature myofibres. It has been shown in isolated muscle fibre cultures that complete skeletal muscle myofibre regeneration can occur in the presence of satellite cells alone (without the need for other stem or progenitor cells) (Zammit *et al.*, 2002).

Animal models which allow for gene manipulation, to delete or inactivate specific genes of interest, make it possible to investigate the specific roles of their products

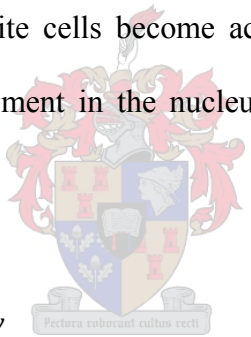
in vivo. For example, by inactivating the fibroblast growth factor 6 gene, Floss and his colleagues have shown that this growth factor is important for muscle regeneration (Floss *et al.*, 1997). It is also possible to modify a specific gene so that cells can be tagged and easily identified. An example of such a gene modification is that of transgenic desmin nls lacZ mice (Lescaudron *et al.*, 1997; Lescaudron *et al.*, 1999), where all cells expressing desmin are positive for lacZ and can therefore be identified by the addition of X-gal. In this model desmin is only expressed during embryonic development and during muscle regeneration in adults. Without, or in combination with these models, it is necessary to identify satellite cells or satellite cells by other means.

1.2.3.3 Identifying satellite cells

A number of techniques can be used to identify satellite cells in skeletal muscle sections. Most commonly, electron microscopy (EM) and light microscopy are used. Labelling certain biological elements with radioactive isotopes or a nucleotide analogue can be used to identify targeted molecules or structures. For example, ^{3}H-labelled thymidine or bromodeoxyuridine (BrdU, a thymidine analogue) will be incorporated into the nuclei of proliferating cells (Hurme & Kalimo, 1992; Rantanen *et al.*, 1995). Both light and electron microscopy are used to visualise these labelled nuclei. However, using only light microscopy will not allow one to distinguish between proliferating satellite cells and proliferating fibroblasts (or any other proliferating cell in close proximity to myofibres), thus care should be taken when using only light microscopy in skeletal muscle sections when investigating satellite cells.

a) Electron microscopy (EM)

Satellite cells were first discovered by Mauro (Mauro,1961) using EM. EM is a very valuable tool to investigate cells at ultra-structural level and to gather morphological information. Using EM, satellite cells are identified by their location between the basal lamina and sarcolemma of a myofibre. Distinction can be made between quiescent and activated satellite cells based on their differences in morphological and morphometric appearances. In transverse sections, quiescent satellite cells' nuclei occupy most (about 60%) of their surface areas with their plasmalemmae separated from their associated myofibres' sarcolemmae. When satellite cells become activated, they enlarge or elongate with an accompanied decrement in the nucleus to cytoplasm ratio (Gregory & Mars, 2004).



b) Light microscopy

Dyes and stains commonly used for light microscopy (e.g. hematoxylin, eosin) do not enable identification of satellite cells. Immunohistochemistry (the use of antibodies in conjunction with microscopy) on the other hand, can be used to identify satellite cells. Antibodies against laminin (located in the basal lamina) (Bischoff, 1986) or dystrophin (located within the sarcolemma) (Ganong, 1997b) are commonly used to identify satellite cells by their location (between the basal lamina and sarcolemma). With the fairly recent discovery of specific markers for satellite cells, especially in the last decade, and the commercial availability of antibodies to identify these markers, it has now also become possible to identify

satellite cells themselves immunohistochemically in skeletal muscle sections. Antibody binding can be demonstrated using non-fluorescent dyes, such as diaminobenzidine (DAB) (bright-field microscopy) (Cooper *et al.*, 1999; Kadi & Thornell, 2000) or fluorescent substances such as Texas Red or FITC (dark-field microscopy) (Cooper *et al.*, 1999; Crameri *et al.*, 2004; Kadi *et al.*, 2004b). Satellite cell markers and their distribution are listed in Table 1.2.

Controversy still exists surrounding some of these markers (and this will be highlighted whenever appropriate in the following sections) due to the recent nature of immunohistochemistry as a technique to identify satellite cells. These controversies are due to different laboratories using different models, e.g. cell cultures versus muscle tissue sections, to investigate expression of these markers. Furthermore, different research groups use antibodies developed by different companies, or some research groups develop their own antibodies. Satellite cell-specific antibodies are a fairly new concept and the development process of more sensitive (and even new) antibodies is quite fast, thus also contributing to the controversies. As can be seen from Table 1.2, markers are localised in various areas of the satellite cell's structure. Some markers are specific for only one phase of the cell cycle, e.g. CD34, whereas most markers are expressed in satellite cells at several stages of their cell cycle, e.g. m-cadherin. Co-staining with two or three antibodies (satellite cell specific or for another protein) is advisable to ensure positive identification of satellite cells. Often laminin, which stains the basal lamina, is used in conjunction with a satellite cell marker to exclude any positively stained cell that lies outside the boundaries of the myofibre (Rantanen

et al., 1995). On the other hand it must be taken into account that both the myonuclei and satellite cell nuclei lie within the boundary of the basal lamina, and although myonuclei lie beneath the sarcolemma and satellite cell nuclei outside

Table 1.2 List of possible satellite cell markers adapted and modified from Hawke & Garry (2001) and Charge & Rudnicki (2004).

Marker	Expression in SC*	Localisation	ref
CD34	Quiescent	membrane	2
CD56 (NCAM, Leu19)	quiescent, activated, proliferating	membrane	1, 2
MyoD (Myf3)	activated, proliferating	nucleus	1, 2
Myogenin (Myf4)	Differentiating	nucleus	
M-cadherin	quiescent, activated, proliferating	membrane	1, 2
MNF	quiescent, activated, proliferating	nucleus	1, 2
Pax7	quiescent, activated, proliferating	nucleus	1, 2
c-Met	quiescent, activated, proliferating	membrane	1, 2
VCAM-1	quiescent, activated, proliferating	membrane	1, 2
Desmin	activated, proliferating	cytoskeletal	1, 2
Myf5	(quiescent), activated, proliferating	nucleus	1, 2
Syndecan-3	quiescent, activated, proliferating	membrane	2
Syndecan-4	quiescent, activated, proliferating	membrane	2
MSTN	Quiescent, (proliferating)	nucleus	2
IRF-2	Quiescent, proliferating	nucleus	2
Msx1	Quiescent	nucleus	2

* Expressed in growing or adult organisms

1. (Hawke & Garry, 2001)
2. (Charge & Rudnicki, 2004)

Abbreviations: MNF, myocyte nuclear factor
NCAM, neural cell adhesion molecule
VCAM, vascular cell adhesion molecule
PCNA, proliferating cell nuclear antigen
MSTN, myostatin
IRF-2, interferon regulatory factor-2

the sarcolemma, the distinction between the sarcolemma and basal lamina is not that clear with light microscopy. Thus, laminin may not be the best co-stain to be used with other satellite cell markers, especially when staining with satellite cell nuclear markers

c) EM versus Immunohistochemistry

Both these methods of visualising satellite cells have their advantages and disadvantages. EM is useful and very reliable for accurate identification of cells and other structures, but is not very useful for quantitative studies as few cells are viewed in each field. Furthermore, EM cannot be used to identify the different phases that a satellite cell may find itself during regeneration, except for quiescent and activated satellite cells (Gregory & Mars, 2004). Immunohistochemistry on the other hand is more useful for quantitative studies (larger number of cells per field of view), but cannot identify satellite cells with the same accuracy as EM. The latter is due to the fact that the basal lamina and sarcolemma are not distinguishable with light microscopy. EM is very time consuming, because the visible field under the microscope is very small, thus it will take longer to view a large area.

1.2.3.4 Regulation of satellite cell activity

The exact mechanisms involved in satellite cell activation are not well understood. Satellite cells on a myofibre can be activated in the absence of necrosis of the myofibre (Darr & Schultz, 1987). Furthermore, activation is not always

accompanied by proliferation or differentiation. For example, a bout of exercise can induce satellite cell activation without evidence of proliferation and differentiation (Crameri *et al.*, 2004).

Satellite cell activation following exercise has been investigated in young (1 month old) compared to mature (3 month old) rats (Darr & Schultz, 1987). At days 1, 2, 3, and 5 post-exercise, the *soleus* and *extensor digitorum longus* muscles were analysed for satellite cell activation making use of autoradiography. Activation peaked a day earlier in mature rats than in young growing rats. In addition, the peak of activation differed between the two muscle groups within the mature group, with the peak occurring at day 1 for the *soleus* and day 2 for the *extensor digitorum longus* muscle. Even though a small number of fibres showed some evidence of degenerative and necrotic features (characterised by infiltration of fibres by macrophages and mononuclear cells), it was not only these fibres that had satellite cell activation. Fibres without lesions also displayed an increase in satellite cell activation post-exercise. Whether this activation is due to the mechanical or cytokine effects of exercise, or both, is not clear. It is also not clear why the different muscles demonstrated different time courses, or why the age of the rats influenced the time course of satellite cell activation.

a) Satellite cell-myofibre contact

Bischoff (Bischoff, 1990) has indicated that the contact between a satellite cell and its mature myofibre might play a role in regulating the state of the satellite

cell. Specifically it seems that the mature myofibre might reduce proliferation of satellite cells in contact with it. This suggests one or more of the following:

1. The myofibre produces a factor(s) following damage that activates the satellite cells.
2. Damage to the sarcolemma itself will disturb contact between the satellite cell and myofibre which will then activate the satellite cell.
3. Another myofibre produce cytokines to activate satellite cells.
4. Friction due to mechanical stretching by exercise may activate satellite cells.

Bischoff *et al.* were unsuccessful in culturing satellite cells in the presence of the sarcolemma, but in the absence of the basal lamina. In the absence of the basal lamina the satellite cells cannot stay intact with myofibres. Crushed muscle extract (acting as mitogen) was required to activate satellite cell growth. However satellite cells in contact with both the basal lamina and plasmalemma (live myofibre) are about 40% less responsive to mitogen compared to satellite cells which are not. Therefore contact is important for satellite cell proliferation, but activation may require the basal lamina to become permeable to certain growth factors.

Once satellite cells are activated, they may start to proliferate and some of the progeny may differentiate into muscle cells. These latter processes are under control of the myogenic regulatory factors (MRFs). The MRFs include Myf5, MyoD (Myf3), myogenin (Myf4), and MRF4 (Myf-6/herculin) (Kerst *et al.*, 2000) which are members of the myogenic basic helix-loop-helix (bHLH) family of

transcription factors (Alberts *et al.*, 1983; Bailey *et al.*, 2001). These transcription factors are involved during the development of skeletal muscle, but are also involved in satellite cell differentiation and maturation postnatally (Table 1.3).

Table 1.3 Summary of myogenic regulatory factors (MRFs).

MRF	Function	Expression during SC cell cycle
Myf5	May be involved in SC self-renewal	quiescence/activation
MyoD	Very important for SC progression to terminal differentiation	proliferation
Myogenin	Important for differentiation	differentiation
MRF4	May play role in muscle fibre maturation	fusion

b) Myf5 and MyoD

Sabourin *et al.* (Sabourin *et al.*, 1999) used primary cultures of wild-type *vs* MyoD deficient mouse skeletal muscle to investigate the differentiation potential of MyoD-/- myogenic cells. The finding that MyoD deficient cells displayed a reduced differentiation potential, substantiates the view of MyoD being an important factor in myogenic determination. Their results also indicated that Myf5, although also a MRF, cannot substitute the function of MyoD.

Cooper and his colleagues (Cooper *et al.*, 1999) showed that activated murine satellite cells express either MyoD or Myf5, or both *in vivo*. They also showed

that MyoD is expressed by all proliferating satellite cells. I propose that the difference between these two populations of satellite cells may be that those satellite cells expressing MyoD early are somehow primed to proliferate and differentiate immediately when stimulated, while those that express Myf5 support the quiescent satellite cell pool. This would infer that those expressing both MyoD and Myf5 can proliferate and differentiate as well as maintain the quiescent satellite cell pool (depending on the stimulus). Apart from the authors' findings and conclusions their results suggest the existence of three distinct populations of satellite cells *in vivo*: $MyoD^{+}$, $Myf5^{+}$, $MyoD^{+}/Myf5^{+}$. However, in cell culture, nearly all (>98%) activated satellite cells were shown to express both MyoD and Myf5 at 24 hours (Zammit *et al.*, 2002). The latter study infers that data obtained from *in vitro* models may not always be absolutely applicable to the *in vivo* situation.

The possibility of more than one satellite cell population was hypothesised a few years earlier by Rantanen and his colleagues (Rantanen *et al.*, 1995). In the study by Rantanen *et al.*, desmin-, MyoD- and myogenin positive nuclei were detected in rat skeletal muscle after damage before BrdU (indication of proliferation) was detected. These authors proposed that one population is ready for immediate differentiation, without proliferation (*'committed' satellite cells*). The other population undergoes mitosis prior to dividing into one daughter cell for differentiation and another for future proliferation (*'stem' satellite cells*). However, they did not address the possibility of unequal expression of receptors for satellite cell activation factors on distinct satellite cell populations. If this was

the case, different activators may stimulate the distinct populations. It would be valuable to determine whether receptor expression levels can be correlated to different satellite cell populations. To date no research group has investigated this possibility. Recently an electron microscopy study of monkey skeletal muscle showed two patterns of satellite cell activation after ischaemia and reperfusion. These authors concluded that these two different responses in satellite cell activation may either represent different functions or may infer the existence of two satellite cell populations (Gregory & Mars, 2004).

The possibility that two distinct satellite cell populations exist should not be confused with the fact that skeletal muscle (and other tissues) also have a pool of side population (SP) cells. This muscle SP cell pool is a population of stem cells that can be purified from skeletal muscle by fluorescence-activated cell sorting (FACS) and that can be identified by their exclusion of the DNA dye, Hoechst 33342. This population expresses the hematopoietic stem cell marker Sca-1 and may also express another hematopoietic marker, CD45. Muscle SP cells are capable of differentiating into hematopoietic cells, muscle cells and satellite cells (reviewed by Asakura, 2003). In contrast, committed satellite cells don't express these particular hematopoietic stem cell markers.

Cornelison and Wold (Cornelison & Wold, 1997) investigated the expression of all MRFs in single murine satellite cells still attached to their myofibres. Their study indicated that c-met mRNA is expressed by all satellite cells in fibre cultures, but m-cadherin mRNA is expressed initially during activation in only a

small fraction (<20%) of satellite cells. They have also shown that there was no significant difference in c-met and m-cadherin mRNA expression between satellite cells from predominantly fast and slow fibres. All MyoD- or myogenin-positive cells expressed c-met. The conclusion was that c-met is expressed by quiescent satellite cells (intact muscle) and during the different phases after activation (fibre culture).

c) Myogenin

It has been shown that myogenin protein expression increases during the differentiation phase of the satellite cell cycle. To date the function of myogenin is unclear. Myogenin knockout animals are not viable. The reason for unsuccessful breeding is due to the fact that myogenin-mutant mice die immediately after birth. These mutant animals also have a reduction in size of all skeletal muscles. This infers the essential role of myogenin for the functional development of skeletal muscle (Hasty *et al.*, 1993).

Myogenin has been analysed in adult rats in response to contusion and toxic muscle injury. Traumatic contusion damages the connective tissue and the vasculature whereas notexin (toxin) only damages the myofibres without damaging the blood vessels and nerves. Although the toxic injury induced less muscle damage, muscle regenerating events after both injury models were very similar. Mitotic activity, as indicated by BrdU-positive nuclei, was detectable at 24 hours post-injury. Myogenin positive satellite cell nuclei were detectable at 12

hours post-injury and expression increased 5-fold at 36 hours post-injury (Rantanen *et al.*, 1995).

d) MRF4

MRF4 (alternatively known as Myf6 or herculin) protein was found to be present prior to and after fusion in regenerating myofibres, but not in satellite cells. This suggests that MRF4 is important for myofibre fusion, but does not play a role in satellite cells during proliferation prior to differentiation (Zhou & Bornemann, 2001). In a recent study it was concluded that MRF4 is also a determination gene for the myogenic lineage. This became evident when Myf5:MyoD double mutant mice developed skeletal muscle only if the MRF4 gene was not deleted as well (Kassar-Duchossoy *et al.*, 2004).

In addition to the MRFs needed for maintaining satellite cells in their cell cycle, other factors are needed to regulate the expression of MRFs as well as the migration and activation of satellite cells. For example, myoblasts are capable of migrating across the basal lamina, even in the absence of muscle damage (Hughes & Blau, 1990). Some of these factors will be discussed in the following section.

e) Growth factors

The majority of studies on growth factors have been conducted using primary satellite cell cultures, or myoblast cell lines derived from satellite cells (e.g. C2C12 cells). Most growth factors have no effect on quiescent satellite cells of adult muscle *in vivo* as long as their myofibres are intact. Plasma membrane

damage therefore in some way primes the satellite cells thereby making them responsive to growth factors. The only exception is hepatocyte growth factor (HGF) (also known as scatter factor, SF) which is able to activate quiescent satellite cells, even in the absence of muscle damage (Tatsumi *et al.*, 1998).

Crushed human muscle has the ability to stimulate proliferation, alignment and fusion of C2C12 cells (Li *et al.*, 2000). These authors identified that this ability could be attributed to a ~ 40 kDa protein in a fraction of the muscle extract. The fraction containing this unidentified protein stimulated regeneration even when injected into rats after strain-induced muscle injury. Tatsumi *et al.* identified the unknown satellite cell activator found in crushed muscle extract to be HGF (Tatsumi *et al.*, 1998). Although HGF is a 90 kDa protein the 40 kDa protein identified by Li *et al*. (Li *et al.*, 2000) may be a subunit of HGF.

In addition to acting as an activation factor, HGF also enhances satellite cell proliferation, but inhibits differentiation (*in vivo* and *in vitro*). The timing as well as the dose of exogenous HGF is important (Miller *et al.*, 2000). In Miller *et al*.'s study it was shown that a dose of 50 ng, and not 6.25 ng, HGF could induce satellite cell proliferation. Also, when 50 ng HGF was added every day to muscles starting on the day of injury (freeze damage), HGF induced significant myoblast proliferation the following day. However, at day 2 or 3 post-injury, myoblast numbers were not significantly increased.

Nitric oxide (NO) is also indicated as a role player in the activation of satellite cells (Anderson, 2000). This author has also shown immediate satellite cell activation upon muscle damage. Stretching seems to play a very important role in initiating the events for satellite cell activation. Activation of satellite cells is not accomplished directly through stretching, but rather indirectly via the release of NO and HGF. Data also indicate that NO is released prior to HGF which then activates satellite cells (Tatsumi *et al.*, 2002).

Bischoff was able to stimulate satellite cells to proliferate on whole single fibres by adding chick embryo extract (CEE). This proliferation was assessed by following the incorporation of [*methyl*-^{3}H]thymidine ([^{3}H]TdR) into satellite cells (not myonuclei). The satellite cells also enlarged after adding the CEE. After 4 days some of the labelled satellite cells fused with each other, but not with their associated fibres (Bischoff, 1986). In the same study several purified growth factors and mitogens were also studied using single fibre-satellite cell units. Only fibroblast growth factor (FGF) was capable of stimulating satellite cell proliferation. However, more recently two other growth factors have been implicated. Insulin-like growth factor I (IGF-I) enhances short-term satellite cell proliferation (Chakravarthy *et al.*, 2001) as well as satellite cell migration *in vitro* and *in vivo* (Lafreniere *et al.*, 2004). Erythropoietin has been shown to stimulate proliferation of C2C12 cell cultures, but to inhibit differentiation of these cells (Ogilvie *et al.*, 2000). Another study has also shown that acidic fibroblast growth factor (aFGF) can enhance proliferation and differentiation of satellite cells *in*

vitro (isolated form *soleus* and *tibialis anterior* rat muscles - a primary cell culture) (Dusterhoft & Pette, 1999).

