The effects of low level laser therapy on satellite cells

GUSTAVUS VAN NIEKERK
B.Sc. Genetics (Hons.)

Thesis submitted in fulfillment of the requirements for the degree Master of Science in Physiological Sciences

Department of Physiological Sciences

Faculty of Science

Dr Robert M Smith

February 2009

Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 20 February 2009

Acknowledgements

I would like to thank Dr Rob Smith, my supervisor who guided my stumbling efforts, through this project- and a special thanks with regard to his epic patience at my ~~blatant butcher~~ “eccentric use” of the English language... all my thanks for salvaging my thesis from an imminent grammatical cataclysm!

Maritza Kruger -my designated patron goddess- who took me under her wing that first year in a strange new department and sharing her knowledge, wisdom, expertise and ultimately, even friendship.

In sight of the monumental list of thanks I would need to recite to do justice to Dr Theo Nell’s endless contributions, I shall simply leave at being an all-purpose-panic-button. Not only me, but our entire department are in your debt!

Then the other ⅔ of the “Tripod of Power”, Mark Thomas and Jamie Imbriolo for the moral support, with a special thanks to Jamie who helped me out with the Western Blots.

I would also like to acknowledge the following institutions for their financial and other support: the MRC (financial) and the CSIR for equipment and technical support.

OPSOMMING

Alhoewel spierweefsel merkwaardige regenerasie kapasiteit vertoon ten opsigte van besering, is hierdie proses stadig en word soms vergesel met die vorming van letselweefsel asook ‘n gevolglike afname in kontaktiele kapasiteit na afloop van regenerasie. Behandelingsmoontlikhede is skaars en meesal ondersteunend van aard. Hierdie proses sluit spierstamselle (satelietselle), wat uiteindelik die ontstaan van die regenerasie van spier tot gevolg het, in.

Die kontroversiële veld van lae vlak laserterapie (Engels: Low level laser therapy (LLLT)) het merkwaardige aansprake in die fasilitering met verskeie sagteweefsel wondgenesing. Nietemin, die meganisme(s) wat voordelige effekte induseer, word nog nie goed begryp nie.

Ons het die effek van LLLT, deur gebruik te maak van ‘n 638 nm laser op kultuur *in vitro* satelietselle sowel *in-vivo,* ondersoek. Deur gebruik te maak van verskeie tegnieke is onder meer die metaboliese, sowel die seinstransduksie weë en antioksidantstatus na laserbestraling, gemeet.

In reaksie op die laserbestraling het satelietselle (in kultuur) ‘n toename in MTT waardes getoon (‘n maatstaf van die metaboliese aktiwiteit) en ‘n afname in die antioksidantstatus (gemeet deur van die ORAC toets). Addisioneel het laserbestraling ook uitdrukking en fosforilering van verskeie proteïene betrokke in seintransduksieweë beïnvloed, insluitend Akt, STAT-3).

Na afloop van hierdie effekte op satelietselle na laserbestraling, is daar gebruik gemaak van ‘n kneusbeseringsrotmodel om hierdie effekte *in vivo* te ondersoek. Geen betekenisvolle verskille in die aantal satelietselle na laserbestraling is opgemerk nie, maar veranderings is wel opgemerk in weefsel- en bloed-antioksidantstatus (gemeet deur van die ORAC toets gebruik te maak).

Gedurende die verloop van die studie is van verskeie standaardtegnieke gebruik gemaak om die effekte van laserbestraling op beide satelietselle *in vitro* en *in vivo* te ondersoek.

Dit het duidelik na vore gekom dat daar wel gepaardgaande probleme met van hierdie tegnieke voorgekom het, en dat van hierdie tegnieke nie gepas is vir ondersoek in laserbestralingsstudies nie. Nietemin, die resultate toon wel dat laserbehandeling satelietselgedrag induseer wat verdere studie in hierdie veld noodsaak.

ABSTRACT

Although muscle tissue demonstrates a remarkable capacity for regeneration following injury, this process is slow and often accompanied by the formation of scar tissue and a subsequent decrease in contractile capacity following regeneration. Treatment options are few and mostly supportive in nature. This regeneration process involves muscle stem cells (satellite cells) which ultimately give rise to the regenerated muscle.

The contentious field of low level laser therapy (LLLT) has made remarkable claims in facilitating wound healing in soft tissue injuries of various types. Yet, the mechanism(s) invoked in these beneficial effects are poorly understood.

We have investigated the effects of LLLT using a 638 nm laser on satellite cells in culture and *in-vivo*. Using an array of techniques we have measured, amongst other things, metabolic responses to laser irradiation, signaling pathways activated/altered and antioxidant status.

In response to laser irradiation satellite cells in culture showed an increase in MTT values (a measure of metabolic activity) and a decrease in antioxidant status (measured using the ORAC assay). In addition laser irradiation also altered the expression and phosphorylation state of several signaling pathways, including Akt and STAT-3.

Following on from this the effects of laser irradiation on satellite cells *in-vivo* was assessed in a rat model of contusion injury. No significant differences in satellite cell number was found following laser irradiation, changes were seen in tissue antioxidant status and blood antioxidant status (measured using the ORAC assay).

In the course of this study several standard techniques were used to investigate the effects of laser irradiation on satellite cells both *in*-vitro and *in*-vivo. It has become apparent that several of these techniques have problems associated with them that possibly make them inappropriate for

further use in studies involving laser irradiation. However the results indicate that laser therapy is induces satellite cell behavior and further study is warranted in this field.

With loving endearment to my parents who made all this possible, never compromising on my dreams

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AAPH	2'-azobis(2-aminodinopropane)-dihydrochloride
AMPK	5' AMP-activated protein kinase
AOX	Antioxidant
APS	Ammonium persulfate
ATP	Adenosine triphosphate
AUC	Area under the curve
bHLH	Basic helix-loop-helix (transcription factors)
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA)	Complementary DNA
COX	cytochrome c oxidase or Complex IV
COX-2	Cyclooxygenase-2
DAPI	4',6-diamidino-2-phenylindole
dH_2O	Distilled water
DMEM	Dulbecco's modified essential medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDL	*Extensor digitorum longus*
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
FGF	Fibroblast growth factor
FAD	Flavin adenine dinucleotide
FITC	Fluorescein isothiocyanate
FL	Fluorescein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSH	Glutathione
H&E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
HAT	Hydrogen atom transfer
HGF/SF or HGF	Hepatocyte growth factor/scatter factor
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule-1
IFN-α	Interferon-α (IFN-α)
IGF	insulin-like growth factors
IL	interleukin
In	Injured (intervention)
iNOS	Inducible nitric oxide
i.p.	Intraperitoneal (injection)
Ir	Irradiated (intervention)
JNK	c-Jun N-terminal kinase
LED	Light-emitting-diodes
LLLT	Low level laser therapy
LMW-PTP	Low molecular weight phosphotyrosine-protein phosphatase

MAPK	Mitogen-activated protein kinase
MEF2	Myocyte enhancer factor-2
MHC	Myosin heavy chain
MLC	Myosin light chain
MPG	N-(2-mercaptopropionyl)-glycine
MRC	Mitochondrial respiratory chain
MRFs	Myogenic regulatory factors
mtNOS	Mitochondrial nitric-oxide syntheses
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-Acetyl-Cysteine
NAIDs	Non-steroidal anti-inflammatory drugs
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
ORAC	Oxygen Radical Absorbance Capacity
$O_2^{\cdot -}$	Superoxide
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCD	Programmed cell death
PDGF	Platelet-derived growth factor
PhR+	Phenol red containing media
PhR-	Phenol red clear media
PI	Propidium iodide
PTP	Phosphotyrosine-protein phosphatase
PVDF	Polyvinylidine fluoride
RIPA bufer	Radioimmunoprecipitation assay buffer
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
S	Sham
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STAT	Signal Transducers and Activator of Transcription
T	injured and irradiated
TB	Trypan blue
TBS-T	Tris Buffered Saline-Tween 20
Texas Red	Sulforhodamine 101 acid chloride
TGF-β	Transforming growth factor-beta
TNF/TNF-α	Tumor necrosis factor-α (Note, the “α” is sometime omitted)
TNFR-1	TNF-receptor 1
Trolox	6-Hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic Acid
UV	Ultraviolet
w/v	Weight per volume
YAG	Yttrium aluminium garnet - $Y_3Al_5O_{12}$

Chapter 1 Introduction

1.1 Skeletal muscle

"Muscle" is not merely a term used for a type of tissue, but can be regarded as an organ with the specific function of enabling a organism to be mobile, facilitate respiration and act as a hydrostatic pump (though, as will later become clear, the role of muscle is not exhausted by its contractile function). Skeletal muscle is derived from the mesoderm layer of the developing embryo (Blau, Pavlath *et al*. 1985) and is a complex tissue arranged with three-dimensional architecture. It is sometimes neglected that the muscle, when considered as an organ, demonstrates a great deal of heterogeneity in tissue constituents in that the "muscle" organ is interwoven with a network of connective tissue, vascular structures and nervous tissue (Hiatt, Regensteiner *et al*. 1996).

Thou the focus of this thesis lay on skeletal muscle, a quick mention regarding the characteristics of other categories of muscles shall be made: Smooth muscle differs from skeletal muscle in both its histological appearance and contractile mechanism. These muscle cells have their own isoforms of contractile proteins and are arranged in a dramatically different manner than in striated muscle. Smooth muscle is notably an involuntary muscle, undergoing contraction in response to mechanical stretching or various chemical signals. Cardiac muscle (also a type of striated muscle) differs from skeletal muscle in that it is not a voluntary muscle (Kreuzberg, Willecke *et al*. 2006). Also, where skeletal muscles are long, linearly arranged fibres, cardiac muscles occasionally "branches out" (Sommer and Scherer 1985).

Furthermore, cardiac muscle cells are usually mono-nucleated and form intercalated discs through which the cardiomyocytes are interconnected. This connection is both mechanical (allowing the physical transmission of force) as well as chemical (facilitating the propagation of the action potential from stimulating

nerves across multiple cells) (Kreuzberg, Willecke *et al*. 2006). Finally, these cells are also metabolically geared towards oxidative phosphorylation and can be considered fatigue resistant (Ventura-Clapier, Garnier *et al*. 2004).

1.1.1 *Morphology of striated muscle*

Terminally differentiated skeletal muscle cells are multinucleated and elongated fibres (myofibers) (Light and Champion 1984; Morgan and Partridge 2003). Each myofibre is bordered by the endomysium which in turn are collectively bundled and surrounded by the perimysium. These bundles of myofibres ensheathed by the perimysium are collectively known as a muscle fascicle. The fascicles forming the muscle group are surrounded by another layer of connective tissue, the epimysium (Maganaris and Paul 2000) (see Figure 1.1). Muscle is anchored to bone or some other structure *via* tendons through which biomechanical force is applied (by the shortening of muscle) to produce locomotion (Huxley 1985; Zajac 1989).This "contractile" process is known as the cross-bridge cycle (Brenner 1986; Gordon, Homsher *et al*. 2000). It might be considered a bit misleading to use the word "contraction", since we are only referring to the generation of force which does not necessarily accompany a shortening of muscle fibres, for example as seen in an isometric contraction.

The most basic contractile functioning unit in a myofibre is known as the sarcomere (see Figure 1.2 for description). The largest known protein to occur naturally, titin, connects the Z-disk (N-terminal) with the M-band (c-terminal) forming a continuous filament across half the sarcomere (Trinick 1994). It is also believed to play a major role in muscle elasticity (Soteriou, Gamage *et al*. 1993; Linke and Leake 2004). The titin expands across the sarcomere and interacts with various other proteins, notably, myosin and other components of the thick (myosin) filament (Rayment, Rypniewski *et al*. 1993).

Myosin itself is a protein consisting of a heavy and light chain. The total protein structure can be divided in to a "head", "neck" and "tail" domain (Korn 2000), were the "head" consist of the "motor" where the hydrolysis of ATP produces the power stroke (Yanagida, Arata *et al*. 1985) which propels actin across the myosin chain.

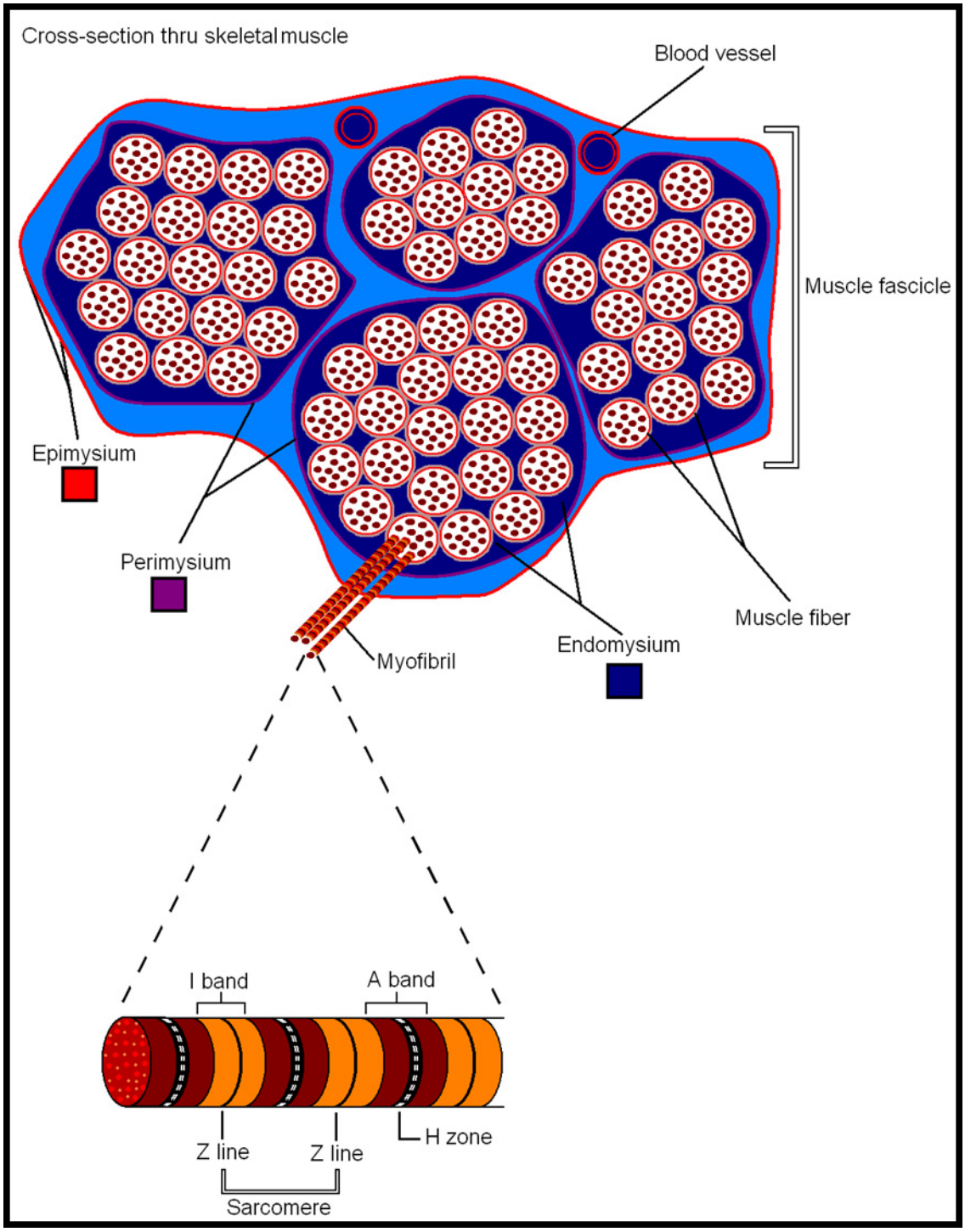


Figure 1.1 Macro-structural organization of a typical striated muscle as seen from a correctional view.

Myosin itself is a protein consisting of a heavy and light chain. The total protein structure can be divided in to a “head”, “neck” and “tail” domain (Korn 2000), were the “head” consist of the “motor” where the hydrolysis of ATP

produces the power stroke (Yanagida, Arata *et al*. 1985) which propels actin across the myosin chain. The structure that forms between the actin and myosin is referred to as the cross-bridge. As the cross bridges apply force on the thick (myosin heavy- and a myosin light chain) and thin (actin) filament of each sarcomere, they produce the “power stroke”. With each power stroke, the filaments slide past each other, giving rise to the “sliding filament” mechanism of muscle contraction. This sliding motion of the filaments effectively draws the Z–lines towards each other (shortening the I-band, as well as the H-zone, but not the A-band or the M-line).

These fibres must be able to withstand immense tensile stress and be able to propagate contractile force uniformly. The propagation of this force from a sarcomere towards a tendon is deceptively complex and will not be dealt with – the interested reader is referred to (Grounds *et al*., 2005; Monti *et al*., 1999). Figure 1.2 illustrates the arrangement of thin and thick filaments to emphasize the 3-dimentional plane in which force is generated.

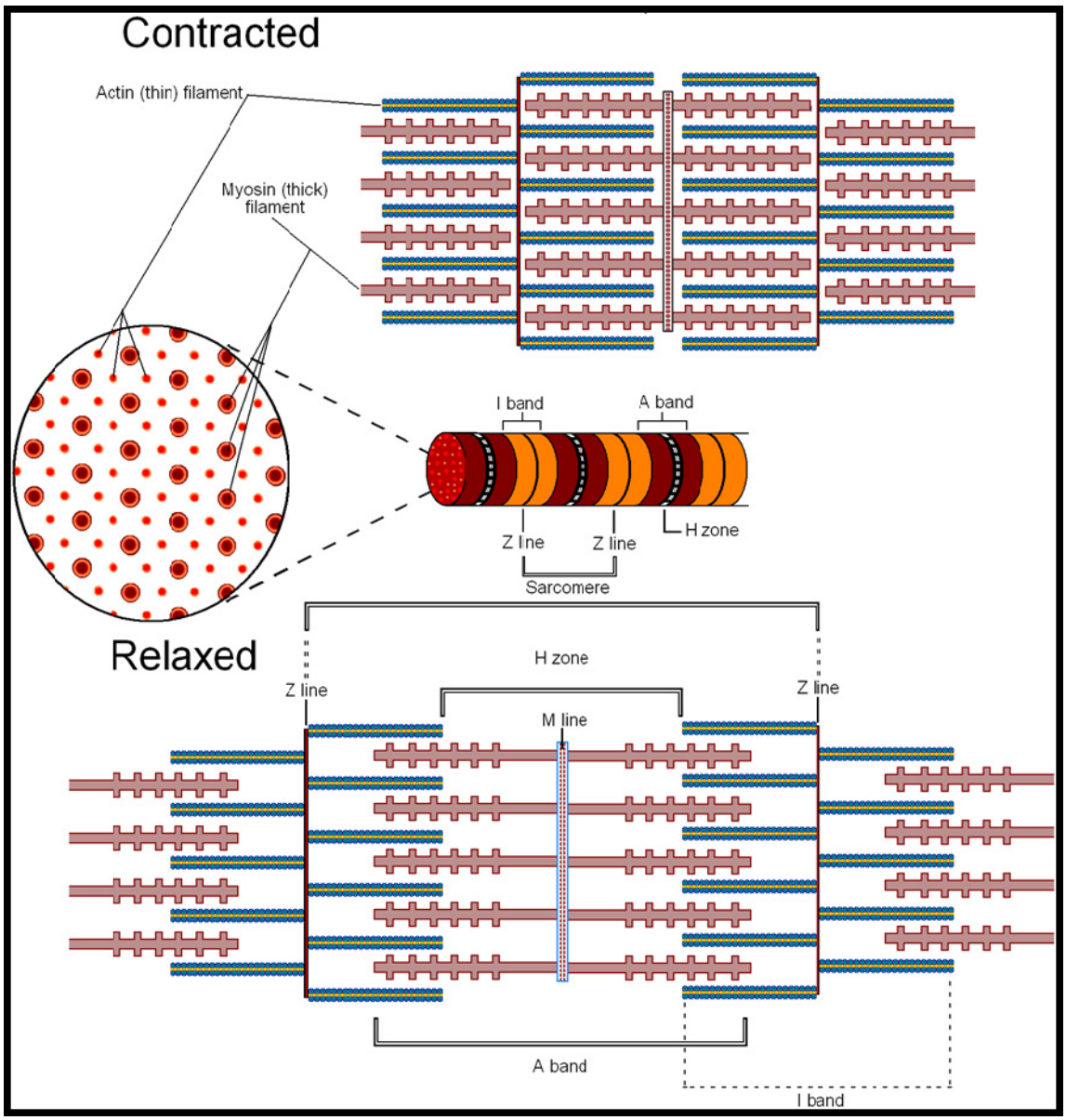


Figure 1.2 Micro-structural organization of a typical striated muscle. Note the 3-dimensional arrangement of the thick and thin filaments. For the sake of clarity some contractile proteins (notably, titin) have been omitted.

1.1.2 *Muscle plasticity and heterogeneity*

Muscle specialization covers a wide verity of contractile parameters. Muscle contraction can be required to be sustained for prolonged periods (for example the stabilizer muscles of the back (Schilling, Arnold *et al*. 2005)), or recruited for production of a sudden burst of force (for example the muscles involved in jaw movement such as the masseter muscle (Horton, Brandon *et al*. 2001)). A fine contrast in extreme forms of adaptation can be seen in muscles used in sound generation (Rome, Cook *et al*., 1999; Conley and Lindstedt 2002; Rome 2006) as opposed to some slow-twitch muscles of sloth (Barany 1967; Hoyle 1969). It is with these specific contractile demands that muscle manifests itself in various forms. These manifestations are due to the isoform types of myosin (both heavy and light chain) expressed in the individual myofibres.

1.1.2.1 **Myosin heavy-/light chain isoforms**

Multiple myosin heavy chain (MHC) isoforms are known to exist, derived from gene duplication and variable splice patterns (Berg, Powell *et al*. 2001; Briggs and Schachat 2002; Desjardins, Burkman *et al*. 2002), each with a characteristic contractile capability. Muscles are also not static in their MHC isoform configuration and can adapt according to mechanical or metabolic demands, such as that imposed by contractile stress (Jansson, Esbjornsson *et al*. 1990; Hicks, Ohlendieck *et al*. 1997; Pedemonte, Sandri *et al*. 1999; Dahmane, Djordjevic *et al*. 2006). But the plasticity observed in muscle organ is not limited to variation in the MHC contractile apparatus.

There also exist different isoforms for the myosin light chain (MLC) (Talmadge, Roy *et al*. 1993; Jostarndt-Fogen, Puntschart *et al*. 1998; Bicer and Reiser 2004) that is able to influence contractile velocity (Sweeney, Kushmerick *et al*. 1988). The MLCs seem to regulate MgATPase kinetics of the myosin motor through its interaction with actin (Timson 2003). This regulatory role has also been observed in cardiac muscles (which, like skeletal cells, are also striated) through modulating contractile force (altering the cross-bridge kinetics) without interacting with the catalytic site

(Yamashita, Sugiura *et al*. 2003). These MLCs have also been categorized according to fast/slow twice and can be paired to a MHC either as “matched” or “mismatched” (Stephenson 2001). Despite the modifying role of MLC, MHC still seems to be the major role player in dictating contractile performance of a muscle group (Ohtsuki, Maruyama *et al*. 1986; Bottinelli, Canepari *et al*. 1996; Pette and Staron 2000).

1.1.2.2 Muscle types and their identification

The first distinction between fibre types was made by Ranvier as early as 1873 when he distinguished between “red” and “white” muscles (Ranvier 1873). Modern techniques used to identify and distinguish fibre types were developed by Sréter (Seidel 1967) and Seidel (Sreter 1969) based on the observation that “fast” (white) and “slow” (red) muscles have different alkaline and acidic sensitivity in terms of ATPase activity . This happened at the same time that a connection between ATPase activity and muscle contraction velocity (or muscle shortening time) was established (Barany 1967).

The difference in ATPase activity is directly dependant on the MHC isoforms being express in that muscle fibre (Fry, Allemeier *et al*. 1994; Staron 1997). This allows the identification of MHC on the basis of the chain isoform's pH-sensitive ATPase activity. Also, since each isoform has a different molecular weight, the identification of fibre type by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is also possible (Pette, Peuker *et al*. 1999). Because of the difference in dominant strategies of cellular ATP production (either oxidative or glycolytic), fibre types can also be distinguish on the bases of relative metabolic enzymes composition (Pette, Peuker *et al*. 1999).

Other techniques include the use of immunohistochemistry with fibre type specific monoclonal antibodies (Havenith, Visser *et al*. 1990), RT-PCR (Lefaucheur, Milan *et al*. 2004) and an *in-vitro* motility assay (IVMA) (Kron and Spudich 1986; Lowey, Waller *et al*. 1993). It is worth noting though that not all the above mentioned techniques correlate with each other (Staron 1997; Lefaucheur, Milan *et al*. 2004).

1.1.2.3 Other adaptive strategies

A muscle cell of a set volume must allocate its space economically to maximize functional utility. It is indeed no coincidence that the myonuclei lay outside the myotubes. The major units between which a myotube must divide its limited space (as dictated by contractile demand) are mitochondria; the sarcolemma and the myofibrils (Josephson 1975; Schaeffer, Conley *et al*. 1996). Consequently, hypertrophic fast twitch muscles, compromise with a low mitochondrial density in favour of more myotubes which in turn translate into a more powerful contractile potential. Cross-section area of a single fibre also correlates with the fibre type: oxidative fibres tend to be smaller when compared to glycolytic fibres which tend to be bigger (Tesch and Karlsson 1985). Also muscles with an extremely fast twitching time (thus requiring a short calcium-clearing period) typically have a larger volume allocated towards their sarcoplasmic reticulum, resulting in weaker contractile force generation (Josephson 1975; Rome and Lindstedt 1998).

Another aspect regarding muscle adaptation is the structural or “macro” changes of muscle fibres in the context of their contractile function. An increase in glycolytic fibres (associated with resistance training) decreases capillary density, where as endurance exercise leads to an increase in the degree of vascularisation and a shift towards oxidative fibres (Tesch, Thorsson *et al*. 1984). Muscle fibres also can augment their molecular “oxygen stores” by increasing the expression of myoglobin. Myoglobin is a 153 peptide long protein, structurally related to haemoglobin (Wittenberg and Wittenberg 1990) and is much more abundant in oxidative (slow, fatigue resistant) muscles (Gayeski and Honig 1986; Gayeski and Honig 1988; Ordway and Garry 2004). Since the heart muscles relay heavily on oxidative respiration, dead or dying tissue will liberate myoglobin. This liberated myoglobin is used as a molecular marker to indicate muscle damage in patients that present in emergency rooms or trauma centres with chest pain or other early symptoms of heart disease (such as a myocardial infarction or heart failure) (Weber, Rau *et al*. 2005).

In the context of the above, human skeletal muscle fibres are classified according to the following “hierarchy” of types:

Type I→ Type IIa→ Type (IIx/d) → Type IIb.

In this order, shortening velocity and maximum power-velocity decrease, whilst fatigue resistance increases (Bottinelli, Canepari *et al*. 1996). This muscle type also correlates with MHC composition (Fry, Allemeier *et al*. 1994; Staron 1997).

One last factor worth a quick mention might as much be a result of muscle adaptation as it could facilitate adaptation. Curiously, it is found that the dispersion of satellite cells between fast- and slow-twitch muscles appears to be asymmetric (Schmalbruch and Hellhammer 1977; Gibson and Schultz 1982; Snow 1983). This seems to hold true even for the distribution of fast-/slow twitch fibres in the same muscle groups (Gibson and Schultz 1982). The significance of this, as well as the possible factors governing the "privileged" allocation of satellite cells remains unclear.

The observation that satellite cells seem to be more common around capillaries (Schmalbruch and Hellhammer 1977) and motor-neuron junctions (Wokke, Van den Oord *et al*. 1989) does hint at some as of yet unidentified influence of these structures. As mentioned previously, slow twitch muscle seem to be more vascularised (Tesch, Thorsson *et al*. 1984). This could possibly account for the increase in satellite cell density in slow twitch fibres.

1.1.2.4 Hybrid fibres

Because of this dynamic plasticity of muscle, the fibre type composition is often complex. The distribution of slow- and fast twitch fibres in a muscle is often described as a "salt-and-pepper" distribution as both fibre types can be present in the same muscle (Schiaffino and Reggiani 1996; Schilling, Arnold *et al*. 2005). This composite distribution of muscle fibre types in a single muscle organ seems to be the product of the functional specialization of the muscle (Otis, Roy *et al*. 2004). Muscles thus present a "graded" organization of fast-to-slow twitch muscle types, as required by the contractile demand of the muscle.

One could imagine the evolutionary advantage of having a contractile apparatus capable of generating a powerful contraction force for a prolonged

period of time. Unfortunately, no such a “magic bullet” exists in the context of contractile utility. As previously mentioned, myofibres face a trade off in function: more myofibrils give rise to greater contractile capacity, but reduce volume for mitochondria, present a larger cross section for oxygen to defuse across and less space for capillaries – thus rendering the muscle susceptible to fatigue. Because of this limitation, muscle cells need to continuously adapt to new contractile challenges and this give rise to hybrid fibres: myotubes expressing more than one type of MHC.

On the level of an individual muscle fibre, adaptation takes place in gradual steps. Were a pure fibre type is considered a muscle fibre that only express one single type of MHC, some muscle fibres co-express various fibre types simultaneously, giving rise to hybrid fibre types (Lutz, Weber *et al*. 1979; Bottinelli, Betto *et al*. 1994; Sant'ana Pereira, Wessels *et al*. 1995). The co-existence of this multiple isoforms is also known to influence contractile velocity (Larsson and Moss 1993; Bottinelli, Betto *et al*. 1994).

In the context of muscle plasticity, hybrid fibre types represent a bridge over the transitionary gap between one muscle phenotype and another (Pette, Peuker *et al*. 1999; Pette and Staron 2000).

1.1.2.5 Factors inducing muscle adaptation

As already discussed, each of the cells in a muscle adapts according to the contractile demands imposed on them (Holloszy and Booth 1976). In terms of resistance training muscle adaptation leads to an increase in muscle mass and a shift towards powerful contractile machinery (namely, type IIb fibres) (Schiaffino and Reggiani 1996; Bottinelli and Reggiani 2000; Fluck and Hoppeler 2003).

In contrast, chronic low intensity contractile stimulation of the *tibialis anterior* muscle in rabbits results in a dramatic switch form fast twitch towards slow twitch with an accompanied increase capacity for oxidative respiration (Pette and Vrbova 1992). The same adaptations have been observed in rat striated muscle (both skeletal and cardiac muscle) by examine modification in oxidative enzymes and muscle contractile characteristics (Baldwin, Cooke *et al*. 1977).

There also exist other metabolic and functional mechanisms by which muscle adaptation can be induced. An early example of muscle plasticity was provided by Buller *et al* when he was able to demonstrate a swap of muscle phenotype from slow twitch to fast twitch after cross-innervation of the respective muscle groups (Buller, Eccles *et al*. 1960). This adaptation mimicked the previously mentioned role of motor unit size in relation to muscle fibre type (Bewick, Zammit *et al*. 1993).

The role of muscle recruitment was expanded upon by studies on the decline in muscle recruitment following a decrease in muscle loading (Thomason, Herrick *et al*. 1987), induced spinal cord damage (Talmadge, Roy *et al*. 1995) or muscle denervation (Carraro, Dalla Libera *et al*. 1982) - all of which shown to induce a shift in the type of muscle contractile machinery (see also (Talmadge 2000) for review on these effects).

These results are similar to other findings on the effects of muscle unloading where a shift from slow to fast MHC isoform was observed (Talmadge, Roy *et al*. 1996; Talmadge, Roy *et al*. 1999). This muscular response seems to be time dependant as the phenotypic change varies upon the duration of unloading. A human study demonstrated an increase in maximum contractile force after 17 days of bed rest (Widrick, Romatowski *et al*. 1997) and presented the same results when duplicated in a space flight model (Widrick, Knuth *et al*. 1999). Similar results were found with a rodent model (Baldwin 1996). However, with increased durations of unloading 6 weeks of bed rest (Larsson, Li *et al*. 1996) and 3 months of immobilization (D'Antona 2000) are associated with a decrease in contraction velocity.

The effect of cancer cachexia on a mice model illustrated an interesting shift form type I MHC towards type IIb MHC (Diffee, Kalfas *et al*. 2002) over a 21 day period following the injection of cancer cells. Chronic heart failure has also been shown to shift skeletal muscle type towards a glycolytic type (Drexler, Riede *et al*. 1992; Wilson, Mancini *et al*. 1993), where as regular aerobic exercise (cycling) seems to increase oxidative capacity *via* increased mitochondrial density, which was strongly correlated to an increase in type I fibres (Hambrecht, Niebauer *et al*. 1995; Hambrecht, Fiehn *et al*. 1997).

Another factor influencing fibre type is age. Aged individuals (88 ± 3 years) show a remarkable shift towards the co-expression of type I and type IIa muscle types, indicating that ageing itself (or an associated "symptom" of aging) seems to induce a transformation of muscle fibre type to a hybrid form (Andersen 2003).

Alcoholism, in the context of malnutrition also seems to specifically deplete type II fibres (Fernandez-Sola, Sacanella *et al*. 1995). Of interest to *in vitro* modelling, it appears that muscle cells in culture always differentiate into fast twitch phenotype (type II) (Rubinstein and Holtzer 1979).

1.1.3 *Muscle from another angle*

Intuitively, the first image that comes to mind in response to the word "muscle" would most probably be in line with some notion of mobility or another representative motion of contraction. It is not without reason that our contractile function impresses us so much, for it is in our ability to manipulate our environment that we express ourselves (for example an artist hand guiding his brush over a piece of canvas) or indulge in recreational activities (for example playing rugby). Besides functioning as a motility organ, muscle also plays an important role in blood glucose metabolism (Kahn, Rosen *et al*. 1992; Wright, Geiger *et al*. 2004; van Loon and Goodpaster 2006). Another aspect of muscle function relates to its "expendability"- muscle tissue acts as a storehouse for amino acids (Lopes, Russell *et al*. 1982).

An additional role of muscle cells in the maintenance of homeostasis involves the endocrine function of this organ. It is know that muscle cells produce a multitude of cytokines and other signalling molecules (Segal 1994; Bruunsgaard, Hartkopp *et al*. 1997; Ostrowski, Rohde *et al*. 1999; Ostrowski, Rohde *et al*. 2001; Febbraio and Pedersen 2002). Considering the contribution muscles tissue makes to total body mass (Proctor, O'Brien *et al*. 1999), the endocrine function of muscle can be substantial. In the light of all this, it has recently been proposed that some cytokines and other peptide hormones produced and excreted by muscle cells be dubbed "myokines" (Pedersen and Febbraio 2005).

Interestingly though it has been shown that muscle cells can perform an endocrine function that is not related to their contractile function. What's more, this endocrine function was found to be fibre type specific (Plomgaard, Penkowa *et al*. 2005). This fibre type specificity was maintained even when different muscle groups (triceps, vastus and soleus) of the same fibre type were sampled. Fibre type specificity was also found in response to muscle exercise were glycolytic fibres express higher concentration of IL-15 (Nielsen, Mounier *et al*. 2007).

The muscle organ also provides the body with information valuable to the maintenance of homeostasis. For instance, muscle activity might communicate signals not induced by nerve impulses that cause metabolic alterations in other tissue (Pedersen and Febbraio 2005). Indeed, muscle derived IL-6 alone seems capable of influencing metabolism on a systemic level (i.e. not only skeletal muscle adaptation) (Febbraio and Pedersen 2002).

1.1.4 *In summary*

Some of the mechanisms by which muscles "gear" their morphological and biochemical machinery towards contractile efficiency have been mentioned. These modifications might include: altering the organelle architecture (not only number), manipulating contractile components, muscle motor unit size, modification in sensitivity towards signalling cascades (e.g. calcium sensitivity and the fibre type-specific distribution of adrenergic receptors), increased sarcoplasmic reticulum volume, recruitment of metabolic machinery, and even adapting on the macro structure by altering capillary density (Buller, Eccles *et al*. 1960; Barany 1967; Henriksson and Reitman 1977; Spamer and Pette 1977; Holloszy 1982; Holloszy and Coyle 1984; Breitbart and Nadal-Ginard 1987; Martin, Murphree *et al*. 1989; Simoneau and Bouchard 1989; Froemming, Murray *et al*. 2000; Fluck and Hoppeler 2003; Novotova, Pavlovicova *et al*. 2006).

Table 1.1 Some parameters by which human fibre types can be compared. Adapted and modified from (Wang, Hikida *et al*. 1993; Fitts and Widrick 1996).

	Slow-oxidative (Type I)	**Fast-oxidative (Type IIa)**	**Fast-glycolytic (Type IIb)**
Metabolic strategy	oxidative phosphorylation	oxidative phosphorylation	glycolytic
Glycogen content	low	intermediate	high
Relative rate of fatigue	slow	intermediate	fast
Myoglobin content	high	high	low
Relative mitochondria	many	many	few
Capillary density	dens	dens	spares
Fiber diameter	small	intermediate	large
Contractile velocity	slow	fast	fast
Motor unite size	small	intermediate	large
Satellite cell density	high	*not specified in literature*	low

1.2 Muscle injury and repair

1.2.1 *Muscle damage*

Muscle tissue is susceptible to a vast array of injuries. Because of its functional nature, injuries inflicted through shear stress are one of the most common types of injury seen, resulting in muscle tears or tendon ruptures (Garrett 1996). This type of injury is also more common in muscles working across two joints as seen in the *femoris*, *gastrocnemius* and *semitendinosus* muscles (Garrett 1996). A less common injury is laceration, produced by a sharp object making a transitional cut through myotubes. These types of injuries are relatively difficult to treat and usually result in the formation of scar tissue (Menetrey, Kasemkijwattana *et al*. 1999).

The functional position of muscle unavoidably places this organ in a precarious position. For one thing, our limbs are used to manipulate our environment and thus more prone to accidental injury. Secondly, as muscles are often flanked to the bone on which force is applied (or adjacent to some other bone), they are liable to be “pinned” between the underlying bone and an external object exerting force on the muscle. This results in a unique type of injury, which also represents the injury model investigated in this thesis’s *in vivo* section, namely contusion injury.

Contusion injuries are generally caused by a compressive force acting on muscle (typically seen in blunt force trauma) without penetration of underlying tissue (Crisco, Jokl *et al*. 1994). Contusion injuries are second only to strain injuries as the dominant cause of sports related injuries (Beiner and Jokl 2001), and are, by nature, more prevalent in contact sports like judo, karate and rugby (Beiner and Jokl 2001). Despite their relative larger size (than strain injuries), contusion injuries generally heal more rapidly than strain injuries (Thorsson, Lilja *et al*. 1997). However, complications can arise, including compartment syndrome (Mulder, Sakoman *et al*. 1991) and pyomyositis (Patel, Olenginski *et al*. 1997).

Besides muscle loss by injury, muscle wasting often accompanies other disease or degenerating states, including: cancer (Tisdale 1997); HIV infection (Engel 1977; Kotler, Tierney *et al*. 1989; Hellerstein, Kahn *et al*. 1990); aging (Lexell, Taylor *et al*. 1988; Pahor and Kritchevsky 1998) and some neuro-degenerative diseases (Bordet, Lesbordes *et al*. 2001; Wang, Lu *et al*. 2002). Muscles are also susceptible to infections like pyomyositis (Patel, Olenginski *et al*. 1997), strains of salmonella (Collazos, Mayo *et al*. 1999) and streptococci (Stevens 1999) as well as parasitic infestation (Beiting, Bliss *et al*. 2004) and damage induced by cytotoxins (Rucavado, Escalante *et al*. 2002).

1.2.2 *Primary and secondary Injury*

In contusion injury, the primary injury to muscle involves the physical and structural disruption of tissue as well as immediate necrotic cell death that is implicated in tissue disorganization (Hurme, Kalimo *et al*. 1991). Following

contusion injury (the primary insult), the physical and localized chemical alterations might ultimately lead to the secondary injury through cell death resulting from the adverse environmental shift taking place at and around the site on injury. Another key aspect of secondary damage relates to subsequent engagement of an inflammatory response and the involvement of immune cells. Artificially dissecting primary and secondary injury would do insult to the intricate physiological relationship between the two injuries. Instead, primary and secondary injury will be discussed consecutively as the occasion arises.

1.2.2.1 Primary injury - tissue disruption and cell death

Following contusion injury of a skeletal muscle there is inevitably some degree of bleeding and in some instances the formation of a hematoma. This is due to the high level of vascularisation seen in skeletal muscle (Levenberg, Rouwkema *et al*. 2005). This process occurs reasonably early following injury (Hurme, Kalimo *et al*. 1991). It has been found that primary spinal afferent neurons release pro-inflammatory neuropeptides capable of increasing inflammatory oedema (Steinhoff, Vergnolle *et al*. 2000).

This early occurrence following injury results in swelling, which expands as the initial inflammatory response proceeds. The increase in pressure as a result of the inflammation and possible hematoma, as well as the vascular disruption, might cause local hypoxic conditions, as well as an accumulation of cellular waste products. In turn, this might result in the induction of secondary injuries, as these adverse conditions increase the amount of secondary cell death.

In addition to the localised bleeding and swelling, the compromise in the myofibre's sarcolemma integrity seen following the contusion results in an influx of extracellular calcium into the damaged myofibre which leads to the disruption of ionic homeostasis within the injured fibres (Trump and Berezesky 1995; Belcastro, Shewchuk *et al*. 1998). At the same time, a host of proteolytic enzymes (proteases liberated from damaged cells, muscle fibres and discharged from leukocytes) begin to digest the macromolecular

contractile machinery into smaller units for further degradation (Frenette, St-Pierre *et al*. 2002).

By virtue of their continuous, elongated structure, muscle cells face a unique threat when damaged. A single rupture point can theoretically compromise an entire cell. To halt the advance of such a necrotic threat, myofibres often form condensing plates of cytoskeletal proteins called contraction bands (Ganote 1983; Hurme, Kalimo *et al*. 1991) preventing the spread of injury and the death of the entire fibre. In addition the surviving myotubes tend to contract (as a result of the increased calcium concentration) (Warren, Hayes *et al*. 1993), thus distancing the surviving tissue from the injury zone. This effectively localizes the site of necrosis and preserves the integrity of the surviving myofibre, minimizing injury.

1.2.2.2 Secondary injury

Secondary damage occurs at, and surrounding the site of the primary injury. This secondary injury is caused by numerous factors including (but not limited to): the disruption of the vasculature and the ensuing ischaemia; ionic disequilibrium; infiltration and activation of the immune cells (see sections below for more detail) and enzymatic degradation (as a result of the release of proteases from the damaged myofibres) to name but a few (also see Figure 1.3).

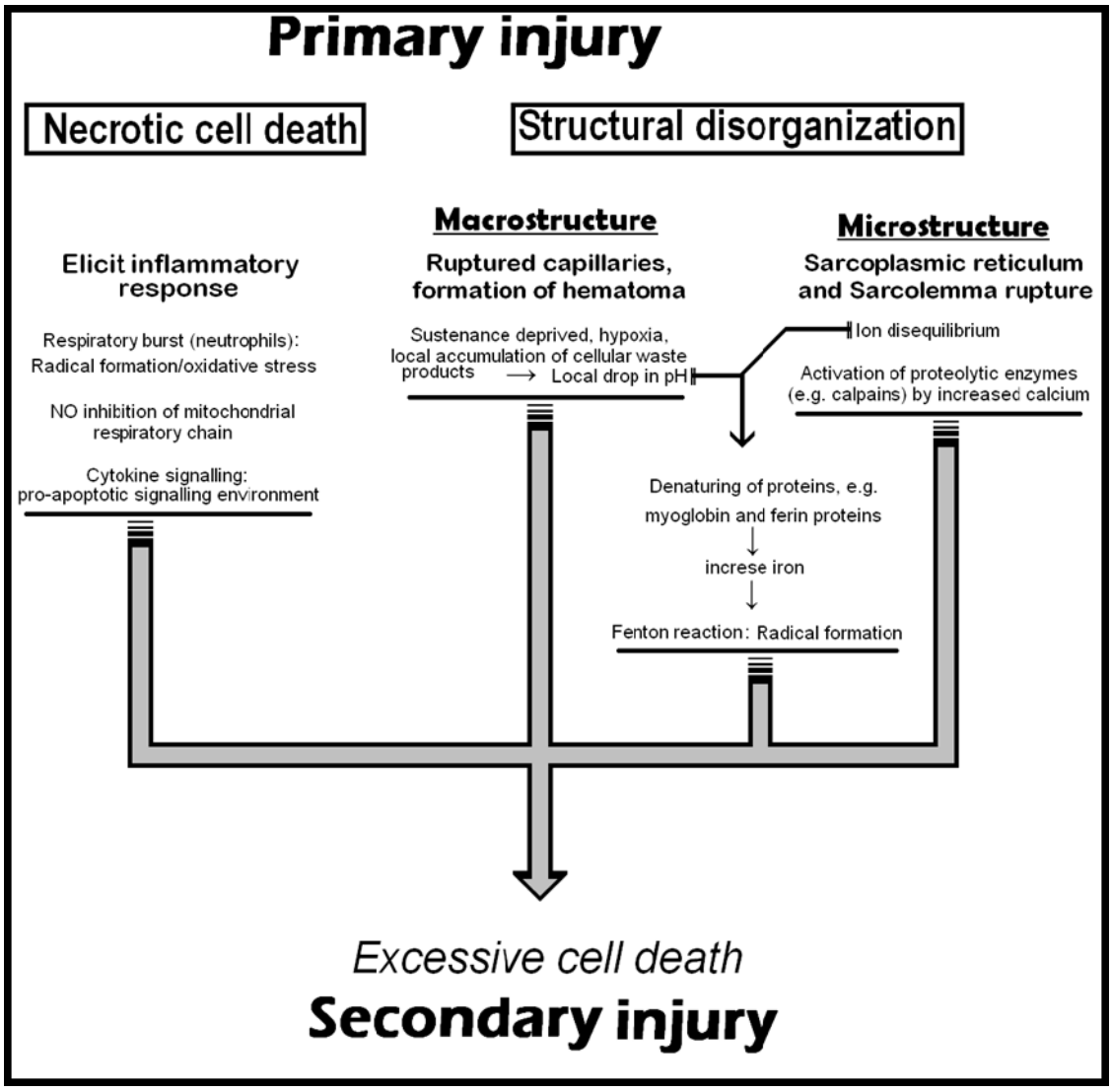


Figure 1.3 A brief illustrative summary demonstrating the homeostatic relationship between primary and secondary injury. This diagram is by no means exhaustive and arbitrarily outlines only some events in muscle injury.

1.2.3 The inflammatory response

An inflammatory response is generally described in the context of three phased events namely “damage”, “repair” and “remodelling” (which tend to overlap both spatially and temporally – see Figure 1.4 for illustration of distinct phases). The immediate function can be described as “homeostatic damage control” followed by the mobilization of immune cells.

The presence of these immune cells represents a prophylactic strategy of the body to remove potential invading pathogens. Some leukocytes (notably,

those of a phagocyte phenotype) are also responsible for clearing up cellular debris (Lescaudron, Peltekian *et al*. 1999) – an important event in wound repair. Furthermore, these cells also contribute to the remodelling stage in various ways (Massimino, Rapizzi *et al*. 1997; Merly, Lescaudron *et al*. 1999; Meszaros, Reichner *et al*. 2000; Cantini, Giurisato *et al*. 2002; Allenbach, Zufferey *et al*. 2006). The inflammatory response is a complex and tightly orchestrated event, which quite often causes more damage, seen as secondary damage, than the initial injury itself (Nathan 2002).

Ruptured and necrotic cells liberate intercellular proteins that act as chemotactic agents (Carp 1982). These chemotactic agents can cause and/or aggravate the inflammatory response (Scaffidi, Misteli *et al*. 2002; Lauber, Blumenthal *et al*. 2004). Within 24 hours, neutrophils, phagocytes and other inflammatory cells invade the injured area and start removing cellular debris and any blood clots formed due to the vascular disruption caused as a result of the contusion (Tidball 2005). The short lived, non-dividing neutrophils (Roitti and Rabson 2000) are the first sub population of white blood cells to arrive at the site of injury (Pizza, Koh *et al*. 2002) and have been found to increase in number at the site of damage as early as one hour after an injury (Fielding, Manfredi *et al*. 1993; Belcastro, Arthur *et al*. 1996).

Connective tissue mast cells colonize the muscle tissue (Galli 1993) in relatively low numbers but are capable of releasing a host of molecules in response to cytokines released by surrounding tissue during an inflammatory response (thus IgE independent) (Galli 1993) that induces and facilitates the chemotaxis of neutrophils (Gaboury, Johnston *et al*. 1995; Kubes and Gaboury 1996). Neutrophils are also capable of releasing a host of inflammatory cytokines including interleukin-8 (IL-8), interleukin-1 (IL-1), tumour necrosis factor-alpha (TNF-α) and Interferon-alpha (IFN-α) (Fielding, Manfredi *et al*. 1993; Cassatella 1995; Moilanen and Vapaatalo 1995; Tidball 1995; Cannon and St Pierre 1998; Suzuki, Totsuka *et al*. 1999; Barbero, Benelli *et al*. 2001) which subsequently also recruit macrophages to the site of injury (Tidball 1995; Cannon and St Pierre 1998).

Although the exact context in which neutrophils might exacerbate muscle injury remains unclear, there is consensus that, at least *occasionally*, the

inflammatory response seems to cause more damage than the injury itself (Nathan 2002). In this regard, in particular, neutrophils appear to be the “black sheep” by allegedly being the major contributor in the exacerbation of secondary damage caused by the inflammatory response seen following injury (Tidball 1995; Tiidus 1998; Pizza, McLoughlin *et al*. 2001; Tidball 2005).

Supporting this view, an increase in secondary damage coincides with peak neutrophil invasion (Brickson, Hollander *et al*. 2001; Schneider, Sannes *et al*. 2002). Indeed, the “excessive nature” of neutrophil induced inflammation is well described in cystic fibrosis, where neutrophils tend to be more of a hinder than a help (Konstan, Byard *et al*. 1995) and where anti-inflammatory medication seems to decrease morbidity (Auerbach, Williams *et al*. 1985; Eigen, Rosenstein *et al*. 1995; Konstan, Byard *et al*. 1995). The direct mechanisms by which neutrophils cause secondary damage seem to be mostly mediated by an increase reactive species production (Welbourn, Goldman *et al*. 1991; Brickson, Hollander *et al*. 2001; Brickson, Ji *et al*. 2003).

However, the role of neutrophils in secondary muscle injury is contentious. Following injury sustained after exercise some results has shown no direct evidence for neutrophil induced secondary damage (Lowe, Warren *et al*. 1995; Lapointe, Frenette *et al*. 2002). In contrast, blocking the respiratory burst in neutrophils prior to stretching of rabbit muscles reduced the occurrence of muscle damage (Brickson, Ji *et al*. 2003), thus implicating neutrophil involvement in the damage seen following injury. These results have been corroborated by another study using a mice knock-out model. In this study it was found that a reduction in neutrophils in muscle tissue resulted in significant lower levels of muscle injury (Pizza, Peterson *et al*. 2005).

Other notable leukocytes involved with inflammation are the longer-lived macrophages. Macrophages accumulate at the site of injury (where they also undergo cell division) (Honda, Kimura *et al*. 1990) and gradually decline in number as the healing process progresses (St Pierre and Tidball 1994). Three distinct populations of macrophages can be distinguished: ED1+ve which are found in circulation and appear early following injury and seem mostly responsible for clearing up debris (Honda, Kimura *et al*. 1990), ED2+ve are “resident” macrophages found in the muscle tissue, but appear only later (St

Pierre Schneider, Correia *et al*. 1999) and ED3+ve which are mostly confined to the lymphatic system (Dijkstra, Dopp *et al*. 1985; Roitti and Rabson 2000). ED3+ve and ED2+ve macrophages only appear later on after the initial inflammatory response (McLennan 1993).

The invasion of tissue by macrophages coincides with the onset of the regeneration phase (Hopkinson-Woolley, Hughes *et al*. 1994; St Pierre and Tidball 1994). Indeed, macrophages appear to support the healing process in numerous ways. The ability of these cells to clear debris seems to be important in the healing process, as studies have shown that macrophage-depleted recipients of myogenic cells undergo decreased levels of muscle regeneration (Lescaudron, Peltekian *et al*. 1999). Also, macrophages are known to induce apoptosis in neutrophils, thus reducing the level of secondary damage brought about by these cells (Meszaros, Reichner *et al*. 2000; Allenbach, Zufferey *et al*. 2006). Furthermore, *in vitro* studies have shown an increase in satellite cell proliferation when co-cultured with macrophages (Massimino, Rapizzi *et al*. 1997; Merly, Lescaudron *et al*. 1999; Cantini, Giurisato *et al*. 2002), although the exact mechanism of action remains unknown.

But the relationship between neutrophils and macrophages in a muscle injury context could well be more complicated. In one study, using a *dmx* mouse model of Duchenne muscular dystrophy, mice with depleted macrophage populations showed dramatically reduced muscle membrane lesions (Wehling, Spencer *et al*. 2001). These results were followed up in a study in which muscle cells co-cultured with neutrophils and macrophages in a physiologically relevant setting (Nguyen and Tidball 2003) showed a nitric oxide dependant, superoxide-independent mechanism of muscle cytotoxicity, implicating a possible role of phagocytes in muscle secondary injury.

Also, it is well known that macrophages also have the ability to “turn nasty”. Activated by lipopolysaccharides (an outer membrane component of gram-negative bacteria) or by the compliment system, macrophages can secret cytokines (e.g. Il6 and TNF-α) which up regulate the expression of adhesion molecules (facilitating neutrophil mobility) and increase capillary permeability (Roitti and Rabson 2000). In this activated state, they also provide a rich

source of nitric oxide (Cannon and St Pierre 1998). Thus, macrophages have the capacity to intensify an inflammatory response in the presence of pathogens.

Finally, there also occurs tissue infiltration of cells involved in the adaptive immune response, most notably T-cells. T-cells are a unique subset of white blood cells which are involved in cell mediated immunity. These cells can be further sub divided into different populations (memory, helper, natural killer and cytotoxic as well as Regulatory and γδ cells) of T-cells with unique specialized functions. It has been shown that these cells might be involved in other non-immune functions in wound healing such as vascular remodelling (Mach, Schonbeck *et al*. 1999). Studies have also indicated that T cells (along with other immunological cells) might contribute towards survival and repair of neurons following peripheral axon damage (reviewed in (Sanders and Jones 2006)).

1.2.4 *Inflammation: role of myotubes*

As previously mentioned, muscle tissue also performs an endocrine function. During inflammation, myotubes interact with immune cells and contribute in facilitating an immune response through the release of numerous cytokines and inflammatory mediators. It has been demonstrated that myotubes release IL1-β and TNF-α upon exposure to pro-inflammatory cytokines (De Rossi, Bernasconi *et al*. 2000). Similarly, incubating myotubes with TNF-α results in a decrease in TNF-receptor 1 (TNFR-1) expression as well as demonstrating a modulating function on cytokine expression (IL-9, IL-10 and IL-15) (Alvarez, Quinn *et al*. 2002).

Another *in vivo* model demonstrated an increase in IL-6, transforming growth factor-beta (TGF-β), and granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as an up regulation of intercellular adhesion molecule-1 (ICAM-1) and human leukocyte antigen (HLA) class I and II in muscle following a pro-inflammatory stimulus (Nagaraju, Raben *et al*. 1998). A recent study performed on an *in vitro* muscle injury model and on a muscle mechanical loading model demonstrated an increased release of neutrophil chemotactic molecules, as well as "priming" (activating) neutrophils toward the

production of reactive oxygen species (ROS) (Tsivitse, Mylona *et al*. 2005). Muscles thus appear to be active participants in the inflammatory response.

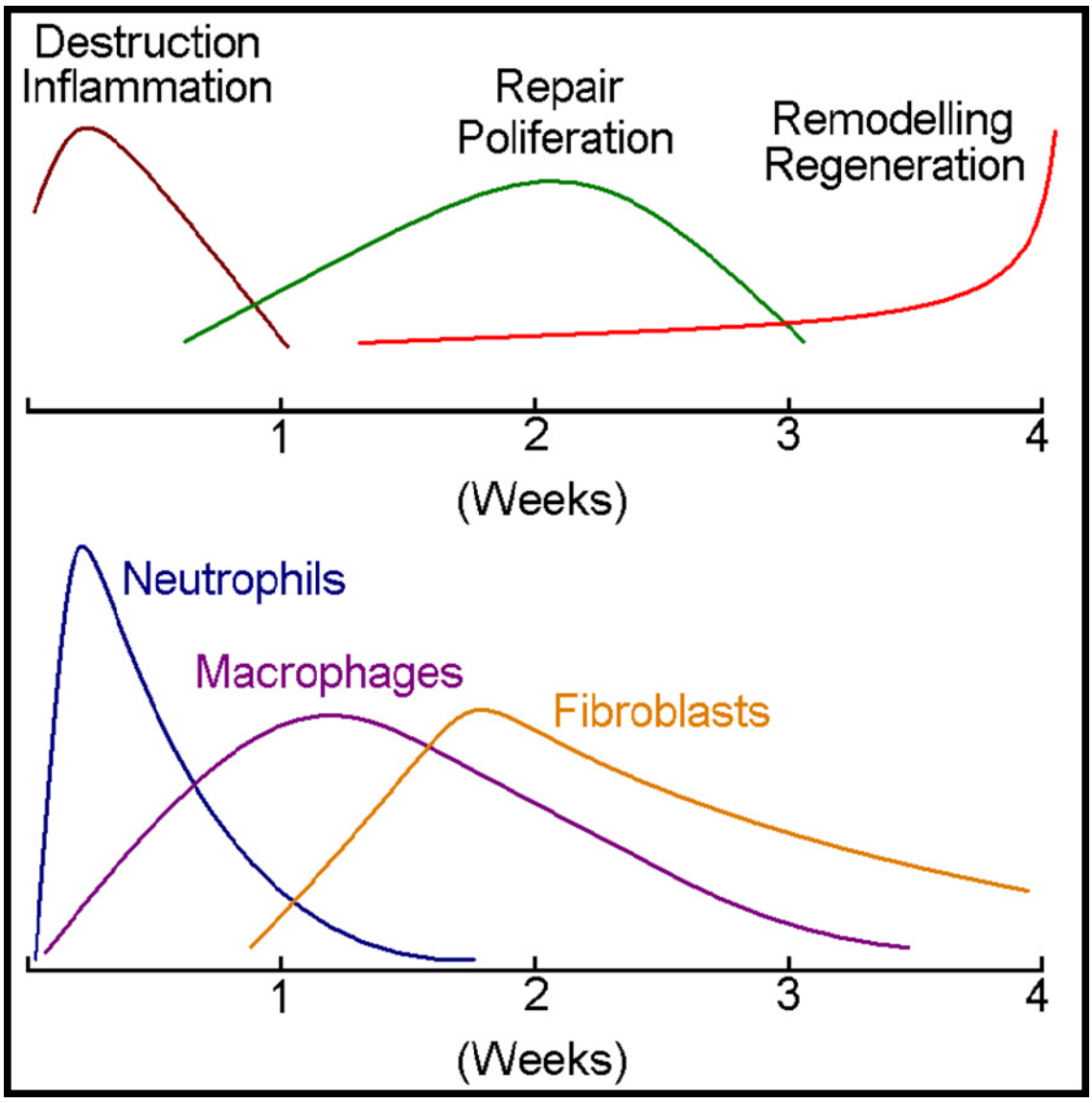


Figure 1.4 Simplified representation of inflammatory progression accompanied by cell involvement. Adapted from (Witte and Barbul 2002) and (Li, Cummins *et al*. 2001)

1.2.5 Effect of fibre type on inflammatory response

Another interesting consideration is the possible role of fibre type on the inflammatory response. As mentioned earlier, oxidative muscle fibres tend to be more vascularised then fast-twitch glycolytic fibres. An injury sustained in these muscle groups are thus much more liable toward excessive haemorrhage brought about by capillary disruption.

This immediately intensifies the potential repercussion of oedema, as disruption of the immediate surrounding vasculature would lead to pronounced haemorrhage and exacerbate the local accumulation of blood. Furthermore, being more vascularised, one could expect an increase in the amount of immune cell trafficking taking place with, possibly, a more rapid (or pronounced) inflammatory response.

On a metabolic level, one might intuitively expect a glycolytic fibre to have a greater marginal resistance to hypoxia, since these cells are geared towards a glycolytic/anaerobic, rather than an oxidative form of energy production. Furthermore, components like myoglobin (which as mentioned, is more abundant in oxidative fibres) might potentially denatured under low pH conditions (brought about by the local increase in lactate and CO_2), liberating its iron centres. This could allow the iron to participate in Fenton reactions and increase oxidative stress through the formation of reactive species.