Another approach to investigate a factor, instead of adding the factor to a culture *in vitro* or injecting it *in vivo*, is to make use of knockout animals. By inactivating the fibroblast growth factor 6 (FGF-6) gene in mice it has been shown that FGF-6 may play a critical role in skeletal muscle regeneration after crush injury (Floss *et al.*, 1997). This growth factor appears to be a necessary activator of satellite cells, and may also possibly play a role in MyoD expression during muscle regeneration. Under normal conditions, FGF-6 mutants (FGF-6 (-/-)) and wild-type mice did not differ in their skeletal muscle immunohistochemistry pattern, myotube ultra-structure, or in their satellite cell morphology. However, the FGF-6 mutant appeared to have a slightly higher amount of satellite cells. After freeze-crush injury in the wild-type mice these authors showed that mRNAs of FGF-6, MyoD as well as myogenin were up-regulated during regeneration. As early as 4 days post-injury, new myofibres were seen in the lesion in wild-type mice. This was in contrast to muscle repair in the mutants, where smaller and fewer newly formed myofibres and more mononuclear cells were observed. Two weeks after injury, wild-type mice myotubes showed no more signs of differentiation and there was almost no indication of any damage. In comparison, the mononuclear cell number in mutants was still high and injury was accompanied by a small number of degenerated myotubes, large amounts of collagen (indicating fibrosis), and a lack of differentiated myotubes. At 3 weeks the lesions in wild-type mice were completely regenerated, whereas in the mutant mice there were two groups:

one presented strong fibrosis with large amounts of mononuclear cells; the second had moderate fibrosis with high numbers of degenerated myotubes and mononuclear cells. Interestingly, the typical pathological reaction was detected in all FGF-6 mutant mice, but the regeneration response in wild-type littermates varied. In the wild-type mice regeneration progressed very well at days 3-8 with complete reconstruction after 2-3 weeks.

f) Chemotaxis (migration)

Satellite cells have a good migrating capacity, but not much is known about the chemotactic signals involved. Bischoff (Bischoff, 1997) used an *in vitro* model to investigate the effects (chemotactic activity) of several growth factors and wound fluid on satellite cells (isolated from rat skeletal muscle). Chemotactic activity was assessed by migration chambers – the more cells that migrated across the membrane dividing the chambers, the higher the chemotactic activity of the specific growth factor tested. Only HGF and transforming growth factor-beta (TGF-β) showed chemotactic activity with a maximum activity range of 1-10 ng/ml for both. HGF concentrations higher than 10 ng/ml inhibited migration of satellite cells. The reason for higher concentrations causing inhibition is not known. This study was the first to show that HGF might be a satellite cell chemoattractant during muscle regeneration. At 5 ng/ml TGF-β stimulated maximum migration but inhibited it to basal level at 50 ng/ml. This study suggests that the chemotactic activity of platelet-derived extracts can be attributed at least in part to TGF-β (platelets are a major source of TGF-β (Ganong, 1997a)). Crushed muscle extract was also able to stimulate chemotaxis of satellite cells in a

dose-dependent manner. Other factors which had no effect on satellite cell proliferation included insulin, dexamethasone, platelet-derived growth factor, epidermal growth factor and luteinising hormone. Taken together, the data of this study show that migration of satellite cells is stimulated within strict concentration gradients of several soluble factors released during muscle injury. Some of these soluble factors are attributable to the immune response to muscle damage.

g) Inflammation (macrophages, cytokines, etc)

In an extreme model of muscle damage it was shown that neutrophils and monocytes appeared 8 hours after contusion injury in the rat *gastrocnemius* muscle. These cells appeared within the basal lamina of necrotic fibres where they phagocytose the debris. The number of these leucocytes increased until the end of the observation period of 48 hours. Following the toxic injury, inflammatory cell influx was similar to that in the contusion injury with macrophages virtually filling the basal lamina cylinders at 24 to 48 hours (Rantanen *et al.*, 1995).

Blood borne macrophages play a key role in the regeneration process (Lescaudron *et al.*, 1999). In addition to removal of debris, these cells also secrete factors such as TGFβ, TNFα, IFNα and -β, IL1α and -β (Ganong, 1997a) which may also promote repair. For example, as mentioned before, TGFβ has been shown to play a role in satellite cell activity (Bischoff 1997).

In the study by Lescaudron *et. al.* (Lescaudron *et al.*, 1999) tissue from transgenic *pHuDes-nls-LacZ* mice (transgenic mice carrying a *lac-Z* reporter gene that is under control of a truncated human desmin promoter - refer to appendix A for more detail) was transplanted into non-transgenic mice and regeneration activity of the transplanted tissue investigated *in vivo*. Tissue incubated with either macrophage inflammatory protein beta (MIP 1-β) or vascular endothelial growth factor (VEGF) prior to transplantation, displayed an enhanced infiltration of host macrophages. Only pre-incubation with VEGF increased myotube formation (6-fold increase) possibly due to an enhanced re-vascularisation which would enhance the transport of growth factors to the transplant site. It may have been interesting to analyse not only satellite cells of the donor tissue, but also host tissue and investigate fusion of host satellite cells with transplant tissue. The authors did not address the possibility that this enhanced myotube formation in the VEGF group could be due to other functional effects of VEGF. Subsequent studies indicate that VEGF stimulates migration of satellite cells, which should lead to an increase in myotube formation, and that it may protect these cells against apoptosis (Arsic *et al.*, 2004; Germani *et al.*, 2003).

An important finding of the original study was that those mice depleted of macrophages (by radiation) did not show any regeneration activity (Lescaudron *et al.*, 1999). This indicates that macrophages do play a key role in the regeneration process. This is of importance in the light of our study as it is believed that cryotherapy reduces inflammation (Knight, 1995). However, it is unclear whether extended cryotherapy decreases macrophage infiltration to such an extent that it

retards the regeneration process, or whether it reduces secondary injury (Merrick *et al.*, 1999; Merrick, 2002) and thus, lessens the required repair.

The rationale for using cold treatment is that it is believed to decrease inflammation as well as the chance of secondary injury (Knight, 1995; Merrick *et al.*, 1999; Merrick, 2002). However, Yackzan and colleagues (Yackzan *et al.*, 1984) concluded that ice massage (whether applied immediately, 24 h or 48 h after exercise) does not alleviate muscle soreness associated with DOMS. However there are certain methodological problems with this study, including the fact that the ice massage was applied for only 15 minutes and that this one application might not be sufficient to be effective. Furthermore, due to massaging, the muscle was not exposed to the cold treatment for a full 15 minutes.

As discussed by Merrick (Merrick, 2002), although it is suggested that cryotherapy may decrease secondary injury, it may be that it only delays the second injury phase. Furthermore, it is difficult to distinguish whether damage has occurred by primary or secondary injury. Although several studies have investigated the effect of different cold interventions on DOMS, all these studies (including most studies investigating other treatment modalities) have focussed only on changes in clinical symptoms of DOMS and or the physical/performance parameters. None of these studies have investigated the effect of cold treatment on the muscle recovery process, specifically the satellite cells which are responsible for muscle repair. It is very important for an elite athlete suffering

from muscle soreness to alleviate the clinical symptoms, but it is also very important that his/her recovery is promoted.

1.2.3.5 Satellite cells in human subjects

a) Distribution of satellite cells

A study by Kadi and Thornell has suggested that the number of satellite cells per myofibre may be related to the number of myonuclei in human muscle biopsy samples (Kadi & Thornell, 2000). Furthermore, Maier and Bornemann (Maier & Bornemann, 1999), who investigated myofibre diameter and satellite cell numbers in normal and diseased muscle, demonstrated that in normal skeletal muscle the number (frequency) of satellite cells is not influenced by the myofibre diameter. In contrast, muscular myopathies (inflammatory, neurogenic, and non-Duchenne myopathies) showed a positive relationship with an increase in fibre diameter.

b) SC in aging muscle

Post-natally there is generally no increase in muscle fibre number, but rather an increase in myofibre length and diameter due to growth and hypertrophy respectively. In animal models it has been shown that fibre hyperplasia can occur with resistance training but data are controversial (reviewed by Abernethy *et al.*, 1994). Also, exercised-induced hyperplasia in humans is unsubstantiated (reviewed by Matoba & Gollnick, 1984; Taylor & Wilkinson, 1986). With an increase in fibre diameter the number of myonuclei also increases, and this is due to the proliferation of satellite cells and their subsequent fusion with existing muscle fibres. Controversies exist about the decrease in satellite cell number with

increasing age. Roth *et al*. have not shown any differences between satellite cell numbers of healthy young and older men and women (Roth *et al.*, 2000), whereas Sajko *et al*. have indicated that the number of m-cadherin^{+} satellite cells decreases with age (Sajko *et al.*, 2004). However, in the latter study muscle samples were obtained at autopsies (6 - 24 hours post-mortem) which may have influenced the outcome of the results.

A further study also demonstrated a significantly smaller satellite cell population in the old compared to young men and women (Kadi *et al.*, 2004a). This discrepancy might be due to several factors, including different histochemical methods, i.e. electron microscopy (Kadi *et al.*, 2004a; Roth *et al.*, 2000) versus immunohistochemistry (Kadi *et al.*, 2004a). In addition, Roth and his group used sedentary subjects, whereas Kadi *et al* used moderately active subjects. Although the ages of the young subjects were similar, Kadi's elderly group were older. Furthermore, different muscle groups were analysed in the investigations.

c) Exercise

Not much is known about the response of satellite cells to exercise, especially in humans. Recent studies (Crameri *et al.*, 2004; Kadi *et al.*, 2004b) have shown that one bout of exercise is enough to activate satellite cells. In the study by Kadi's group, muscle biopsies were taken immediately (within 10 - 60 seconds) after the exercise bout (which consisted of one-leg cycle ergometry for 30 minutes at 40% and 75% of VO_{2max}) and myonuclei stained positively for myogenin. In contrast, Crameri and her group found no positive staining for myogenin.

Crameri and colleagues concluded that one bout of exercise (consisting of one bout of high intensity eccentric exercise with one leg: 50 one-leg 'drop down' jumps from a platform, 8 x 10 maximal eccentric knee extensions at -30 degrees.s^{-1} and 8 x 10 maximal eccentric knee extensions at -180 degrees.s^{-1}) might not be sufficient to stimulate satellite cells to differentiate. Apart from baseline biopsies, Crameri *et al*. took biopsies 5 hours post-exercise, and on days 2, 4, and 8 post-exercise. Taken together, data from both these studies may suggest that one bout of exercise is enough to stimulate transient myogenin protein expression in the myonuclei, but that this is unlikely to be sustained and may be completely unrelated to satellite cells.

1.2.3.6 Application/Biotechnology

Due to practical and ethical issues, skeletal muscle regeneration has been studied most frequently in animal and cell culture models. However, although *in vitro* studies have proved to be very helpful in the understanding of satellite cells, results are not always applicable to the *in vivo* situations. One must bear in mind that not only one factor (e.g. growth or stimulating factor, or cytokine) is present in a mammalian whole body, but each factor is part of a complicated physiological system. This means that the activity of a factor *in vitro* versus *in vivo* may be completely different. Thus there is a need to investigate factors not only *in vitro*, but in conjunction with other factors *in vivo* to fully understand satellite cell activity. Satellite cell activity can be studied *in vivo* in different myopathies (Bornemann *et al.*, 1999; Laule & Bornemann, 2001). Although myopathies are a serious problem, they are applicable to a smaller percentage of

the human population. It is also necessary to investigate circumstances that are applicable to the whole population. DOMS may be a good model to investigate satellite cell activity as most of us have experienced it at some stage in our lives. In addition, information on satellite cell activity gathered from such a study would also be applicable to understanding satellite cells in general.

In summary, DOMS is associated with muscle micro-injury and upon muscle damage the recovery pathway will be switched on. However, satellite cell activity during and after DOMS is not well established. Currently cryotherapy is a very popular treatment used by athletes, but the use of this therapy is unsubstantiated by scientific research. Importantly, so far no human study conducted to investigate the effect of cryotherapy on DOMS has investigated the effect it may have on skeletal muscle repair. In fact, no human study so far has addressed the effect of any DOMS therapy on muscle recovery at tissue level. This is very important since the competitiveness in today's sports participation creates the need for athletes to recover faster and not just to alleviate their pain levels. Thus the aims of this study were as follows:

1) To characterise the satellite cell activity in humans with DOMS, as well as
2) To investigate the effect of an ice application on this satellite cell activity.
3) To investigate the efficacy of the set of antibodies used in this study, making use of an *in vitro* model, i.e. C2C12 cells under normal conditions.

CHAPTER 2: MATERIALS AND METHODS

2.1 *In vivo* (Human subjects)

2.1.1 Subject recruitment & inclusion criteria

This study was approved by Sub-committee C of the Research Committee of the University of Stellenbosch. Six male human subjects were recruited by means of advertisements and word of mouth. The subjects were informed of the procedure verbally and in written form. They signed an informed written consent before they were allowed to start with the protocol. Subjects were included if they fulfilled the following criteria:

- age: 18 – 35 years
- regular participation in recreational physical activities, excluding regular downhill running or walking
- informed written consent was obtained

The exclusion criteria were:

- regular, systematic training
- regular participation in competitive events
- participation in hiking or hill walking in the 3 months prior to the study
- treatment for an inflammatory condition
- muscle injury in the previous 3 months
- a cardiovascular condition
- informed consent was not obtained

2.1.2 Induction of delayed onset muscle soreness (DOMS)

At least two weeks prior to inducing DOMS the subjects completed a peak treadmill speed (PTS) test. During this test, both VO_{2max} and maximum speed attainable during an incremental treadmill test to fatigue, were determined. To induce DOMS subjects performed a 40-minute intermittent downhill run (DHR) protocol. The protocol consisted of five 8-minute bouts at –10% (-5.7 degrees slope) on a motorised treadmill (RUNRACE HC1200; Technogym, Italy). Each bout was followed by a 2 min standing rest. The speed of the treadmill was set at 70% (two subjects with $VO_{2max} < 50$) or 80% (four subjects with $VO_{2max} > 50$) of each subjects' own PTS. Subjects were instructed not to exercise for the duration of the study after they had completed the DHR. All laboratory testing, including the ice treatment, occurred at an ambient room temperature of 18 ^{o}C.

The DHR protocol used in this study was modified from Eston *et al*. (Eston *et al.*, 1994; Eston *et al.*, 1996a; Eston *et al.*, 1996b). To our knowledge all DHR protocols have previously made use of an individual's heart rate (HR) (Byrnes *et al.*, 1985b) or predicted maximum heart rate (Eston *et al.*, 1996b), or VO_{2max} (Schwane *et al.*, 1983) to determine their DHR treadmill speed. In our laboratory we found that the HR and VO_{2max} drift with time making it difficult to determine a constant speed for the test. Thus, it was decided to use each individual's PTS to determine their individual DHR speed. The final protocol had been used to induce DOMS in a previous unpublished study in our laboratory (I. Neethling, Honours student 2002).

2.1.3 Ice treatment

Immediately after cessation of the DHR each subject received their first ice application on only one leg. The ice treatment involved the placing of a plastic bag (400 x 340 mm; Jiffy Freezer bag, Transpaco Flexibles) filled with crushed ice, weighing approximately 2.5 kg, directly onto the upper leg (quadriceps muscle group) and without any interface between the plastic and the skin. The aim was to place the ice pack in a position that included the typical biopsy areas. Each ice application lasted 30 minutes and was repeated every two hours, five times a day for three days on the same leg. Subjects received ice treatment for a total of three consecutive days, including the day of the DHR (Figure 2.1). The researcher applied all ice treatments to ensure correct application. The leg receiving ice was randomly selected to be the dominant or non-dominant leg.

Subjects were informed not to use any medication for the duration of the study, especially after receiving biopsies. This was to ensure that only the effect of the ice treatment would be investigated.

2.1.4 Muscle biopsies

In total five biopsies were obtained from each subject. A baseline biopsy was obtained from each subject (from the dominant leg) at least four weeks before inducing DOMS. The remaining four biopsies were obtained either on days 1 and 7 post-DHR (three subjects), or days 2 and 9 post-DHR (three subjects). At each of these time points two biopsies were obtained from a subject, one from each leg (treated and untreated leg) (Figure 2.1).

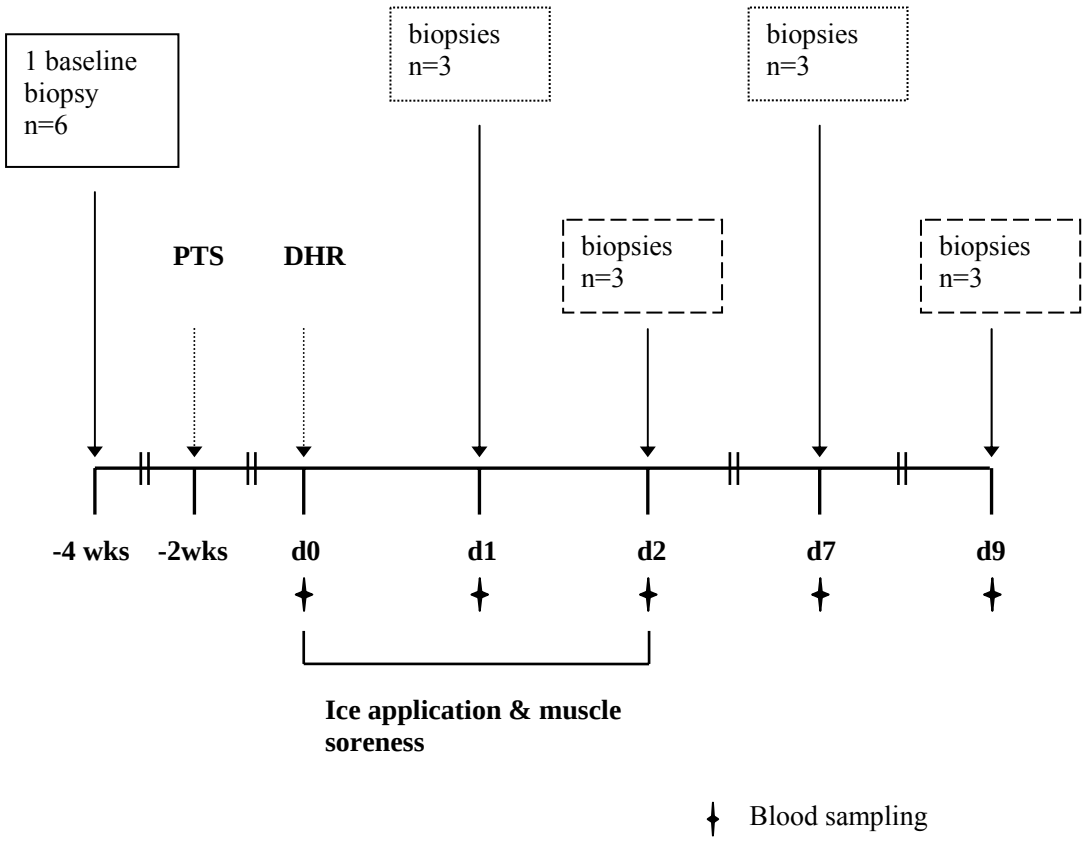


Figure 2.1. Summary of the study design.

All muscle biopsies were performed by the same experienced general practitioner throughout the entire study. Biopsies were obtained from the *vastus lateralis* muscle. To ensure that post-DHR biopsies were taken at 24-hour intervals these biopsies were taken between 7:30-8:00 in the morning. The location of baseline biopsies was determined as follows: subjects lay on their backs with their upper body slightly elevated and with the arms alongside the body; where the thumb touched the upper leg the biopsy was taken. The second pair of biopsies was taken 2 cm below that point with the third pair 2 cm above that point.

Biopsy procedure: A biopsy area was cleaned with an alcohol swab before commencing with the biopsy procedure. A local anaesthetic, Xylotox (containing 2% Lignocaine HCL, m/v, and 1:80 000 noradrenaline) (Adcock Ingram Ltd.), was injected (subcutaneously and then intra-muscular) before the incision with a scalpel blade was made. The needle biopsy method was used (5 mm Bergström biopsy needle (STILLE, Sweden)). The biopsy was orientated on a piece of cork and embedded in a tissue freezing medium (Jung; Leica Instruments GmbH, Germany) and frozen in isopentane cooled in liquid nitrogen. Frozen muscle samples were stored at –80 ^{o}C until further analysis.

2.1.5 Blood sampling

Although not directly related to the extent of muscle damage (Manfredi *et al.*, 1991; Nosaka & Kuramata, 1991), a significant increase in serum CK activity is often used in DOMS studies as an indicator that muscle damage occurred (Schwane *et al.*, 1983; Tiidus & Ianuzzo, 1983). Thus, blood samples were collected to analyse serum CK activity using an automated enzymatic method. The normal range of serum CK activity is 15 – 195 u/L (at 37 ^{o}C).

All blood samples were drawn from the forearm vein by a qualified phlebotomist in SST (serum separating tube) (BD Vacutainer, Becton Dickinson and Company). Blood samples were collected immediately pre- and post-DHR, and on days 1, 2, 7, and 9 post-DHR (Figure 2.1). All samples were collected between 7:30-8:00 am (except post-DHR) before biopsies were taken, after a five minute rest in the supine position.

2.1.6 Muscle soreness

Soreness was assessed immediately pre-DHR, and on days 1 and 2 post DHR (Figure 2.1) before starting the ice treatment sessions of those days. Questionnaires (Appendix B) and a muscle pain probe (developed by the Department of Electrical and Electronic Engineering, University of Stellenbosch) were used to estimate the extent of muscle soreness. Subjects were asked to use a scale of 1-5 to rate their soreness with a score of 1 equalling no pain and 5 equalling very severe pain (Table 2.1).