Unfortunately, the literature did not afford much in terms of study results to indulge this curiosity. Two studies performed in rat models both mentioned a lower level of neutrophil invasion in white (fast-twitch) as opposed to oxidative muscles in an eccentric-biased contractile model (Tiidus, Deller *et al*. 2005) and in an excessive, high intensity exercise model (Morozov, Tsyplenkov *et al*. 2006).

1.3 Satellite cells

Muscle fibres are terminally differentiated cells and thus, are unable to re-enter the cell cycle. The fact that myotubes are not capable of proliferating brought up the question of a possible mechanism whereby post-natal muscle growth and regeneration could take place. Muscle mass can be expanded by increasing the amount of myofibres (hyperplasia) or by increasing the size of existing muscle fibres (hypertrophy). It was long known that myofibres seemed capable of increasing in size without undergoing nuclear division (Capers 1960), and today, hypertrophy is a well documented phenomena (Frontera, Meredith *et al*. 1988; Lowe and Alway 1999).

The mechanism by which hyperplasia could take place eluded researches. However, one early experiment found that, muscle fibres could “split up” and each separately undergo hypertrophy (Reitsma 1969), thus increasing total muscle mass. But this was a relative rare event and could not completely account for the capacity of muscle to regenerate or increase in mass.

In 1961 a huge contribution towards our understanding of the regenerative capacity of muscle was made. Utilizing electron microscopic, a small cell with a minimal amount of cytoplasm, tightly wedged between a muscle fibre’s plasma membrane and the basement membrane was discovered (Mauro 1961).

With regard to their cellular morphology and resident location, theses cells were subsequently dubbed *satellite cells*. Today, these cells are known to provide a replenishing pool of cells by which muscle tissue can recruit new myofibers (Collins, Olsen *et al*. 2005). These cells also act as nuclear donors to already existing muscle fibres and contribute towards muscle hypertrophy (Russell, Dix *et al*. 1992).

This recruitment of myonuclei during hypertrophy takes place in the context of muscle adaptation: As previously described, muscle fibres bearing excessive contractile stress adapt by increasing the amount of contractile machinery which inevitably lead to an increase in cell volume. As each myonuclei is only capable of governing the transcriptional activity in a finite amount of space (Cheek 1985) additional growth thus requires the recruitment of extra myonuclei to accommodate hypertrophy. This recruitment is influenced by muscle fibre type, which is highly sensitive to contractile demand and is orchestrated by paracrine signalling factors (Allen, Monke *et al*. 1995).

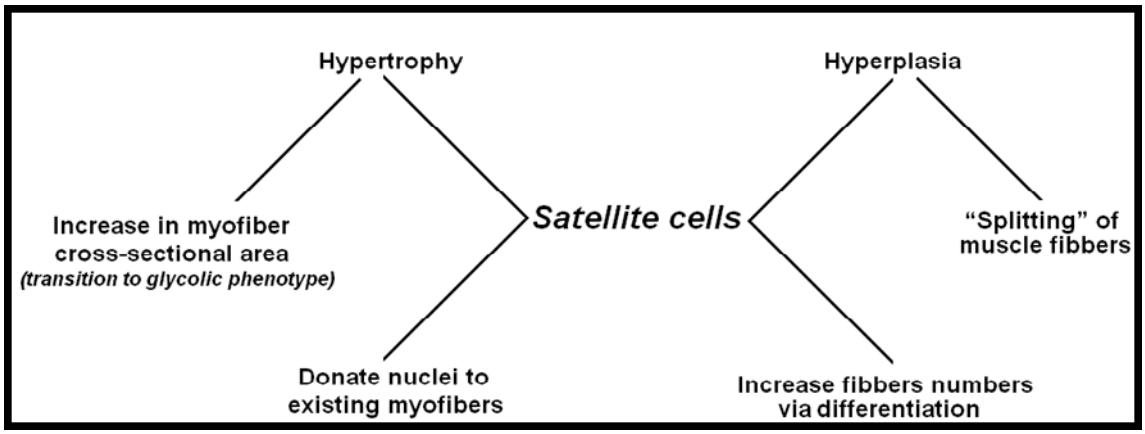


Figure 1.5 Mechanism by which muscle tissue mass can be expanded with special reference to the role of satellite cells.

Satellite cells are stem cell like progenitor cells of skeletal muscle with a multi-potent phenotype (Zammit and Beauchamp 2001). Under normal conditions in matured muscle tissue these sells are quiescent (Schultz, Gibson *et al*. 1978), but can undergo vigorous proliferation in a regenerative context.

This was demonstrated in a recent study were seven satellite cells associated with a single transplanted myofibre generated over a 100 myofibres, totalling thousands of myonuclei (Collins, Olsen *et al*. 2005). It has been shown that these cells, once activated, are capable to migrate to the site of injury (Schultz, Jaryszak *et al*. 1985). It has also more recently come to light that factors like nitric oxide and insulin-like growth factor (IGF) play some role in this mobilization process (Anderson and Pilipowicz 2002).

1.3.1 *Indentifying satellite cells*

In the past, electron microscopy was utilized to identify satellite cells on the basis of their cellular morphology and placement beneath the basal lamina. The advancement of immuno-histochemical techniques made it possible to identify satellite cells on the basis of their (semi-)unique transcriptional activities. Also, depending on the cell-cycle status, satellite cells might express unique proteins that can be used as markers to identify cell status. It should be noted though that this transcriptional activation takes place across a spectrum with no exact boundary. It is also generally accepted that there exists no all-encompassing marker for satellite cells (Montarras, Morgan *et al*. 2005).

Table 1.2 Adhesion molecules, membrane proteins and transcription factors typically used to identify satellite cells. The expression of each molecule is temporally associated with different stages of differentiation.

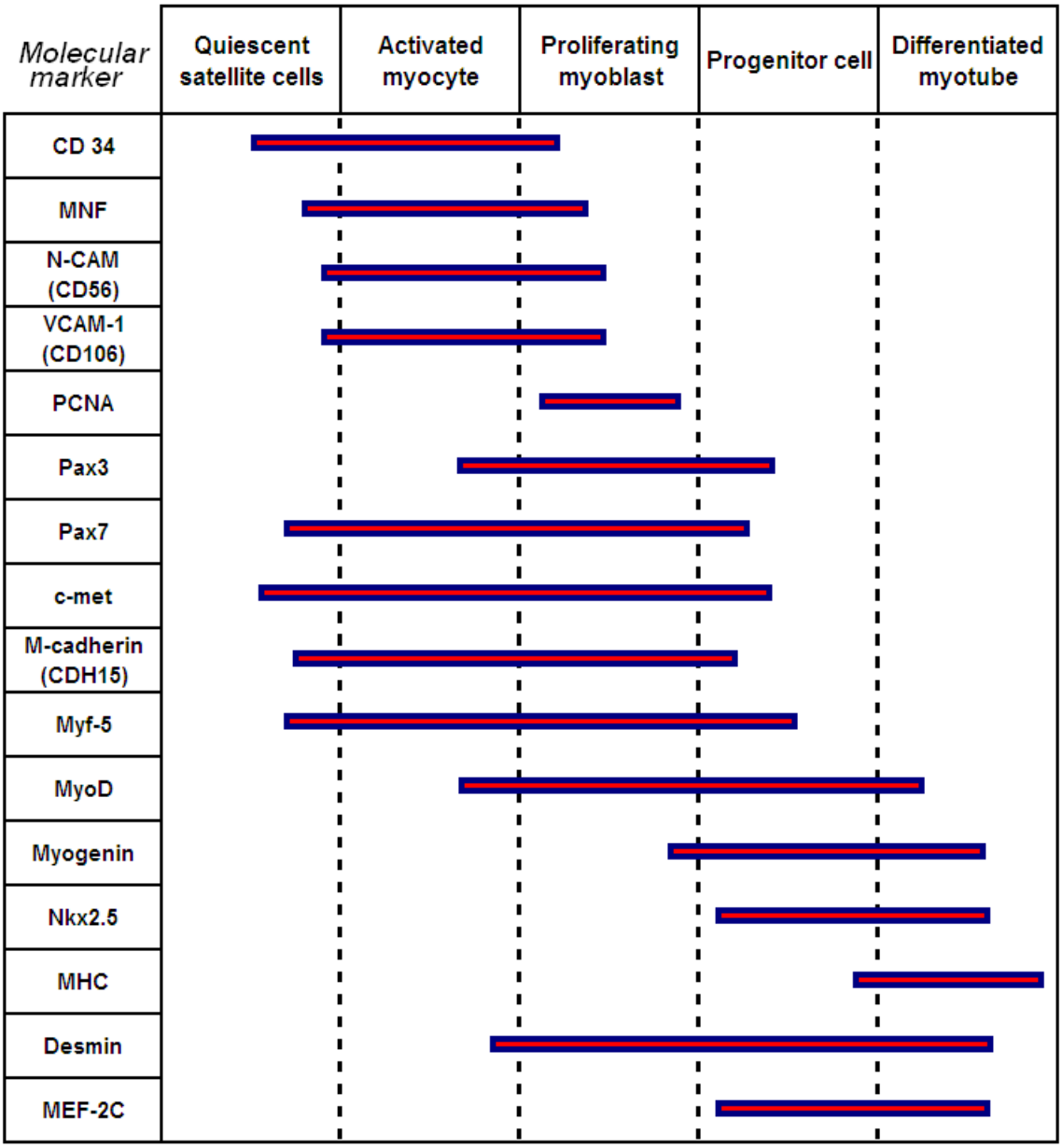


Table 1.3 List of some transcription factors, adhesion molecules and structural proteins expressed by satellite cells and used to indicate metabolic state of cell as depicted in Table 1.2. Indicated by gray scale are members of myogenic regulatory factors (MRFs) and grouped under basic helix-loop-helix (bHLH) transcription factors.

	CD 34	Trans membrane protein with unknown function. Proposed functions include: (1) Inhibition of differentiation 2) promoting of proliferation (3) adhesion molecule (4) an anti-adhesion molecule (Furness and McNagny, 2006).
Myocyte nuclear factor	MNF	Winged helix transcription factor expressed selectively in satellite cells. Gnomically coordinate the proliferation and differentiation of myogenic stem cells after muscle injury (Garry et al., 2000).
Neural cell adhesion molecule	N-CAM (CD56)	Glycoprotein that plays a role in cell-cell and cell-matrix adhesion (Rabinowitz et al., 1996).Regulate myoblast fusion (Suzuki et al., 2003).
Vascular cell adhesion molecule 1	VCAM-1 (CD106)	Cell surface glycoprotein, promoting cell adhesion in various tissues (Garmy-Susini et al., 2005) . (Possibly, adhesion of satellite cell to basal lamina?)
Proliferating Cell Nuclear Antigen	PCNA	Acts as a "DNA clamp"- Increase processivity on DNA polymerases (Bowman et al., 2004).
Paired box 3	Pax3	Member of the paired box (PAX) family of transcription factors. Stem cell transcription factor that simultaneously determine cell fate wile maintain an undifferentiated state resulting in a cell "primed or external stimuli (Lang et al., 2005).
Paired box 7	Pax7	Expressed by cells that appear to be maintained as a proliferating pool of embryonic and foetal muscle cells (Relaix et al., 2005) (i.e. inhibit differentiation).
mesenchymal-epithelial transition factor	c-met	c-Met is the cell-surface receptor for hepatocyte growth factor (HGF)(Bottaro et al., 1991). Expressed by quiescent and activated satellite cells (Tatsumi et al., 1998; Wozniak et al., 2003).
cadherin 15	M-cadherin (CDH15)	Transmembrane glycoproteins with Ca-dependent intercellular adhesion properties. M-cadherin mRNA unregulated in myotube-forming cells (Donalies et al., 1991).
myogenic factor5	Myf-5	regulatory factor (MRF) family (Tajbakhsh and Buckingham, 2000). Act upstream of MyoD, in concert with other transcription factors, to direct embryonic multipotent cells into the myogenic lineage (Kassar-Duchossoy et al., 2004).
Myogenic differentiation	MyoD	Activate differentiation-specific genes, will transcriptional repressing proliferating myoblasts (Mal and Harter, 2003).
Myogenic factor 4, MYOG	Myogenin	Up regulate (along with MyoD) muscle-specific microRNAs responsible for modulating myoblast-myotube transition (Rao et al., 2006).
NK2 homebox 5	Nkx2.5	Crucial role in myocardial development (Hiroi et al., 2001).
Myocin heavy chain	MHC	Contractile protein. Expressed by differentiated or differentiating myotube (Anderson and Davison, 1999; Staron, 1997).
	Desmin	Desmon attach terminal Z disc and membrane-associated proteins to form a conductive structure through which force can propagate (Tidball, 1992).
MADS box transcription enhancer factor 2, polypeptide C	MEF-2C	MEF2C is transcription factor, member of the MEF family of MADS (MCM1, agamous, deficient, serum response factor). By bind to A-T rich DNA sequence of muscle-specific genes. (Leifer et al., 1993; Ornatsky and McDermott, 1996; Yu et al., 1992).

1.3.2 *Satellite cell response to injury*

Upon muscle injury, satellite cells are activated and recruited for muscle repair (Hill, Wernig *et al*. 2003). However, the exact processes involved in this satellite cell recruitment, and the subsequent modulation between proliferation and differentiation still remains to be fully elucidated. Injury induced by which ever means, result in a magnitude of cytokines and signalling molecules to be released. Strenuous exercise can give rise to elevated plasma levels of cytokines IL-8, IL-6, IL-1ra, IL-1β, IL-10 and TNF-α (Ostrowski, Rohde *et al*. 1999; Ostrowski, Rohde *et al*. 2001). These effects can be compounded by the presence of neutrophils which, as stated, are capable of releasing IL-8, IL-1, TNF-α and IFN-α (Fielding, Manfredi *et al*. 1993; Cassatella 1995; Tidball 1995; Cannon and St Pierre 1998; Suzuki, Totsuka *et al*. 1999; Barbero, Benelli *et al*. 2001) as well as some unknown stimuli from macrophages (Massimino, Rapizzi *et al*. 1997; Merly, Lescaudron *et al*. 1999; Cantini, Giurisato *et al*. 2002).

1.3.2.1 **Satellite cell activation**

Despite this complex signalling environment, two major factors are well known to activate satellite cells. These are the signalling molecule nitric oxide (Anderson 2000) and the paracrine signalling peptide, hepatocyte growth factor/scatter factor (HGF/SF –hereafter simply revered to as “HGF”) (Tatsumi, Anderson *et al*. 1998; Miller, Thaloor *et al*. 2000). It should be noted that both neutrophils and macrophages (Salvemini, de Nucci *et al*. 1989; Moilanen and Vapaatalo 1995; Roitti and Rabson 2000) along with the endothelium (Salvemini, de Nucci *et al*. 1989) and mast cells (Salvemini, Masini *et al*. 1990) are capable of releasing nitric oxide.

It has been shown that at least some macrophages (Morimoto, Amano *et al*. 2001; Khan, Masuzaki *et al*. 2005) and neutrophils (McCourt, Wang *et al*. 2001; Taieb, Delarche *et al*. 2002) are capable of secreting HGF. Many cytokines released during muscle injury might not have any mentionable effect on satellite cell activity, but might through their interaction with other cells

cause a release of both nitric oxide and HGF which might act on neighbouring satellite cells.

Other factors of interest are fibroblast growth factor (FGF) which has been found to activate quiescent satellite cells (Johnson and Allen 1995). Also, satellite cells deficient in the heparin sulfate proteoglycan, syndecan-4 (a transmembrane protein) has been shown to have a delayed onset of satellite cell proliferation (Cornelison, Wilcox-Adelman *et al*. 2004).

Both exercise (Keller, Steensberg *et al*. 2001; Tomiya, Aizawa *et al*. 2004) and inflammation (Kaplanski, Marin *et al*. 2003) induce a marked increase in local as well as systemic IL-6 concentration. Besides being released by cells involved in immunological responses, IL-6 are also found to be released by vascular endothelial, fibroblasts (Akira, Taga *et al*. 1993), myofibers exposed to non-injuring contraction (Brenner, Natale *et al*. 1999) and even satellite cells (Cantini, Massimino *et al*. 1995). The effect of IL-6 on muscle is complex, demonstrating almost conflicting results. A slight increase in basal IL-6 levels is associated with muscle atrophy (Tsujinaka, Ebisui *et al*. 1995; Haddad, Zaldivar *et al*. 2005) while IL-6 have also been implicated in satellite cell activation (Cantini, Massimino *et al*. 1995).

This proliferating effect seems to be mediated through NF-κB inhibition of differentiation (Langen, Schols *et al*. 2001). IL-6 also seem to have beneficial effects through its ability to attenuate an inflammatory response by stunting TNF-α production (Starkie, Ostrowski *et al*. 2003). Yet, the mediated physiological response might not be as straight forward, for TNF-α have been shown to exert mitogenic effects on satellite cell (Li 2003) and are also implicated in myogenic differentiation (Li and Schwartz 2001).

Irrespective of the preceding events, once activated, satellite cells exit the G_0 phase of the cell cycle and commit to a state of proliferation (Grounds and McGeachie 1989). Orchestrating the recruitment of cell cycle machinery, the activated cells up-regulate their expression of a group of basic-helix-loop-helix (bHLH) transcription factors known as myogenic regulatory factors (MRF)

(Perry and Rudnick 2000) as well as myocyte enhancer factor-2 (MEF2) (Naya and Olson 1999).

Activated satellite cells also become highly mobile. Satellite cell's sensitivity to chemotactic agents seem to be induced by growth factors like basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and insulin-like growth factor 1 (IGF-1) and mediated by signaling molecules, Ras and Ral (Suzuki, Yamazaki *et al*. 2000) . The ability of satellite cells to migrate to the site of injury is crucial, for it is in their ability to replace damaged or lost myotubes by differentiating into myotubes that satellite cells express their physiological relevance.

1.3.2.2 Regulating satellite cell proliferation and self renewal

The cell cycle-inhibitor p27Kip1 (also denoted as $p27^{Kip1}$) (a cyclin-Cdk inhibitor) has emerged as a strong candidate for regulating cell cycle progression. It has been found to inhibit cell proliferation, even in the presence of a strong mitogenic stimulus (Spangenburg, Chakravarthy *et al*. 2002). Other growth factors also play a role, IGF-1 has the ability to down regulate p27Kip1, thereby increasing the proliferation capacity of satellite cells (Chakravarthy, Abraha *et al*. 2000). The presence of HGF also seems to contribute to satellite cell proliferation (Tatsumi, Anderson *et al*. 1998).

Activated satellite cells must also have the ability to return to a quiescent state after proliferation in order to replenish the pool of cells. Little is known about how this return to a pre-proliferating state is orchestrated. Two models proposed for satellite cell renewal have been presented: a "stochastic" model by which cells randomly revert to a quiescent state without differentiating and an "asymmetric" model (Dhawan and Rando 2005). In this model, the first round of cell division gives rise to a daughter cell which might continue dividing whilst the mother cell exits the cell cycle and enters a state of quiescence again.

Recently it has been demonstrated that satellite cells undergo self renewal via asymmetric segregation of template DNA (Shinin, Gayraud-Morel *et al*. 2006). What's more, this selective segregation of "immortal" DNA template strands coincides with the polar segregation of the "cell-fate determinant" Numb

molecule. The dividing cell has a “Numb pole” which co-segregates with the daughter sell inheriting the original template DNA. This daughter cell also express the self renewal marker, Pax7 and does not differentiate.

1.3.2.3 Satellite cell differentiation

As mentioned, our interest in satellite cells is not in the fact that they are undifferentiated cells. Rather, it is their ability to differentiate and there by replenish lost tissue that they arouse our interest. This “replenishing capacity” effectively ordaining satellite cells as agents of homeostatic plasticity. In a way then, differentiation can be considered the most important aspect of satellite cells. After exiting the cell cycle, satellite cells (myoblasts) begin to fuse with each other, forming new myotubes. The cells start to produce the machinery necessary for contractile function: Contractile proteins are synthesized (Anderson and Davison 1999) and an increase in creatine kinase activity is observed (Florini and Magri 1989).

All the events involved from activation to differentiation are carefully managed through a host of signalling events and are the collective result of multiple participating cell groups’. As a closing note, it is worth mentioning the exceptional sensitivity satellite cells have towards their environment. This was exquisitely demonstrated in a recent study where Conboy and co-workers evaluated the regenerative capacity of aged rats when cross-circulating bloods supply with a young rat. Monitoring different parameters of satellite cell physiology, it was shown that exposure of the old rat to factors in the young rats serum lead to a drastic increase in regenerative capacity (Conboy, Conboy *et al*. 2005). This study again demonstrated the significance of the environmental context in which physiological responses take place.

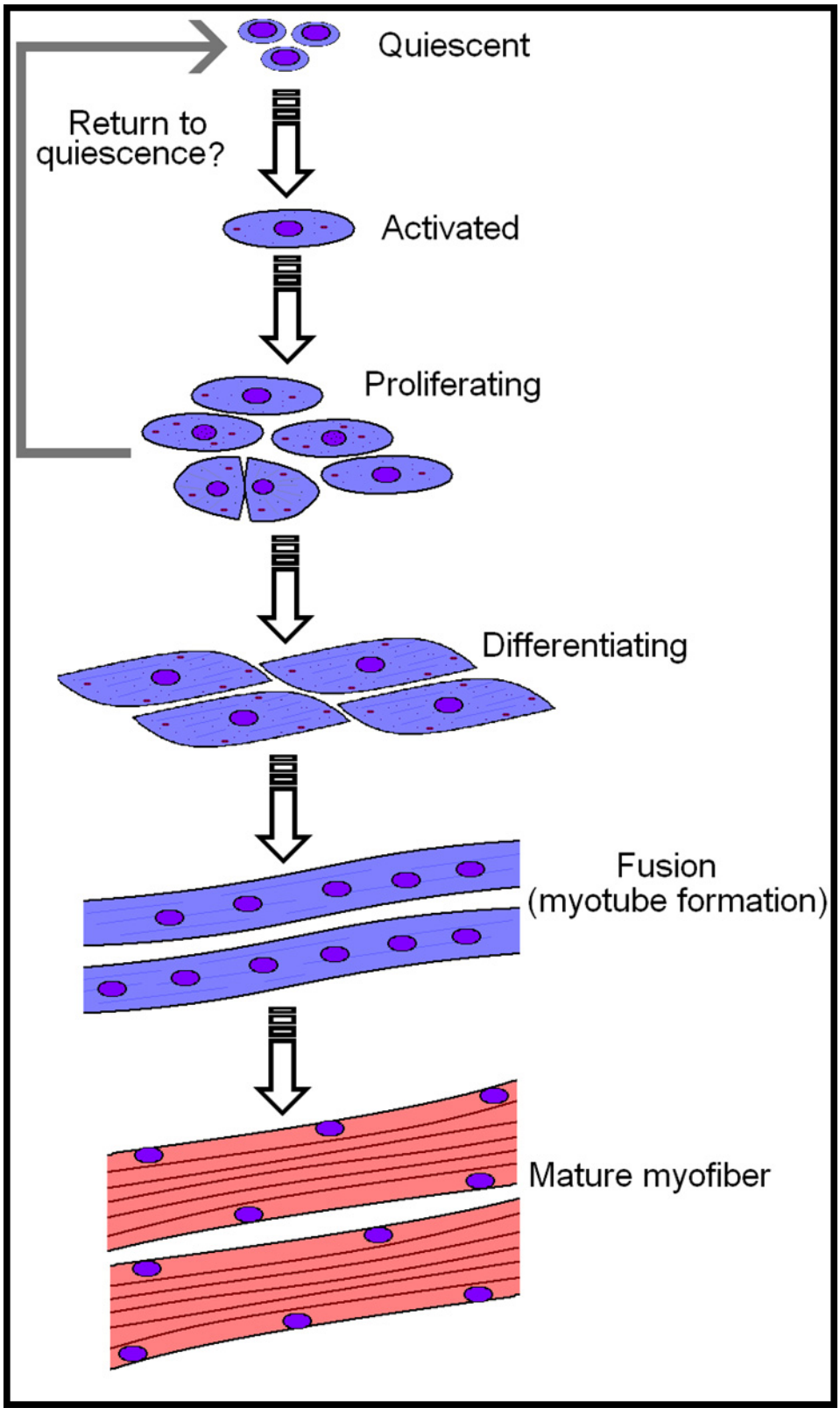


Figure 1.6 A basic schematic representation of sequential events taking place during satellite cell activation and the subsequent possible "routes" satellite cell progression might follow.

1.4 Treatment options

A few clinical treatment modalities do exist to augment and facilitate muscle regeneration following contusion injury and these will very briefly be discussed here.

1.4.1 *Conventional intervention: supportive*

In general, muscle with or without any treatment will eventually (with respect to the extent of the injury) regenerate with a relatively good outcome. As such, most intervention is attempts to "tweak" the healing process in terms of the time it takes to regenerate or to minimize formation of scar tissue as well as secondary damage.

Immediate treatment mostly comprises the RICE principal (rest, ice, compression, and elevation) which is aimed at reducing local haemorrhage. Currently there exist no proper (controlled) study to evaluate the effectiveness of RICE, although some of the independent components have been validated (Bleakley, McDonough *et al*. 2004). As far as rest is concern, immediate immobilization early on might be advantageous (Jarvinen 1975; Jarvinen and Lehto 1993). In a recent study, ice-massage of exercise-induced muscle damage seem to have no effect on muscle regeneration (Howatson, Gaze *et al*. 2005). Compression seemed to be beneficial in one study (Kraemer, Bush *et al*. 2001) whilst appearing to have no effect in another (Thorsson, Lilja *et al*. 1997).

Early mobilization (depending on the extent of the injury) has long been considered the best treatment after muscle trauma (Jarvinen 1976; Buckwalter 1995; Kannus, Parkkari *et al*. 2003). Mobilisation has been found to induce vascularisation, earlier gain in muscle-fibre density (Jarvinen 1975; Jarvinen 1976) and leads to improvement of contractile force generation in the injured muscle (Jarvinen 1976). This is of course dependant on the type and severity of the injury. It has also been shown that immediate mobilization can be detrimental to muscle regeneration and result in scar formation (Jarvinen

1975). In general, it is difficult to decide how early is too early and clinical experience seems to be of the utmost importance in evaluating each injury on a case by case basis.

1.4.2 *Therapeutic ultrasound*

Another prospective treatment modality to consider is therapeutic ultrasound. The exact mechanism of action regarding therapeutic ultrasound are not fully understood and the application and dosage seem somewhat arbitrary (Warden and McMeeken 2002). Typically the beneficial effects are found with regard to contusion injury in a rat model (Rantanen, Thorsson *et al*. 1999; Karnes and Burton 2002) where an increase in collagen deposition during the repair of tendon injuries was observed (da Cunha, Parizotto *et al*. 2001).

1.4.3 *Antioxidant therapy*

The rationale behind antioxidant therapy/antioxidant supplementation is the fact that excessive induced reactive oxygen species production is seen in the inflammatory response. Also, as mentioned, the secondary damage induced by neutrophils seems to be mostly mediated by an increase reactive species production (Welbourn, Goldman *et al*. 1991; Brickson, Hollander *et al*. 2001; Brickson, Ji *et al*. 2003). Neutralizing these radicals could reduce the degree of oxidative stress the muscle is exposed to and help prevent excessive muscle damage.

Antioxidant therapy has been shown to reduce inflammatory damage (Cuzzocrea, Thiemermann *et al*. 2004). A more recent study has shown that muscle damage, induced by eccentric resistance training, can increase circulating bio-markers of oxidative stress. Treatment intervention involving antioxidant supplements (in the form of vitamin E and C) can attenuate the rise of these markers (Goldfarb, Bloomer *et al*. 2005). Antioxidant homeostasis is a complex system of which reactive species production is a totally normal (and indeed a necessary) part. It is yet unclear under which circumstance, and, in what extent antioxidant supplementation should be followed (Urso and Clarkson 2003).

1.4.4 *Non-steroidal anti-inflammatory drugs*

Non-steroidal anti-inflammatory drugs (NSAIDs or NAIDs) are often prescribed for a vast array of muscle or other soft tissue injuries. The main controversy around NSAIDs is which parts of the inflammatory response is inhibited. As stated earlier, neutrophils are often associated with exacerbatiion of tissue damage, where as arrival of macrophages often herald the beginning of the reconstruction phase (by phagocytising cellular debris). It is preferential to inhibit the one but not the other, though it is questionable whether NSAIDs exhibit this specificity in terms of their pharmacodynamics.

In a rat model, the effects of NSAIDs were evaluated after inflicting a contusion injury. Both satellite cell and fibroblast proliferation as well as capillary formation seemed unaffected (Thorsson, Rantanen *et al*. 1998). In a more resent study where a range of NSAIDs was injected, muscle injury was induced in mice by either injecting myotoxin or via crush injury. The application of NSAIDs did not seem to have any long-term negative effects on muscle recovery (Vignaud, Cebrian *et al*. 2005).

Also, another study demonstrated increases in the microcirculatory system after administrating NSAIDs with specific cyclooxygenase-2 (COX-2) inhibitory capacity (Gierer, Mittlmeier *et al*. 2005). These results were accompanied by a reduction in platelet-dependent secondary damage and support tissue repair.

Yet, NSAIDs might not be the panacea they appear to be. A freeze injury model performed on a knock-out mouse model demonstrated the requirement for COX-2 during early stages of muscle regeneration. In this study a decrease in inflammatory cells were observed as well as a reduction in the number of myoblasts (Bondesen, Mills *et al*. 2004). This result was expanded upon by recent finding that some NSAIDs involved with COX-2 inhibition might mitigate the repair process by limiting myogenic precursor cells from fusing (Shen, Prisk *et al*. 2006).

The COX-family of enzymes performs a catalytic function in the formation of prostaglandins. Prostaglandins in turn contribute to the regulation of multiple

physiological roles (basically all mammalian cells, with the exception of red blood cells, produce prostaglandins). These effects include the induction of pain and fever, regulation of blood pressure, induction of blood clotting, labour (among other reproductive functions), regulating circadian rhythm and of course, modulating the inflammatory response (Voet and Voet 2004).

Recently if had been found that COX pathway play a role in muscle atrophy and possibly exert a regulating function on satellite cell activation and proliferation (Bondesen, Mills *et al*. 2006) hinting that inhibiting the COX-2 pathway might compromise muscle growth. Furthermore, the extent to which inflammation is impaired could be detrimental towards wound healing. As an example, inhibiting macrophage infiltration would result in a reduction in the phagocytosis of cellular debris and delay wound repair.

Currently, there is a substantial margin of incongruence in the literature regarding the efficacy of NSAIDs. If nothing else, caution should be applied in the general use of NSAIDs in treating muscle injury.

1.4.5 *Satellite cell grafting*

Muscle fibres have been successfully grafted from one muscle to another (Snow 1978; Mong 1988). The observed regeneration of grafted tissue is believed to be facilitated by the resident satellite cells co-grafted with muscle. The transplantation of viable satellite cells is another possible treatment option. Transplantation of satellite cells into extensor *digitorum longus* (EDL) muscle after “irreversible damage” results in remarkable (though not complete) recovery (Alameddine, Louboutin *et al*. 1994).

The potential benefit of transplanting satellite cells was again dramatically demonstrated in a recent publication. Using a dystrophic dog model, it was shown that transplanted satellite cells expressing wild type allele greatly alleviated many of the symptoms induced by Golden retriever muscular dystrophy (GRMD) (Sampaolesi, Blot *et al*. 2006). The prospect of transplanting genetically modified satellite cells aside, this also demonstrates the ability of transplanted satellite cells to colonize and repair damaged tissue in a realistic *in vivo* model. Also, the transplantation of satellite cells in order to promote cardiac muscle regeneration is also considered (Menasche 2003).

Unfortunately, there are some limitations, one being immune rejection (which can be circumnavigated by performing autologous transplants) (Brent, Brooks *et al*. 1976; Partridge 2003). Furthermore, the spread of these cells cannot be localized and cell survival seem not to be optimum (Partridge 2003). Finally, the time and cost involved might currently not allow these procedures to become routinely performed as a treatment option for muscle injury.

1.4.6 *Photo-biostimulation*

The contentious field of low level laser therapy (LLLT) has, over the past decade, made striking claims in the fields of orthopaedics, dermatology, dentistry and physiotherapy (in both medical and veterinary disciplines). With regard to recent findings, LLLT has been shown to decrease the inflammatory response following muscle contusion injury in a rat model (Rizzi, Mauriz *et al*. 2006), reduce the formation of fibroses (Fillipin, Mauriz *et al*. 2005) and even delay the onset of muscle fatigue in conjunction with a reduction in muscle injury during episodes of excessive muscle contraction, albeit in an artificial *ex-vivo* setting (Lopes-Martins, Marcos *et al*. 2006). With regard to the shortcomings of many of the treatment modalities, this noninvasive procedure has, unlike many other forms of pharmacological intervention), very few contraindications (Navratil and Kymplova 2002) and would be a welcome addition to the currently, somewhat, limited repertoire of treatment options available for treating muscle injuries.

1.5 Low level laser therapy

Both the phenomenon of photosynthesis as well as a child burning a piece of paper by focusing sunlight through a magnifying glass rest upon the principle: namely that light is propagating energy. In recent years, our ability to manipulate and direct this energy embodied in light has given rise to a host of innovations with which we have greatly increased our quality of life. The application of lasers in science has greatly expanded our experimental "toolkit". With lasers, membrane protein diffusion has been observed and (Yguerabide, Schmidt *et al*. 1982) and retinal nerve fiber layer measured (Lleo-Perez, Ortuno-Soto *et al*. 2004), nanotubes have been and continue to

be “forged” (Scott 2004), molecules dissected (Schiller, Suss *et al*. 2004), genomes are scrutinized (Ragoussis, Elvidge *et al*. 2006) and it has even enable the curious to intrude on the private lives of cells (Afonin, Ho *et al*. 2006).

In modern medicine, the dominant application of lasers is in their capacity as laser scalpels. Instead of mechanically cutting tissue, these surgical instruments can ablate tissue with micrometer diameter resolution, minimising “collateral” tissue damage as well as being perfectly sterile. The excellent precision and minimum damage make them especially popular in both neuro- and ocular surgery.

In stark contrast to the powerful, high intensity YAG and CO_2 lasers applied in tissue ablation, the power outputs used in LLLT are much lower. Indeed, LLLT induces its effect by non-thermal means. Moreover, LLLT is immersed in controversy and haunted by a lack of understanding as to how LLLT induces the biological effects seen following tissue irradiation.

Regarding the cellular effects of LLLT, studies have shown an increase in collagen production (Reddy, Stehno-Bittel *et al*. 1998; Pereira, Eduardo Cde *et al*. 2002), increase in wound tensile strength (Stadler, Lanzafame *et al*. 2001), promotion of regeneration of bone fractures (Trelles and Mayayo 1987), a boost in DNA synthesis (Callaghan, Riordan *et al*. 1996), improvements in sperm viability (Ocana-Quero, Gomez-Villamandos *et al*. 1997) as well as motility (Corral-Baques, Rigau *et al*. 2005) and altered the expression of over a 100 genes in human fibroblasts (Zhang, Song *et al*. 2003). The clinical literature is full of reports showing the beneficial effects of LLLT, boasting literally thousands of articles on its seemingly miraculous healing effects. Yet, justifying these results by the mechanism invoked by LLLT are painfully amiss in the literature.

1.5.1 *Historic overview*

It is probably difficult to pin-point the exact time and manner in which humans used the sun in the treatment of their ailments. Though earlier reports of heliotherapy (the medical application of sun light) are known, the first systematically documented use of sun in healing skin disease was by the

Hindu's, described in the sacred text "*Atharva-Veda"*, more specifically, *Ayurveda*. The *Ayurveda* is a subsection of *Atharva-Veda* dealing with "wellbeing" and health (Fitzpatrick and Pathak 1959). This Hindu text dating back 1400 BC described the preparation of ointments prepared from the seeds of *Psoralea corylifoliu* were applied to vitiliginous skin before exposing the skin to direct sun light.

During Napoleon's campaign in Egypt (1798-1799) an astute man named Dominique Jean Larrey (who was responsible for many battlefield-medical initiatives, including the principal of triage) noted that ulcers healed more successfully if exposed to the sun (Bernhard 1926). Up to the time of the Second World War, this principal of "open air treatment" was followed until antibiotics became readably available.

In 1903 Niels Ryberg Finsen received the Nobel Prize in Medicine and Physiology for his successful treatment of *lupus vulgaris*. What makes this such a unique incident is the fact that this would be the only time in the history of the Noble prize (for medicine) that it has been awarded in the field of dermatology. What is peculiar is the nature of the treatment: high dose UV radiation from a carbon arc lamp.

Not so much of immediate importance to the medical discipline was August 6, 1960. After being turned down by *Physical Review Letters* the first official publication of a laser was made (Maiman 1960) in *Nature*. This was after Theodore Maiman demonstrated the first working laser at Hughes Research Laboratories on May, the 16th 1960. It was remarked once that the laser is "a solution looking for a problem" (Garwin and Lincoln 2003). Today lasers are employed in a vast array of applications ranging from warfare to household appliances.

In 1967 whilst exploring the carcinogenic effect of intense photon bombardment from a ruby laser on shaven mice, Mester noticed the irradiated group had their hair grow back faster than the control group (Mester, Szende *et al*. 1968). This was the first report of laser bio-stimulation to be published. In the subsequent year, his group published more results (Mester, Ludany *et al*. 1968; Mester, Ludany *et al*. 1968) with the first publication of laser light as

a treatment modality in wound heal in 1971 (Mester, Spiry *et al*. 1971). Laser therapy was born!

Here it should also be noted that LLLT involves a shift in the application of light. Whereas helio- and photo- therapy utilized damaging properties of light to treat infections, LLLT harnesses the stimulating effect of light. Indeed, at high dosages, LLLT seem to be useless or even detrimental (van Breugel and Bar 1992; Lopes-Martins, Marcos *et al*. 2006).

1.5.2 *Terminology*

Low level laser therapy, low power laser therapy, low level laser irradiation, low energy laser irradiation, photo bio-stimulation and some other associated terms are used interchangeably. This might lead to some confusion as some of the terms are also used in other fields with a different application of the phrase (e.g. “light therapy” is used to treat seasonal affective disorder and has absolutely nothing to do with photo-biostimulation). The phrase “*laser* therapy” would technically not be appropriate since light-emitting-diodes (LEDs) are also utilized, which produce incoherent light of a narrow-spectrum through electroluminescence and not by photon pumping as in lasers.

Furthermore, it might be more prudent to emphasize the action rather than the instrument. Lasers and diodes are used in many other applications outside photo-biostimulation. Nevertheless, out of convention, these terms will be used interchangeably in this thesis.

In the (serious) scientific literature, very little effort is made to distinguish between phototherapy and heliotherapy (the use of this terminology is usually at the discretion of the author). Technically, all forms of heliotherapy are indeed phototherapy as both use photons to treat patients. But heliotherapy, as the name implies, makes use of the sun as it source where as phototherapy make use of an artificial light source. In effect, the “active ingredient” in sun light is the antimicrobial effect of UV rays. Eukaryotes generally have more sophisticated genomic repair mechanisms to handle ionizing radiation (Brown 2002). What’s more, the host (in this case humans) consists of a much larger bio-mass. Therefore, bombarding the infected wound with ionizing (UV) radiation is figuratively a “war of attrition”. Both

heliotherapy and phototherapy (as used in this context) utilize the same central aspect namely, UV lights' antimicrobial properties.

In a similar way, the phrase "light therapy" (also, more ambiguously called phototherapy) is sometimes confused with LLLT. Light therapy as used in the treatment of seasonal affective disorder has absolutely nothing to do with LLLT: where as light therapy can influence circadian rhythm as well as other psychological mechanisms in an individual, LLLT influences cellular metabolism directly by somehow influencing the molecular machinery of individual cells.

Finally, phototherapy and heliotherapy also have nothing to do with LLLT (though the term "phototherapy" is also sometimes correctly, but ambiguously, used in the context of LLLT). Whilst photo- and heliotherapy act via antimicrobial means, LLLT works by stimulating metabolic aspects of cellular routines. Furthermore, UV light is sometimes used in LLLT, but rarely, as ionizing irradiation is mostly applied for its antibacterial properties.

1.5.3 *Resolving the controversy?*

Much of the support for LLLT rest upon findings in clinical trials. In essence, these studies are correlation studies where the irradiation of tissue, usually in some stressor context (e.g. inflammation of tendons, infection of gums, auto-immune induced inflammation, contusion injury etc.) and the subsequent improvement is causally correlated to benefits endowed by LLLT. Expanding these studies from correlations to working hypothesis entering on a mechanism of action will give credibility to these types of studies. It is with this in mind that the field of laser therapy finds itself desperately pressed for an operational explanation to describe both the so-called "primary" and "secondary" mechanisms of action of LLLT.

The absence of a mechanism of action also manifests in other challenges to the field of photo-biostimulation. There is great diversity in the experimental parameters used by both clinicians and researchers, including: wavelength; power density (intensity or "brightness" of the laser); total radiant exposure (total "dosage") not to mention cell-/animal-/tissue model and intervention

(toxicity, contusion injury, hypoxia etc) used. This makes it rather difficult to compare the published studies and therefore the results.

Because of the “aura of mystery” surrounding “laser therapy” (with special regards towards mechanism of action), many charlatans and proponents of pseudoscience find it fertile ground for their quackery. Amazing results can be published without giving explanations or predictions (in some instances peer review appears to be sadly lacking). The huge variations in parameters used and biological effects seen (in conjunction with the resulting confusion) present excellent camouflage for unscrupulous and fraudulent results. A concrete mechanism of action might do much to remedy this problem since the physical events involved will dictate the parameters required for a given response and thus help develop a environment were studies can be readily reproduced by others in the field.

Also, more insight is required on the “secondary” downstream effects of photo-biostimulation. What interaction takes place between cells and their environment? Besides the signalling cascades, how does the cellular context influence the effect of LLLT? For instance, how does LLLT affect a myotube under normal conditions versus at the same cell in an injury setting (probably under ischaemic, low pH conditions, deprived of nutrients and exposed to high concentrations of calcium from ruptured sarcolemma)? Also, the micro-environment of a cell changes as inflammation progresses (in fact, even the local composition of cell types changes as inflammation progresses). How should treatment modalities be modulated over time? These are complex questions. Undeniably, the inflammatory response itself is not fully understood, l*et al*one the effect of a treatment modality like LLLT on the inflammatory process.

1.5.4 *Healing with light?*

Conceptually, using light as a treatment modality is somewhat of a departure from our current pharmacological perception, nurtured over the past century and still dominating our medical science. And clearly with good reason so. This paradigm has served us well and indeed it has increased our quality of life greatly over the last 100 years or so. With this in mind, it is somewhat

understandable that claims about LLLT are greeted with such scepticism by the medical fraternity, and even over the past 20 years, is still considered “fringe science” (or worse) in most scientific circles.

If the field of LLLT are to dispel the mystic undertone imbued by it’s application of “glorified torch lights”, LLLT must be approach from a rigid scientific point of view. To genuinely complement our understanding, a new theory or (paradigm) must show a certain degree of connectivity with previously established empirical facts (see Popper (Popper 1972)). In this regard, LLLT has to uphold the previously established knowledge. If LLLT was a “tiny inland of knowledge” on its own, isolated from other fields and disciplines in science, it would be little more than an elaborate version of “chromo therapy”.

All attributes of LLLT must therefore follow –unconditionally- the pre-existing laws of nature. One such law useful to our examination of LLLT biochemical mechanisms is the first law of photochemistry (also known as the Grotthuss-Draper law)(Negi and Anand 1985). Simply stated, for all photochemical reactions to take place, light (photons) must be absorbed.

In this regard, LLLT have earned it’s right to be legitimately investigated by a scientific methodology: it demonstrate connectivity with previously established scientific findings and make falsifiable claims which can be empirically investigated. Science is first and foremost a methodology. It does not regard *what* is investigated, but *how* it is investigated. If we forgo our squeamishness about using photons (which should not be of concern in the first place since photochemistry is a well established field) and look at the *science*, it should be clear that LLLT deserve an earnest and sincere consideration.

1.5.5 Action- and absorption spectrum: primary and secondary mechanism of action

The basic tenant behind LLLT rests upon the absorption of a photon by a molecule or molecular accessory-group acting as a photoacceptor (“pigment” or term used to describe chlorophyll, “photon antenna”). This absorption of a photon (also to be consider an event in which energy is transferred) must illicit

some biochemical effect and not simply sublimate the transferred energy as heat. The events leading form the absorbance and chemical transfer of energy, ultimately manifesting in some biological effect (increase H_2O_2, singlet oxygen formation, alteration in redox state of elements in the repertory chain etc.) is called the primary mechanism of action (Karu 1999).

As with most other homeostatic mechanisms, downstream amplification-loops are believed to be involved. Whatever primary effect invoke by the absorption of a photon, this effect must ultimately have some effect on cellular metabolism (increase ATP or Ca^{+2}) (Karu 1999). This “effect” is referred to as the secondary mechanism of action and ultimately lead to a phonotypical effect in cell biology.

If photochemical reactions involve the absorption of light to mediate an effect, the fundamental question arises: *what molecules* absorb light at which *wave lengths* and give rise to what kind of *biological effect*? This biological effect can be plotted as an “biological action spectrum” (Karu and Kolyakov 2005). The *biological* action spectrum refers to the biological effect induced as a function of a specific wave length. In other words, it is the biological effect observed at each possible wave length. The criteria for an action spectrum are diverse. Sensibly, it must measure “units” which are indicative of an adaptive or advantageous process. As such, cell adhesion, DNA/RNA synthesis, ATP production, cell proliferation rate etc. have all been used.

The “absorption spectrum” refers to the frequency (wave length) of light at which a molecule absorbs this light. To elicit some effect, a molecule must act as a photoacceptor. This photoacceptor must then somehow relay an effect to induce some biological outcome. Ideally, the molecule would absorb at the same spectrum that induces a biological action spectrum, i.e. it must produce some quantifiable biological response. Such scenario is illustrated in Figure 1.7.

Identifying these absorption-action spectrums is somewhat challenging. The problem lays in the complexity of the cell (both biochemically and optically). Many molecules interact with each other and thus change their optical properties. Also, the redox state of a cell might also influence the absorption

spectrum (Voet and Voet 2004). Biochemically, it is difficult to causally link a specific event to a unique result as many downstream effects are networked with a multitude of metabolic and signalling cascades.

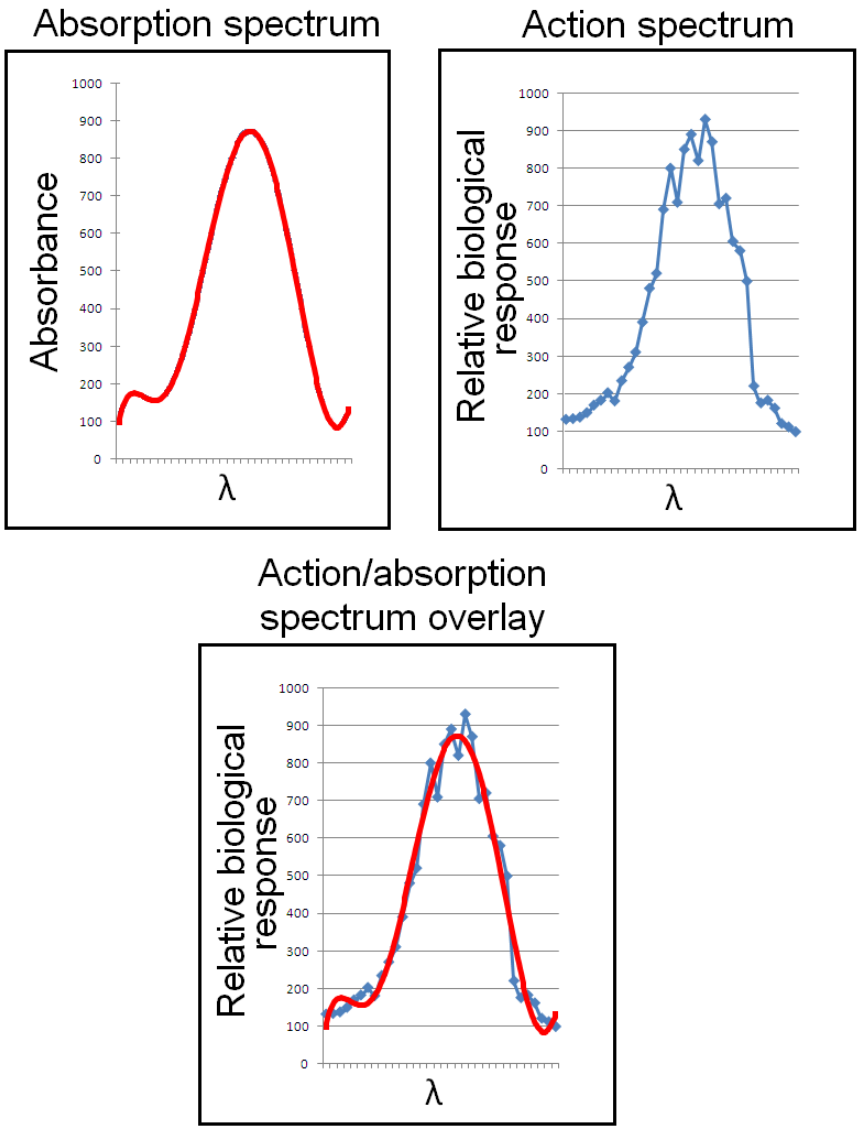


Figure 1.7 Schematic representation of a hypothetical absorption spectrum (red) with associated biological action spectrum (blue) of a candidate photoreceptor. This relationship between an absorption spectrum and biological action spectrum represent the basic principal through which photochemical mechanism can induce a biological response.

1.5.6 *LLLT: signalling and mediators of effect*

Work elucidating the action-absorption spectrum's existence was pioneered by Karu (Karu, Kalendo *et al*. 1982; Karu 1984; Karu, Kalendo *et al*. 1984; Karu, Pyatibrat *et al*. 1987) who looked at DNA synthesis, (followed in later

studies by cell proliferation and adhesion) as possible candidate markers for a biological action spectrum. In these early studies, flavoproteins (like NADH dehydrogenase) were hypothesised to act as photo acceptors in the blue light range. It was not long though until another strong candidate as photoacceptor emerged.

1.5.6.1 Mitochondrial photon antenna: cytochrome c oxidase?

It is well known that the mitochondria possess a group of chromophors (photo-acceptors). The haem-groups of cytochrome exhibit highly characteristic absorbance spectra, depending on their redox state. Reduced haem-groups (Fe II) show specific peaks namely α, β and γ (Soret) bands (absent in the oxidized state).

Table 1.4 Absorption spectrum of a few cytochromes in the electron transport chain (Voet and Voet 2004).

	α	β	γ
Cytochrome a	439 nm	-	600 nm
Cytochrome b	429 nm	532 nm	563 nm
Cytochrome c	415 nm	521 nm	550 nm
Cytochrome c_1	418 nm	524 nm	554 nm

This chromophore, in conjunction with the mitochondrion's position central to energy production (as well the fact that it acts as a calcium store), has made it an attractive candidate as a mediator of photo-biostimulation (Passarella, Ostuni *et al*. 1988; Greco, Guida *et al*. 1989). Cytocrome c specifically was long considered as a major mediator of LLLT (Pastore, Greco *et al*. 2000) and recently, a strong case was made for the legitimacy of cytochrome c oxidase as the photoacceptor in the near infrared spectrum (Wong-Riley, Liang *et al*. 2005).

It has been established that mitochondrial chromophore, cytochrome c oxidase is sensitive to He-Ne laser light (wavelength 632.8 nm) (Pastore, Greco *et al*. 2000). Recently, it has been observed that light of a certain wave length are able to induce the reduction of the photo-acceptor (wave length

might be dependent on cellular environment, for example the local redox state) (Karu, Pyatibrat *et al*. 2005).

The author speculates that the rate limiting step in the electron transfer process within cytochrome c oxidase might be accelerated to provide more electrons available for the reduction of oxygen, as it is well know that electron excitation by light does in fact stimulate redox processes in other organic reactions. By increasing this flux in the rate limiting step, energy production can possibly be augmented. Though speculative, an increase in ATP production (capacity) could explain many of the global beneficial effects observed by LLLT.

1.5.6.2 LLLT- an increase ATP production?

ATP, as the final product of cellular respiration is principally employed a cellular "currency" of energy. In our daily lives the activity of ATP is most salient in the role it plays as the energy molecule driving the molecular machinery which makes locomotion possible. Each power stroke of the cross-bridge cycle ends when an ATP molecule binds to the catalytic site of the MHC "head" section, allowing the actin-myosin complex to disengage. Depletion of ATP renders this process incomplete and the MHC and actin filament remain bound, leaving the muscle "rigid" and giving rise to the post-mortem phenomenon of *rigor mortis*.

Besides muscle contraction, cellular motility (assembly and disassembly of cytoskeleton) and intercellular motility (e.g. cell division) also demand ATP. ATP also plays an important role in signal transduction. For example, adenylate cyclase uses ATP as substrate in the production of the signalling molecule cAMP, as do kinases in the phosphorylation of proteins. ATP is also utilised in DNA polymerisation as well as transcription. Indeed, ATP is utilized in a multitude of cellular activities.

It is also interesting to note that the first published study done on the beneficial effect of red light on ATP production was published about 3 months before the first working laser was published (Gordon and Surrey 1960) (and about 7 years after the dawn of LLLT). In the field of LLLT, an increase in ATP production following LLLT has been observed *in vitro* (Karu, Pyatibrat *et al*.

1995) and it was recently found that LLLT was able to delay skeletal muscle fatigue in an *in vivo* model (Lopes-Martins, Marcos *et al*. 2006). These results would be consistent with an increase ATP flux model. Furthermore another recent study demonstrated the protective effect of LLLT after irradiating neurons poisoned with cyanide (Wong-Riley, Liang *et al*. 2005). It was found that neurons treated with cyanide showed increased viability after receiving LLLT. Since the emission spectrum of their lasers coincide with the absorption spectrum of cytochrome c, it was hypothesised that this increase in cell viability was induced by an increase in cellular respiration to compensate for cyanide poisoning.

Cellular death usually takes on the form of either apoptosis or necrosis (another "type" of cell death is autophagy but is not relevant to this thesis). Apoptosis, also known as programmed cell death (PCD), is, as the name implies, a well orchestrated process which requires energy (ATP). Its hall mark is the "neat-and-tidy" death of a cell in which cellular components are "packaged" in membrane vesicles and "tagged" by phosphatidylserine (Li, Sarkisian *et al*. 2003) for non-inflammatory phagocytosis (Majno and Joris 1995). Necrosis on the other hand is well known to induce inflammation by the spilling of obnoxious cellular debris (Majno and Joris 1995). Thus, an increase in ATP production might facilitate a speedy resolution of an inflammatory response by facilitating apoptotic cell death.

As the cellular utility of ATP is truly immense, any modulating effect on ATP production could induce profound effects. As ATP is of such importance, an increase in ATP could greatly benefit cellular wellbeing and possibly make cells more resistant to homeostatic challenges. Alternatively, it could also facilitate a shift in cellular death mechanism from necrotic to apoptotic which might attenuate subsequent inflammatory damage in an injury setting.

1.5.6.3 Increased reactive species production?

Reactive species (RS), including reactive oxygen species (ROS) have been implicated in a host of cellular responses, ranging from inflammation to cellular proliferation. The cellular response to ROS seems to be mostly dependant on the dose, where a low, transient increase seems to be more beneficial toward "cellular wellbeing" (Thannickal and Fanburg 2000; Hancock, Desikan *et al*.

2001; Pantano, Reynaert *et al*. 2006). These effects are also influenced by cell type and probably, the signalling context and cellular micro-environment. It is also believed that the effects of LLLT might be mediate through radical signalling mechanisms. These mechanisms as well as evidence pertaining to the role of LLLT in this ROS signalling induction will be briefly discussed.

1.5.6.4 ROS as a signalling molecule

It is no coincidence that ROS evolved as a signalling molecule. By virtue of its chemistry, is an excellent cell signalling molecule, able to have short quick responses which can just as quickly be removed. H_2O_2, not being a free radical, is relatively stable with a half life of about 1 ms as compared to $O_2^{-}\cdot$ with a half life of about 1 μs (Reth 2002). Also, since the molecule is not charged, it is one of the few reactive species that will readily diffuse across membranes.

H_2O_2 also demonstrates a level of specificity, mostly targeting cysteine containing proteins (more specifically, cysteine thiolates) (Forman, Fukuto *et al*. 2004). Under physiological conditions, cysteine can take on the oxidative state as disulfide (-S-S) or sulfenic acid (-SOH). Since most cysteine amino acids have a pKa of about ~8.5, they are protonated. However, if the cysteine residue is in close proximity to a positively charged residential amino acid, the pKa can be dropped and the cysteine takes on its thiolate anion (-S-) form, rendering itself susceptible towards oxidation by hydrogen peroxide (Reth 2002). These chemical aspects insure a level of signalling specificity for H_2O_2 induced signal transduction.

Finally ROS lies central to energy production and can relay important information regarding the state of cellular metabolism and ATP production. Thus, ROS acts as the principal signal transduction molecule in mitochondrial retrograde cell signalling. In a sense, ROS acts as a metabolic “trip-switch”, capable of influencing multiple signalling cascades in orchestration with other cellular signalling events.

1.5.6.5 ROS signal transduction

ROS, as well as the change in cellular redox state, have been shown to modulate cell responses in numerous models. Interestingly, a host of

cytokines and growth factors have been found to increase cellular ROS production (reviewed by (Finkel 2000)). ROS also have a multitude of downstream signalling targets and effectors. As an example, NF-κB, a transcription factor influencing gene expression of over a 100 proteins, has been found to be sensitive to cellular redox state and ROS production (reviewed by (Pantano, Reynaert *et al*. 2006)).

In an insightful study recently performed on melanoma cells using an *in-situ* photo-generation bacteriochlorophyll-based sensitizer, the effect of intrinsically produce ROS was monitored. Controlling the level of ROS production by modulating the dose of light, ROS was observed to dose dependently modulate p38 and its cellular distribution in a phosphorylated state.

Other cellular signalling responses were also investigated by monitoring the behaviour of mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and Akt (Posen, Kalchenko *et al*. 2005). A dose dependant reaction was observed, where vigorous generation of ROS seemed detrimental and mild dosage beneficial.

Extreme proliferation via radical induction has also been shown to take place independent of JNK or MAPK. By stably transforming fibroblasts with a constitutively active isoform of p21 (Ras), H-RasV12, it was shown that large amount of superoxide (·O2-) was produced. This radical production coincided with intense proliferation which could subsequently be abolished by the addition of antioxidant N-acetyl-L-cysteine (NAC). It was also found that MAPK activity decreased while JNK remained inactive in the H-RasV12-transformed cells, indicating that these responses took place independent of JNK/MAPK activity (Irani, Xia *et al*. 1997).

In order to maintain proper homeostasis, signals must be able to be “turned-on and off”. One such family of molecules responsible for “turning off” activated signals are protein phosphatase (PTP). These enzymes are, as the name indicates, capable of de-phosphorylating molecules. By such action, low molecular weight phosphotyrosine protein phosphatase (LMW-PTP) are capable of inactivating the receptors of platelet-derived growth factor (PDGF) (Berti, Rigacci *et al*. 1994) and insulin (Chiarugi, Cirri *et al*. 1997). LMW-PTP

is known to be reversibly inhibited by H_2O_2-induced oxidation of two amino acids (Cys-12 and Cys-17) in its catalytic pocket (Caselli, Marzocchini *et al*. 1998). It thus follows that an increase in H_2O_2 could effectively prolong a PDGF and insulin mediated effect by increasing the half-life of their phosphorylated (activated) state. This same example of redox signalling have more recently also been observed in other PTP's (Kamata, Shibukawa *et al*. 2000; Lee and Esselman 2002). Considering the important role that phosphorylation plays in cell signalling, these redox sensitive manipulations of receptor phosphorylation could have a drastic impact on cellular metabolism. Also, since H_2O_2 is dominantly derived from mitochondrial activity, it also represents a mitochondrial retrograde signalling pathway.

1.5.6.6 ROS and the mitochondria

Some reactive species are too reactive and unstable to either diffuse far or exhibit reasonable specificity (both essential characteristics of signalling molecules). In general, it is difficult to elucidate the type of ROS being produced given that these reactive molecules are quickly transformed. In the mitochondria, superoxide ($\bullet O_2^-$) is quickly dismutased to hydrogen peroxide (H_2O_2), a more stable molecule able to defuse across the inner mitochondrial membrane.

Central to the *in-vivo* function of ROS are mitochondrial respiration. The electron transport chain (ETC) "leaks" reduced oxygen, namely superoxide, which under normal conditions undergoes rapid conversion to hydrogen peroxide, in a reaction catalysed by superoxide dismutase (SOD), an enzyme found in both mitochondria and cytosol. H_2O_2 thus represents the major radical derivative of mitochondrial ROS production found in the cytosol of the cell.