The muscle probe can be applied to a small area with different magnitudes of force. We chose to apply forces that ranged from 1 kg to 5 kg. The probe is connected to an internal force transducer and the analogue data of the force applied are converted to digital data and displayed. It was not necessary to calibrate the muscle probe as the force transducer is very robust (personal communication: Mr W. van Rooyen, Central Electronic Services, Stellenbosch University). For assessment with the muscle probe, three areas on each leg of a subject were marked with a pen. The three areas were determined as follows: with the subject seated and his knee joints flexed 90^{o}, the researcher used a measuring tape to measure each subjects' upper leg length from the mid-superficial part of the patella to the mid part of the inguinal fold of the hip joint (hip joint also flexed 90^{o}). Then the three areas were marked at 20%, 50%, and 80% (measured from the mid-proximal patella) of the upper leg length. With the subject still in the seated position, the researcher applied a force equal to 1 kg to

each of the three marked areas on the *quadriceps* muscle group of each leg with the probe held 90 degrees to the horizontal plane of the dorsal aspect of the upper leg. As soon as the 1 kg reading was achieved, the probe was removed from the leg. Immediately after each force application the subject was asked to rate the soreness experienced in the specific area during the force application using the same soreness scale as for the soreness questionnaires (Table 2.1). After 1 kg was applied the same procedure was repeated for 2, 3, 4 and 5 kg. Although the use of this muscle probe was not validated, the use of this method to determine muscle soreness has been used by other authors (Cleak & Eston, 1992a; Eston *et al.*, 1996b; Newham *et al.*, 1987; Semark *et al.*, 1999; Tiidus & Ianuzzo, 1983).

2.1.7 Sample analysis

2.1.7.1 Serum analysis

Total serum CK was determined by *PathCare* pathology laboratory (Stellenbosch Medi Clinic, South Africa).

Table 2.1. Rating of perceived pain.

Score	Worded equivalent
1	No pain
2	Slight pain
3	Moderate pain
4	Severe pain
5	Very severe pain

2.1.7.2 Immunohistochemistry

Embedded muscle samples were cut into 8 μm transverse sections with a cryostat (Leica CM1100, Leica Microsystems Nussloch GmbH, Germany) at temperatures between -20 ^{o}C and -22 ^{o}C. Sections were air-dried at room temperature before storing at -20 ^{o}C. Three serial sections per slide were cut and eight slides per time point for each subject were analysed.

At each time point, two slides for each subject were stained for each of the following markers – i.e. two slides per marker at each time point for each subject (refer to Table 2.2 for details of the primary antibodies): CD34, CD56, myoD1 and myogenin. Sections stained for myoD1 and myogenin were also co-stained for the satellite cell marker m-cadherin. CD34, CD56, MyoD1 (referred to as only MyoD hereafter) and myogenin primary antibodies were raised in mouse, and m-cadherin antibody was raised in rabbit. The immunostaining procedure is summarised in Appendix C. Slides were allowed to thaw before fixing sections in 100% acetone for 2 minutes (CD34 and CD56) and allowed to dry, or without fixing (myoD and myogenin). Non-specific binding sites were blocked by incubating sections with 5% donkey serum at room temperature. After 30 minutes the serum was drained off before adding the primary antibody (see Table 2.2 for dilutions) for four hours (CD34 and CD56) or twenty-four hours (myogenin and myoD1) at 4 ^{o}C.

Table 2.2. Primary antibodies used to stain skeletal muscle sections.

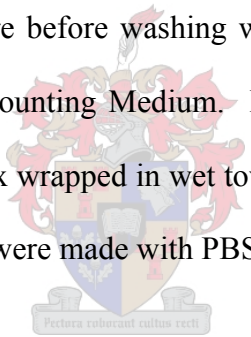
	Clone	Company	[Stock] (μg/ml)	Dilution
CD34	581	BD PharMingen	100	1/200
CD56	MOC-1	DAKO	170 x 10^3	1/100
MyoD1	5.8A	DAKO	2303	1/25
Myogenin	F5D	DAKO	819	1/25
M-cadherin	H-71	Santa Cruz	200	1/50

Slides were washed with phosphate-buffered saline (PBS, 0.1 M, pH 7.4) before adding the biotinylated secondary antibody, raised in horse (1/500, CD34 and CD56) or donkey (1/1200, myoD1 and myogenin), for 40 minutes at room temperature (refer to Table 2.3 for details about secondary and tertiary antibodies).

Table 2.3. Secondary and tertiary antibodies were all obtained from Vector Laboratories, Inc., USA.

	Dilution
Biotinylated secondary Ab	
Horse anti-mouse	1/500
Donkey anti-rabbit	1/1200
Donkey anti-mouse	1/1000
Tertiary Ab	
Fluorescein Streptavidin (FITC)	1/500
Texas Red Streptavidin	1/500

Slides were washed again with PBS whereafter a FITC-labelled tertiary antibody (1/500) was added for 30 minutes at room temperature. The nuclear stain Hoecsht (1/200) was added for 10 minutes to the CD34 and CD56 slides. Slides were washed with PBS. CD34 and CD56 slides were mounted with DAKO Fluorescent Mounting Medium. M-cadherin antibody (1/50) was added to the myoD1 and myogenin slides and incubated overnight at 4^{o}C. The following morning slides were washed with PBS before incubation with a biotinylated secondary antibody raised in donkey (1/1200) for 40 minutes. Slides were washed 3 times before adding Texas Red-labelled antibody (1/500) for 30 minutes at room temperature. The nuclear marker Hoechst (1/200) was added for 10 minutes at room temperature before washing with PBS and mounting the slides with DAKO Fluorescent Mounting Medium. During this whole protocol slides were enclosed in a black box wrapped in wet towels to keep it in a dark and moist environment. All dilutions were made with PBS.



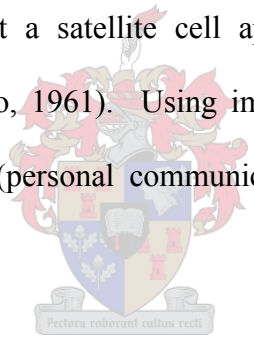
2.1.7.3 Imaging and image analysis of stained biopsy sections:

Stained sections were viewed with a microscope (Nikon ECLIPSE E400) set at 40x enlargement and photos were taken with a digital camera (Nikon DXM1200) that added no further enlargement. Photos were used to count positively stained satellite cells as well as total nuclei and myofibre numbers per field of view using the computer programme *Simple PCI* version 4.0 (Compix Inc., Imaging Systems, USA). If necessary, photos were enlarged after importation into *Simple PCI* to assist with identification. Satellite cell data were expressed as the number of satellite cells per myofibre.

2.1.7.4 Satellite cell counting in muscle biopsy sections

CD56 and CD34 data:

As mentioned before counting was done using the Simple PCI programme. The CD56 (or CD34) image (refer to Figures 3.4 and 3.9 for examples) was merged with the Hoechst stained photo from the same area. A CD56 positive stained area was only identified positively as a satellite cell if it was co-localised with a Hoechst stained nucleus. The CD34 antibody also stains other cells, including hematopoietic cells, thus making it more difficult to specifically identify satellite cells. Because we did not use a co-stain with this antibody, for positive identification we also relied on characteristic visible features: using electron microscopy it is clear that a satellite cell appears to make a groove in its associated myofibre (Mauro, 1961). Using immunohistochemistry, this groove appears as an indentation (personal communication: Prof L-E Thornell, Umea University, Sweden).



MyoD and myogenin:

The same procedure was followed as with CD56 and CD34 stained slides. MyoD and myogenin are nuclear stains that are easily identified. When a MyoD (or myogenin) nucleus overlapped with that of a Hoechst positive nucleus, the separate photos (refer to Figure 3.16 for an example of identifying myogenin$^+$ satellite cells) were enlarged to make sure that the MyoD (myogenin) and Hoechst nucleus had the same shape. When the nucleus was identified to be positive for both, the following was used to elucidate whether this positive nucleus belonged to a satellite cell: both MyoD (myogenin) and Hoechst images were separately

merged with the m-cadherin image. If the MyoD (myogenin) nucleus was co-localised with m-cadherin the nucleus was identified to belong to a satellite cell, otherwise it was identified to be a myonucleus.

2.1.8 Statistical analysis

Data for the human study are presented as means ± SD. Muscle soreness ratings (ADL questionnaire) and the serum CK data were analysed using repeated measures one-way ANOVA. The Bonferroni post-hoc test was conducted in order to distinguish at which time points the statistical difference was. The results for the muscle probe data are complicated (3 sites; 3 different pressures; 2 legs; baseline and 2 days). Therefore, general statistical tests could not be done on the raw data – the sample size was too small for the assumptions made by most statistical tests for data with this complex design. Furthermore, all the muscle probe data of a particular subject were dependent on each other in several different ways, e.g. over time, for position and for the treated and untreated legs, which also contributed to the complexity of this data set. For this analysis a statistician advised the following: The change in soreness rating (delta) from baseline was determined for both time points post-DHR. The changes at each time point post-DHR were then analysed separately for a treatment effect using repeated measures one-way ANOVA. If the error bars of the box-and-whisker plots did not overlap with zero, then the change in soreness at that time point was considered significantly different from baseline (no change will occur at baseline). Thus, the visual results were used to see whether there were time effects. T-tests

(dependent samples) were used to determine the P-values for these visual differences.

Due to the small sample size (n=3) non-parametric tests were conducted for the muscle biopsy data (CD56 and CD34 data). The Friedman ANOVA was used to test for time effects. When a time effect was found, the Wilcoxon matched pairs test was conducted to determine at which time points a difference existed – the P-values were then multiplied by three and compared at a significance level of 0.05 (to follow the same assumption made with a Bonferroni post-hoc test for three groups). Another way to approach this would have been to set the acceptable limit for statistical significance at 0.05 divided by 3; the statistical outcome would be the same. The effect of the ice treatment was analysed using the Wilcoxon's matched pairs test at each time point separately. All statistical analyses were done with the aid of the computer software *Statistica* version 6. The level of significance was accepted at $P \leq 0.05$.

2.2 *In vitro* (C2C12 cell cultures)

2.2.1 Standardised conditions:

C2C12 cells (kindly donated by the Cape Heart Centre) were used to investigate profiles of specific satellite cell marker expression during the different stages of their cell cycle. These cells were grown and maintained in a basal medium consisting of Dulbecco's Modified Eagles Medium (DMEM) (Highveld Biological (Pty) Ltd, RSA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (*PenStrep*, Highveld Biological (Pty) Ltd, RSA) in T75 flasks (75 cm^2 flasks). When the cells reached about 70% confluence (covering approximately 70% of the flask's surface), they were plated onto glass cover slips (22 x 22 mm) in each well of 6-well plates. When these cells on the cover slips, in the 6-well plates, reached about 70% confluence they were harvested as either myoblasts (day 0 before adding differentiation medium) or they were induced to differentiate. The 10% FBS was substituted with 2% horse serum (donor herd, Sigma) to induce differentiation (differentiation medium). All C2C12 cell cultures were incubated at 37 ^oC in an environment of 95% humidity and 5% CO_2, and all mediums were changed every second day. Every apparatus and each tool used during the cell culture study were sterilized by autoclaving.

2.2.1.1 Baseline

This protocol was to set up a baseline for satellite cell markers expression. Cover slips from days 0, 1, 3, 6, and 8 were collected and stained immunohistochemically for the different cell-cycle (satellite cell) markers.

2.2.1.2 Immunohistochemistry

Satellite cell activity was investigated by staining with antibodies against the following markers: CD34 (quiescent), CD56 (activated), MyoD1 (proliferation) and myogenin (differentiation), and M-cadherin (all satellite cells) (refer to Table 2.3 for dilutions and suppliers). Five cover slips per time point were stained for each of the following antibodies, CD34, CD56, MyoD1 and myogenin. M-cadherin was used as co-stain for all of these markers. At each time point cover slips were gently lifted out of the wells and washed with sterile PBS before fixing with methanol-acetone (1:1, v/v). These fixed cover slips were stored at -20 ^{o}C until used to stain for above-mentioned markers.

Table 2.4. Primary antibodies used to stain C2C12 cells.

	Clone	**Company**	**[Stock] (µg/ml)**	**Dilution**
CD34	ICO115	Santa Cruz	200	1/200
CD56	MOC-1	DAKO	170 x 10^3	1/200
MyoD1	5.8A	DAKO	2303	1/100
Myogenin	F5D	DAKO	819	1/100
M-cadherin	H-71	Santa Cruz	200	1/50

2.2.1.3 Imaging and image analysis:

Six photos per slide were captured using a microscope (Nikon ECLIPSE E400) and digital camera (Nikon DXM1200) at 20x enlargement. Staining intensities of the different antibodies at the different time points were analysed using the

computer software *Simple PCI* version 4.0 (Compix Inc., Imaging Systems, USA). Data gathered from images of individual markers at specific time points were pooled together for statistical analysis of the specific time points and individual markers.

2.2.2 Statistical analysis

Data are presented as means ± SD. One-way ANOVA, with Bonferroni post-hoc test, was used to analyse C2C12 data. Statistical tests were all done with the aid of the *Statistica* (version 6) programme. The level of significance was accepted at $P \leq 0.05$.

CHAPTER 3: RESULTS

3.1 *In vivo*: human study

The subject characteristics for all six subjects are presented in Table 3.1. Although age and BMI had little variability, there was quite a range for VO_{2max}. Two subjects who did the DHR test at 70% of their PTS had an average VO_{2max} of 42.7 ± 0.8 ml/min/kg (mean ± SD). The average VO_{2max} of the remaining four subjects who ran at 80% of their PTS during the DHR test was 54.3 ± 2.0 ml/min/kg.

Table 3.1 Subject characteristics.

Age (yr)	24 ± 3
Height (cm)	181 ± 8
Weight (kg)	77 ± 8
BMI (kg/m^2)	24 ± 2
VO_{2max} (ml/min/kg)	50.4 ± 6.5
DHR speed (km/h)	12.0 ± 2.4

Data presented as mean ± SD.
Abbreviations: BMI - body mass index; DHR – downhill-run

The DHR protocol in this study was that of Eston *et al*. (Eston *et al.*, 1994; Eston *et al.*, 1996a; Eston *et al.*, 1996b), with a single modification (see methods). To our knowledge this is the first study to use a subject's individual PTS to determine his DHR speed. The muscle soreness indicates that this modified protocol was successful in inducing DOMS.

3.1.1 Muscle soreness

The muscle soreness ratings assessed using the 'active daily living' (ADL) questionnaire (Appendix B) indicated that subjects experienced significant soreness a day after the DHR (Figure 3.1) in both the treated and untreated legs. This soreness was sustained for a further 24 hours.

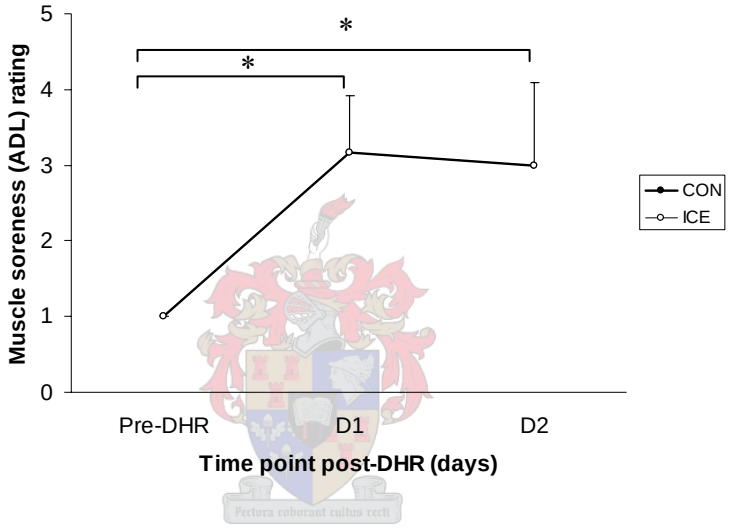


Figure 3.1 Soreness ratings (mean ± SD) assessed with the 'active daily living' (ADL) questionnaire before and after the downhill-run (DHR). The rating in the treated and untreated leg was exactly the same, thus only one line is clearly visible. Statistical analysis: repeated measures ANOVA, * P<0.01 *vs* pre-DHR (Bonferroni post-hoc test).

In fact, the scores for the treated and untreated legs were exactly the same for all subjects at all time points. Due to the fact that muscle soreness was only assessed for two days after the DHR the total number of days that muscle soreness persisted could not be determined.

Muscle soreness was also determined using the muscle pain probe. The pain probe was used to investigate whether different areas of the *quadriceps* muscle group were affected differently by the DHR. Force magnitudes of 1-5 kg were applied to three areas of the superior aspect of the *quadriceps*, i.e. proximal (80%), middle (50%), and distal (20%). The percentages in brackets indicate the relative distance on an individual's upper thigh measured from the mid-superior edge of the patella to the mid part of the inguinal fold of the hip joint (with the subjects seated on a chair with their knee and hip joints bent 90^{o}). No difference in soreness from baseline was experienced when applying 1 or 2 kg. Only the applied pressures of 3-5 kg elicited significant pain. Only the results of 5 kg applied pressure are presented here (please refer to the appendix D for results of 3-5 kg). Also, only the results of the middle and distal areas (for 5 kg) are presented here. The reason for the latter is due to the facts that a) we obtained biopsies from the middle area of the *vastus lateralis* and b) muscle damage in animal models predominantly occurs at the distal musculo-tendon junction (reviewed in Graham, 1990) and a study using DHR to induce DOMS in humans subjects, has shown that the distal and proximal musculo-tendon junction of the *quadriceps femoris* is more sensitive to pain than the mid-belly of the *quadriceps femoris* (Baker *et al.*, 1997). Thus, we considered it important to show a) whether significant DOMS was induced in the middle area after the DHR and b) whether the distal area experienced more pain. We found that pain was significantly increased in the middle portion at day 2 post-DHR in both the treated and untreated legs (Figure 3.2). No significant treatment effect was found at any time point for any of the forces applied.

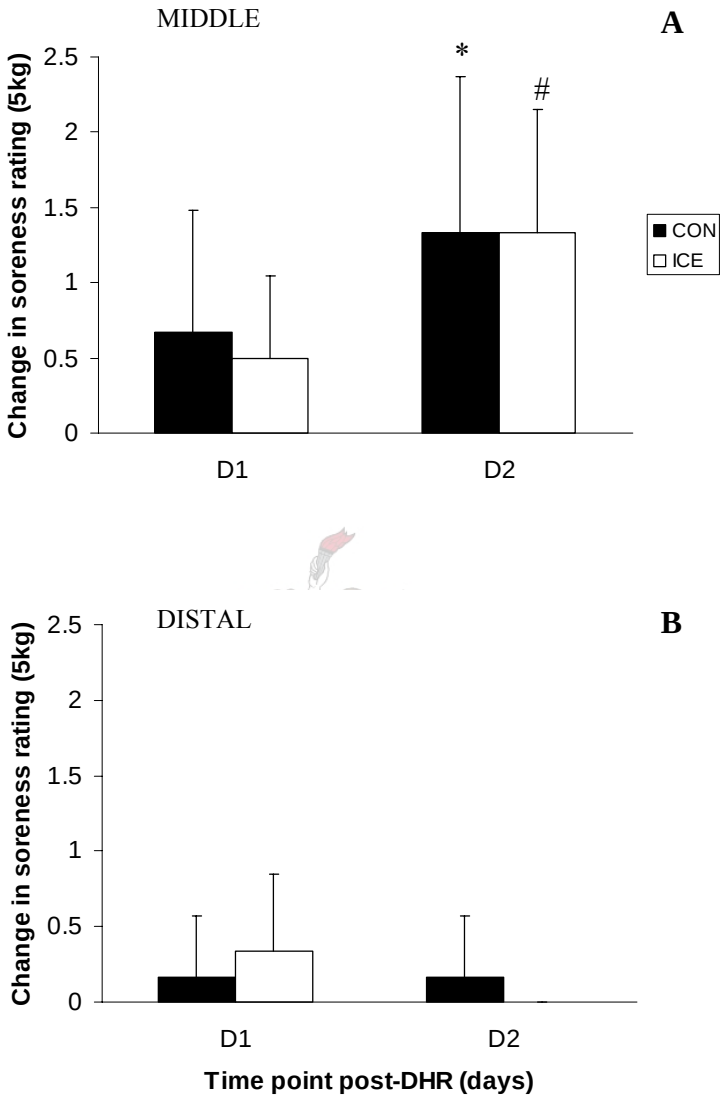


Figure 3.2 The change (rating at time point minus baseline) in pain ratings (mean ± SD) determined using the muscle pain probe, applying a force of 5 kg in the middle *quadriceps* (panel A), and (panel B) the distal *quadriceps*. The absence of error bars indicates a standard deviation of zero. Statistical analysis: t-test for dependent samples, * $P < 0.005$, # $P < 0.05$ *vs* baseline.