Mitochondrial activity has been shown to be necessary in ROS signalling in cardiac myocytes. During short-term exposure to hydrogen peroxide, the ERK/MAPKs signalling cascades were activated, but subsequently loss this activation function after inhibition of complex III of the ETC by administrating myxothiazol (Bogoyevitch, Ng *et al*. 2000). Similarly, mitochondrial ROS have also been shown to activate c-Jun N-terminal kinases (JNK) via increased release of mitochondrial H_2O_2 (Bogoyevitch, Ng *et al*. 2000).

A fascinating study by Hughes *et al*. 2005 (Hughes, Murphy *et al*. 2005) made use of a mitochondria specific antioxidant (Mito Vit E) in order to explore specifically the role of mitochondrial ROS in apoptotic signal transduction. Administration of Mito Vit E enhanced TNF-induced apoptosis in U937 cell. It was found that under normal conditions following TNF stimulation, maximal binding of NF-κB to transcription motifs was induced within 15 min.

Treating cells with Mito Vit E (and thus depleting ROS of mitochondrial origin) resulted in a substantial delay, only reaching maximum DNA-binding activity 60 min after TNF treatment. The researchers concluded that ROS are produced in the mitochondria in response to TNF treatment, and, that by scavenging these mitochondrial ROS, NF-κB activation was down regulated. NF-κB, in turn, can no longer bind as efficiently to transcription motifs of anti-apoptotic genes, thus resulting in a defective anti-apoptotic signal transduction.

1.5.6.7 Reactive Species - mediators of LLLT?

Reactive species have also long been considered a possible agent for mediating signal transduction events induced by LLLT (Karu 1989) and many studies have provided support for photochemical induction of ROS, believed to be mostly mediated through changes in mitochondrial systems (Karu, Pyatibrat *et al*. 1987 ; Callaghan, Riordan *et al*. 1996; Grossman, Schneid *et al*. 1998; Alexandratou, Yova *et al*. 2002)

Recently it was found that LLLT (here using a 904 nm, 45 mW laser with a dose of 5 J/cm^2) modulated the inflammatory response by attenuating ROS production and inhibiting NF-κB activation (Rizzi, Mauriz *et al*. 2006). There was also a reduction observed in the activity of iNOS. The author concluded that LLLT reduced oxidative stress, thereby down regulate NF-κB activation and thus inhibited NO production (via reduced iNOS expression).

These results are difficult to interpret and the conclusion drawn from these results should be done with caution. For one thing, LLLT need not have any direct effect on ROS production to observe these results. As a hypothetical possibility, LLLT could have promoted an increase flux of electron and thus increase ATP production (similarly, as will be discussed below, LLLT could

have disassociated NO from cytochrome c, leading to an increase in ATP production).

With more energy, cells could engage in apoptosis instead of necrosis which would dramatically reduce inflammatory response. With a decreased inflammatory response, the subsequent activation of NF-κB and iNOS production would not take place -not because of any direct effect of LLLT on them, but simply because the signal to invoke an immune reaction was down played by a shift in cellular death from necrosis to apoptosis.

1.5.6.8 Local transient thermal effect?

Part of the energy passed on to an electron when absorbing a photon is converted to heat. This causes a local increase in the heat at the point of the chromophore. It has been hypothesized that this localised, transient increase in temperature could result in the conformational change of some enzymes (Letokhov 1991). This might either activate or inactivate them and thus influence metabolism.

1.5.6.9 Antimicrobial effect?

Acne vulgaris (or simply acne) is usually treated with topical bactericidal agents or antibiotics as well as hormonal treatment to control sebum secretion. It has also been found to be successfully treated with intense blue light (wavelength 407–420 nm)(Kawada, Aragane *et al*. 2002; Ashkenazi, Malik *et al*. 2003). Coproporphyrin III (a porphyrin) produced within the acne causing bacterium *P. acnes* has been found to produce free radicals when irradiated by light at the near UV spectrum (<420nm) (Kjeldstad 1984; Nouri and Villafradez-Diaz 2005). It is believed that this increase in reactive species ultimately cause cell death by means of excessive oxidative stress and so reduce the incidence of acne. Interestingly, superior results were obtained when used in conjunction with red light (Papageorgiou, Katsambas *et al*. 2000; Charakida, Seaton *et al*. 2004; Elman and Lebzelter 2004; Goldberg and Russell 2006). It is rather tempting to speculate on the possible mechanistic nature of this synergism between blue and red light. While blue light might actually kill the bacterium via the photochemical induction of radicals, the red light might ease an inflammatory response or simply incite

epithelial cell rejuvenation, facilitate deposition of protein matrix forming the basal lamina or by some other means.

1.5.6.10 NO inhibition of cytochrome c

Nitric oxide is chemically rather peculiar compound, and physiologically of great importance, so much so that it was named molecule of the year in 1992 (Koshland 1993). It has a single unpaired electron on the nitrogen atom which affords NO it's signalling and functional specificity to bind to iron in haem-groups. NO has been shown to increase fibroblast proliferation (Du, Islam *et al*. 1997) as well as be involved in satellite cell activation (Anderson 2000).

Recently, using a new bioluminescence imaging technique, it has been shown that LLLT decreases the number of inflammatory cells, whilst simultaneously increasing the expression of iNOS (Moriyama, Moriyama *et al*. 2005). Furthermore, NO has been shown to play a possible role in the induction of apoptosis via mitochondrial retrograde cell signalling (Brown and Borutaite 2002; Moncada and Erusalimsky 2002; Brune 2003). Adding conviction to this view is the discovery of a unique mitochondrial nitric-oxide synthase (mtNOS) (Elfering, Sarkela *et al*. 2002). Also, the ability of NO to induce mitochondrial biogenesis is also well established (Clementi and Nisoli 2005).

Nitric oxide's capacity to inhibit cytochrome c oxidase has been well known for more than a decade (Brown and Cooper 1994; Cleeter, Cooper *et al*. 1994) and its ability to reversibly attenuate mitochondrial respiration has since been verified (Solien, Haynes *et al*. 2005). Both oxygen and NO compete for the same binding site, resulting in the reversible inhibition of the ETC by NO. Because of this reversibility, the inhibition of mitochondrial respiration is postulated to be a physiological mechanism by which cellular energy production can be modulated (Brown and Borutaite 2002; Moncada and Erusalimsky 2002).

Since this process is reversible, the amount of oxygen available in the system under investigation should be kept in mind when considering the inhibitory effect of NO on the ETC (Solien, Haynes *et al*. 2005; Mason, Nicholls *et al*. 2006). Most *in vitro* studies done on isolated mitochondria are performed at un-physiologically high levels oxygen saturation (Solien, Haynes *et al*. 2005).

Indeed, studies done in a more physiological range have shown that the inhibitory effect of NO can be amplified under hypoxic conditions (Solien, Haynes *et al*. 2005; Mason, Nicholls *et al*. 2006).

This is of great interest in muscle injury, especially in a contusion injury model. As mentioned, ruptured arteries and the hematoma that arise reduce the amount of oxygenated blood reaching the cells in the surrounding contusion injury. One would naturally expect the micro-environment of these cells to be hypoxic in nature and would thus lead to a greater susceptibility of cytochrome c inhibition by NO competition. As mentioned, neutrophils, macrophages (Salvemini, de Nucci *et al*. 1989; Roitti and Rabson 2000), endothelial cells (Buga, Gold *et al*. 1991) and mast cells (Salvemini, Masini *et al*. 1990) are capable of releasing NO during an inflammatory response, making the inhibition of ETC more likely.

Finally, NO can also lead to the irreversible inhibition of cytochrome c. This irreversible inhibition is believed to be mediated through the conversion of NO to peroxynitrite (via radical interaction) and the subsequent damaging effect of the peroxynitrite on the respiratory machinery (Sharpe and Cooper 1998; Mason, Nicholls *et al*. 2006). It should be noted that the inhibition of the ETC by NO might increase the leakage of superoxide from within the mitochondria, a process which might allow the superoxide to interact with NO to form peroxynitrite.

It has long been known that light can cause NO to dissociate from cytochrome c oxidase (Boelens, Wever *et al*. 1983), but only at low temperatures (less than -223 ˚C). Of special interest is the more recent finding that relatively low levels of light under physiological temperature (37˚C) appear to be able to reverse the inhibitory effect of NO on respiration (also note, this study was not performed in a LLLT context) (Borutaite, Budriunaite *et al*. 2000). Of note, the author did not systematically investigate the wavelength of the light source. It was found that a band pass filter with a maximum transmission at 425 nm (specifically, <390 nm and >480 nm exclusion width) was still effective at abolishing the NO-induced respiratory inhibition.

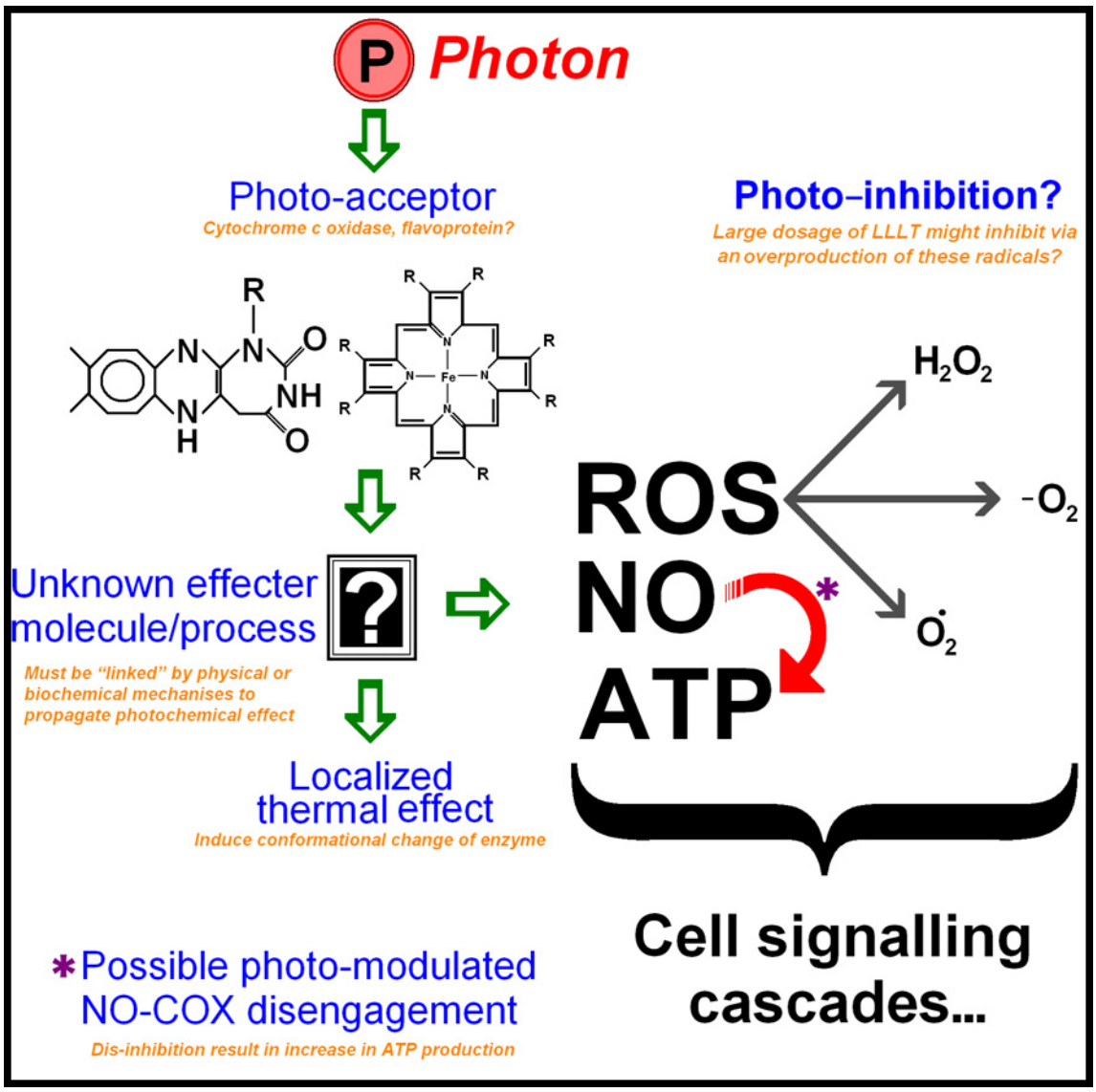


Figure 1.8 Short illustration of postulated primary and secondary mechanisms by which LLLT may exhort a photo-biostimulatory/inhibitory effect. Abbreviations: ROS- reactive oxygen species; COX- cytochrome c oxidase.

1.5.7 *Influence of muscle fibre type on LLLT*

The simple view that “a muscle is a muscle” simply is not true. In the previous section, some variation on fibre type and the physiological adaptation this tissue undergo was mentioned. This was not done purely out of academic interest, but also to voice the importance and the (somewhat neglected) need to accommodate tissue specific interaction with light.

Many mechanisms of action centre on the mitochondria and its associated chromophors. As mentioned, oxidative fibres tend to have a higher mitochondrial density. This immediately implies a possible increase in photo acceptor capacity of these cells and the accompanying increase in sensitivity towards photo-biostimulation. To date, not a single article has been produced investigating the effects of LLLT on different fibre types in skeletal muscle. Similarly, other structural and metabolic considerations are also neglected. The differences in vascularisation and satellite cell density (to name but a few) are not addressed in the literature with regard to LLLT.

1.6 Hypothesis

Muscle is one of the few tissue types that can substantially regenerate itself. Though muscle regenerative capacity is considerable, it is not always perfect (Plant, Beitzel *et al*. 2005). Furthermore, it is slow, and, especially in the context of professional athletes, augmenting the regeneration process is highly sought-after.

We hypothesise that the photo-biostimulatory effects of LLLT (at a wavelength of 638 nm) can incite cellular events involved in energy metabolism and cell viability/proliferation in skeletal muscle stem cells (satellite cells) both *in vitro* and *in vivo*. Specificly, it is hypothesised that these even

1.7 Aims

1. Establish an *in vitro* model of satellite cells in culture in order to evaluate the effects of LLLT, specifically measure LLLT's effects on cell viability and metabolic activity and investigate possible mechanisms of action of any effects seen.
2. Establish an *in-vivo* (Wistar rat) model of contusion injury to explore the effects of LLLT.

Chapter 2

Materials and Methods

2.1 C2C12 in vitro model

For the *in-vitro* part of this project, C2C12 cells, obtained from European Collection of Cell Culture (ECACC), were utilised. C2C12 cells are muscles progenitor cells, allowing us to explore certain aspects of muscle regeneration in this model. Furthermore, there already exists a robust protocol set up in our laboratory for growing and maintaining these cell cultures.

Cells were maintained in T75 flasks (Greiner, France) prior to experimental procedures. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, South Africa) supplemented with 10% foetal calf serum (Highveld Biologicals, South Africa) and 4 mM GibCo L-glutamine (Invitrogen, Carlsband, CA, USA). Cells were passaged at a confluence of 60% ~ 65%.

Cells between passage 8-12 were used for experiments. With the exception of the experimental treatment, all cells were maintained at 37°C, in a 5% CO_2 and 95% relative humidity incubator.

For experimental purposes all cells were maintained in a phenol red free DMEM supplemented as above (Higveld Biologicals, South Africa). To the best of our knowledge, the effect of phenol red in cell culture is negligible, although it has been found to be a weak agonist of the oestrogen receptor (Berthois, Katzenellenbogen *et al*. 1986). All cells used as control groups were also incubated in the phenol red free media.

2.1.1 Experimental Protocols

Experiments were performed in a 37°C incubator without any manipulation of humidity, CO_2 or O_2. Control groups were exposed to identical conditions for the same time interval as corresponding experimental groups. Laser power

output was checked prior to the commencement of the experiment using an Ophir laser power meter and a calibrated power head (Ophir, Israel). The arrangement of equipment used during irradiation is illustrated in Figure 2.1. Irradiation time was adjusted to ensure that all cells received equal "doses" (Table 2.1) of laser irradiation (60 mW). Power dosage was calculated as follows:

Laser power: 60 mW

Petri dish diameter: 60 mm = 6 cm

Petri dish area: $\pi d^2/4 = \pi \times (6cm)^2 /4$ (π = Pi)

= 28.27 cm^2

Power density on Petri dish = 60 mW/28.27 cm^2

= 2.12 mW/cm^2

W = J/s [Power (in Watt) = Energy (in Joules) / time (in seconds)]

Dosage for treatment is always reported on in J/cm^2

Dosage = Power density x irradiation time

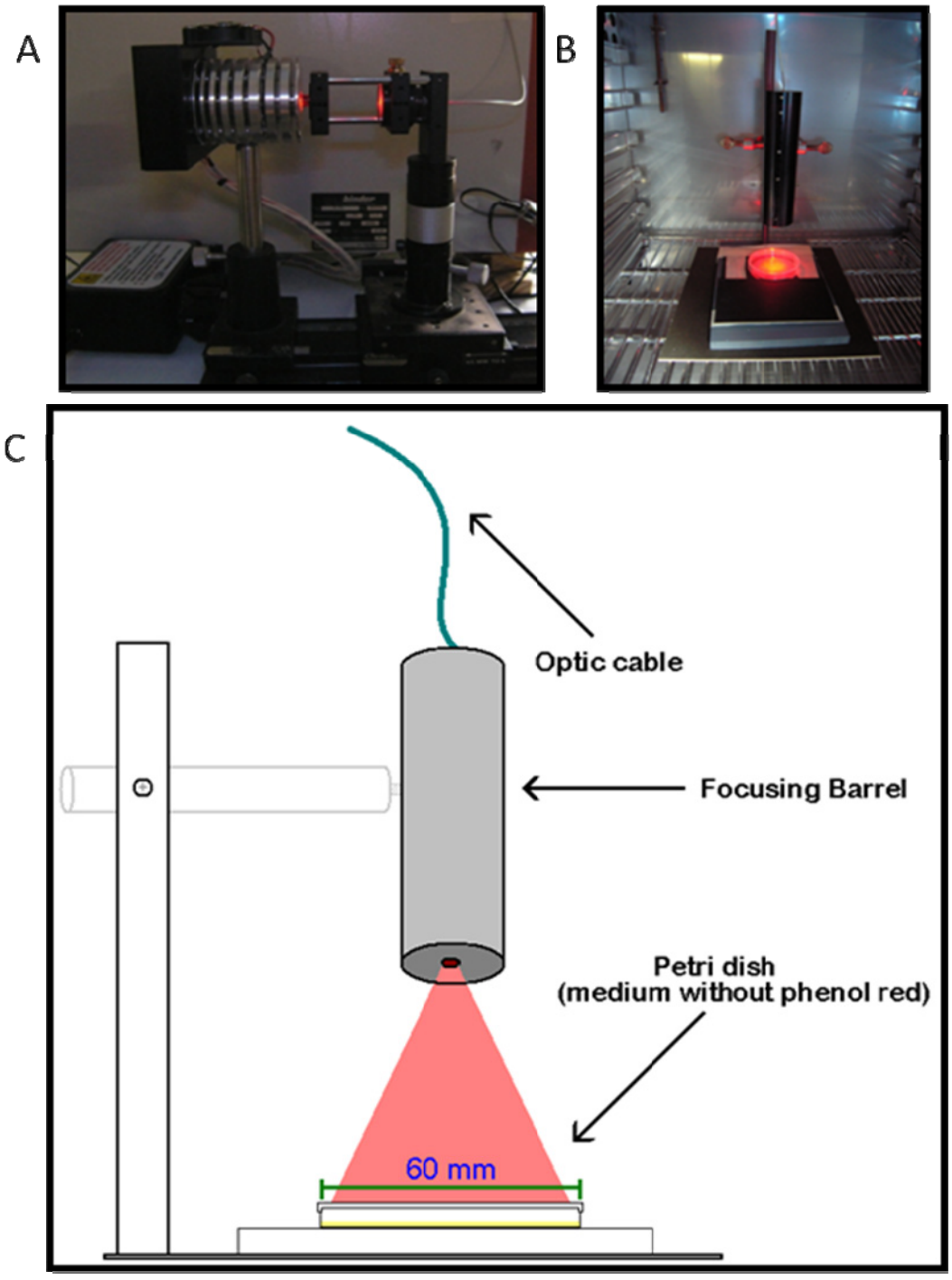


Figure 2.1. Diode laser focusing barrel inside modified incubator (A). The actual laser itself was mounted outside next to the experimental incubator (B). Schematic representation of irradiation rig inside modified incubator (C).

Table 2.1 Basic parameters of diode laser and dosage calculations

Irradiation time (min)	***Dose ($J\ cm^{-2}$)***
5	0.636
15	1.908
30	3.816

2.1.2 Cell Irradiation

Cells were grown up to appropriate confluency (~65%), trypsinized, counted and diluted in medium, to provide a seeding density of 1100 cell/cm^2 in 60/15 mm Petri dish (Greiner Bio One,) with 3 ml medium. The Seeded cells were left to adhere for 6 hours after which cells were irradiated for the required time at 37°C.

Thirty (30) minutes of irradiation as the optimal dose and as such, all experiments were performed accordingly (refer Chapter 3). After irradiation, cells were placed back into in environment controlled incubator and a MTT assay was performed 24 hours later.

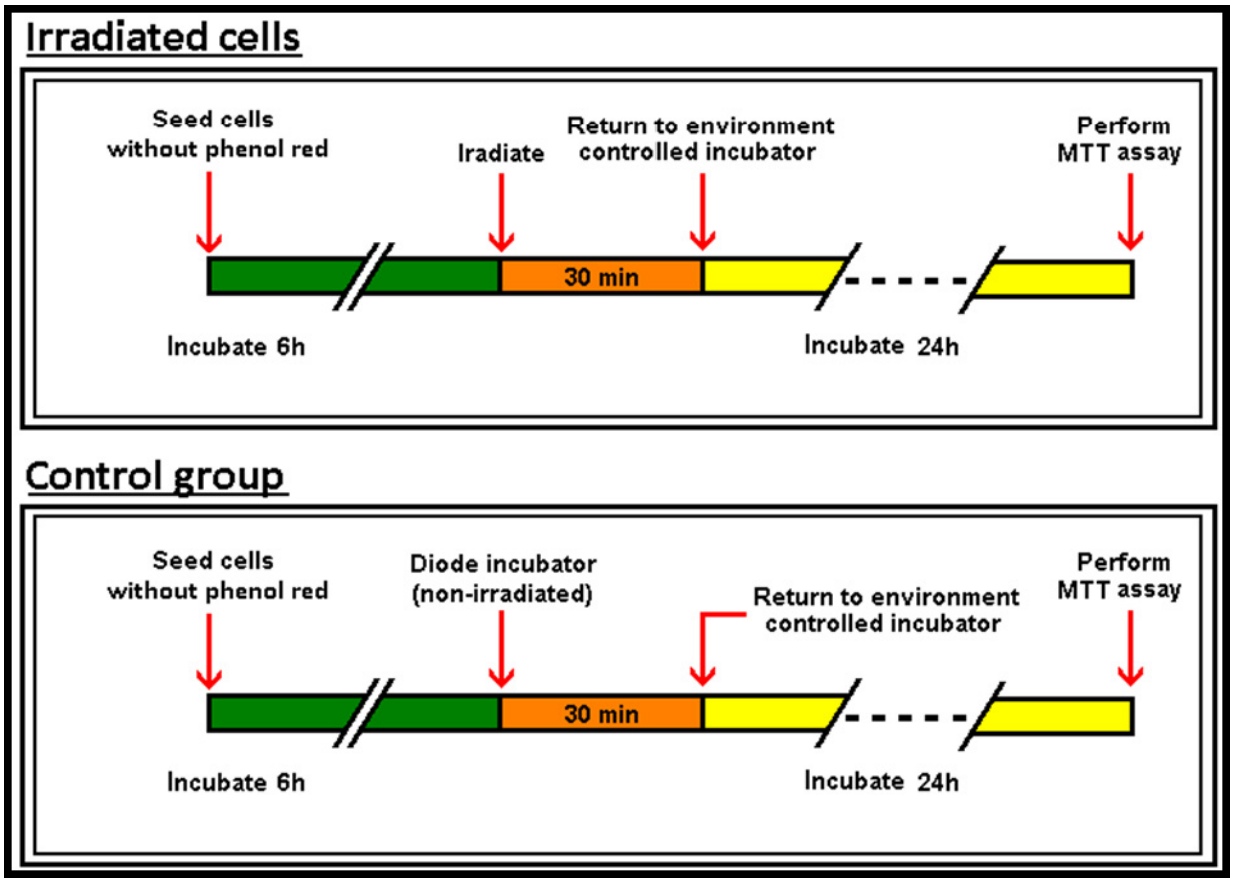


Figure 2.2 Basic outline of laser intervention on C2C12 cells.

2.1.3 *Effect of Antioxidants on irradiated cells*

To observe the effect of antioxidants on irradiated cells, two membrane-permeable antioxidants, namely N-Acetyl-Cysteine (NAC) and N-(2-mercaptopropionyl)-glycine (MPG) were used. The respective concentration (NAC: 30 µM and MPG 10 µM) were chosen as they have been previously shown to be effective at scavenging mitochondrial ROS (Lacerda, Smith *et al*. 2006).

Cells were grown up to appropriate confluency (~65%), trypsinized counted and diluted in medium to provide a seeding density of 1100 cell/cm^2 in 60 mm Petri dish (Greiner Bio One) with 3 ml medium. The intervention setup is outlined in Figure 2.3 and will briefly be discussed here. Seeded cells were left to adhere for 6 hours after which medium was aspirated and the cells washed three times with PBS before adding medium containing the antioxidant (either NAC or MPG). Control cells also had a media changed with new media as well as washing steps, but without any antioxidant supplementation in the media. Cells were left for a pre-irradiation incubation period of 15 min. Thereafter, cells were either irradiated for 30 minutes or incubated under the same conditions (control) for 30 minutes as previously described.

After the intervention session, the antioxidant media was aspirated and cells were gently washed with PBS (three times) and new media was added. Cells were again incubated for 24 hours before the MTT assay was performed.

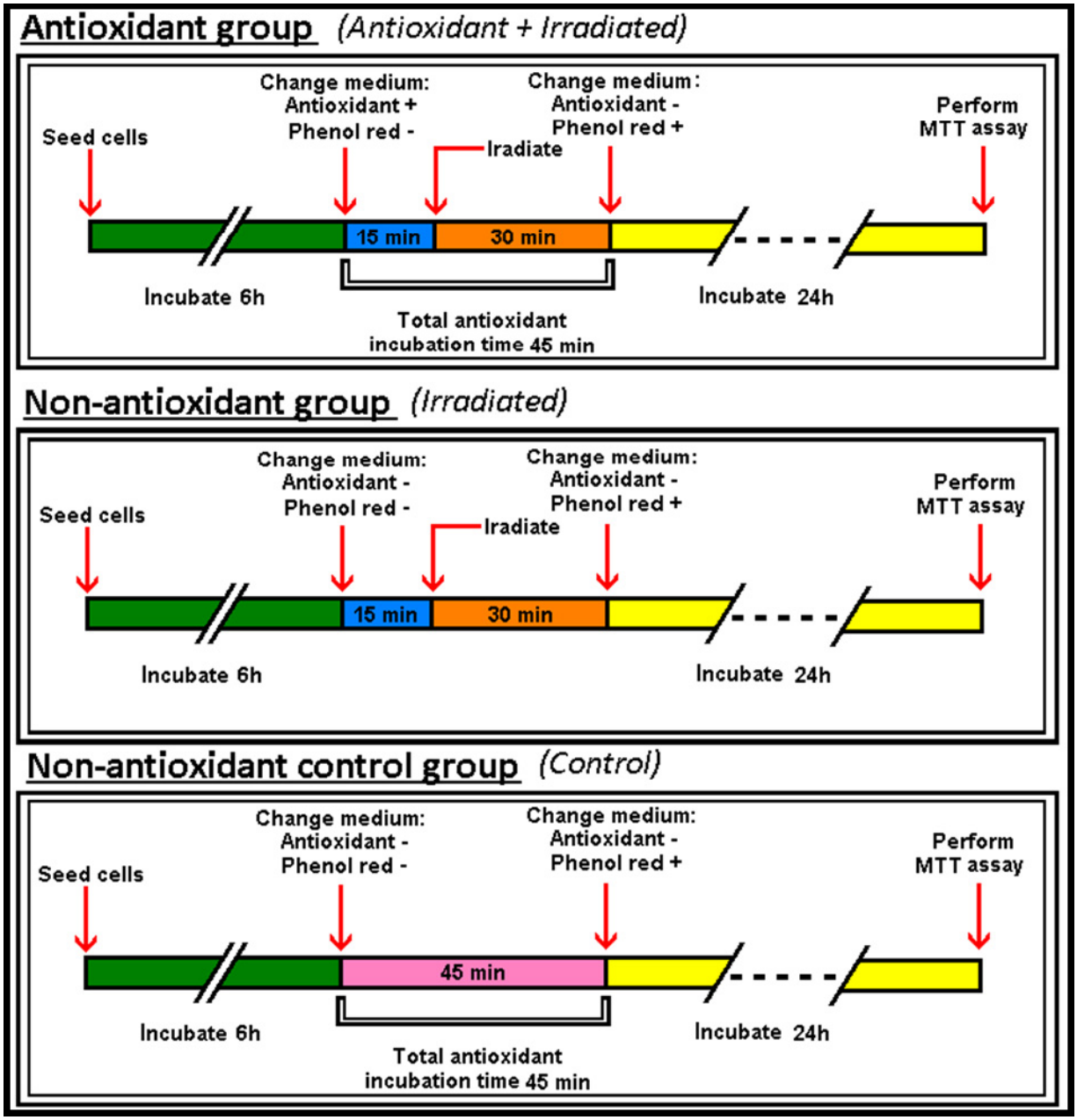


Figure 2.3 Basic outline of laser intervention on C2C12 cells treated with antioxidants. PhR+ and PhR– represent media change with (+) and without (-) phenol red in media respectively.

2.1.4 MTT assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is an indirect, high-throughput approach to evaluating cell viability/cell metabolism (Mosmann 1983; Janjic and Wollheim 1992). The assay depends on the ability of mitochondrial succinate dehydrogenase to reduce the MTT, thereby cleaving the tetrazolium rings forming blue-purple formazan crystals which are water insoluble and membrane impermeable. It should also be

noted that some formazan is also produced in cytosolic fractions of cells (Berridge and Tan 1993; Gonzalez and Tarloff 2001; Bernas and Dobrucki 2002).

As a result, viable cells (which, by implication, have uncompromised membrane integrity) will accumulate formazan. Dead cells will either not be able to produce formazan, or will have any formazan produced removed during decantation step. The cells are lysed by applying a detergent, releasing the formazan which is dissolved in an appropriate solvent (in this case, acidified isopropanol). The amount of formazan can then be calculated spectrophotometrically at an absorption maximum roughly between 500 and 600 nm (depending on solvent applied).

Since the assay depends on mitochondrial activity, it is use to deduce cell viability. Introducing different control groups allow the standardization of cell viability. By comparing MTT absorbance of control groups with corresponding intervention groups, a dose response curve can be deduced to evaluate the effect of different treatment regimes. Factors that might compromise MTT accuracy include cell volume, reagents in culture media as well as chemicals applied in intervention groups (Wang, Ge *et al*. 2006). Overall, the MTT assay has been show to be robust indicator of cell viability (or at the least cellular metabolism) (Mosmann 1983; Janjic and Wollheim 1992; Gonzalez and Tarloff 2001).

2.1.5 *MTT procedure*

Media was decanted and cells were washed with pre-warmed PBS (37°C) after which cells were incubated in 0.25% m/v MTT solution (MTT dissolved in PBS) for 2 hours in an incubator at 37˚C. The MTT solution was then decanted (if cells remained adhered to plate) and 1.5 ml solvent (0.1% Triton-X added to 1% isopropanol in a 1:51 ratio) is then added. Cells were left on a shaker for 5 minutes to ensure the formazan product is thoroughly dissolved. Dissolved formazan solution is then transferred to 2 ml opaque/lightproof tube and centrifuge (multispeed refrigerated centrifuge: ALC international SRL, Milano, Italy —model PK121R) for 2 min at 109,564 g. The absorbance of the

supernatant is then measured at 540 nm (blanked with solvent) in Varian 50 spectrophotometer.

2.1.6 *Effect of LLLT on Cell signalling events*

As previously described cells were handled, seeded and irradiated according to the normal irradiation protocol described earlier. At time intervals of 5 min, 15 min, 60 min, 24 and 48 hours post-irradiation, medium was decanted from the cells and they were then washed with ice cold PBS. Ice cold lysis buffer (RIPA buffer -see Addenda for chemical constituents) was added and cells were scraped manually. Cell debris in the RIPA buffer suspension was sonicated before freezing aliquots at -80ºC. Protein content was determent by Bradford technique (Bradford 1976).

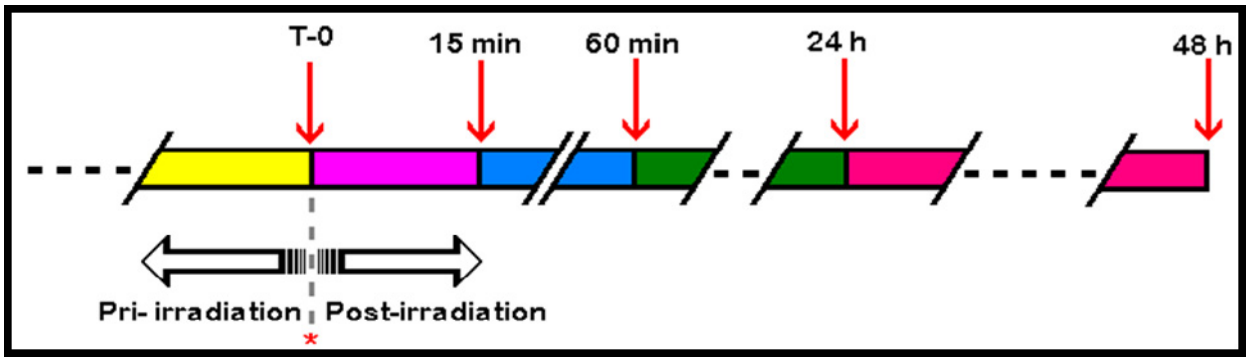


Figure 2.4 Basic outline of protein harvesting time points for Western blot analysis (irradiation procedure as described in Figure 2.1 was followed). Red arrows denote time points at which cells were sacrificed. T-0 (red asterisk) denotes time after irradiation.

2.1.6.1 Western blot analysis

Either 20 or 30 µg of whole cell protein was loaded onto a 10% polyacrylamide gel (with a 4% stacking gel to load samples) and separated according to molecular weight by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In order to assess band weight, a pre-stained protein ladder (Bio-Rad Laboratories, California, USA) was loaded onto each gel.

Gels were run at 200 V (400 mA) for 1 hour and subsequently transferred to a polyvinylidine fluoride (PVDF) membrane (Immobilon, Millipore, USA) using a semi-dry method (Bio-Rad Laboratories, California, USA) at 15V, 500 mA for 60 minutes (see Addenda).

Membranes were blocked in a 5% (w/v) Elite© non-fat milk powder prepared in Tris Buffered Saline-Tween 20 (TBS-T, 0.05% v/v) for at least 2 hours at room temperature. Membranes were washed 3 times in TBS-T prior to incubating with primary antibodies (1:1000, diluted in TBS-T) overnight at 4 ˚C. Following 5 washes (0.05% TBS-T), membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:1000 diluted in TBS-T) (anti-mouse: Dako Cytomation, Denmark) for 2 hours at room temperature.

After washing membranes, an enhanced chemiluminescence kit (Amersham Bioscience, UK) was used to detect protein-antibody localization on the membrane. Bands were visualized on x-ray film (Hyperfilm, Amersham Bioscience, UK) and quantified by densitometry with UN-SCAN-IT© (Silk Scientific, Utah, USA).

2.1.7 ***Effect of LLLT on antioxidant status***

There exist many seemingly conflicting reports on the role of LLLT on ROS production. Some studies claim LLLT to increase ROS production (Callaghan, Riordan *et al*. 1996; Grossman, Schneid *et al*. 1998; Kim, Pak *et al*. 2000) while other advocate an attenuating effect on ROS production (Vladimirov, Gorbatenkova *et al*. 1988; Berki, Nemeth *et al*. 1991; Potapov, Trepilets *et al*. 1995; Fujimaki, Shimoyama *et al*. 2003). The basic mechanism and chemistry of the ORAC assay itself is described in the Chapter 4 of this thesis. Here the procedure followed in ORAC assay will be briefly discussed.

2.1.7.1 Reagents preparation of ORAC

Standards were made up fresh for each reaction from aliquot Trolox stock solutions. A standard curve was prepared by making a dilution range with 75 mM phosphate buffer (pH 7.4) at according dilution points: 200, 100, 50, 25 and 12.5 µM.

Fresh 153 mM 2,2'-azobis(2-aminodinopropane)-dihydrochloride (AAPH) was made up for each reaction. 0.414 g AAPH was dissolved into 10 ml ice cold 75 mM phosphate buffer (pH 7.4) and kept on ice.

A 5×10^{-3} mM fluorescein stock solution was prepared and kept in a light protected container at 4 ◦C which, if stored as explained, is viable for several months (Huang, Ou *et al*. 2002). A fluorescein working solution (8.16×10^{-5} mM) was made up fresh for each run.

Cell samples as well as blood plasma were diluted ×75, while tissue were diluted ×200 (w/v) in 75 mM phosphate buffer (pH 7.4). Both cell and tissue sample were sonicated (Versonic 300; The Virtis Company, Inc; Gardiner, N.Y;seting 3). Tissue samples were spun down via centrifugation for 30 min, 8853 g at 4ºC (multispeed refrigerated centrifuge: ALC international SRL, Milano, Italy —model PK121R). Supernatant was immediately used for ORAC assay. Protein content of snap-frozen samples as well as cell lysate were measured by Bradford technique (Bradford 1976).

Basic plate loading "architecture" is described in Chapter 4. A 96 well black plate was used, but only 48 (half the plate) was employed in each run. Samples were run in triplicate. Into each well 25 µl of sample standard or blank (phosphate buffer) were added. With the aid of a multi-pipette, 150 µl fluorescein working solution (8.16×10^{-5} mM) was added. The plate was then incubated at 37◦C inside the temperature controlled plate reader for 10 minutes before adding 25 µl AAPH. Fluorescent intensity was logged at one minute intervals for 180 minutes (thus, 180 readings).

2.1.7.2 ORAC calculations

By plotting the relative florescent intensity over time, a curve is generated from which the area under the curve (AUC) can be calculated, according to the formula: $AUC_{total} = 0.5 + f_1/f_0 + ... f_i/f_0 + ... + f_{179}/f_0 + f_{180}/f_0$

(Were f_0 representing initial fluorescence at time point zero, f_i after *i* amount of reading intervals)

To compensate for any background fluorescent signals produce by scattering, reflection or nonspecific excitation, the value obtained from a blank was subtracted from each sample or Trolox standard:

Net AUC = ($AUC_{sample\ or\ Trolox} - AUC_{blank}$)

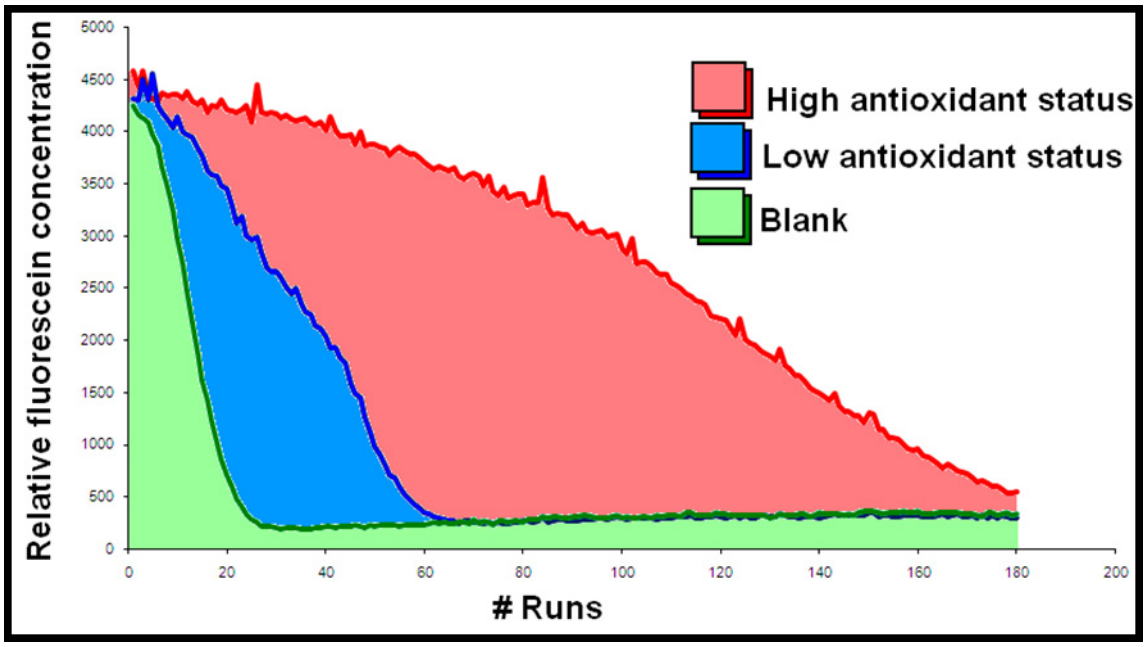


Figure 2.5 Typical ORAC curves. If Blue sample represent a blank, Red's net AUC would be obtained by (value of Red – value of Blue). Applying the same formula for Green sample (value or Green – value of Blue) would produce Greens sample's net AUC. Calculating the net AUC, antioxidant status of different samples can be compared. Graphs of samples with lower antioxidant capacity would have a steeper decay profile, resulting in a smaller AUC.

The Trolox standard AUC was plotted against a corresponding Trolox concentration to produce a regression curve. The equation of this curve was used to express ORAC results as Trolox equivalent units: Substituting net AUC value into X produce Trolox equivalent value expressed as µmol Trolox per litter of sample. Homogenized muscle was expressed as Trolox per litter of sample per µg protein (see Addenda). Finally, all sample values were multiplied by the dilution factor.

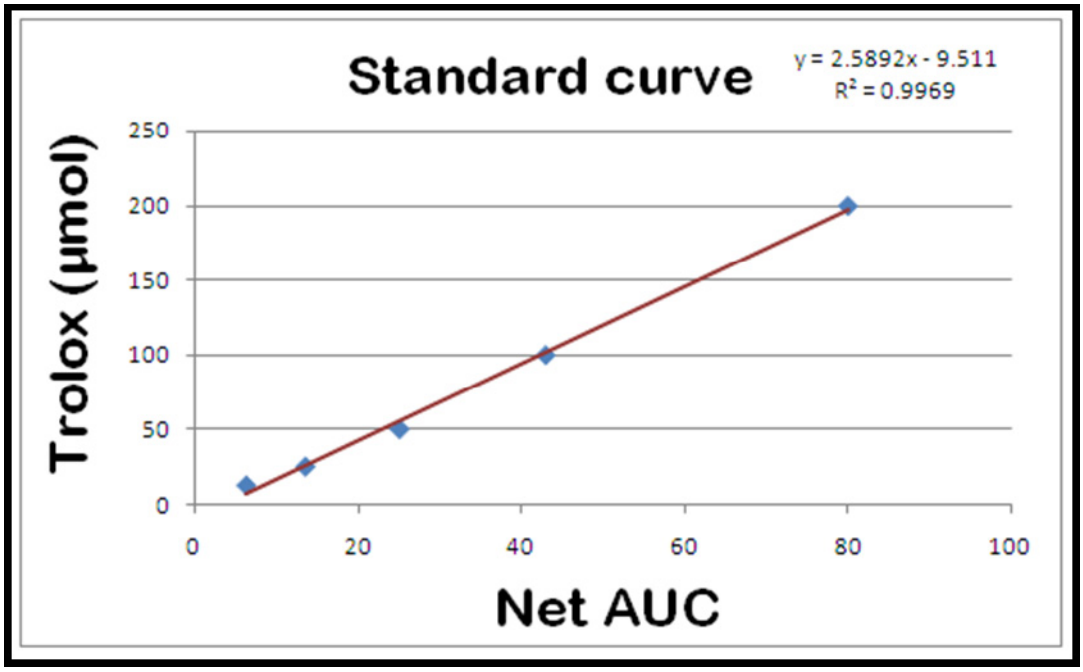


Figure 2.6 Antioxidant (trolox) standard curve of respective sample concentrations plotted against Net area under the curve (AUC). Because of the large gap between 200 µmol and 100 µm, samples were inspected for linearity by omitting the 200 µmol concentration interval.

The exact same protocol as in the previous irradiation studies was followed. Cells were harvested at the same time points as the Western blot protocol to investigate different possible alterations in cellular redox state over time. Cells were scraped in ice cold PBS.

2.2 *In vivo* experimental model

All procedures on experimental animals were performed according to the guidelines set forth by the Animal Ethics Committee (Sub-Committee B) of Stellenbosch University. All animals were provided and housed by the Small Animal Unit at the Departments Physiological Science at University of Stellenbosch.

Our department has a well established protocol for the handling and processing of Wistar rodents. Wistar generally have a good temperament and are easily handled. Furthermore, for the purpose of intervention (contusion injury and laser irradiation) Wistar rats were of optimal size. Male Wistar rats were used in this study.

Animals were randomly allocated into groups of four rats per cage. Temperature was kept constant a 21ºC with room ventilation rate of 10 changes/hour. A 12/12 light-dark cycle was maintained (light 6:30-18:30). Animals had access to food (standard rat chow) and water *ad libitum.* To allow the rats to get accustomed to the researcher, animals were handled daily and weighed three times a week. Mean weight was 371.03g ± 49.24 g at the start of the experimental protocol.

2.2.1 Experimental groups

As already mentioned animals randomly allocated into groups of four and were housed in standard cages (Techniplast, Italy). Animals exhibiting any signs of illness or injury were excluded from the study.

The animals were divided in to one of four groups:

- Injured control (In)
  - Rats only received contusion injury
- Irradiated control (Ir)
  - Rats only received irradiation session (30 min)
- Irradiated and Injured (Ir+In)
  - Contusion injury was inflicted, folowed by irradiation session (30 min) on the injured leg
- Sham (S)
  - No injury or irradiation, but rats were anesthetised and leg was shaven.

This basic group allocation was repeated to produce a corresponding time point on 4 hr, 24 hr, 48 hr and 72 hr, with each group consisting of 4 animals. Thus, four interventions groups each at four time frames with four animals in each group resulted in a total of 64 rats used in this study.

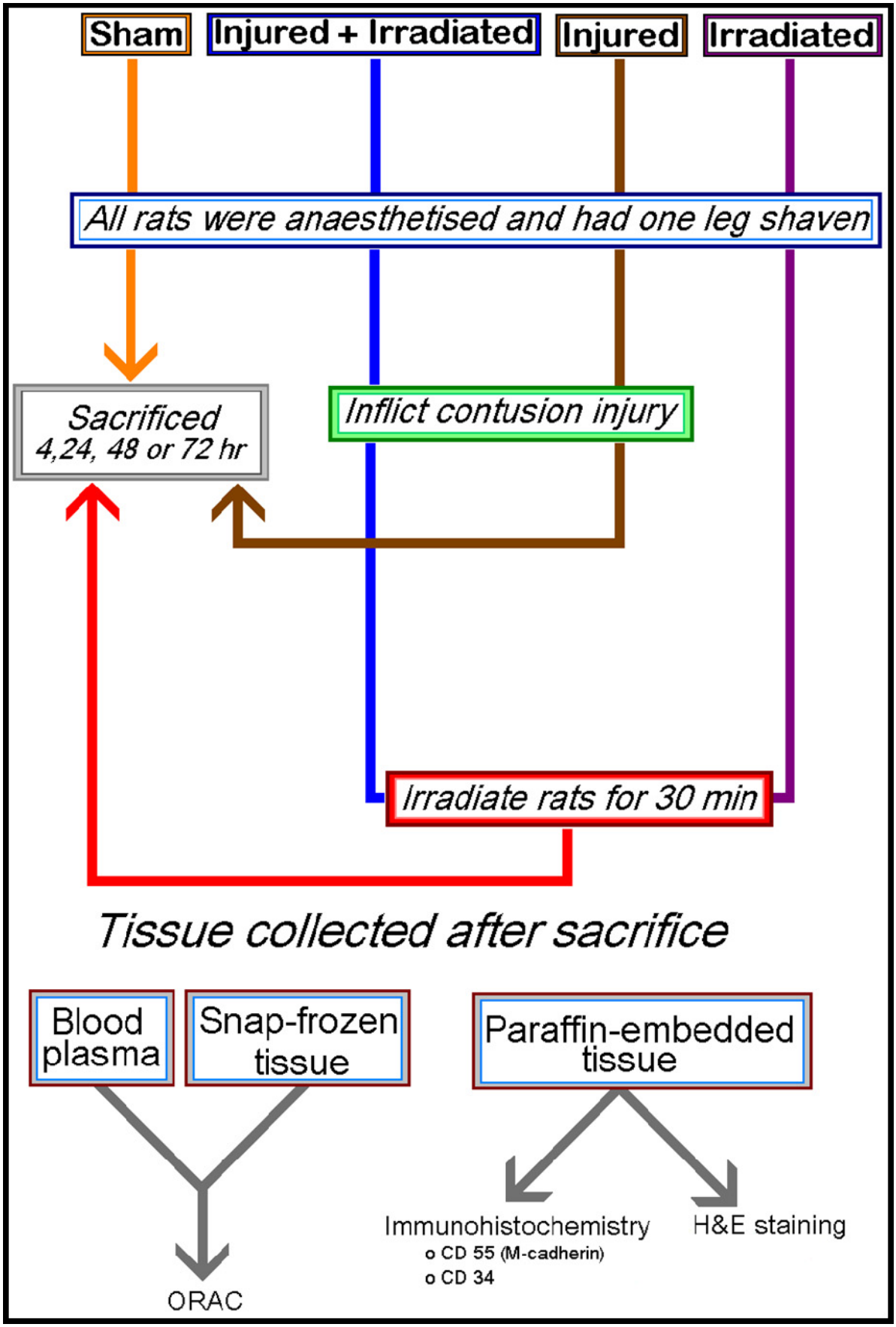


Figure 2.7 Basic outline of experimental group allocation, intervention and subsequent analysis.

2.2.2 Anaesthetics

Prior to any intervention (irradiation and/or contusion injury) animals were anaesthetised (75 mg/kg ketamine, 0.5 mg/kg medetomidine, *i.p.*). Whilst anaesthetised, one hind limb was shaved clean using an electric clipper.

2.2.3 Irradiation

Before intervention, laser power output was confirmed using an Ophir laser power meter fitted with a calibrated probe (Ophir, Israel). The temperature of the animal was maintained by placing the animal on paper towels and covering the body of the animal with bubble-wrap. The shaved limb was manoeuvred into position, supported by folded tissue paper, under the diode focus barrel. A flexible, thin sheet of cardboard with a circle cut out in the centre (diameter = 2 cm) was placed against the shaven leg to insure only the section of leg protruding from the circular gap was irradiated. The leg was irradiated for 30 min with a 60 mW diode laser, providing the following dosage:

Table 2.2 Dosage calculation for irradiated groups.

Irradiation time (min)	***Dose ($J\ cm^{-2}$)***
30	34.28

2.2.4 Injury

In collaboration with the Central Engineering Service, Stellenbosch University, a drop-weight system was designed to induce a contusion injury. The system allows one to vary the weight and height of a metal bar that runs in a guide to have an impact on the tissue placed underneath it, thus inducing a contusion injury. The system has been characterised in our department and the height and mass requirements previously determined. This system permit for a highly standardized contusion injury with a high degree of reproducibility. A schematic of the injury rig is shown in Figure 2.8.

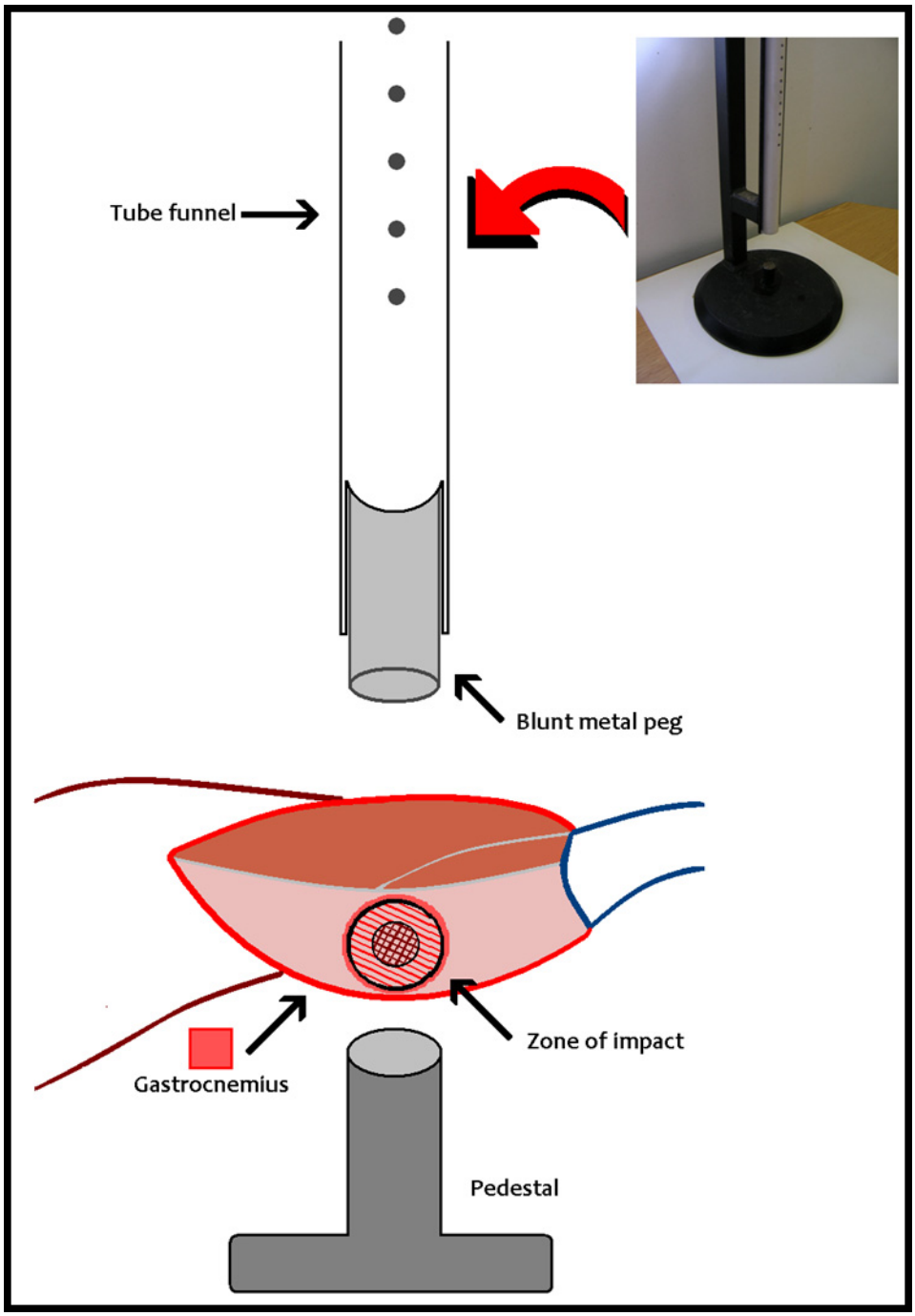


Figure 2.8 Basic setup of contusion injury rig. Illustrated is a diagrammatical representation of skinned rat leg, showing rudimentary anatomical landscape with *gastrocnemius* muscle indicated in light red.

2.2.5 Sample acquisition

Tissue was collected from animals at the following time points: 4 h, 24 h, 48 h and 72 h. Animals were euthanized by administering an overdose of Pentobarbitone sodium (200 mg/kg *i.p.*).

Blood was collected by cardiac puncture into the right ventricle through direct visualisation of the heart following opening of the chest, using a 20 gauge needle and a 5 ml plastic syringe. Blood was immediately placed into heparinised Vacutainers (Becton-Dickinson, South Africa), gently mixed and placed on ice. Rats were sacrificed in groups of 2 to ensure blood (as well as tissue) samples collected could be frozen as quickly as possible (sample collection was completed within 90 minutes).

After sample collection, the whole blood was centrifuged at 3000 g for 10 minutes at 4 ºC. Plasma was aliquotted into 1.5 ml tubes and stored in -80 ºC for analysis.

Directly after collecting blood, the *gastrocnemius* muscle was processed. Access to the clean muscle was gained by cutting through the pelt, fat and connective tissue with a sharp scissor. Afterwards the leg was skinned by pulling back on the loosened pelt. The muscle was removed by cutting through the tendons before being immersed into ice cold PBS saline to wash of any hair or debris. The muscle was cut with a scalpel till only the central median section remained.

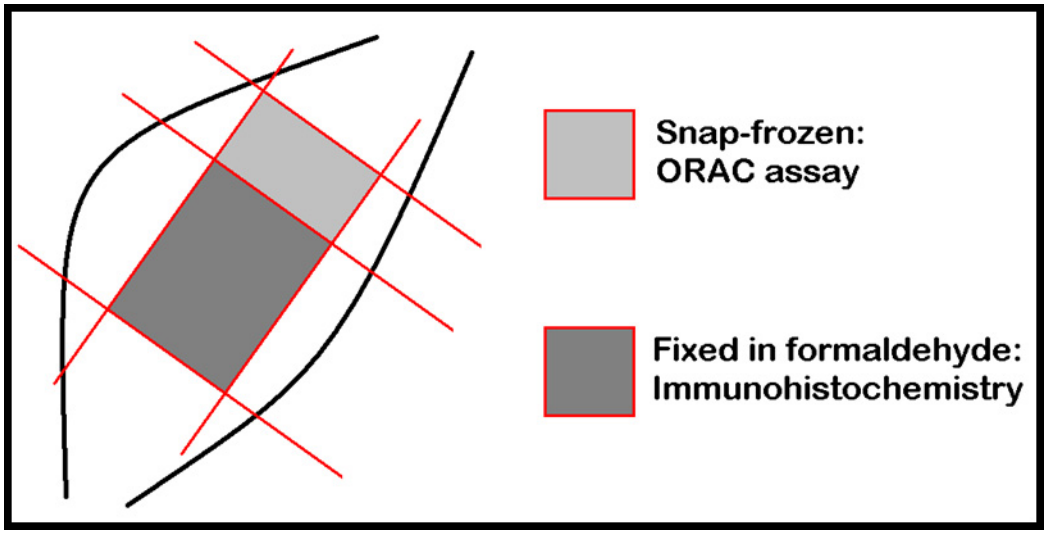


Figure 2.9 Diagrammatic representation of a *gastrocnemius* muscle. Red border indicate the excision boundaries of a typical biopsy. Dark-gray scale correspond to the section used for immunohistochemical analysis were as the light-gray scale represent section snap frozen in liquid N_2.

2.2.6 Histology

During biopsy, the excised *gastrocnemius* muscle is cut as indicated in Figure 18 and placed in a 10 % formaldehyde-saline solution for 72 hours at room temperature. It is placed afterwards into an embedding cassette before being positioned into the tissue processor (TISSUE TEK III, model 4640B, Lab-Tek division, Miles Laboratories Inc, Napervile, IL). The precise process is discussed in the Addenda. In brief, the formaldehyde fixed tissue is dehydrated, impregnated with paraffin wax (Histosec, Merck). Following this the sections are blocked in more paraffin wax, mounted and sectioned into 5 µm diameter slides on a rotary microtome (Reichert Jung, Heidelberg, Austria).

2.2.7 H&E staining

Haematoxylin and eosin progressive staining or simply, H&E staining was used for histological inspection contusion injury and the accompanying inflammatory response. The procedure is discussed in the Addenda. Haematoxylin stains basophilic structures (acidic cell components which most notably include nucleic acids in the cell nucleus and the endoplasmic reticulum) a blue-purplish colour. The fluorescent red dye, eosin is used as a counter stain which binds to basic cell components (e.g. proteins in cytoplasm and red blood cells).

The staining procedure is described in the Addenda. After staining, slides were air-dried and a cover slip mounted on the sections using mounting media. Sections were, and visualised at 200 and 400 x magnification on a Nikon Eclipse E400 microscope equipped with a Nikon DXM1200 colour camera. Photographs were taken of the main anatomical structure of the tissue, but no formal analysis was performed on H&E staining samples. These sections were used to investigate the extent and severity of the injury and to ensure that the injuries were consistent in all animals.

2.2.8 Immunohistochemistry

There currently exists no all-encompassing marker for satellite cells (Montarras, Morgan *et al*. 2005). However, CD 34, a glycoprotein functioning as a cell adhesion factor (Beauchamp, Heslop *et al*. 2000; Hwang, Yuk *et al*. 2004; Shi and Garry 2006) and N-CAM (CD56) (Crameri, Langberg *et al*. 2004; Sinanan, Hunt *et al*. 2004; Olsen, Aagaard *et al*. 2006) another glycoprotein involved in cell adhesion, have both been shown to be, at the very least, reasonable markers and are regularly applied (as observed in the literature). Satellite cells were thus co-stained with these two markers to increase the fidelity of our analysis. Nuclei was stained for with 4',6-diamidino-2-phenylindole (DAPI). Muscle fibres are easily identified by their structure and do not require any staining.