3.1.2 Serum CK activity

In addition to inducing muscle soreness this modified DHR protocol also caused a significant increase in total serum CK activity on day 1 post-DHR (Figure 3.3A). CK activity peaked the day after DHR which was uninfluenced by any muscle biopsies (baseline biopsies were completed 1 month previously and day 1 post-DHR biopsies were taken after blood sampling). Thereafter it receded toward baseline level again. However, the CK activity results of days 2, 7 and 9 could have been affected by the muscle biopsies and hence the dashed line in Figure 3.3A. Recovery was significant by day 9 post-DHR.

A muscle biopsy itself will cause an increase in serum CK activity due to the muscle damage caused. In subjects receiving biopsies at 24 h post-DHR, serum CK activity did not further increase on day 2 or later. However, in subjects whose biopsies were taken 2 days (48 h) post-DHR, the serum CK activity tended to be increased at day 7 (i.e. 5 days post-biopsies). The significance of this finding is not clear as serum samples were not obtained at days 3-6 and 8 post-DHR. The effect of biopsies on serum CK activity is demonstrated in Figure 3.3B.

3.1.3 Satellite cell analysis

The satellite cell data were obtained by analysing two sections (8 μm apart) from each subject's biopsy, at each time point for each antibody. Five to six fields of view per section were analysed (a few biopsy samples were too small to count 6 views). Due to difficulty obtaining a muscle biopsy from the treated leg from one

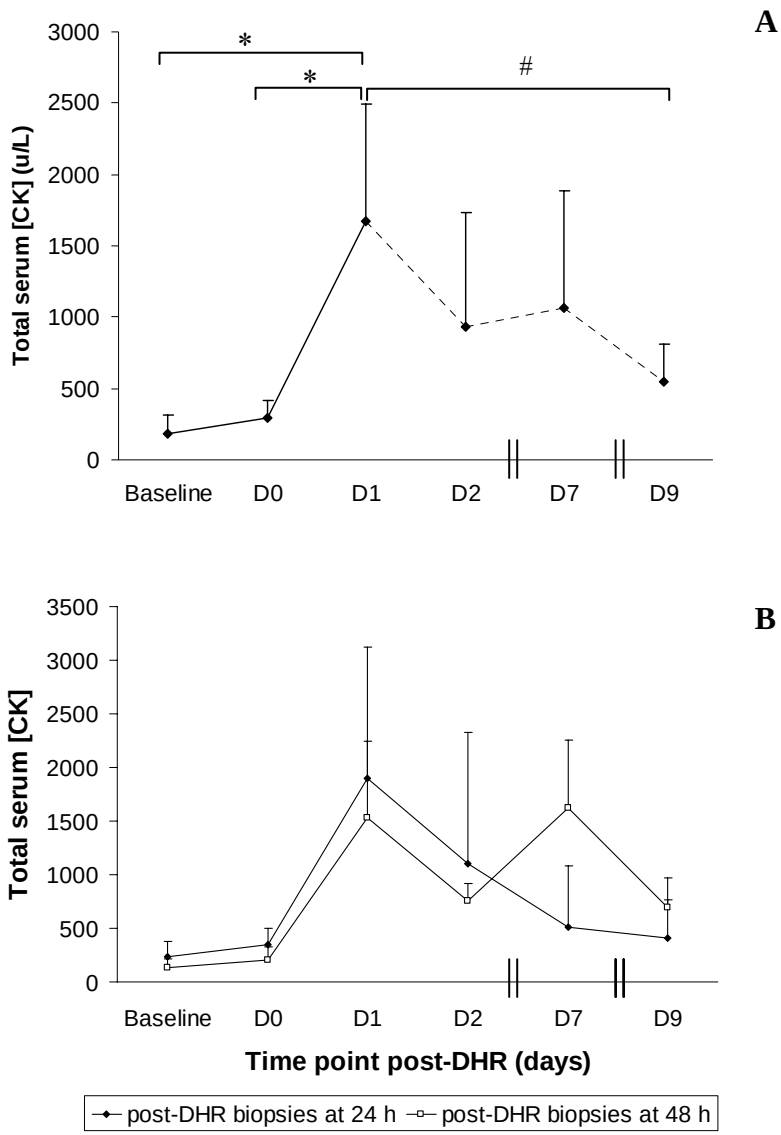


Figure 3.3 Total serum creatine kinase (CK) activity (mean ± SD) of all six subjects before and at several time points after the DOMS inducing exercise (panel A). Statistical analysis: Repeated measures one-way ANOVA, * $P < 0.01$, # $P = 0.05$ (Bonferroni post-hoc test). Mann Whitney U-test showed no significant differences between the group receiving their post-DHR biopsies at 24 hours and those receiving it at 48 hours (panel B).

subject (biopsy sample too small to analyse), the day 7 post-DHR (ICE group) could not be statistically analysed.

3.1.3.1 CD34

CD34 expression in muscle sections was quantified as the number of $CD34^+$ satellite cells per myofibre number. Due to the fact that other cells, including fibroblasts and hematopoeitic cells, also express CD34, only $CD34^+$ cells nestled close to the sarcolemma and causing an indentation in the myofibre were recorded as $CD34^+$ satellite cells (Figure 3.4, white arrows). Other $CD34^+$ cells that were not associated with such an indentation were not counted. Usually the cells which do not make an indentation in a myofibre appear round and/or lie outside the myofibre (Figure 3.4, red arrows). Sometimes a nucleus may seem to make an indentation, but the staining for CD34 is not intense enough to be counted as $CD34^+$ and also, the nucleus is not surrounded by a very thin layer of $CD34^+$ cytoplasm (Figure 3.4, red dashed arrows). Another example of a $CD34^+$ non-satellite cell is where there appears to be $CD34^+$ staining and an indentation, but there is no $CD34^+$ cytoplasm separating the nucleus and myofibre (Figure 3.4, red arrow head).

Only the untreated legs at days 1 and 7 post-DHR showed a time effect ($P = 0.05$, Friedman ANOVA). $CD34^+$ cell number decreased at day 1 post-DHR in the control leg but was not significant on post hoc analysis, whereas in the treated leg no decrease at all was apparent (Figure 3.5). $CD34^+$ satellite cell number in the

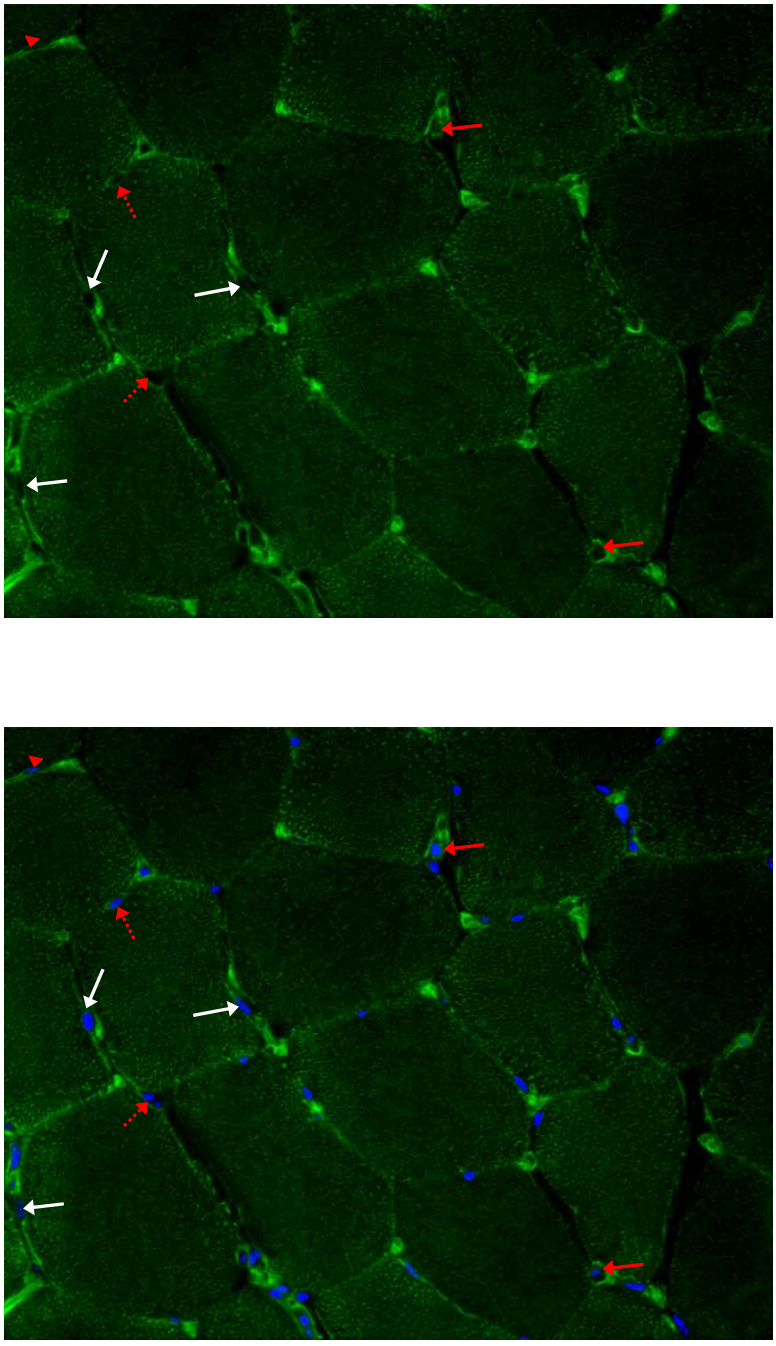


Figure 3.4 CD34 staining in a biopsy at day 1 post-DHR from the control leg of a subject (original magnification x 40). FITC (green) represents CD34 expression (panel A, B) and Hoecsht (blue) stains all nuclei (panel B). The white arrows indicate $CD34^+$ satellite cells whereas the red arrows demonstrate $CD34^+$ cells not identified as satellite cells (see text).

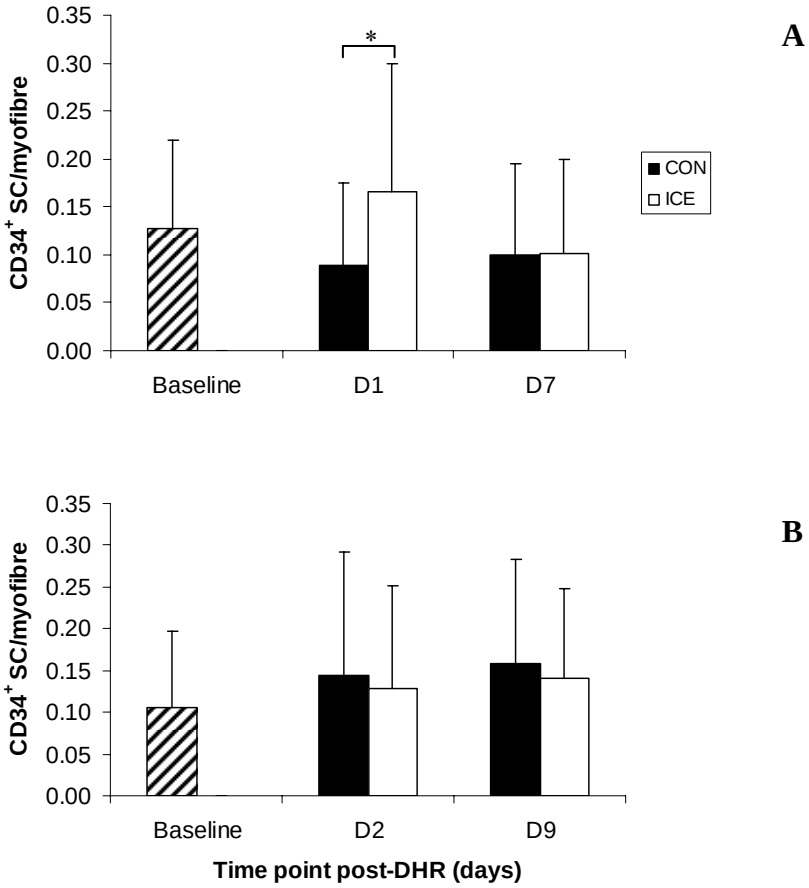


Figure 3.5 $CD34^+$ satellite cell count (SC) normalised for the myofibre number (SC/myofibre) (mean ± SD) at days 1 and 7 (D1, D7; panel A), and days 2 and 9 (D2, D9; panel B) after the DHR. Statistical analysis: Wilcoxon matched pairs test, with post-hoc significance demonstrated between the two specific data points, * $P < 0.05$.

control leg was therefore significantly less compared to the ice leg at day 1 post-DHR. This treatment difference was only evident on day 1. Inter-individual variability of CD34 expression is shown in Figures 3.6 and 3.7. These inter-individual responses may have abrogated a time and treatment effect in the $CD34^+$ satellite cell number. The minimum and maximum values for the $CD34^+$ satellite

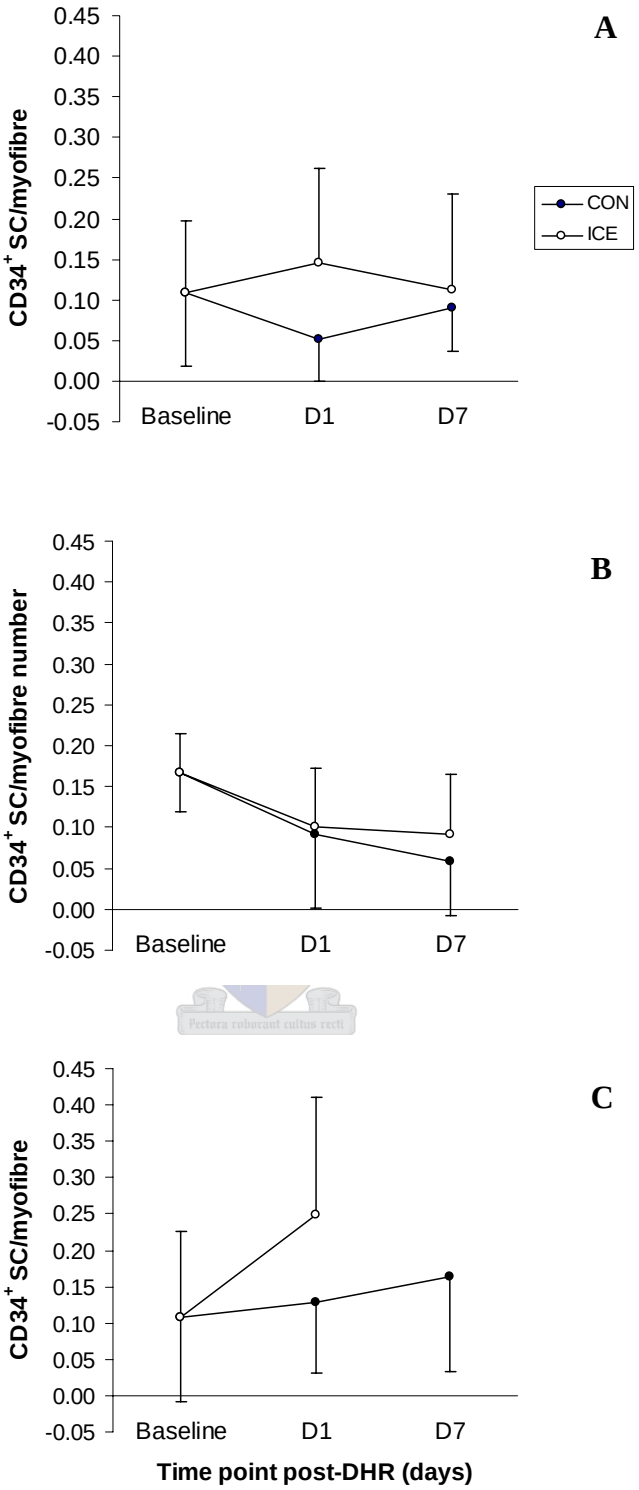


Figure 3.6 CD34 expression in muscle sections of individuals at baseline and days 1 and 7 post-DHR (mean ± SD for ~12 fields of view); (A) subject 1, (B) subject 2, (C) subject 3.

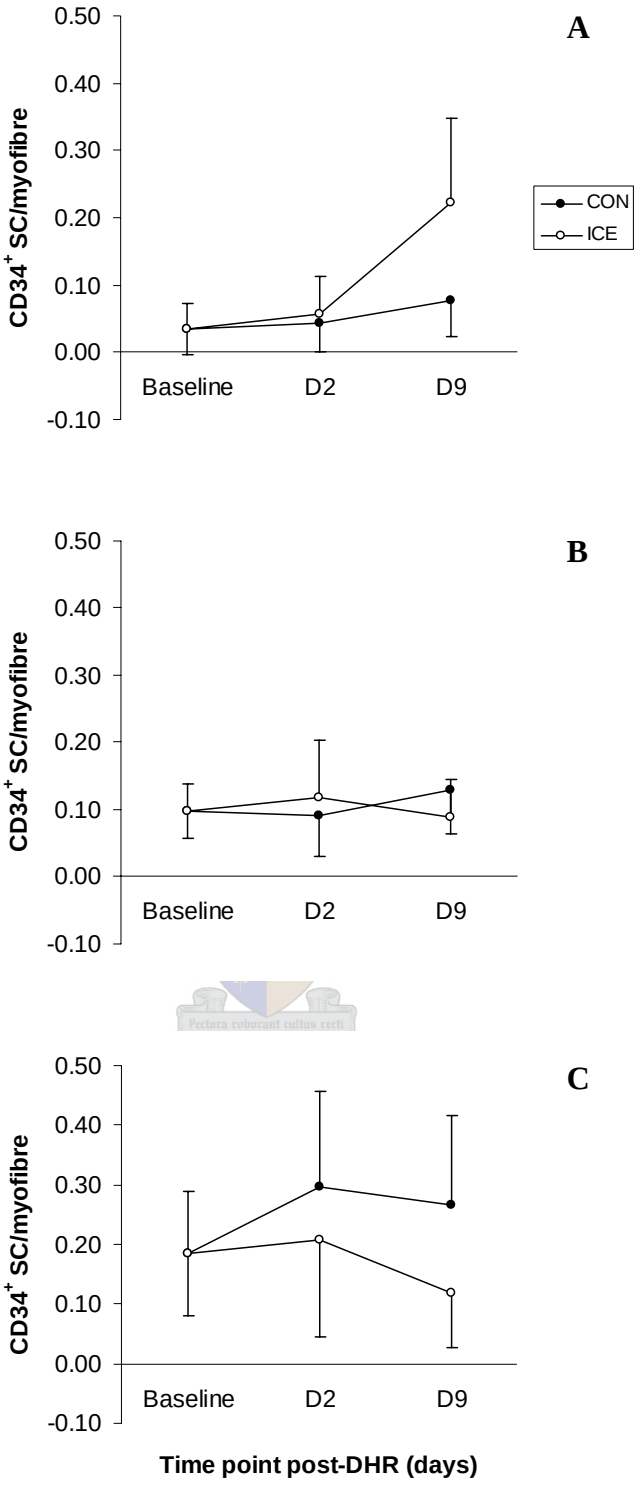


Figure 3.7 CD34 expression in muscle sections of individuals at baseline and days 2 and 9 post-DHR (mean ± SD for ~12 fields of view); (A) subject 4, (B) subject 5, (C) subject 6.

cell number per myofibre number were as follows: baseline (0.01 - 0.18), day 1 CON (0.05 - 0.13) and ICE (0.10 – 0.24), day 2 CON (0.02 - 0.31) and ICE (0.07 – 0.20), day 7 CON (0.06 – 0.15) and ICE (0.09 – 0.11), day 9 CON (0.09 – 0.27) and ICE (0.09 – 0.22).

3.1.3.2 CD56

CD56 was used as a marker to investigate the number of activated satellite cells. The only time effect found was in the control legs at days 2 and 9 post-DHR ($P < 0.005$, Friedman ANOVA). Also, there was a significant difference between the treated and untreated legs after the DHR (Figure 3.8), with muscle from the control leg having significant satellite cell activation 48 hours after inducing DOMS (Figure 3.8B). This activation of satellite cells was sustained up to nine days after the DHR. In contrast, muscle from the treated leg showed no significant increase in the frequency of activated satellite cells over time. Furthermore, at the specific time points days 2 and 9 post-DHR, the treated leg had significantly fewer $CD56^+$ cells compared to the untreated leg.

Figure 3.9 shows examples of CD56 expression found in individual fields of view at baseline (panel A, B), and in a untreated (C, D) and treated (panel E, F) leg at day 2 post-DHR. As can be seen in panels B, D and F, CD56 is co-localised to satellite cell nuclei.