2.2.8.1 Slide preparation and staining

Each slide, containing ~4 cross sections, was dewaxed and rehydrated before being incubated in a 0.1 % Trypsin bath for 20 minutes at 37 ºC. The immunostaining procedure is stipulated in the Addenda, but will briefly be discussed here. At all time exposure to intense light was avoided as all fluorescent probes being used during staining are liable to photo-bleaching. Also, sections were never allowed to dry out and were kept in a humidified container. Sections on slides were encircled with a wax pen to isolate sections and prevent unnecessary spillage of applied reagents. Nonspecific binding sites were blocked by incubating the sections with 5% serum of animal in which secondary antibody was raised (in this case, donkey serum) for 30 minutes at room temperature before draining off serum.

Primary antibody (rabbit-anti-NCAM) was added and left at room temperature to incubate for 4 hours. The primary antibody was then washed off with 0.1 M PBS and sulforhodamine 101 acid chloride (Texas Red) conjugated secondary antibodies (donkey anti-rabbit) was added and incubated over night at 4ºC. The following day, sections were rinsed with PBS and incubated with a second primary antibody (mouse anti-CD34) for 40 minutes at room temperature. After rinsing the section, it was incubated for 30 min at room

temperature with the second secondary antibody (donkey anti-mouse) which was conjugated to fluorescein isothiocyanate (FITC). Directly afterwards (without rinsing of the previous antibody), the fluorescent stain 4',6-diamidino-2-phenylindole (DAPI) was added and incubated for 10 minutes. The slide was then rinsed in PBS and gently flicked to remove exess PBS before adding florescent mounting media (Dako, Diagnostech) and a cover-slip. PBS negative controls (PBS without primary antibody) were included to test the specificity of antibodies.

2.2.8.2 Image visualization

Sections were visualized under Nikon ECLIPSE E400 Fluorescence Microscope, equipped with a colour camera (Nikon DXM 1200) for photomicroscopy. Each section had six fields-of-view imaged and photographed randomly (injured muscles were photographed near the border zone of the injury). Pictures were taken using a 40× objective under all three excitation beams (by manipulating excitation wavelength filter) to visualize DAPI, FITC and Texas Red florescent-probes.

Table 2.3 Absorption-emission spectra of florescent probes.

	Absorption	Emission
DAPI:	358 nm	461 nm
FITC:	488 nm	520 nm
Texas Red:	595 nm	615 nm

Using the computer program *Simple PCI* (version 4.0, Compix Inc., Imaging Systems, USA), the separate images were overlaid to produce a multi-coloured image, containing the blue (DAPI), red (Texas Red) and green (FITC), allowing the images to be viewed as a single image. This allowed for scoring of satellite cells by counting cells co-stained for N-Cam (Texas Red), CD34 (FITC) and visible nuclei (DAPI). Muscle fibres were also counted and the final satellite cell score was stated as satellite cells per myofibers.

Scoring was done by inspecting overlaid photos taken of cells. Co-staining with a red and green probe produced a strong yellowish signal when overlaid, with a small nucleus outside the myofibrillar space (but slightly indented). In

case of multinucleated satellite cell “niches”, each nucleus was counted as a satellite cell.

2.2.9 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were analysed using one way analysis of variance (ANOVA) followed by the Dunns post-hoc test (nonparametric) or a Bonferroni post-hoc test (parametric). $p<0.05$ was considered significant.

Chapter 3

Results

3.1 *In vitro* results

Murine C2C12 cells grew well in our hands in the laboratory. Cells were utilized between passages 8 and 12. Cells which had become more than 70% confluent were not used in setting up experiments. Cell cultures were visually inspected for signs of infection or contamination. Figure 3.1 shows a representative photo of healthy cells in culture.

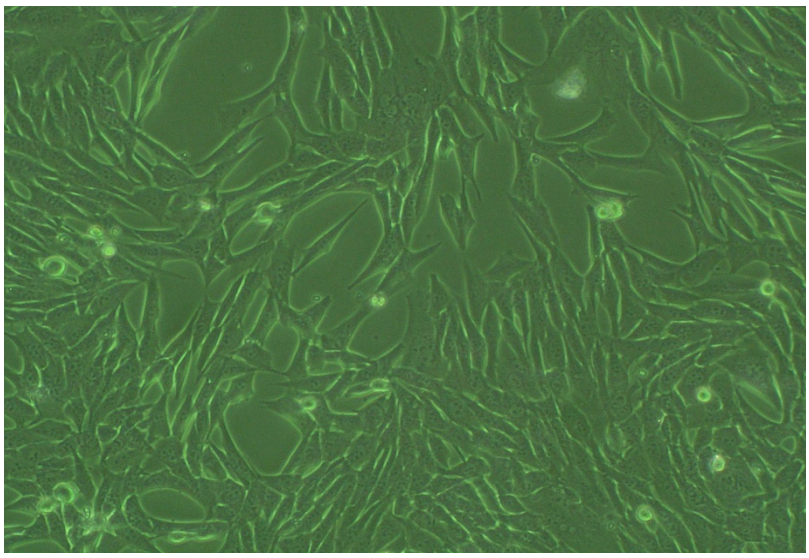


Figure 3.1. Representative image of murine C2C12 satellite cells in culture. Phase contrast image with a green filter (20 x objective).

3.1.1 MTT activity in irradiated satellite cells

The activity of the mitochondrial enzyme measured by the MTT assay increased in an irradiation-time-dependent manner, with a significant increase in MTT activity seen with 30 mins of 638 nm laser irradiation (196.8 ± 16.2% vs control values of 100%, n=6, p<0.001, see Figure 3.2). As a significant increase was seen at 30 mins (3.816 J cm^{-2}) this irradiation time was utilized in all subsequent experiments.

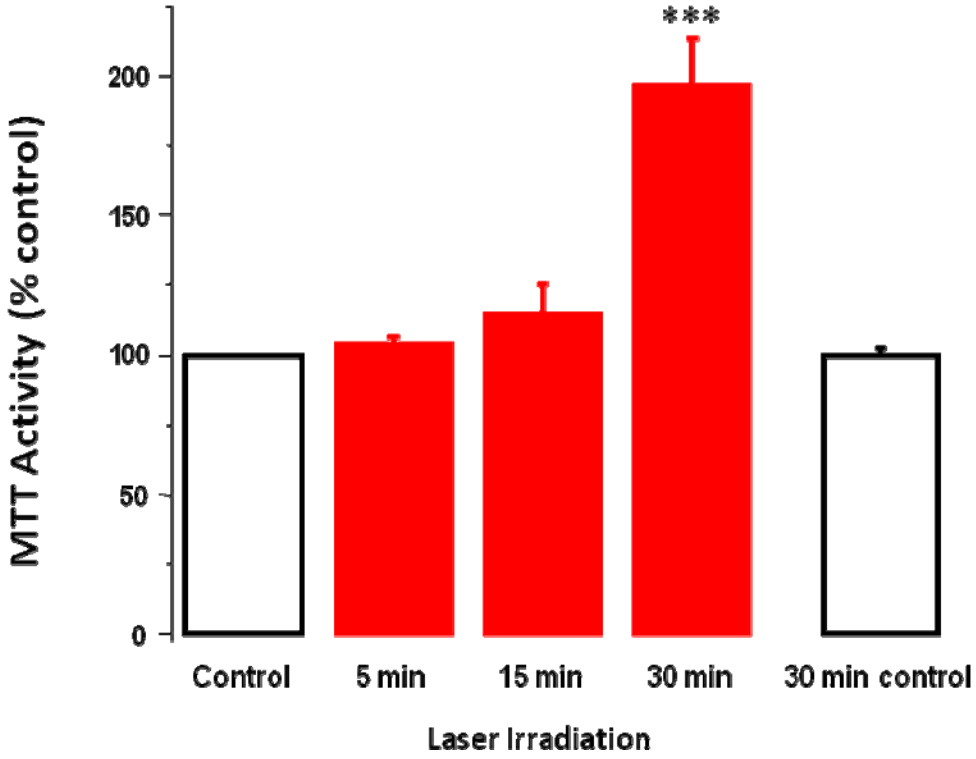


Figure 3.2 Effect of 5, 15 and 30 minute 638 nm laser irradiation on MTT conversion in murine C2C12 satellite cells. n=6-9, *** p<0.001 *vs* Control.

3.1.2 MTT-antioxidant study

Inclusion of the antioxidants MPG and NAC in the cells for 15 mins prior to irradiation resulted in a decrease in the MTT activity measured (to 114.6 ± 4.9% and 121.8 ± 3.3% respectively, normalized to control). The MTT activity was significantly decreased, when compared to 30 min laser irradiation, in the MPG treated group (p<0.05, n=9). However the result with NAC was not

significant when compared to the 30 min laser irradiated group (p>0.05, n=9). There was still a significant increase in MTT activity in the NAC treated group (p<0.05, n=9) when compared to the control group (see Figure 3.3).

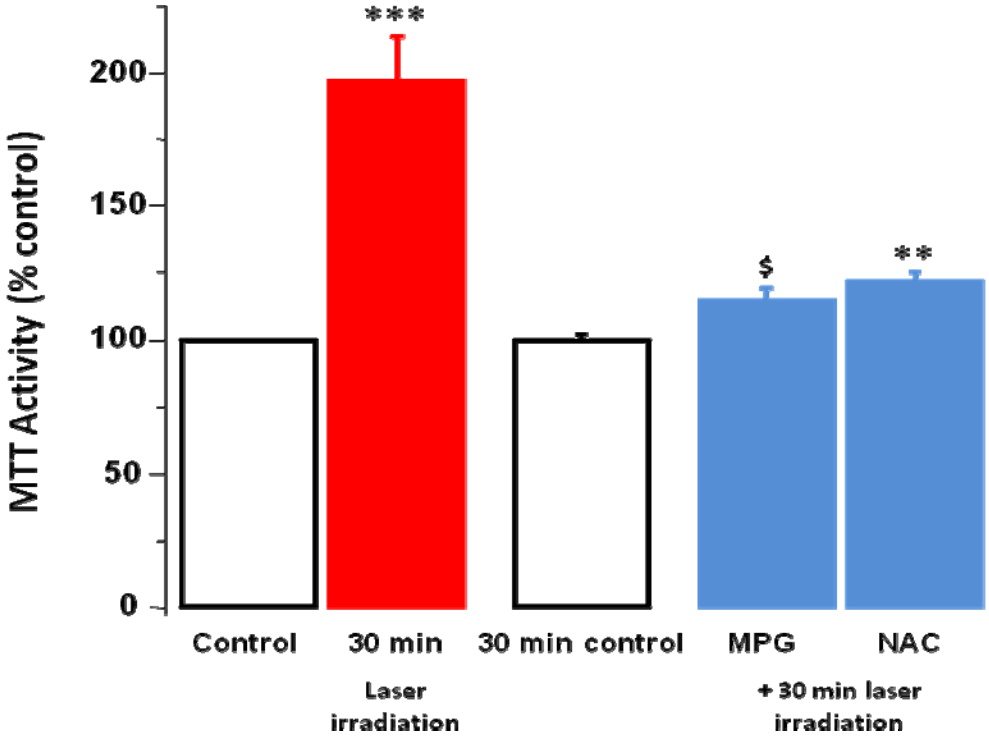


Figure 3.3 Comparative analysis of MTT values between irradiated but not antioxidant treated (laser/no-AOX) groups and two irradiated groups which have been treated with one of two antioxidants (either NAC or MPG). Laser groups receiving antioxidants demonstrated significant (P =0.0365) increases in MTT values.

3.1.3 Cellular antioxidant status (ORAC ASSAY)

The ORAC assay on the irradiated cells showed that the laser irradiation lowered the cellular ORAC value by 47.6 ± 18.3% from the control value 5 minutes after a 30 min irradiation by a 638nm laser (3.816 J cm^{-2}). This decrease was however not significant (p>0.05, n=4). In addition a rebound increase in the ORAC value was seen 15 min post laser irradiation, with an increase of 51.7 ± 47.3% from control values. Again this was not significant (p>0.05, n=4). The ORAC value returned to control levels 60 min post irradiation (see Figure 3.4).

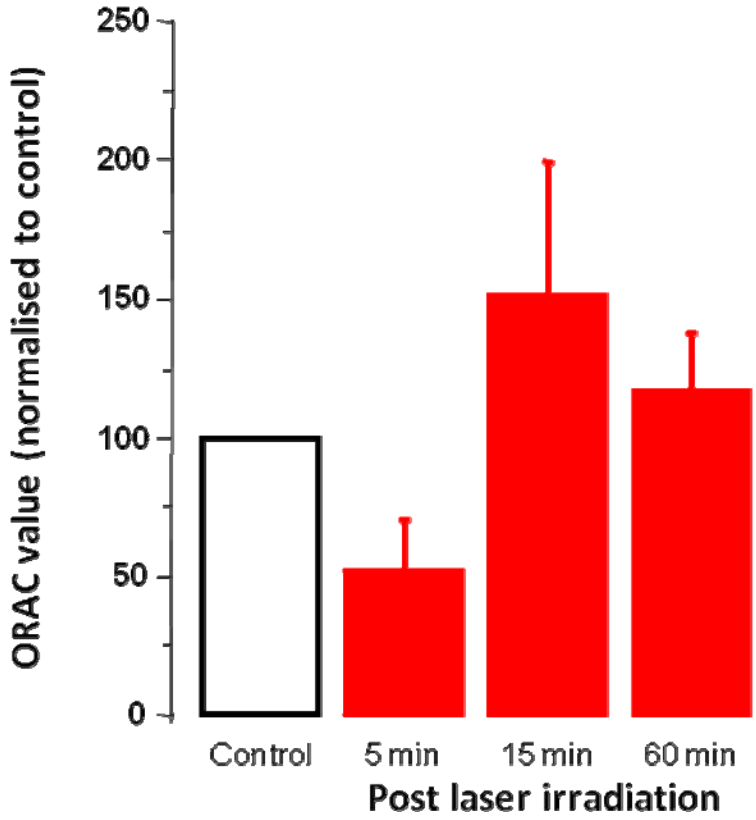


Figure 3.4 ORAC values for cells irradiated for 30 min at 638 nm (3.186 J cm^{-2}). Cells were harvested and assayed at the time points indicated after the completion of the laser irradiation. n=4.

3.1.4 Western blot analysis

Following the findings given above (that the laser irradiation appeared to be having some effect on cellular function), it was decided to look at the activation state of several well known intracellular signalling pathways. Of the pathways investigated (Akt, STAT-3, AMPK and p38 MAPK) Akt and p38 showed immediate increases in phosphorylation state following 30 mins of laser irradiation at 638 nm (3.186 J cm^{-2}), with relative increases of 88.6 ± 26.3% ($p<0.05$, n=4; see Figure 3.5) for Akt phosphorylation and 23.0 ± 5.1% ($p>0.05$, n=3; see Figure 3.6) for p38 phosphorylation. STAT-3 phosphorylation showed no change (data not shown), however total STAT-3 levels increased by 17.2 ± 11.4% at 60 min post laser irradiation ($p>0.05$, n=3; see Figure 3.7). Phosphorylated AMPK levels showed no significant change at any of the time points investigated (data not shown).

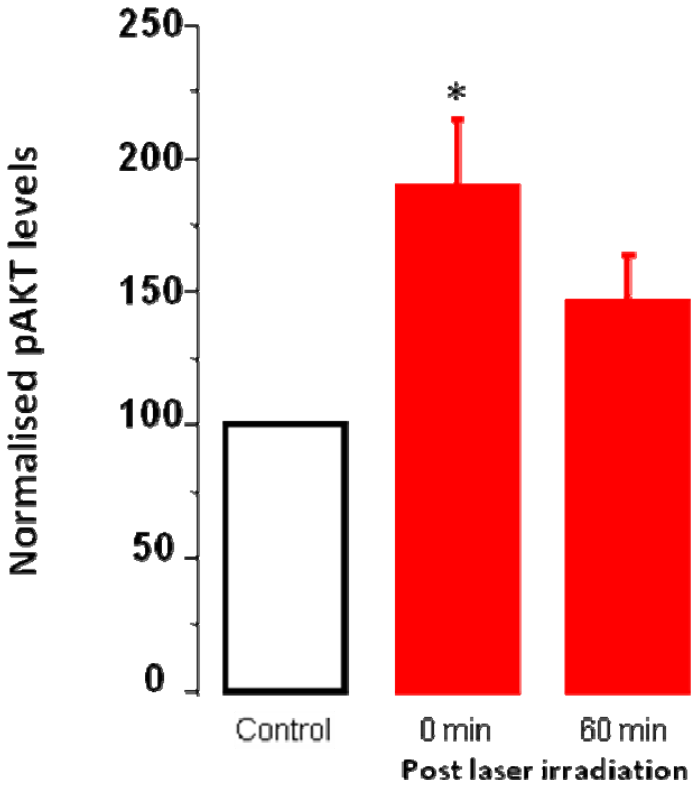


Figure 3.5 Relative increase in Akt phosphorylation status across indicated time points. * $p<0.05$ *vs* Control, n=4.

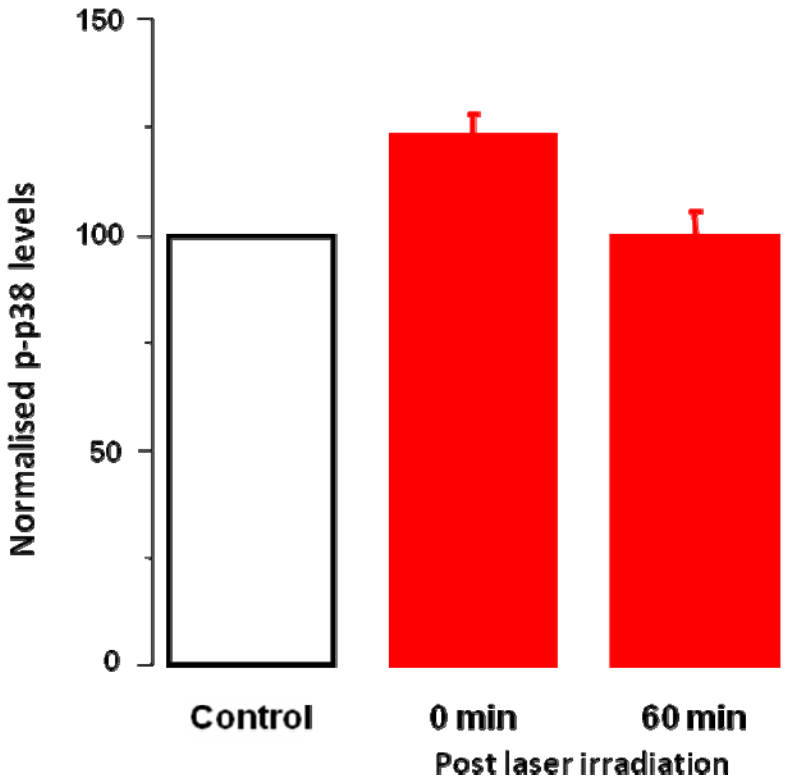


Figure 3.6 Relative increase in p38 MAPK phosphorylation status across indicated time points. No significant differences were found.

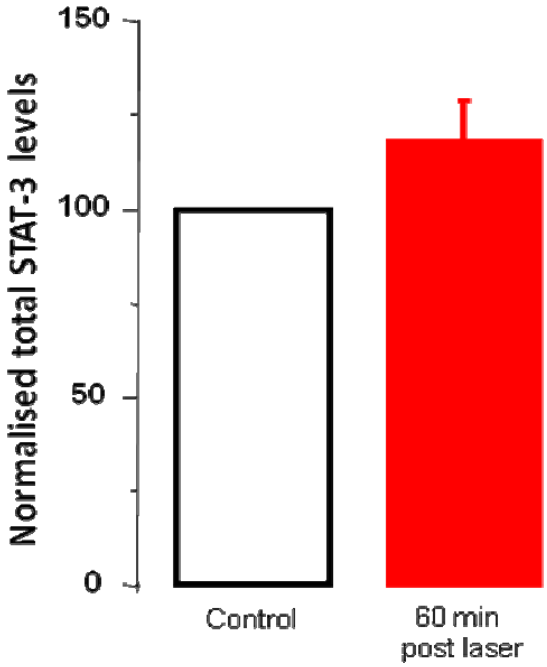


Figure 3.7 Relative increase in total STAT-3 at 60 min post laser irradiation. $p>0.05$, $n=3$.

3.2 *In vivo* model

The contusion injury model has been developed and tested in house, with validation of the model and the effects *in-vivo* being carried out by another post-graduate student in the department. As a result of the findings in this study the following parameters were chosen for the injury:

Drop height: 50 cm

Drop mass: 200 g

These parameters gave a reproducible contusion injury which was healed over a period of 14 days.

Excision of the injured muscle and subsequent histology (using H & E staining) was used to confirm the injury (see Figure 3.8).

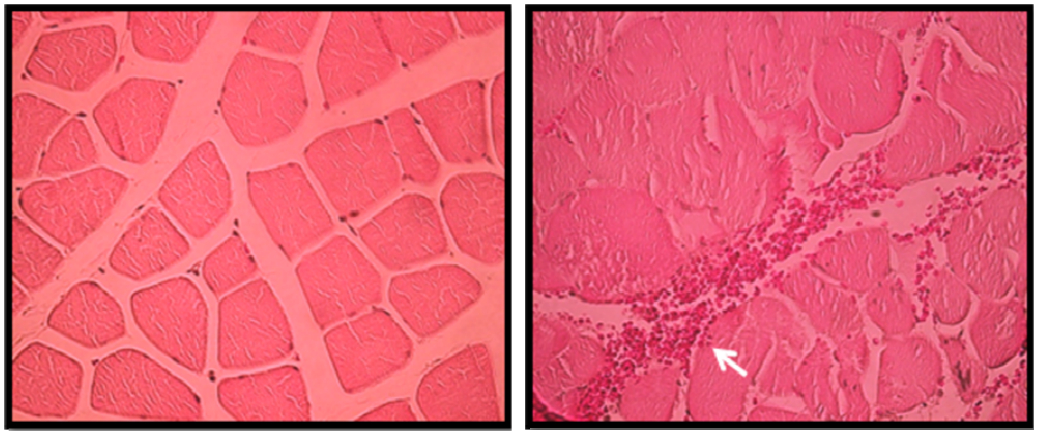


Figure 3.8 H&E stain of normal uninjured muscle (left) and injured (right) (400 x magnification). Note red blood cells, a result of vascular disruption (arrow).

With the use of the laser *in-vivo*, and with the time constraints in mind, a single irradiation time of 30 mins (giving a dose of 34.28 J cm^{-2}) was chosen. All animals tolerated this level of irradiation with no macroscopic signs of damage to the skin.

3.2.1 ORAC results

Following injury, blood and tissue was collected at various time points to allow for determination of both plasma and muscle antioxidant status using the ORAC assay. As can be seen in Figure 3.9 both irradiation and injury resulted in changes in ORAC values at both 4 and 24 hours, although these changes were not significant ($p>0.05$, $n=4$).

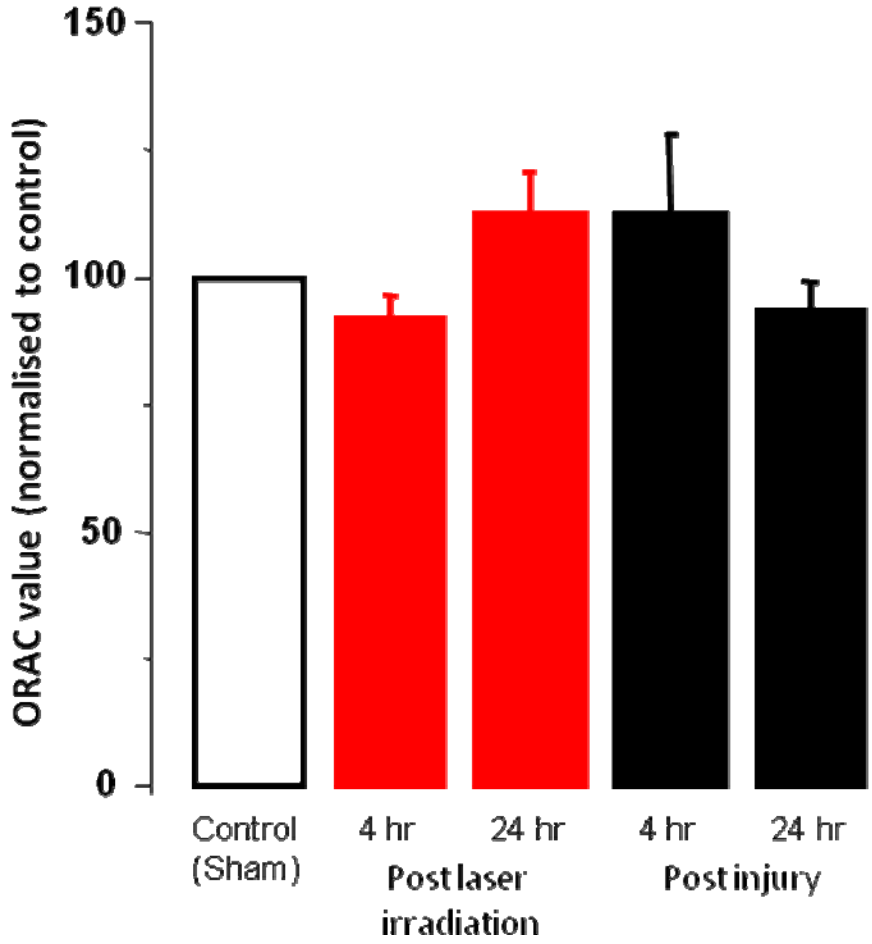


Figure 3.9 Normalised ORAC values for plasma from Sham (control) , irradiated and injured animals. Samples were collected at the time points indicated ($p>0.05$, $n=4$).

When the injured animals were treated (30 min irradiation, 34.28 J cm^{-2}) there was a (non-significant) decrease in plasma ORAC values (see Figure 3.10).

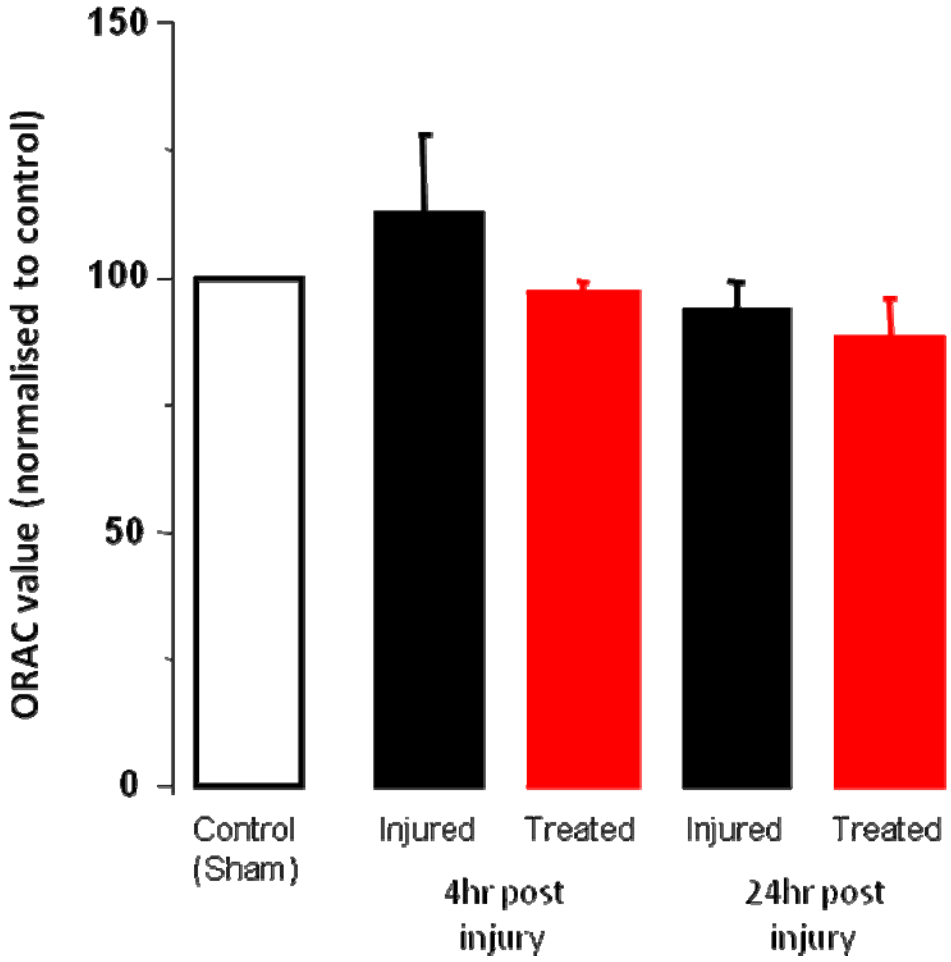


Figure 3.10 Normalised ORAC values for plasma from Sham (control), injured and injured/irradiated (treated) animals. Samples were collected at the time points indicated ($p>0.05$, $n=4$).

In accordance with the results seen in the plasma, changes in the ORAC values in the muscle tissue also changed. As can be seen in Figure 3.10 both irradiation and injury resulted in changes in the ORAC values of the muscle at both 4 and 24 hours, although these changes were not significant ($p>0.05$, $n=4$).