As with CD34 expression, there were inter-individual responses to the DOMS-inducing exercise when the activated satellite cells were analysed (Figures 3.10 and 3.11). The following minimum and maximum values were obtained for the $CD56^+$ satellite cells per myofibre number: baseline (0.04 - 0.26), day 1 CON

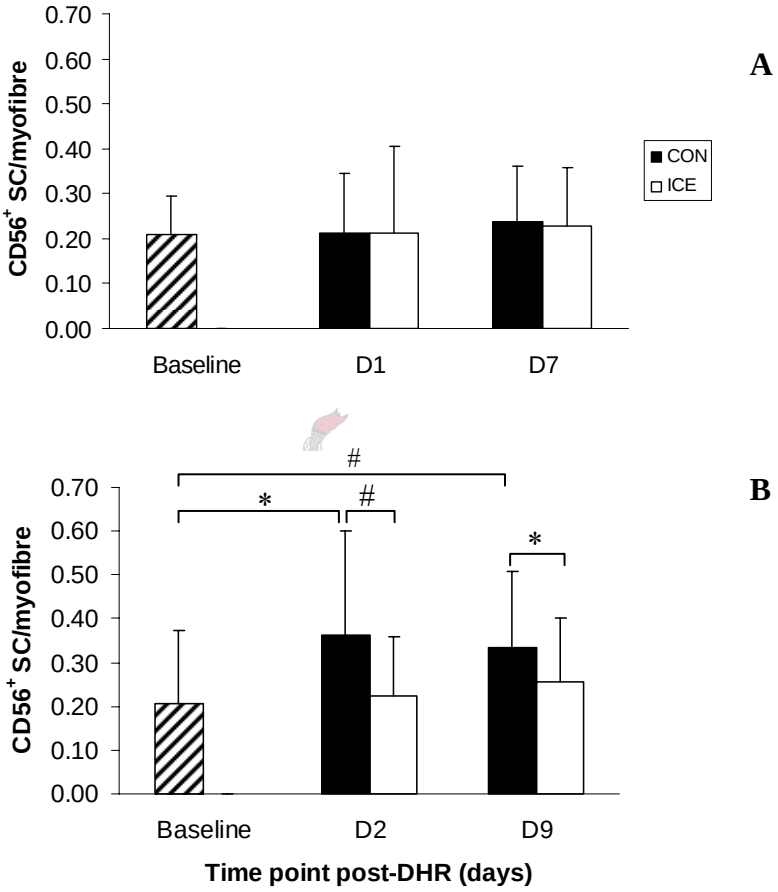


Figure 3.8 CD56 positive satellite cells expressed per myofibre number ($CD56^+$ SC/myofibre number) (means ± SD) at days 1 and 7 (D1, D7; panel A) and days 2 and 9 (D2, 9; panel B) post-DHR. Statistical analysis: Wilcoxon matched pairs test, * $P < 0.01$, # $P < 0.001$.

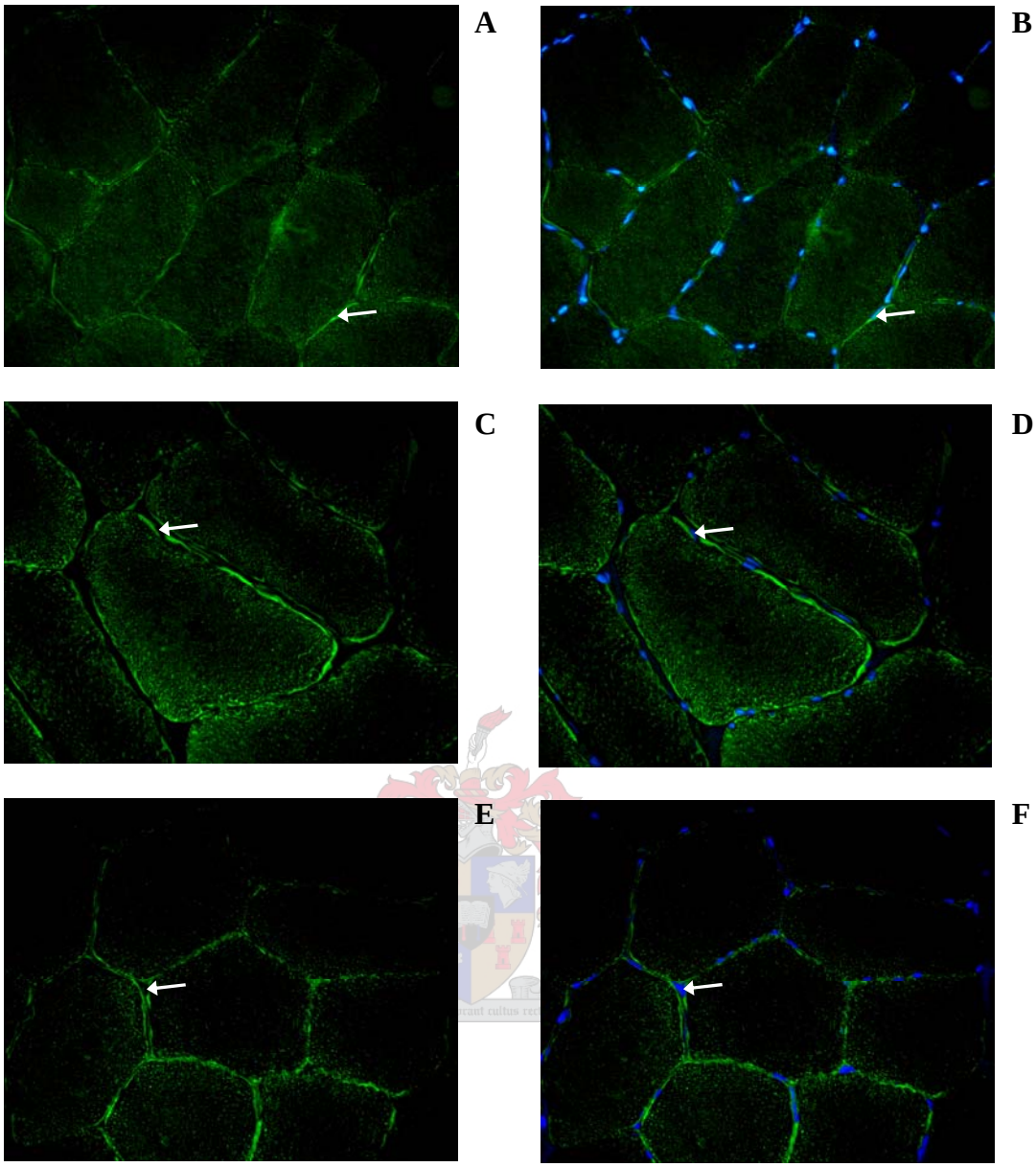


Figure 3.9 CD56 expression (green, FITC) in biopsies taken at (A) baseline and at day 2 post-DHR in the control (C) and ice leg (E) of the same subject. The photos on the right hand side (B, D, F) correspond to the time points of the photos on the left hand side, representing merged images of the CD56 expression and that of Hoechst (blue) that stains all nuclei (original magnification x 40). Examples of $CD56^{+}$ satellite cells are indicated by the white arrows.

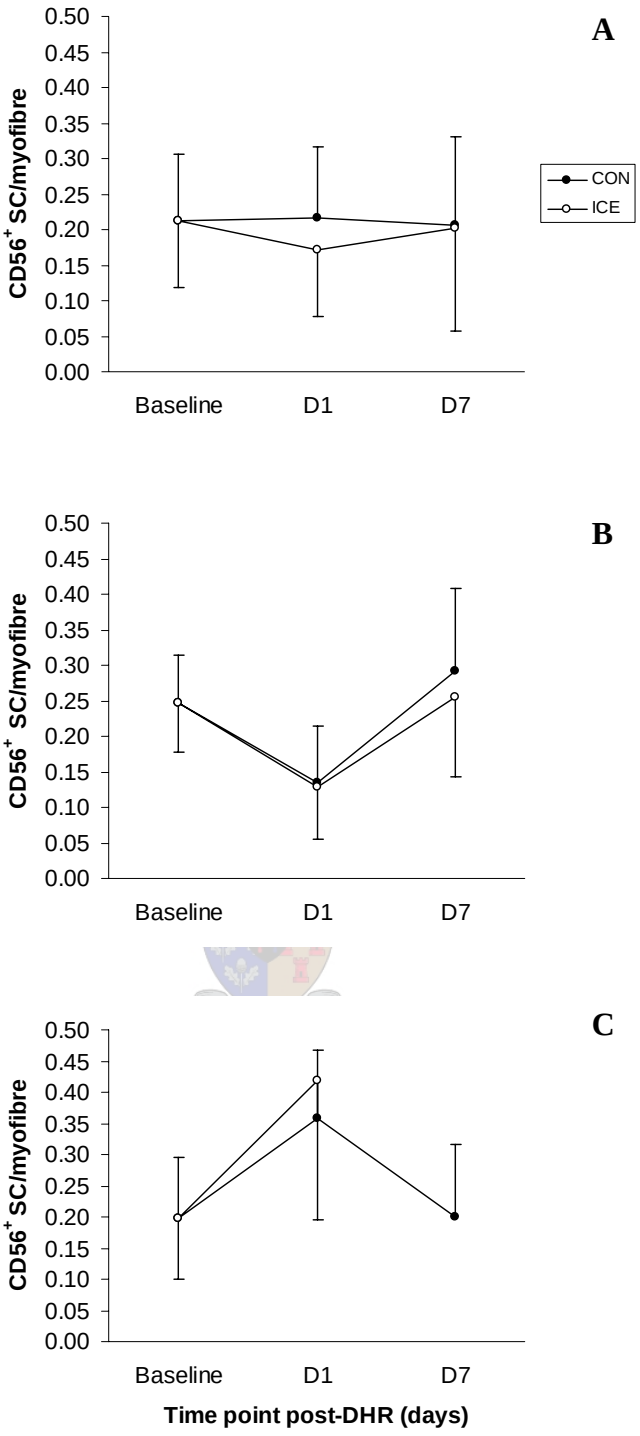


Figure 3.10 CD56 expression (mean ± SD) in muscle biopsy sections of individuals at baseline and days 1 and 7 post-DHR; (A) subject 1, (B) subject 2, (C) subject 3.

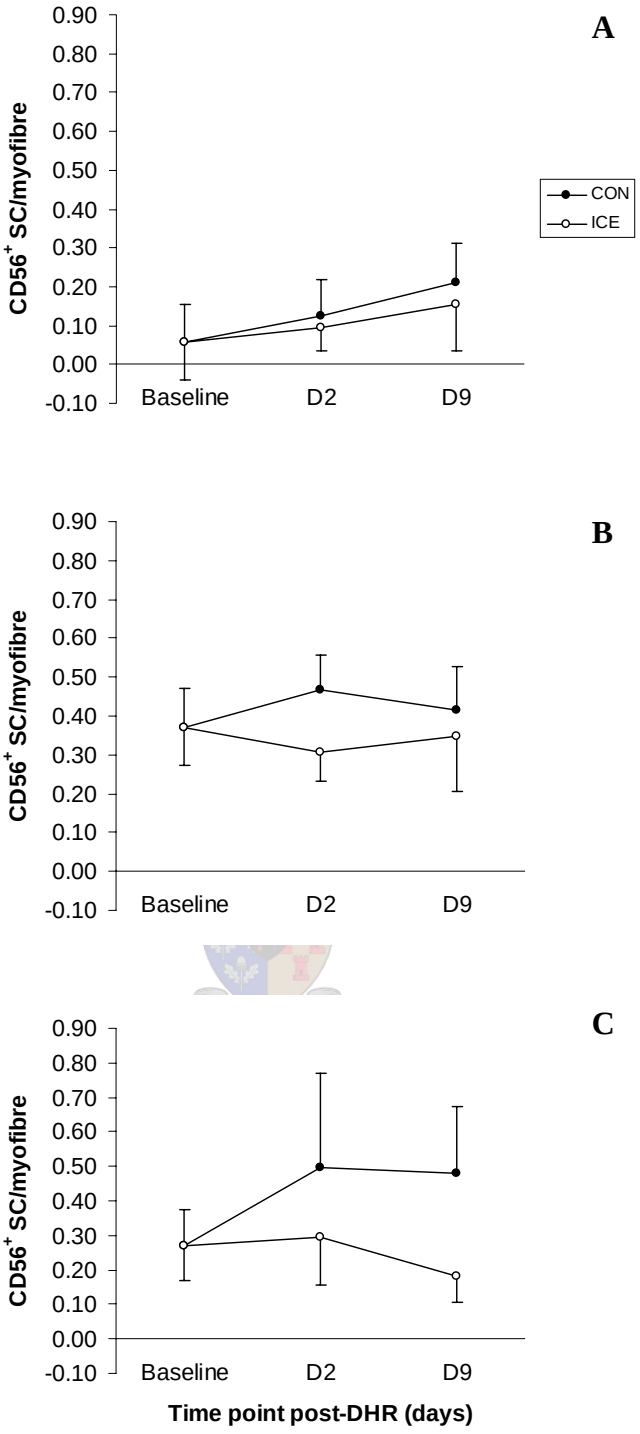


Figure 3.11 CD56 expression (mean ± SD) in muscle biopsy sections of individuals at baseline and days 2 and 9 post-DHR; (A) subject 4, (B) subject 5, (C) subject 6.

(0.13 - 0.21) and ICE (0.13 – 0.42), day 2 CON (0.12 - 0.50) and ICE (0.10 – 0.30), day 7 CON (0.19 – 0.28) and ICE (0.19 – 0.25), day 9 CON (0.21 – 0.47) and ICE (0.16 – 0.26).

Figure 3.12 shows the combined data from all subjects for CD34 (Figure 3.12A) and CD56 (Figure 3.12B). In this figure it is apparent that, in the control leg, there is a tendency for a decrease in number of quiescent ($CD34^+$) satellite cells at day 1 to be followed by an increase in activated ($CD56^+$) satellite cells (day 2 post-DHR). However, the same tendency is not visible the treated leg. Rather, there could be a tendency for an earlier increase in $CD34^+$ satellite cell number in the ICE condition (day 1 post-DHR), with no activation following. These observations could infer that the treatment with ice abrogates the relation between quiescent and activated satellite cells. However, large variability between subjects and low subject numbers prevented a clear and unambiguous result. The data also suggest a possible biphasic response in both the CD34 (in treated and untreated legs) and CD56 (untreated legs) expression. However, the importance of analysing multiple subjects, time points and fields of view in order to obtain conclusive results must be underscored. Although more subjects at each time point would be possible and more time points preferable, this would not be easy to achieve. Similarly, matching biopsies (same subjects) at all time points would be preferable, but not ethically achievable.

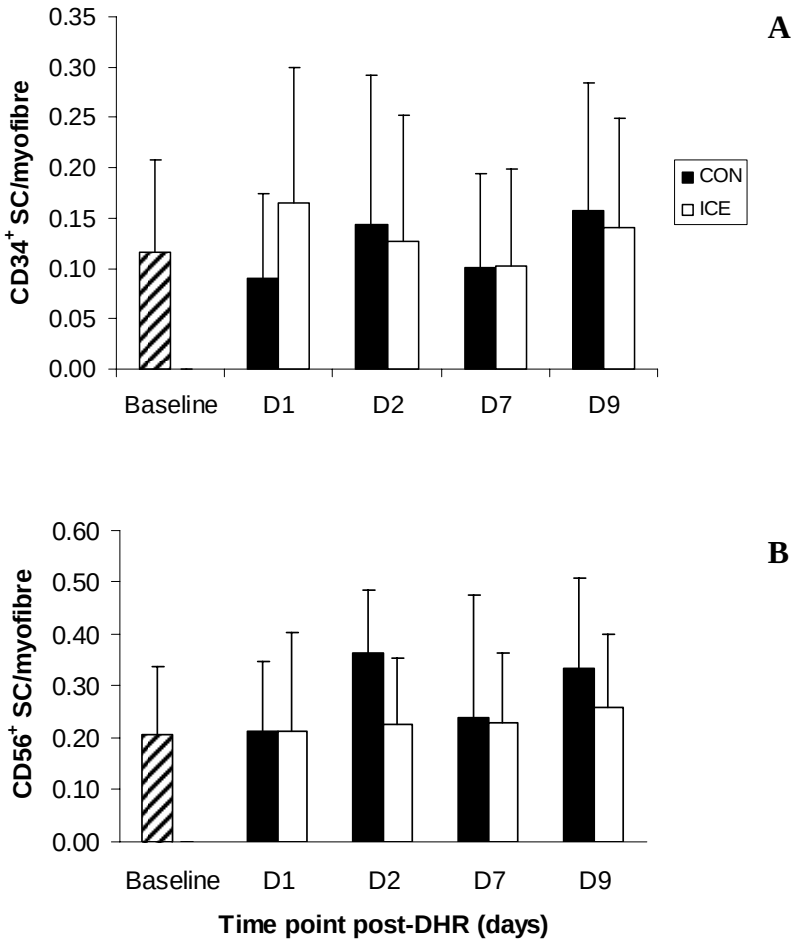


Figure 3.12 CD34 (A) and CD56 (B) data of muscle biopsies grouped together for all time points.

Correlation matrixes were used to determine whether possible relationships existed between the VO_{2max} and the $CD34^+$ satellite cell numbers at baseline as well as the $CD56^+$ satellite cell number at baseline. No significant correlation was found between the $CD34^+$ satellite cell number and the VO_{2max} ($r = 0.65$, $P = 0.16$). Although not significant ($r = 0.78$, $P < 0.07$) the $CD56^+$ satellite cell number and VO_{2max} showed a trend for a possible relationship (Figure 3.13). The small sample size ($n = 6$) may have abrogated the possibility of a significant relationship.

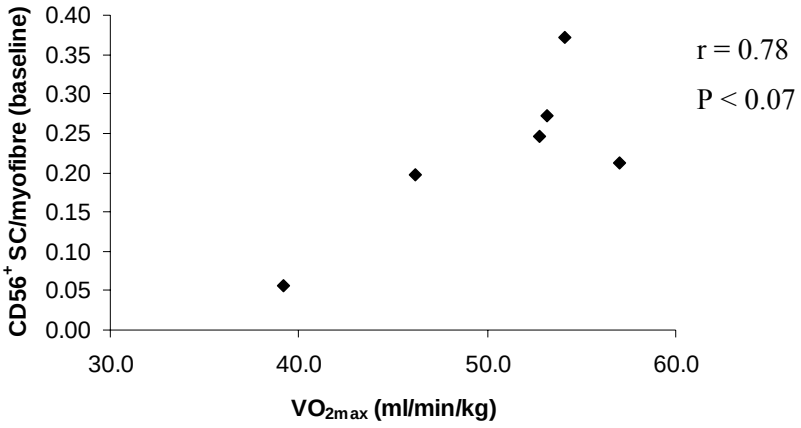


Figure 3.13 Comparison of the $CD56^+$ satellite cells at baseline and the VO_{2max}.

Furthermore, the satellite cell activation responses (delta CD56, time point minus baseline) were determined for early (days 1 and 2 grouped for n = 6) and late (days 7 and 9 grouped for n = 6) time points for the control legs. Some of these activation responses were related to other parameters. The late change in $CD56^+$ satellite cell number was significantly ($r = 0.93$, $P < 0.01$) related to the ADL soreness ratings at day 2 post-DHR (Figure 3.14).

As with the CD56 data, the satellite cell response was also determined for the CD34 data (delta CD34) at early and late time points. A positive relationship was found to exist between the early change of $CD34^+$ and the early change of $CD56^+$ satellite cell numbers ($r = 0.92$, $P < 0.01$) (Figure 3.15A). The early change in $CD34^+$ satellite cell number was also significantly ($r = 0.86$, $P < 0.05$) correlated to the overall change (late time point minus baseline) in $CD34^+$ cell number (Figure 3.15B). This shows that an early change in $CD34^+$ satellite cell number was still evident at the later time point, whether positive or negative.

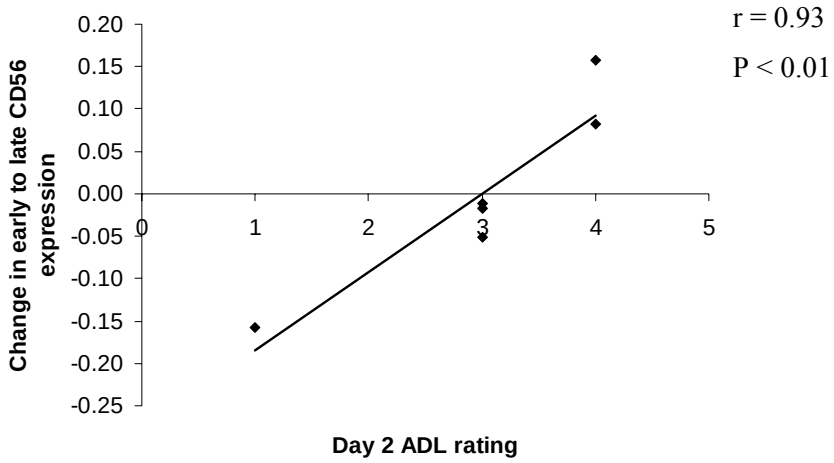


Figure 3.14 The relationship between the late change in CD56 expression and soreness at day 2 post-DHR (determined by the active daily living questionnaire = ADL rating).

We also recorded the total myofibre-associated nuclei (Hoechst$^+$) expressed per myofibre (nuclei/myofibre) in each muscle biopsy section of the control legs (Table 3.2). No time effect of the nuclei/myofibre existed for days 1 and 7 post-DHR (Friedman ANOVA, $P < 0.1$), and also for days 2 and 9 post-DHR (Friedman ANOVA, $P < 0.7$). The early change in nuclei/myofibre showed no relationships with neither the early change in CD34 expression ($r = 0.46$, $P = 0.36$) nor the early change in CD56 expression ($r = 0.34$, $P = 0.50$). To be able to compare our satellite cell data with that of other studies, we also determined the percentages CD34$^+$ and CD56$^+$ satellite cells at each time point for the control legs (Table 3.2). For example, the percentage CD34$^+$ satellite cells was calculated as CD34$^+$ satellite cells/(total number of myofibre-associated nuclei) x 100.

Table 3.2 Data of the total myofibre-associated nuclei expressed per myofibre and the percentage of $CD34^+$ and $CD56^+$ satellite cells for all time points in the control legs.

	Baseline	**Day 1**	**Day 2**	**Day 7**	**Day 9**
Nuclei/myofibre					
%$CD34^+$	4.4 ± 3.6	3.2 ± 3.2	3.8 ± 3.4	2.9 ± 2.3	5.0 ± 2.3
%$CD56^+$	7.1 ± 4.2	7.6 ± 3.9	10.9 ± 6.8	7.3 ± 4.0	11.0 ± 5.3

Footnote: Data presented as means ± SD. Co-expression of these markers is possible.

3.1.3.3 MyoD and myogenin

MyoD and myogenin expression was found in both satellite cells (Table 3.3 and 3.5 respectively) and myonuclei (Table 3.4 and 3.6 respectively). However, very Few positively-stained cells were observed. The majority of satellite cells expressing myogenin were found at day 9 post-DHR. Figure 3.15 is an example of myogenin expression in a nucleus of a satellite cell (also $Hoechst^+$ and expressing m-cadherin). Similar staining was seen with MyoD expression.

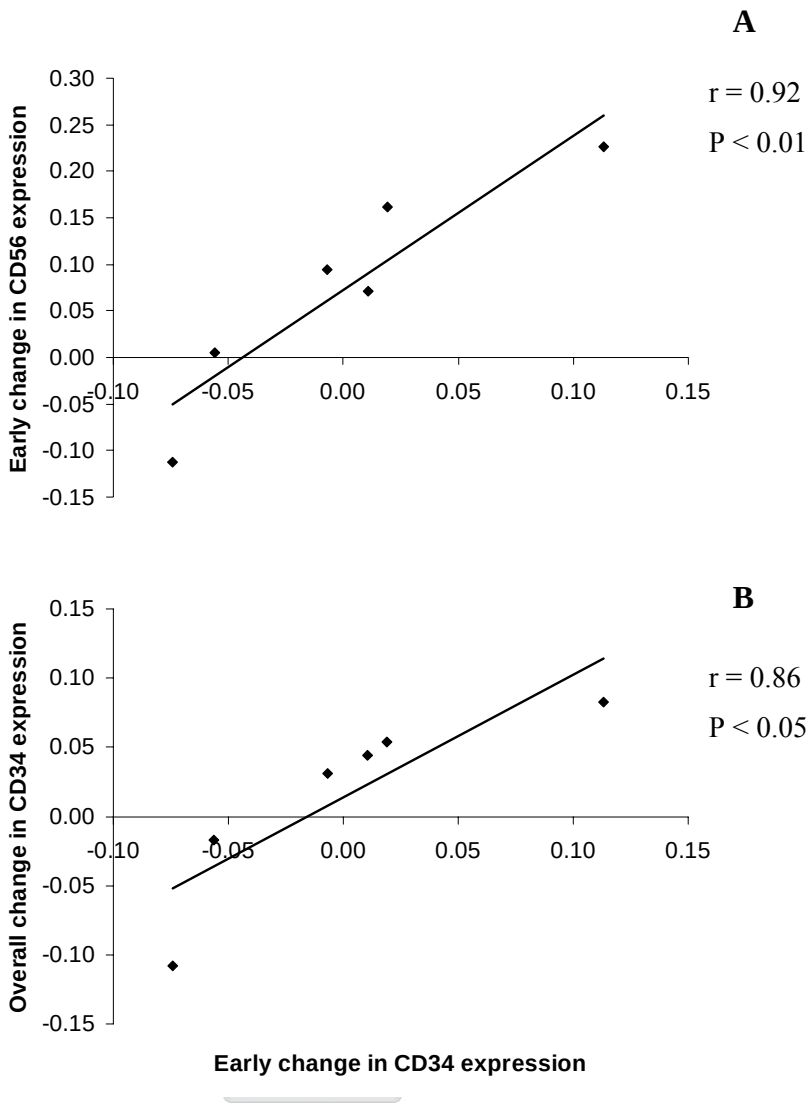


Figure 3.15 Presentation of the relationships between the early changes in CD34 expressions between both the early change in CD56 expression (panel A) and the overall change (days 7 and 9 minus baseline) in CD34 expression (panel B).

Table 3.3 Number of $MyoD^+/m\text{-}cadherin^+/Hoescht^+$ satellite cells expressing MyoD in individual muscle biopsies.

	Baseline	Day 1		Day 2		Day 7		Day 9	
Subject		**CON**	**ICE**	**CON**	**ICE**	**CON**	**ICE**	**CON**	**ICE**
1	-	1	-			1	-		
2	-	-	-			2	-		
3	-	-	-			-	-		
4	-			-	1			-	-
5	-			1	-			-	-
6	-			-	-			-	-

Footnote: - indicates 0 observations in the 10-12 fields of view analysed per subject. 1 or 2 indicates number of $MyoD^+/m\text{-}cadherin^+/Hoescht^+$ cells in the 10-12 fields of view per subject. Cells of the table containing – or nothing indicate no observations or no biopsy was taken on that day respectively.

Table 3.4 Number of $MyoD^+/m\text{-}cadherin^-/Hoescht^+$ cells non-satellite cells expressing MyoD in individual muscle biopsies.

	Baseline	Day 1		Day 2		Day 7		Day 9	
Subject		**CON**	**ICE**	**CON**	**ICE**	**CON**	**ICE**	**CON**	**ICE**
1	-	-	-			-	1		
2	-	-	-			-	-		
3	-	-	-			-	-		
4	-			-	-			-	-
5	1			-	-			-	-
6	-			-	-			-	1

Footnote: see table 3.2 for key to interpretation of the data ($MyoD^+/m\text{-}cadherin^-/Hoescht^+$).

Table 3.5 Number of myogenin^{+}/m-cadherin^{+}/Hoescht^{+} cells satellite cells expressing myogenin in individual muscle biopsies.

	Baseline	Day 1		Day 2		Day 7		Day 9	
Subject		**CON**	**ICE**	**CON**	**ICE**	**CON**	**ICE**	**CON**	**ICE**
1	-	-	1			-	-		
2	-	-	-			-	-		
3	-	-	-			-	-		
4	-			-	-			-	-
5	-			-	-			2	-
6	-			-	-			1	2

Footnote: see table 3.2 for key to interpretation of the data (myogenin^{+}/m-cadherin^{+}/Hoescht^{+}).

Table 3.6 Number of myogenin^{+}/m-cadherin^{-}/Hoescht^{+} non-satellite cells expressing myogenin in individual muscle biopsies.

	Baseline	Day 1		Day 2		Day 7		Day 9	
Subject		**CON**	**ICE**	**CON**	**ICE**	**CON**	**ICE**	**CON**	**ICE**
1	-	-	-			-	-		
2	-	-	1			-	-		
3	-	1	1			-	-		
4	-			-	-			-	-
5	-			-	-			-	-
6	-			-	-			-	-

Footnote: see table 3.2 for key to interpretation of the data (myogenin^{+}/m-cadherin^{-}/Hoescht^{+}).

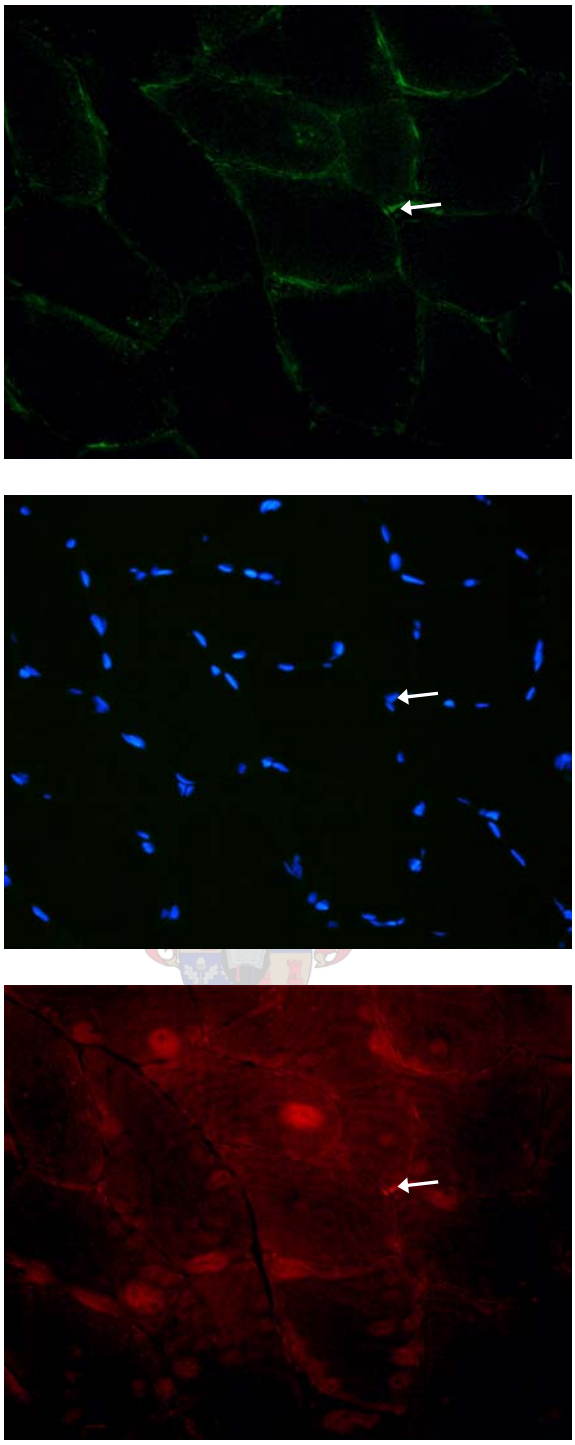


Figure 3.16 Myogenin expressed in the nucleus of a satellite cell (white arrow). This image was taken from a muscle section from the control leg at day 9 post-DHR. FITC (green) indicates myogenin expression (A), Hoecsht (blue) stains all nuclei (B) and Texas Red (red) shows m-cadherin staining (C) (original magnification x 40).

3.2 *In vitro:* C2C12 cells

The expression profiles of the markers used to analyse satellite cells *in vivo* were assessed in differentiating C2C12 cell cultures. The aims of this experiment were to analyse the activation and differentiation *in vitro* using a valid satellite cell model, as well as to test whether our set of antibodies was successful in staining satellite cells at the appropriate, expected times in the cell cycle in these differentiating cells. Apart from testing the validity of the antibodies used in this study, we also wanted to investigate the expression patterns of these markers. Finally, this model could indicate an increase in CD56 expression which was accompanied by a decrease in CD34 and also, a down-regulation in MyoD expression preceding an increase in myogenin expression.

The appearance of the C2C12 cells differs at distinct time points of differentiation (Figure 3.17). Before inducing differentiation the cells contain very little cytoplasm, but following activation the cytoplasm becomes more abundant. At day 3 these cells appear to form extensions (panel B). By day 6 both differentiation and fusion have clearly occurred (panel C). At day 8, myotube formation as well as myotube alignment is clearly visible (panel D).

The expression of CD34, CD56, MyoD and myogenin was quantified by determining staining intensity (Figure 3.18). A low CD34 expression (Figure 3.18A) was sustained over the first three days after inducing differentiation, though a slight, statistically insignificant elevation seems possible for day 1.

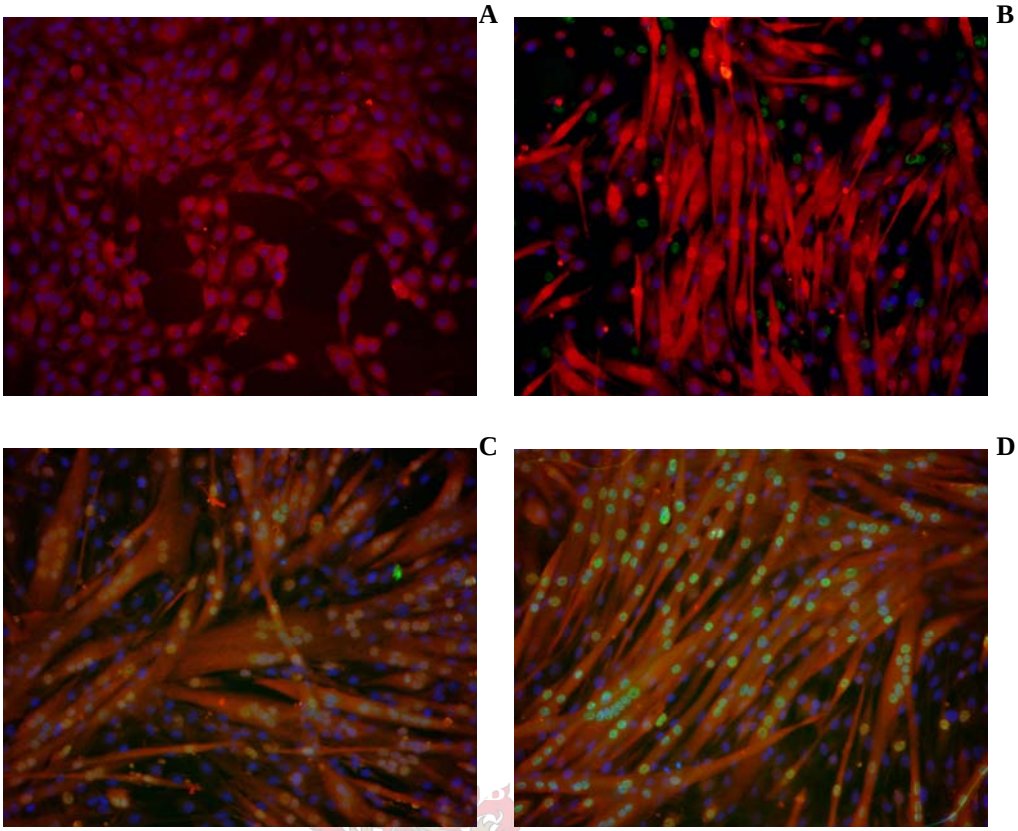


Figure 3.17 Differentiation in C2C12 cells: (a) day 1, (b) day 3, (c) day 6, (d) day 8. Myogenin is indicated with FITC (green), nuclei stained with Hoescht (blue) and m-cadherin is indicated with Texas Red (red) (original magnification x 20).

These results are similar to those found in another study (Beauchamp *et al.*, 2000). CD34 expression had increased significantly at both days 6 and 8, suggesting an increase in the population of quiescent C2C12 satellite cells. Under control conditions (D0), CD56 expression was also low. Following the differentiation stimulus, the expression of CD56 showed a trend to increase until day 6, but showed a significant increase at day 8 with day 8 being significantly different to all other time points ($P < 0.01$). This indicates that, although a small population

of C2C12 cells are already activated prior to differentiation, further activation occurs in response to the differentiation stimulus (Figure 3.18B), and is highest only 2 days after the highest $CD34^+$ staining intensity (although day 7 was not assessed). MyoD expression only increased significantly at day 8 (D0 vs D8, Figure 3.18C; $P < 0.01$), although a trend for an increase could be seen at day 1 (similar in profile to CD34). This is surprising in light of the fact that myogenin expression (indicating differentiated cells) was also significantly increased at day 8 (D0 vs D8, Fig 3.18D; $P < 0.01$). However, both myogenin and MyoD may display a biphasic mode of expression (Figures 3.17C & D) that was not synchronised. The data suggest an increase in myogenin (D3) follows after an increase in MyoD. It is possible that a larger increase in myogenin expression may have followed at e.g. day 10 or 12. Furthermore, within the C2C12 culture there may be distinct populations of satellite cells at different stages of the cell cycle. In the current study we did not synchronize the cells prior to differentiation. Hence, some cells may have been responsive at day 1 (MyoD) and day 3 (myogenin), but the majority at day 8. Alternatively proliferation was still occurring.

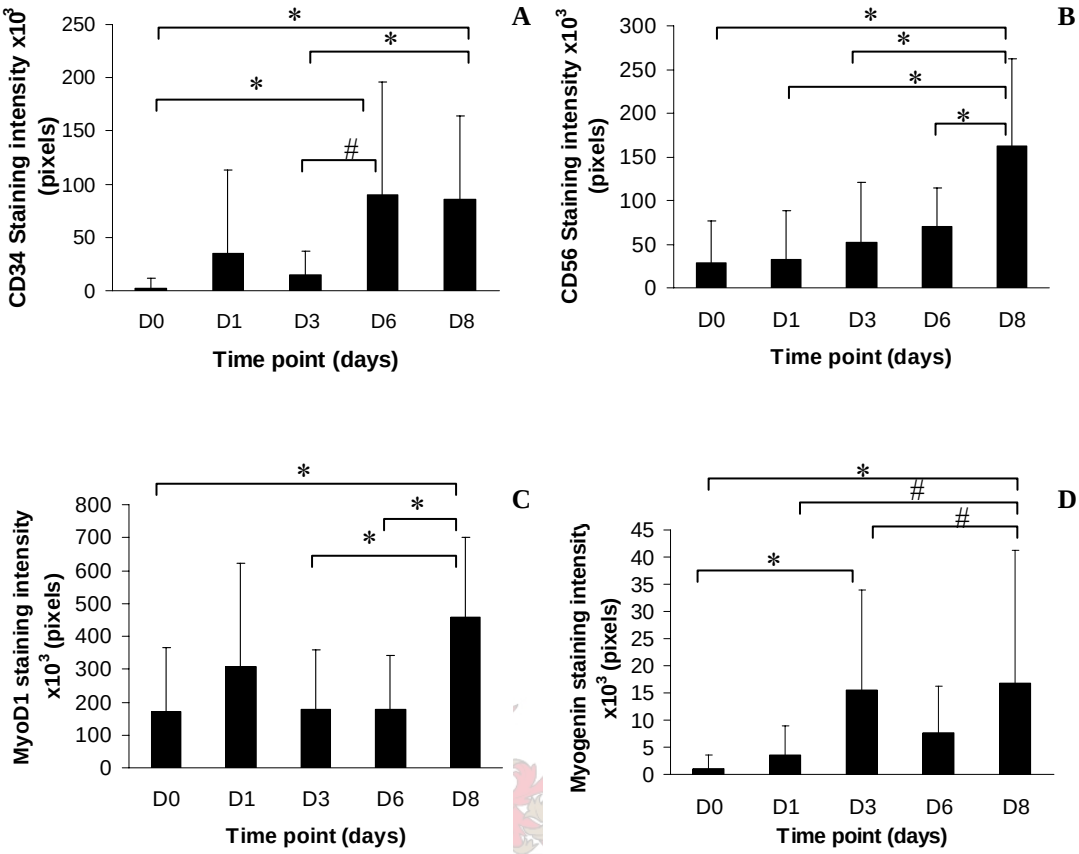


Figure 3.18. Expression of CD34 (A), CD56 (B), MyoD1 (C) and myogenin (D) (means ± SD) in C2C12 cells. Statistical analysis: One-way ANOVA, * $P < 0.01$, # $P < 0.05$ (Bonferroni post-hoc test).

CHAPTER 4: DISCUSSION

In vivo

The aims of the study in human subjects were 1) to induce delayed onset muscle soreness (DOMS) in recreationally active individuals using a modified downhill-running (DHR) protocol, 2) to characterize satellite cell activity in muscle biopsies taken from these subjects and 3) to investigate the effect of an ice application on this satellite cell activity. In general, DOMS studies in human subjects have most frequently addressed the clinical symptoms and have used several serum parameters to investigate the effects of their respective therapeutic interventions (e.g. Smith *et al.*, 1994b; Tokmakidis *et al.*, 2003; Yackzan *et al.*, 1984). However, although the alleviation of the clinical symptoms of DOMS is important, so is the recovery of the affected muscle(s). To our knowledge this is the first study to investigate the effect of cryotherapy on DOMS at the tissue level, specifically on skeletal muscle satellite cell activity, in humans.

Several DOMS-inducing protocols have used specialised equipment such as eccentric cycle ergometers (Kadi *et al.*, 2004b) or extreme protocols such as multiple repetitions of eccentric exercise in a small muscle (e.g. elbow flexors) (Paddon-Jones & Quigley, 1997). Although those models have successfully induced DOMS, they do not represent typical exercise patterns in recreational or elite athletes. Therefore, we decided to use a running protocol that could be completed within one hour. It has previously been shown that DHR, but not level running, induces DOMS (Pizza *et al.*, 1995; Schwane *et al.*, 1983) thus it was

decided to use a DHR protocol. However, it is important to take into account the fitness level of each individual to standardise the intensity of the DHR. Existing DHR protocols make use of individuals' VO_{2max} or estimated maximum heart rates from which to determine a sub-maximal intensity for the DHR (Byrnes *et al.*, 1985b; Eston *et al.*, 1996b). However, past studies report that some, but not all individuals' VO_{2max}'s plateau and hence a linear relationship is not always present (Usaj & Kandare, 2000; Zoladz *et al.*, 2002). But, during incremental tests, workload itself increases linearly up to the end of the test. Thus, we used each individual's peak treadmill speed (PTS) to determine their individual submaximal DHR speed. Our modification to the existing DHR protocols was successful, as seen by both the soreness ratings and the serum CK activities post-DHR (Figures 3.1, 3.2 and 3.3).

Similar to other DHR studies (Pizza *et al.*, 1995; Schwane *et al.*, 1983), the perceived muscle soreness ('active daily living' questionnaire ratings) was significantly increased at 24 and 48 hours post-DHR. Cryotherapy did not result in a difference between the treated and untreated leg, using the soreness data collected in response to standardised pressure applied by the muscle probe. Using the same technique, and using a force of 5 kg, we showed that the proximal (80% from patella) and middle (50% from patella) sites of the *quadriceps* muscle had more soreness than at baseline on day 2, but the distal area (20% from patella) did not differ in soreness from baseline. It is advised for future studies to evaluate soreness (with the muscle pain probe) beyond 48 hours after inducing DOMS, because this may elucidate whether a difference between the treated and untreated

legs become evident, or not. The muscle probe results also indicate that 5 kg might be the most effective force to be applied to indicate possible pain differences. Because muscle soreness was not assessed beyond two days after the DHR, no conclusive deductions can be made about the effect of ice treatment on recovery of muscle soreness.

As mentioned in Chapter 1, DOMS is associated with muscle micro-damage in the affected muscles and serum CK activity is often used as an indicator of the presence of muscle damage. In our study the serum CK activity was significantly elevated one day after DHR, whereafter it started to resolve again (normal serum CK activity range from 15 – 195 u/l at 37 ^{o}C). The CK activity response is similar to that seen in some other DHR studies, however, the CK activity peak at day 1 post-DHR in our study was much greater (almost twofold) when compared with some other DHR studies (Pizza *et al.*, 1995; Schwane *et al.*, 1983). Although one is tempted to infer that our modification to the DHR protocol resulted in a more extreme DOMS-inducing protocol, one cannot do so as it is generally accepted that CK activity is not related to the extent of muscle damage (Eston *et al.*, 1994; Manfredi *et al.*, 1991; Nosaka & Kuramata, 1991). Furthermore, we did not measure the heart rate and VO_{2max} during the DHR, and thus were not able to estimate the percentage heart rate or VO_{2max} at which the individuals completed their DHR. Consequently we cannot conclude whether our modification to the DHR did in fact result in a more extreme DHR intensity relative to the physiological rather than the mechanical maximum.

A skeletal muscle biopsy is an extreme form of muscle damage, thus it is feasible that this may have influenced the serum CK activities measured after day 1. In our study, only the group receiving their post-DHR biopsies 48 hours after the DHR seemed to show an effect of the muscle biopsy on the CK activity at day 7 post-DHR (5 days after the biopsies) (Figure 3.3B). However, no significant difference was found at day 7 post-DHR between the group receiving their post-DHR biopsies at 24 hours (6 days after the biopsies), and the group receiving their post-DHR biopsies at 48 hours. The implication of this observation is unclear and to clarify this we suggest that in future studies the serum CK activity is determined every day for the time-course of the study. Due to the fact that systemic levels of CK activity were measured in our study, we could not determine the effect of the ice application on the CK activity as this would have required two DOMS subject groups: one group receiving ice on both legs and the other no treatment. The study design chosen, however, allows for paired observations and removes the confounding effect of different inter-individual variations in baseline and the response to DHR.

CD34

As far as we know, this is the first study to use CD34 as a marker of quiescent satellite cells *in vivo* in humans. Other studies have used only CD56 as a satellite cell marker without attempting to distinguish between the quiescent and the activated satellite cell pools (Charifi *et al.*, 2003; Crameri *et al.*, 2004; Kadi *et al.*, 2004a; Malm *et al.*, 2000). However, CD34 has been shown to mark most

quiescent adult satellite cells *in vitro* in cell culture (Beauchamp *et al*., 2000). We showed almost no time or treatment effects in the CD34 expression at all time points *in vivo*. Only at day 1 post-DHR the untreated leg expressed significantly less $CD34^+$ cells compared to the treated leg. Other cells in muscle also express CD34 making it difficult to identify satellite cells. Also, we did not use any co-marker to identify satellite cells, thus it is advised to co-stain with another satellite cell marker in future studies. This may result in time and treatment differences in CD34 expression that were not evident in this study. Alternatively, since great care was taken to use morphological features to co-identify $CD34^+$ cells as satellite cells, these data may reflect that one bout of DHR does not increase or decrease the pool of quiescent satellite cells significantly.

In contrast to CD34, CD56 has been used in at least four other studies to assess satellite cells in human muscle biopsies (Charifi *et al.*, 2003; Crameri *et al.*, 2004; Kadi *et al.*, 2004a; Malm *et al.*, 2000).

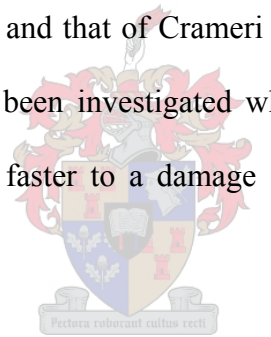
CD56

The percentage CD56 positive satellite cells at baseline was very similar to the percentage found in the *tibialis anterior* muscle at baseline in a study by Kadi *et al.* in which subjects were recreationally active males (Kadi *et al.*, 2004a). However, the percentage of $CD56^+$ satellite cells at baseline in Kadi's study was much higher than that found in other studies (Charifi *et al.*, 2003; Crameri *et al.*, 2004). Although controversial, it has been indicated that satellite cell number decreases with age in humans (Sajko *et al.*, 2004). This may explain the very low

percentage CD56 satellite cells (*vastus lateralis* muscle) found in the biopsies of the subjects (average age: 73) in Charifi's study (Charifi *et al.*, 2003). Another difference between these related studies was the normal activity level of the subjects. Crameri's study used sedentary individuals suggesting that they may have a lower number of $CD56^+$ satellite cells than active subjects. We observed large standard deviations, thus large inter-individual differences in both $CD34^+$ and $CD56^+$ satellite cell numbers per myofibre at baseline. The different percentage of satellite cells present in baseline biopsies in our study may be related to the fitness level of the subjects. The tendency for a correlation between VO_{2max} and $CD56^+$ satellite cell number ($P < 0.07$) supports a relationship between normal activity level and satellite cell activation status.

In our study we report a significant increase in satellite cell activation indicated by the significant increase in CD56 positive cells in muscle sections. This significant increase was seen only at days 2 and 9 post-DHR in the control leg, with no significant increase at days 1 and 7 post-DHR in the other three subjects. In Crameri *et al.*'s study (Crameri *et al.*, 2004), two days after a high intensity eccentric exercise in one leg of each subject, the percentage $CD56^+$ satellite cells ((positive cells/positive cells + myonuclear number) x 100) was 6.4% which is less compared to our study when it was 10.7% at day 2 post-DHR. This smaller percentage in Crameri *et al.*'s study may be due to the difference in exercise protocols. Another possibility is that the change in the percentage CD56 expression may be similar for both studies, but it was not possible to determine this change for Crameri *et al.*'s study due to unreported baseline values for the

exercised leg. The fact that their subjects were sedentary prior to eccentric exercise may also have influenced the satellite cells' sensitivity to respond to the stimulus. Also, whether satellite cells respond differently to distinct types of exercise (e.g. aerobic, anaerobic, power, and high intensity exercise) has not yet been investigated. We cannot explain the difference in activation between the two groups in our study. It is possible that CD56 expression only becomes evident two days after DHR. However, this does not seem likely, because the increase was sustained at day 9 post-DHR and yet there was no evidence at day 7 in the other subjects. Possibly inter-individual differences in satellite cell response may explain our data (no increase in $CD56^+$ satellite cells in subjects receiving biopsies at days 1 and 7 post-DHR) and that of Crameri *et al*. (Crameri *et al*., 2004). To our knowledge, it has not been investigated whether an active, fit individual's satellite cells will respond faster to a damage stimulus when compared with a sedentary individual.



To further try to understand our results, we determined whether or not there were associations between changes in CD34 expression and CD56 expression. The positive relationship between the early changes in $CD34^+$ and $CD56^+$ satellite cells (Figure 3.15A) was unexpected. This finding suggests co-expression of CD34 and CD56 during the early activation stages of satellite cells. Thus, this possibility of co-expression should be investigated further *in vivo*. In Figure 3.15A, two subjects had a decrease in their $CD34^+$ satellite cells with a decrease or no change in the $CD56^+$ satellite cells. This raises the question of what happened to these satellite cells. Very little is still known about satellite cells, and

especially their markers, thus making it difficult to explain this finding. However, we propose a few possible explanations that may explain this finding. It may be that those 'missing' satellite cells fused with existing myofibres at an early stage or, they were lost due to apoptosis. However, no correlations were found between the total myofibre-associated nuclei (expressed per myofibre number) and the early changes in CD34 and CD56 expression, thus apoptosis may not be an explanation. Furthermore, it is also possible that these 'missing' satellite cells may have migrated to an adjacent area of damage not 'caught' by the biopsy. Another explanation may be that the 'missing' satellite cells represent a population that lost their CD34 and CD56 expression due to proliferation, but this possibility seems highly unlikely due to the result of very few $MyoD^{+}$ (representing proliferation) satellite cells detected in the muscle sections. However, it may be that the antibody we used to detect MyoD was not sensitive enough, thus other antibodies should be tested to elucidate whether the latter explanation for the 'missing' satellite cells holds true.

Ice application

Cryotherapy is believed to be an effective intervention to decrease inflammation (Knight, 1995). One question is whether a decrease in inflammatory cell infiltration, specifically macrophages, would be advantageous to the skeletal muscle repair/recovery process. In fact, it has been shown that at least some macrophages are essential for regeneration in muscle transplants in a murine model (Lescaudron *et al.*, 1999), but this may not be applicable to all types of repair. Whether this is due to the fact that macrophages have to remove cellular

debris and/or that the factors secreted by macrophages can stimulate satellite cell activation, is not known.

Several researchers have attempted to investigate the effect of cold therapies on the clinical symptoms of DOMS (Paddon-Jones & Quigley, 1997; Yackzan *et al.*, 1984), but none investigated whether this kind of intervention is advantageous or detrimental to the recovery process after DOMS. In the first study (Paddon-Jones & Quigley, 1997), DOMS was induced in both arms of the subjects, whereafter only one arm per subject received cryotherapy. The cryotherapy protocol consisted of placing the entire forearm and upper arm in a mixture of ice and water (5 ± 1 ^{o}C) for 20 minutes immediately after the DOMS-inducing exercise. In total, five ice-water immersions were performed with an hour of rest between immersions. Muscle soreness was assessed for 6 days after the exercise bout with no significant difference between the treated and untreated arm at any time point. Yackzan *et al*. (Yackzan *et al.*, 1984) also induced soreness in both arms of their subjects. With one of the arms serving as control, the other received an ice massage of 15 minutes. In this study there were three treatment groups: the first group received treatment immediately after the exercise bout (within 3 minutes after cessation of the exercise), the second received treatment at 24 hours after the exercise bout and the third group received an ice massage at 48 hours after the exercise bout. Muscle soreness was assessed for 3 days after the DOMS-inducing exercise with no significant difference between the treated and untreated arms in any of the treatment groups. Although both of these studies suggest that ice treatment is not effective in preventing pain, or alleviating pain after DOMS, there

were some problems in the design of these studies. In the first study described above, the treatment would be more accurately described as cold treatment, rather than ice treatment. In the second study, although the pain rating scale ranged from 0-6, the pain induced was maximally 3 and hence likely to be insensitive to interventions. It could also be argued that due to the circular movements of the massage, total contact time with the ice was less than 15 minutes which may have influenced the results.

At day 1 post-DHR there was no significant increase in $CD56^{+}$ satellite cell number in the treated or untreated muscle samples. However, by day 2 post-DHR, there was a significant increase in the untreated leg. This increase was prevented by the application of ice. The implication of this prevention of satellite cell activation (at day 2 post-DHR) is that ice application may be detrimental to any change in satellite cell status. In contrast, it has been shown that a bout of exercise can activate satellite cells to the same level as seen in the control leg in this study (Kadi *et al.*, 2004b; Malm *et al.*, 2000; Crameri *et al.*, 2004). Taken together, these results might infer that the ice application used in this study prevented even normal exercise-induced satellite cell activation which could have negative implications (negative effect). This is plausible since the extent of ice application in our study was quite extreme. Alternatively, it could be argued that if cold therapy decreased secondary muscle damage (Merrick *et al.*, 1999), the extent of satellite cell activation (CD56) required would be less (seen as a positive effect). Merrick has suggested that ice application may decrease the type of secondary injury caused by enzymes released due to damaged membranes

(Merrick *et al.*, 1999), but this interpretation may not be correct. It may be that cryotherapy retards the progression to cell death caused by primary injury, or that it influences other types of secondary injury. The quantification of both primary and secondary injury is difficult and some authors believe it is not possible to distinguish between primary and secondary injury (Merrick, 2002). Thus it is important to discover a way to distinguish between these two classes of injury or to find another way to investigate the effect of interventions (such as cold therapy) on muscle damage. This highlights the importance of our current study which begins to lay a basis for investigating the response of muscle to damage via quantification of satellite cell activation, a process that is essential for satellite cells to assist in skeletal muscle repair. Our current study has also raised several suggestions for further research. Future studies should include some assessment(s) that could distinguish between these two intracellular effects of ice therapy, e.g. quantification of intra-muscular water content (magnetic resonance imaging, MRI).

Finally, the use of less extreme ice applications (shorter ice application time period and/or fewer application), such as that used by athletes to promote recovery, may not have the same effect at tissue level. Consequently, shorter duration times of ice application should also be investigated at skeletal muscle tissue level. This result also raises the question of whether an individual receiving ice would respond differently to a second bout of exercise compared to an individual receiving no treatment. It may be important to investigate this, as it could be an experimental design to indicate whether satellite cell activation is a

requirement for the "second bout" adaptation effect shown by others (Byrnes *et al.*, 1985b; Donnelly *et al.*, 1990; Eston *et al.*, 1996b) in response to eccentric exercise.

The fact that there were inter-individual differences in satellite cell responses to ice application was clear especially when looking at the CD56 expression in the muscle sections (Figures 3.6 and 3.7). This could be explained by differences in individual subjects' adipose tissue in the upper leg area. Myrer and his colleagues (Myrer *et al.*, 2001) have shown that adipose tissue is a very important factor in determining the amount of cooling induced by an ice-pack application of 20 minutes. Unfortunately no skinfold thicknesses of the upper leg were taken. In future studies we will therefore include skinfold measurements when investigating the effect of cryotherapy on DOMS.

CD56 expression in the control biopsies seemed to display a biphasic trend, but data are not sufficient to comment on this. This does emphasise the importance of choosing the correct time point(s) to investigate satellite cell-cycle markers in order to prevent false deductions. However, the biphasic response seen in Figure 3.12B should be considered with caution, because different subjects' responses are presented together in this figure without taking into account that no subject had biopsies on all four post-DHR biopsy days. Nevertheless, the possibility that a decrease in $CD34^+$ satellite cells is followed by an increase in $CD56^+$ satellite cells, should be investigated.

MyoD and myogenin

In the MyoD- and myogenin stained slides we investigated all nuclei (Hoechst$^+$) for MyoD and myogenin expression respectively, whilst distinguishing between satellite cells (m-cadherin$^+$) and myonuclei (m-cadherin$^-$) expressing these markers. Myogenin positive nuclei (both m-cadherin$^+$ and $^-$) were detected at days 1 and 9 post-DHR. Myogenin$^+$/m-cadherin$^+$/Hoechst$^+$ cells were detected in both treated and untreated legs. Also, intra-myofibrillar myogenin$^+$ nuclei that were negative for m-cadherin (and therefore are likely to be myonuclei expressing myogenin) were also detected. Cooper and colleagues (Cooper *et al.*, 1999) have previously shown *in vivo* (during regeneration after toxic damage to murine muscle) that MyoD can be expressed in myonuclei (m-cadherin$^-$) and not just in the nuclei of satellite cells. In a very recent rat study it has also been shown that both MyoD and myogenin are expressed in myonuclei, satellite cells (m-cadherin positive) and undefined myogenic cells (Ishido *et al.*, 2004) after overloading in the rat *plantaris* muscle.

Very few studies on muscle samples from human subjects have been done using myogenin as a marker for satellite cells. Kadi *et al*. (Kadi *et al.*, 2004b) found myogenin positive nuclei in biopsy samples taken immediately (within one minute) after a bout of exercise in human subjects. These nuclei were identified as myonuclei and not satellite cells by the absence of CD56 expression. In contrast, Crameri and her colleagues (Crameri *et al.*, 2004) did not detect myogenin positive nuclei in seven of the eight muscle biopsy samples taken from

eight subjects at five hours after an exercise bout. These contrasting findings may be due to the different time points after the exercise bout at which biopsies were taken. Another possibility may be due to the fact that the different research groups purchased their myogenin antibody from different companies which may have resulted in differences in binding capacities of the antibodies. Also, the dilutions of the antibodies (final concentrations) were not mentioned. However, in Crameri *et al.*'s (Crameri *et al.*, 2004) study, one subject's biopsies did have myogenin positive nuclei (days 2, 4 and 8 post-exercise) and this subject also showed evidence of myofibre necrosis (desmin-negative, dystrophin-negative, but fibronectin-positive). Thus, the latter two possibilities may not have a great influence on the controversial results of the two studies. Unfortunately it was not mentioned whether these myogenin positive nuclei were satellite cells or not.

It is unclear exactly when satellite cells that have fused with each other or with myofibres lose their satellite cell characteristics (including e.g. m-cadherin expression). Therefore, if the myogenin^{+}/m-cadherin^{-} nuclei in our study are not part of the original pool of myonuclei, another explanation may be that they are the nuclei of satellite cells that fused with the myofibres and consequently no longer have detectable m-cadherin expression, but are still expressing myogenin.

It is difficult to interpret the MyoD and myogenin data in more detail due mainly to the limited expression of these myogenic factors and the few time points at which biopsies were taken, as well as the small pool of subjects at each time point post-DHR. In future the use of other MyoD and myogenin antibodies should be

investigated to make sure that the most suitable antibody is used. Although C2C12 cell cultures do not resemble the *in vivo* situation, the C2C12 results infer that MyoD and myogenin expression may be transiently elevated at first (Figure 3.18) and may follow a biphasic pattern with greater expression later if stimulated to differentiate sufficiently. This may also hold true for the *in vivo* situation, and if so, it may explain the difficulty in detecting the expression of these two markers. In addition to staining for myogenin as an indicator of the differentiation process, it is advised to stain for other markers of muscle damage (e.g. fibronectin (Crameri *et al.*, 2004)). This will aid in elucidating whether cryotherapy has prevented secondary injury or suppressed muscle recovery. It is unclear at this time how long after induction of an injury, like the micro-damage of DOMS, it takes to regenerate muscle in humans, even without ice therapy. Our study is the first study to take biopsies up to day 9 following DOMS.

As mentioned before, several studies have shown that one bout of exercise is capable of activating satellite cells (Kadi *et al.*, 2004b; Malm *et al.*, 2000; Crameri *et al.*, 2004). These three studies involved three different exercise modalities, namely one-leg cycle ergometry for 30 minutes at 40% and 75% of VO_{2max} (Kadi *et al.*, 2004b), eccentric cycling for 30 minutes at peak workload obtained in concentric cycling VO_{2max} test (Malm *et al.*, 2000) and a bout of one-legged high intensity eccentric exercise (one bout of high intensity eccentric exercise with one leg: 50 one-leg 'drop down' jumps from a platform, 8 x 10 maximal eccentric knee extensions at -30 degrees.s^{-1} and 8 x 10 maximal eccentric knee extensions at -180 degrees.s^{-1}) (Crameri *et al.*, 2004). And it is known that satellite cells are

activated upon muscle damage. Our data showed a positive relationship between the late change (late minus early and thus, no influence of the baseline data taken into account) in $CD56^+$ satellite cells and soreness experienced at day 2 post-DHR (Figure 3.14). Although muscle damage and soreness ratings may not coincide, this finding suggests that muscle damage may be responsible for the sustained increase of activated satellite cells at a later time point (day 9 post-DHR), rather than the exercise bout itself. To shed light on this finding, it is recommended for future studies to include investigation and quantification of muscle damage, for example making use of electron microscopy.

This also raises the question of the actual role of the CD56 expression increment after a bout of exercise. In an *in vitro* study it was shown that CD56 expression enhances fusion of myoblasts to form myotubes (Dickson *et al.*, 1990). Thus, this suggests that CD56 has a role in myoblast fusion. Therefore, the increase in CD56 expression after a bout of exercise may prepare the satellite cells for fusion. CD56 has been shown to be expressed in denervated and re-innervated human skeletal muscle (Gosztonyi *et al.*, 2001). Thus, an increase in CD56 expression after an exercise bout may change due to a change in neural stimulation or release of neural factors during the exercise bout.

In summary, although our data are difficult to interpret due to a lack of other related studies, if the soreness ratings were the only data, the conclusion would have been that the ice application had no effect on DOMS, but investigating DOMS at tissue level shows that cold therapy has an effect. This is the first study

to show an effect of cryotherapy at tissue level in human subjects after inducing DOMS. Thus, this shows the importance of investigating other parameters in addition to that of the clinical symptoms and serum parameters. These results do not exclude the use of ice application for shorter time periods or for a shorter time course.

In vitro

In our *in vitro* model of satellite cell differentiation, we were successful in showing that the set of antibodies used in this study did stain the C2C12 cells and that the binding of these markers to the C2C12 cells occurred at time points in the cell cycle corresponding to those expected.

A low CD34 expression was sustained over the first three days following induction of differentiation. These results are similar to those found in another C2C12 culture study (Beauchamp *et al.*, 2000). The significant increase in CD34 expression at days 6 and 8 suggests an increase in the population of quiescent C2C12 satellite cells. Although it remains to be determined what factors so late in differentiation induce this increase, two possibilities exist. Firstly, proliferation of existing quiescent satellite cells or secondly, a return to quiescent status of previously activated but unfused satellite cells.

Under control conditions, CD56 expression was similar to that of CD34. Following the differentiation stimulus, the CD56 expression showed a steady

increase up to day 8. This indicates that, although a small population of C2C12 cells are already activated prior to differentiation, further activation occurs in response to the differentiation stimulus.

MyoD expression only increased significantly at day 8 of differentiation. This is surprising in light of the fact that myogenin expression was also significantly increased at day 8. Differentiated cells generally show a down-regulation of myoD expression (Yoshida *et al.*, 1998). Our data suggest that within the C2C12 culture, there may be distinct populations of satellite cells at different stages of the cell cycle. This shows the need to analyse for other markers of proliferation and also time points after day 8 to determine at what point proliferation may be decreased.

In the current study we did not synchronise the cells prior to the differentiation stimulus. Thus, the time course of CD34, CD56, MyoD and myogenin expression should also be determined with synchronisation of C2C12 cells prior to induction of differentiation to elucidate possible differences in expression of the different cell cycle markers in synchronised versus unsynchronised cells. Another explanation for the non-significant trends in expression patterns could be due to the fact that different cover slips with plated cells were analysed at each time point for each of the different satellite cell markers. This can be resolved by doing more experiments (with different batches of C2C12 cells) as has been done in this study. A further recommendation would be to also include nuclear counts

and to normalise the staining intensities of the different antibodies (at the different time points) relative to the number of nuclei.

Both MyoD and myogenin seemed to display a biphasic mode of expression. To investigate this possible biphasic expression it would be necessary to increase the sample size and to make use of other investigative techniques, such as Western blotting.

So far it is not known what the direct effect of cold temperatures might be on satellite cell activity. This could be investigated *in vitro* using a cell line, e.g. C2C12 cells, to investigate certain cell cycle markers after different durations of exposure to decreased temperatures (resembling the temperatures reached in skeletal muscle after different cryotherapies). However, the control model should first be more clear and consistent with regard to synchronised versus non-synchronised cultures and whether markers are expressed biphasically, or not.

CHAPTER 5: SUMMARY

It is important to understand satellite cell activity in the healthy individual as this will aid in understanding the shortcomings of satellite cells in different myopathies, as well as in aging. For this purpose, it is appropriate to investigate the muscle phenotype associated with muscle soreness at cellular and tissue level. Since muscle soreness is not correlated very well with serum CK activity and it resolves early after micro-damage, it cannot serve as a marker for satellite cell activation or muscle repair. Therefore, analysis of biopsies is important when investigating the effect of an intervention. This is the first study to investigate the effect of an ice application on satellite cell activity in human subjects. We have shown that an ice application of three days, as used in this study, may delay muscle recovery after DOMS. However, it does not discredit the use of ice for shorter durations or application times. Further studies are necessary to investigate time points later than 9 days after a DOMS-inducing exercise.

Furthermore, we did establish a baseline differentiation model for C2C12 cell cultures. In future, we can use this model to explore the effects of several interventions on satellite cell activity. One of these interventions could include investigating the direct effect of decreased temperatures on satellite cell activity.

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Appendix A: Detecting β-galactosidase with X-gal

β-galactosidase (lactase) is an enzyme isolated from *E. coli.* *LacZ* is a subunit of β-galactosidase, i.e. it is a β-galactosidase monomer. β-galactosidase hydrolyses 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal), forming a blue precipitate. This characteristic can be used to monitor genetic activity (transcription) (1). For example, transgenic animals carrying a *lacZ* reporter gene under the control of a desmin promoter can be used to detect any activation and activity of the desmin gene (2). If the desmin gene is activated, or is active, β-galactosidase will be expressed. This means that β-galactosidase is targeted to the nucleus, thus in the presence of X-gal the nuclear product will become blue if the desmin gene is active. This technique can also be used to monitor other genes' activities.

1. Garrett, R.H. & Grisham, C.M. (1999). *Biochemistry*, 2nd ed. Saunders College Publishing, USA.

2. Lescaudron, L., Peltekian, E., Fontaine-Perus, J., Paulin, D., Zampieri, M., Garcia, L., & Parrish, E. (1999). Blood borne macrophages are essential for the triggering of muscle regeneration following muscle transplant. *Neuromuscul .Disord.* 9, 72-80.

Appendix B: Muscle soreness questionnaires

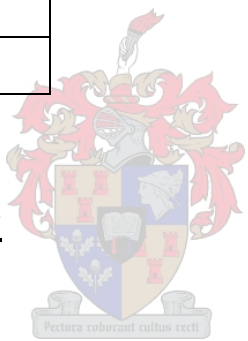
Muscle soreness ("rating of perceived pain")

Subject code: _____ **Starting date: _____**

Dominant leg: _____

Muscle Pain

Hours from exercise protocol	Pain during ADL	
	Left	Right
0		
24		
48		



"Rating of perceived pain"

1 no pain

2 slight pain

3 moderate pain

4 severe pain

5 very severe pain

Hours from exercise protocol: ..…

Pressure pain (pressure probe)

Subject code: _____ **Starting date: _____**

Dominant leg: _____

Muscle Pain

	1 kg		2 kg		3 kg		4 kg		5 kg	
Part of muscle	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
A										
B										
C										

A groin
B middle
C knee

"Rating of perceived pain"

1 no pain

2 slight pain

3 moderate pain

4 severe pain

5 very severe pain

Spierseerheid ("skatting van waargenome pyn")

Proefpersoon kode:_____ **Aanvangsdatum_____**

Dominante been:_____

Spierpyn

Ure vanaf oefen protokol	Pyn gedurende ADL	
	Links	**Regs**
0		
24		
48		

"skatting van waargenome pyn"

1 geen pyn

2 effense pyn

3 matige pyn

4 erge pyn

5 baie erge pyn

Ure vanaf oefen protokol: ……

Drukpyn (druksondeerder)

Proefpersoon kode:_____ **Aanvangsdatum:_____**

Dominante been:_____

Spierpyn

	1 kg		2 kg		3 kg		4 kg		5 kg	
Deel van spier	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
A										
B										
C										

A lies
B middel
C knie

“skatting van waargenome pyn”

1 geen pyn

2 effense pyn

3 matige pyn

4 erge pyn

5 baie erge pyn

Appendix C: Immunohistochemical staining diagram.

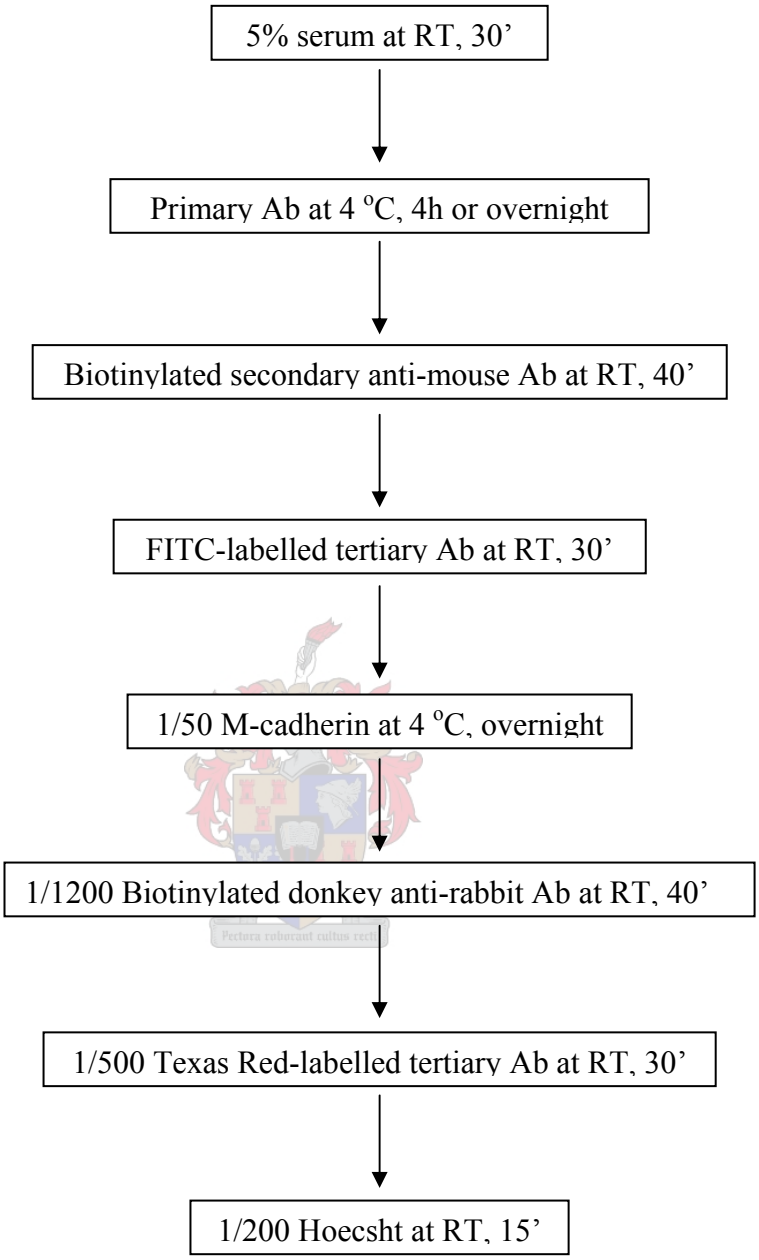


Figure 1. Flow diagram of the general immunohistochemical staining protocol followed in this study for both skeletal muscle biopsy sections and C2C12 cells.

Appendix D: Muscle probe (3 – 5 kg) results

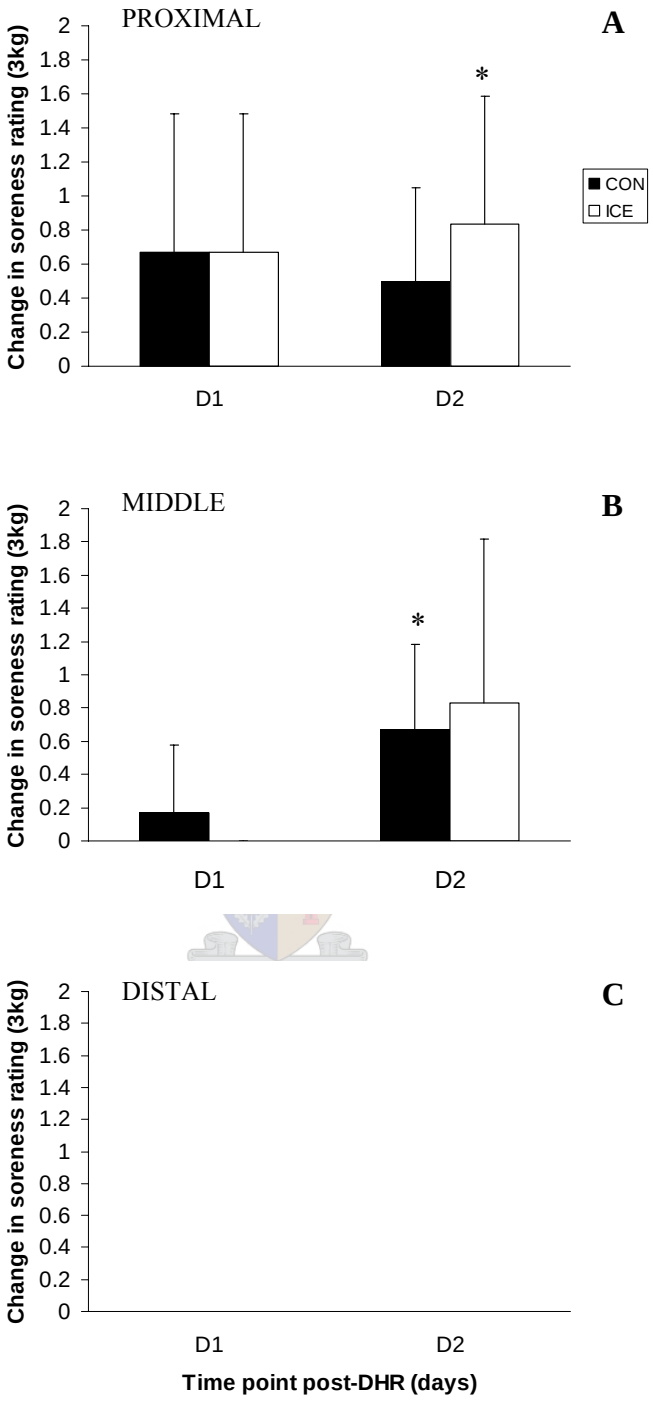


Figure 1 Changes in soreness ratings (mean ± SD) using the muscle probe while applying a force of 3kg. In panel C, the absence of change indicates the absence of soreness. Statistical analysis: repeated measures one-way ANOVA, analysing each time point separately and t-tests (dependent samples). Statistical analysis (comparing each time point with baseline): t-test for dependent samples, * $P < 0.05$.

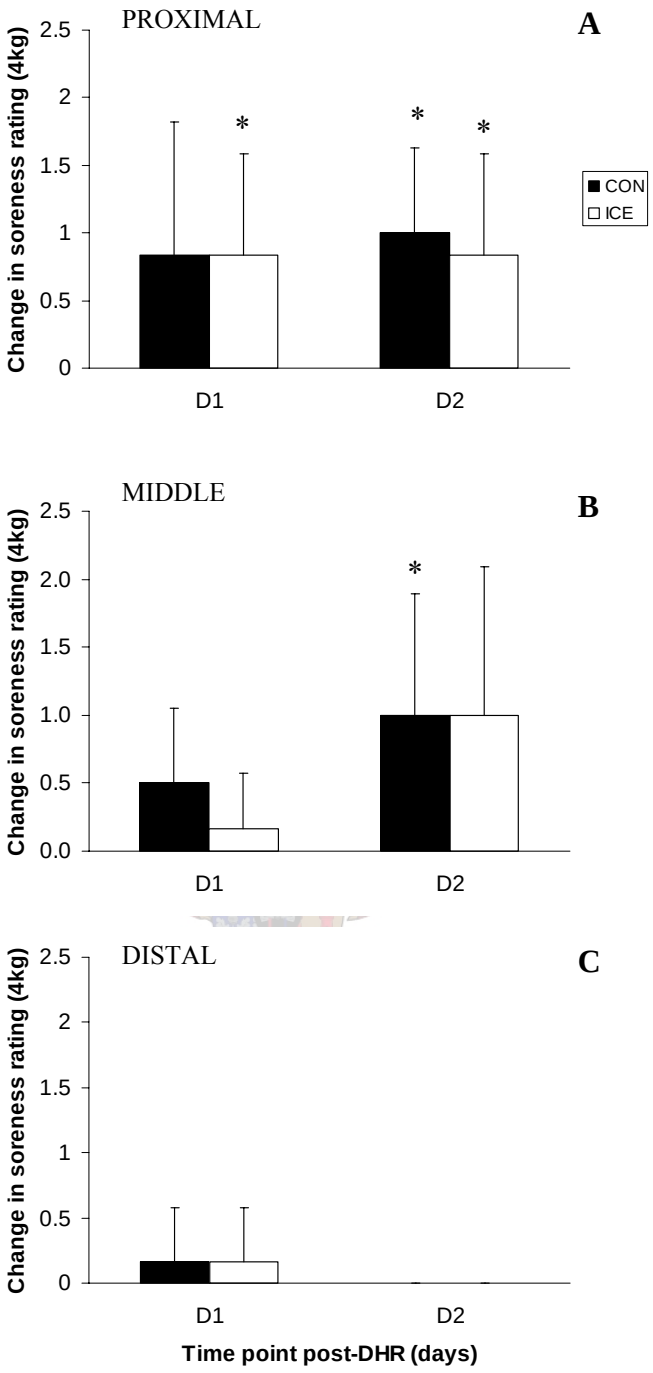


Figure 2 Changes in soreness ratings (mean ± SD) using the muscle probe while applying a force of 4kg. Statistical analysis (comparing each time point with baseline): t-test for dependent samples, * $P < 0.05$.

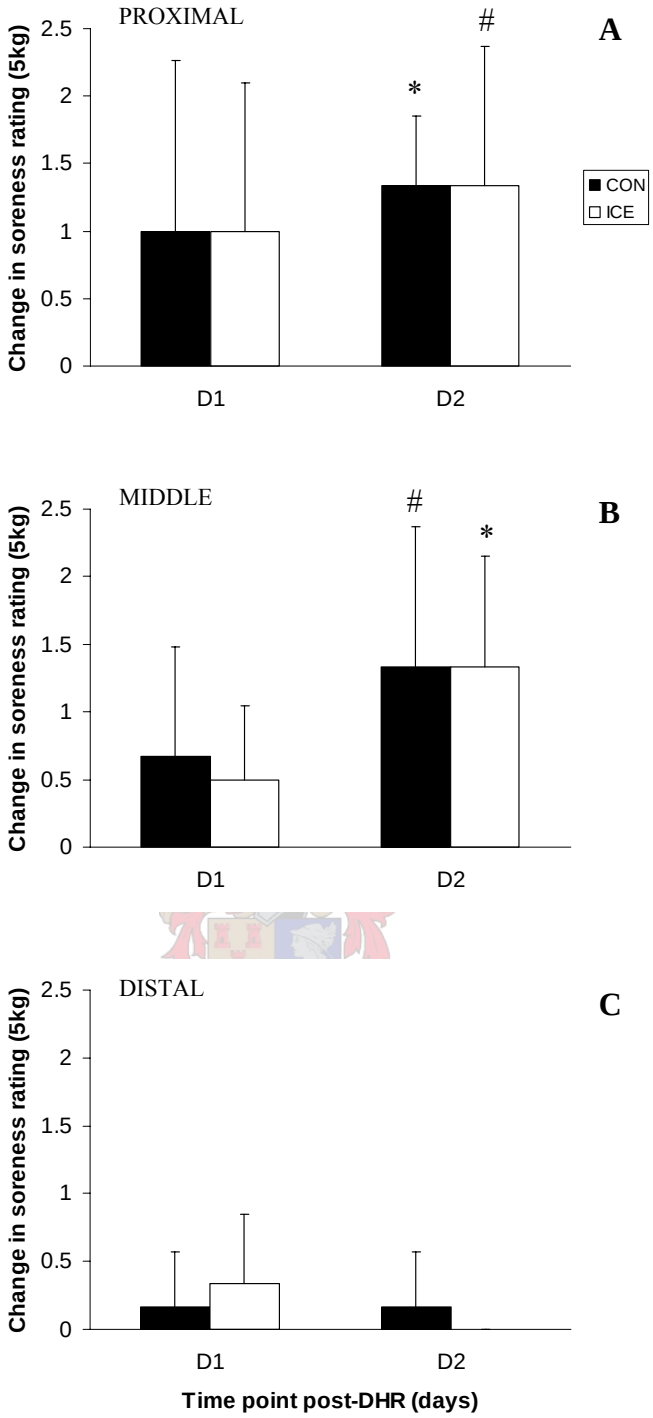


Figure 3 Changes in soreness ratings (mean ± SD) using the muscle probe while applying a force of 5kg. Statistical analysis (comparing each time point with baseline): t-test for dependent samples, * $P < 0.05$, # $P < 0.005$.