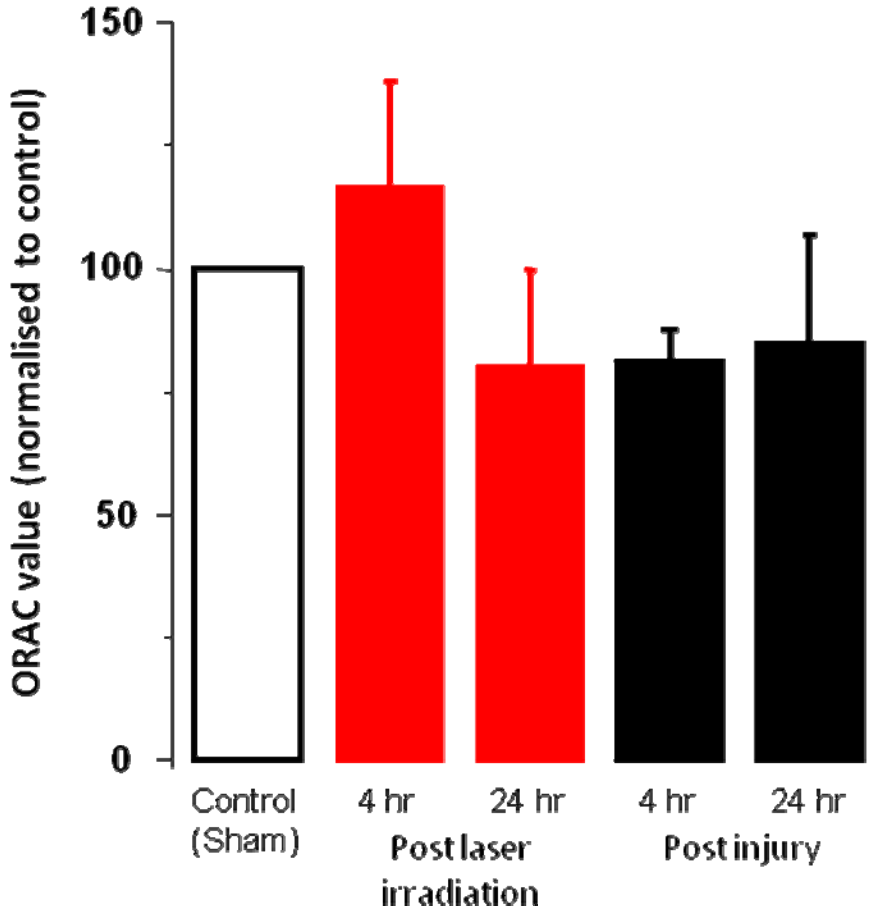


Figure 3.11 Normalised ORAC values for muscle tissue from Sham (control) , irradiated and injured animals. Samples were collected at the time points indicated ($p>0.05$, $n=4$).

In addition ORAC values also changed in the injured animals following treatment at both 4 and 24 hours (see Figure 3.11). As with the plasma values these changes were not significant ($p>0.05$, $n=4$).

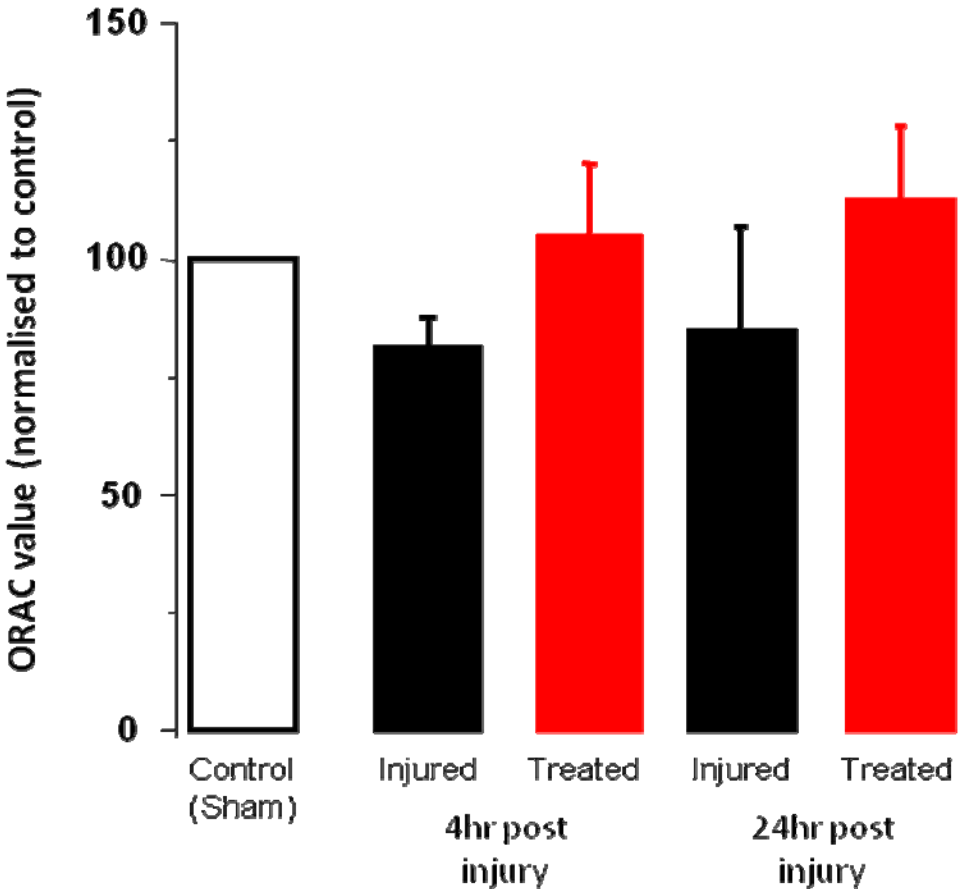


Figure 3.12 Normalised ORAC values for muscle from Sham (control), injured and injured/irradiated (treated) animals. Samples were collected at the time points indicated ($p>0.05$, $n=4$).

3.2.2 Satellite cells

Following injury satellite cell number (measured as CD34/CD56 positive cells per myofibre) was assessed. For a cell to be counted as a satellite cell it had to contain a nucleus and stain positive for CD34 and CD56. Examples of positive cells are shown in Figure 3.12.

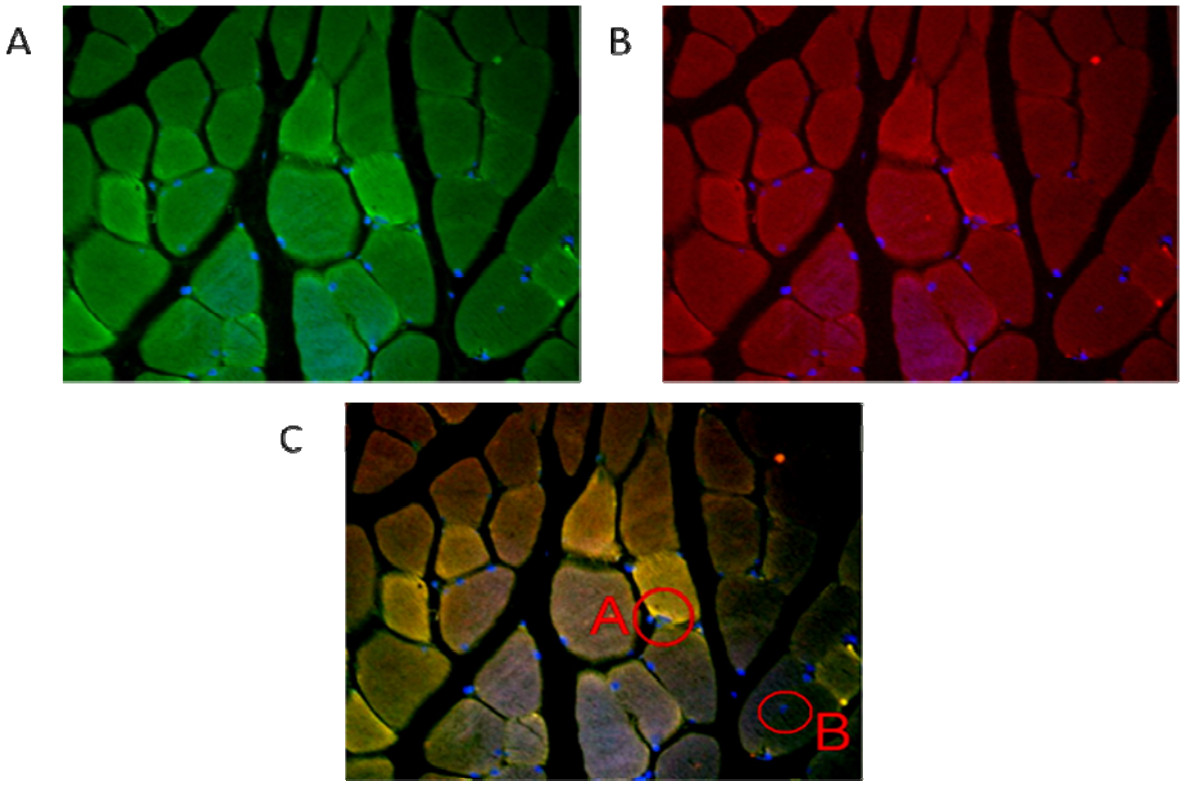


Figure 3.13 Markers of satellite cells: A) CD34, (FITC - green), B) N-Cam/CD56, (Texas Red - red), (DAPI – blue, a nuclear stain).C) Micrographs above are overlaid (representation of A and B). Indicated at "A" is a satellite cell stained positive for both markers. Encircled at "B" is a central myofibre nuclei – indicative of a newly regenerated fibre.

Following injury satellite cell numbers increased at 4 hours (from 0.021 ± 0.002 to 0.028 ± 0.004 satellite cells per myofibre; $p>0.05$, n=4-16). This transient increase appeared to be sustained for 72 hours (see Figure 3.13).

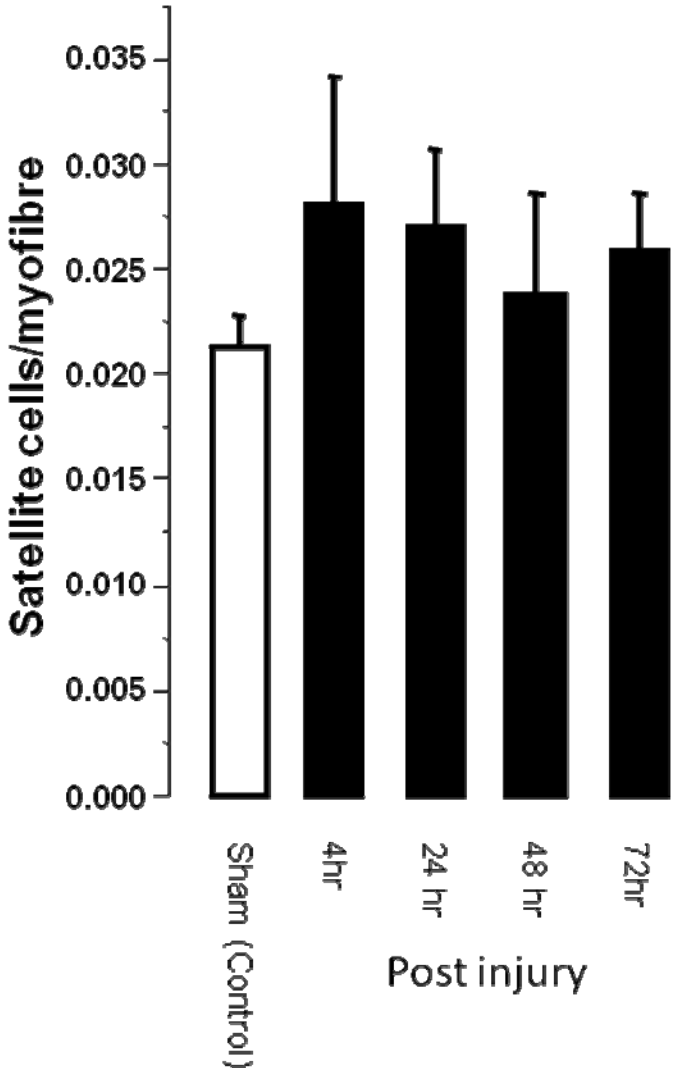


Figure 3.14 Satellite cell number per myofibre at the given time points following injury.

Laser irradiation (638 nm) of the hind limb containing the *gastrocnemius* muscle for 30 min (giving a dose of 34.28 J cm^{-2}) had no effect on satellite cell numbers, as determined by CD34 and CD56 co-staining (see Figure 3.14).

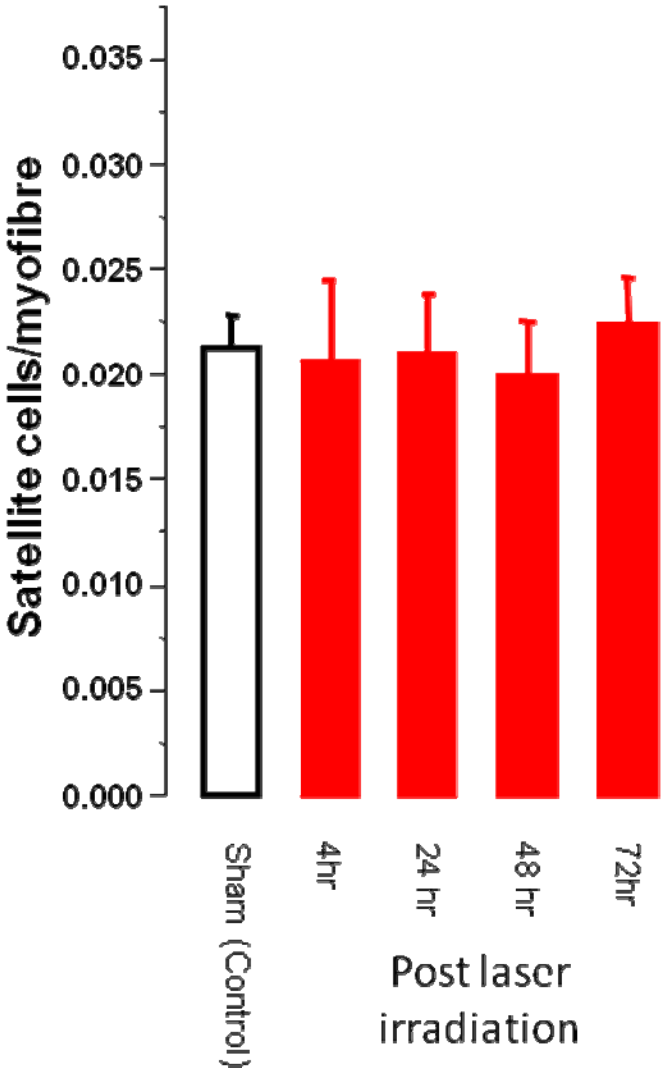


Figure 3.15 Satellite cell number per myofibre at the given time points following 638 nm laser irradiation.

Laser irradiation of the injured muscle (one treatment of 30 min with a 638 nm laser giving a dose of 34.28 J cm^{-2}) had no effect on satellite cell number in the first 48 hours post treatment. At 72 hours a slight (non-significant) decrease in cell number was seen (see Figure 3.15)

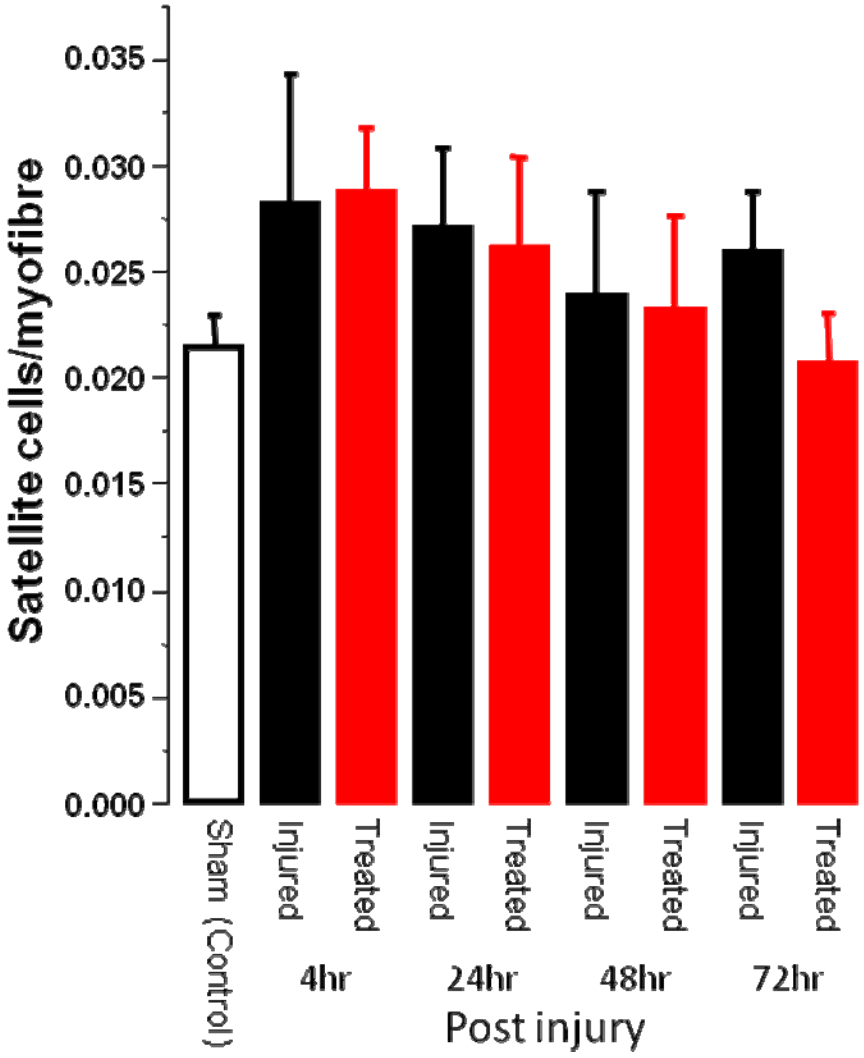


Figure 3.16 Satellite cell number per myofibre in injured and treated (638 nm laser irradiated, 34.28 J cm^{-2}) at the given time points following injury.

Chapter 4

Discussion

4.1 MTT results: laser irradiation and antioxidant treatment

The laser irradiation study, in conjunction with the antioxidant study clearly demonstrated an increase in MTT value in irradiated groups, whilst antioxidant treatment seems to abolish the major beneficial effects. The results can be summed up by five critical observations:

- 30 minutes seems to be the optimum dose.
- The magnitude of change (almost 200% above baseline for irradiated cells).
- The fact that the responses were persistent and still manifested 24 hours post irradiation. Antioxidants abolished this effect.
- Antioxidant groups demonstrated a (modest) increase in MTT values.

Antioxidants are known to abolish cell signalling involving reactive oxygen species (e.g. Mito Vit E (Hughes, Murphy *et al*. 2005) and NAC (Irani, Xia *et al*. 1997)). Since antioxidants abolish the increase in MTT values seen following irradiation, it is reasonable to expect some form of redox signalling is involved in these cellular effects. In this regard, NF-κB (Pantano, Reynaert *et al*. 2006), MAPK, ERK, JNK (Bogoyevitch, Ng *et al*. 2000) and Akt (Posen, Kalchenko *et al*. 2005) have been implicated in ROS signalling. Furthermore, the ability of H_2O_2 to reversibly inhibit LMW-PTP (Caselli, Marzocchini *et al*. 1998) could directly influence the phosphorylation status of a multitude signalling receptors. Deducing *which* of these signalling cascades are involved

and are responsible for the biological effects observed is open for future studies.

By whichever mechanism invoked, this effect must also give rise to longer term alterations in cellular events, as the MTT results were measured 24 hour post irradiation. The pronounced increase in MTT results is perplexing. At first glance, one would expect the result to be indicative of cell proliferation. This however becomes somewhat unlikely when one is confronted with the fact that this would imply the cells are doubling at a rate which will make all but the most prolific eukaryote cells shy. Furthermore, considering the spatial restriction in a Petri dish, cells would most probably be too confluent and stop dividing (*via* contact inhibition).

But if cell proliferation on its own cannot account for the increased MTT values, an alternative must exist. Before embarking on a discussion of these results, explaining the assay itself might illuminate some aspects of what exactly was observed in relation to the characteristics of the assay.

4.1.1 The MTT assay

In essence, the MTT assay makes use of two attributes of a cellular status to infer cell viability and proliferation: total activity of mitochondrial succinate dehydrogenase and membrane integrity. The amount of cell viability is deduced form the “total output” of the mitochondrial succinate dehydrogenase (also known as SQR or Complex II). As more cells will have a higher total turnover of ATP, “more cells” correlates with “more mitochondria”. Finally, since Complex II is a structural entity of mitochondria, it follows that “more mitochondria” will lead to “more Complex II”. A high total succinate dehydrogenase activity is thus indicative of cell proliferation and/or cell viability (more mitochondria and hence more cells in any case).

A second concept used to insure cell viability is membrane integrity. Even truly apoptotic/necrotic cells might still have some viable enzymes and as such, these enzymes might apathetically go about reducing MTT (indicating the cell

is “viable”). But, since formazan is not cell membrane permeable, cells with intact membranes will accumulate formazan inside the cell. On the other hand, cells with disrupted membrane integrity will spill their contents into the buffer along with the formed formazan (which will be decanted and thus will not contribute to the photometrical quantization).

The validity of MTT assay can thus be judged on the combined grounds of how good these two “indicators” correlate with their respective variables (namely viability and total cell mass). Obviously, a cell with a compromised membrane will not be able to maintain any chemical gradients and will speedily descend towards thermodynamic equilibrium (i.e. homeostasis will be compromised). All things considered, a cell’s inability to accumulate formazan does indeed seem to be a good predictor of cell viability.

The reduction of MTT as an indicator of cell proliferation is a complex matter. By measuring the total activity of a metabolic enzyme (i.e. complex II of the electron transport chain), the MTT assay immediately makes itself more identifiable as a *metabolic* assay rather than a *viability* assay. Thus, a more appropriate approach would be to state that the MTT assay associates with cellular metabolism. The counter case could be made that, since “metabolism” denotes the unique chemistry that we identify as “life”, metabolisms should causally follow the measured viability/proliferation index and should therefore be deduced from the MTT assay.

Obviously, this is not necessarily the case. A high metabolism need not imply a large amount of life- it could also mean a low amount of life that just happens to be very busy (i.e. large numbers of highly active mitochondria). Conversely, a low metabolic turn over need not imply a low amount of cells, following the same logic (i.e. low numbers of mitochondria). Different cells have different metabolic outputs depending on their environment. Using a metabolic marker as an indicator of cell proliferation/viability is thus hazardous as it is neither necessary nor sufficient to indicate proliferation/viability.

This being said, there is no reason to completely denounce the MTT assay per se. The assay seems reasonable as an indicator of cellular *metabolism(s)*.

Of note though, complex II is not the only catalytic site at which the reduction of MTT to formazan takes place (Berridge and Tan 1993; Gonzalez and Tarloff 2001; Bernas and Dobrucki 2002) and the other sites do not necessarily correlate with mitochondrial activity, thus complicating the picture even further.

The validity of the assay as a cell viability/proliferation indicator is not totally compromised as long as one remains wary of the parameters of the assay and how the experimental conditions impact upon these. Stress conditions that involve inhibition of cellular oxidative phosphorylation (e.g. hypoxia or starvation) might give false-negative results (or more formally, a type II error) and indicate cell death in cells which have cells simply reverted to lower metabolic output as substrate is limited.

4.1.2 Explaining the results

4.1.2.1 Mitochondrial biogenesis?

In light of the above, a different approach to the results is required. Instead of imagining cells proliferating at a enormous rate, it could be that the cell's prokaryotic symbiot were busy propagating - more mitochondria would imply more mitochondrial protein, and by proxy, more succinate dehydrogenase. Interestingly, no study (to the best of my knowledge) exists that investigates the possibility of mitochondrial biogenesis induced by LLLT.

Yet, by observing the literature, a causal link could be established: nitric oxide has been shown to induce mitochondrial biogenesis (Nisoli and Carruba 2006; Wadley and McConell 2007). In turn, LLLT have recently been shown to increase transcription of iNOS (Moriyama, Moriyama *et al*. 2005). Thus, LLLT can potentially increase mitochondrial biogenesis, *via* LLLT stimulated iNOS activity.

Alternatively, AMPK has also been shown to be activated by LLLT (Makela 2006) which intern also plays a key role in mitochondrial biogenesis (Zong, Ren *et al*. 2002). In any case, these two potential pathways both represent

possible mechanisms by which LLLT could induce mitochondrial biogenesis. An increase in mitochondrial number would also be coherent with a prolonged 24 hour effect.

4.1.2.2 Increased succinate dehydrogenase?

As mentioned, the reduction of the MTT-formazan is catalyzed (mostly, but not exclusively) by succinate-coenzyme Q reductase (succinate dehydrogenase, is in complex with other three subunits in the inner membrane of the mitochondria to form complex II of the electron transport chain). As an alternative, it could be that cells did not proliferate at all, but simply increased expression of the enzyme over time. This increased enzyme transcription would account for the fact that these results were observed 24 hour post irradiation.

But what would be the benefit of increasing the expression of *this* enzyme? Investigating the metabolic position of succinate dehydrogenase presents two curious facts. Firstly, succinate dehydrogenase catalyses the oxidation of succinate, yielding fumarate and so doing also reduces flavin adenine dinucleotide (FAD) to $FADH_2$ (which, by its reduction (gain of two electrons) is now an "energy transporting" molecule). Secondly, the position of succinate dehydrogenase in the citric acid cycle is also possibly of significance. The fumarate product for succinate dehydrogenase is then enzymatically processed to malate which in turn is catalyzed to oxaloacetate (Krebs and Johnson 1980). Oxaloacetate is then "cycled" in the citric acid cycle and forms the very first substrate for Acetyl-CoA. Thus, besides supplying the cell directly with energy rich molecules ($FADH_2$), succinate dehydrogenase could facilitate the flux of Acetyl-CoA through the citric acid cycle and thus also facilitate energy production.

4.1.2.3 Increased electron flow through the ETC: Cytochrome c involvement?

It is also tempting to contemplate an increased flux through the electron transport chain. As mentioned, cytochrome c oxidase has been proposed to be the rate limiting step in the ETC (Karu, Pyatibrat *et al*. 2005). It is also proposed to be an active chromophore, the primary photon acceptor in photo-

biostimulation. If the rate-limiting step is accelerated, it follows that the total flux in the system would increase. Though this implies an increase in metabolic rate, it would not necessarily describe the increased reduction of MTT (succinate dehydrogenase reduction of MTT is not dependant on how many electrons is passed on through the ETC).

4.1.2.4 Analysis of antioxidant results: a possible indication of mitochondrial biogenesis?

The partial increase in the MTT values of the antioxidant treated cells was a curious anomaly. This could however be due to a sub-optimal concentration of antioxidant being used in the experiments. The concentrations used were selected from previous experience using these compounds (Lacerda, Smith *et al*. 2006).

This yet again brings into play another level of analysis on the results: Typically, the mitochondria are viewed as the cellular powerhouse. This however is not the whole story. Eukaryotic cells are in no way capable of "burning" (oxidising) our substrate (i.e. eukaryotes are not capable of performing oxidative phosphorylation). In fact, non-fermenting metabolites like fatty acids are totally and utterly useless to us, since they cannot be fermented. Our prokaryotic symbiotes (mitochondria) on the other hand are particularly fond of fatty acids and are well equipped for oxidative phosphorylation.

The reason why eukaryotes never evolved the highly efficient mechanism of generating energy (oxidative phosphorylation is much more efficient than fermentation) is that we never had reason too: we had our prokaryotic symbiotes performing the task for us. Oxygen is incredibly toxic and indeed hazardous toward cellular machinery. That is why it is left totally and utterly, up to the mitochondria to perform oxidative phosphorylation. In a way, the mitochondria protect eukaryotes by acting as "oxygen vacuums": the mitochondria capture and chemically process (burn) oxygen, releasing energy (e.g. ATP *via* continuation of a proton-motive-force) in the process. Thus, the

mitochondria not only produce ATP – they actively protect the cells from oxygen.

As mentioned, it could be that LLLT causes mitochondrial biogenesis, with this increase in mitochondrial number protecting the cell from a hyper-oxygenated environment: with more mitochondria, their “oxygen vacuum capacity” would increase. This would explain why the LLLT group perform so much better than the non-irradiated group: the mitochondria protected the cells by chemically processing oxygen.

Indeed, the control group probably lost cells as a result of oxidative stress. Whilst the MTT value of the irradiated group increased, the MTT value of the non irradiated groups could actually of decreased as a result of cellular death induced by oxidative stress.

4.1.3 Summary: MTT

In the end, a most probable interpretation involves an integration of some of the above mentioned mechanisms that would lead to an increase in MTT values. It is reasonable to expect that an increase in metabolism, either *via* mitochondrial biogenesis, increases in succinate dehydrogenase activity and/or increases in electron flux by augmenting cytochrome c oxidase activity would ultimately lead to an increase in the MTT value. In turn, this could possibly stimulate cell proliferation. It is thus reasonable to expect at least some of the increased MTT value to be indeed attributed to cell proliferation. This could be confirmed in future studies by accurately counting cell numbers (both live and “dead”) and correlating this with the MTT value. This cell proliferation is also in line with the signalling molecules discussed (MAPK, Akt etc) which can be activated *via* ROS mediated mechanisms. In this regard, follow up studies investigating ROS formation (by example utilizing live cell imaging techniques (Alexandratou, Yova *et al*. 2005)) would be important in validating the antioxidant/ROS mechanisms involved.

Yet still, it seems highly unlikely that cell proliferation on its own could account for the observed MTT values, and the mitochondrial biogenesis hypothesis seem more likely. Future studies should look at employing more direct markers of cell proliferation (e.g. BrdU labelling) as opposed to mitochondrial proliferation (actually, the relationship between nuclear and mitochondrial DNA could also be investigated which might be more descriptive (Strand, Ingebretsen *et al*. 2008)) as well as possibly investigating mitochondrial metabolic activity (e.g. measuring membrane potential (Matsumoto-Ida, Akao *et al*. 2006)).

4.2 ORAC ASSAY

4.2.1 Cell ORAC results

Though not significant, an interesting trend was observed with the irradiated cells, in that irradiation lowered the ORAC value obtained. As LLLT has been shown to induce ROS production (Karu, Pyatibrat *et al*. 1987; Callaghan, Riordan *et al*. 1996; Grossman, Schneid *et al*. 1998; Alexandratou, Yova *et al*. 2002) it is tempting to speculate how laser induced ROS production could result in the observed decrease in cellular antioxidant status. This is, however speculative, especially given the variability in the results (which could be reduced by increasing the n).

Nevertheless, the “rebound” effect seen at 15 minutes pos irradiation and the subsequent return to baseline levels does seem to indicate that something is happening within the irradiated cells. It is possible that the rebound is as result of a normal cellular homeostatic response, in which the chemical endogenous antioxidant capacity of the cell is regenerated following depletion during and immediately after irradiation.

4.2.2 Blood plasma ORAC results

Plasma ORAC levels of injured rats indicated a drop in antioxidant status for irradiated groups followed by a rise 24 hours post irradiation (Figure 3.9). Though it could be argued that LLLT decreased antioxidant status post irradiation, this seems unlikely (it requires validation which simply is not afforded by the literature). In any case, the results were insignificant. The injured group showed a decrease in antioxidant status 24 hours post injury. These result were, again, not significant, but would possibly be easier to explain, as neutrophils (activated in reaction to the inflammatory response in the injured tissue) might increase ROS production through the respiratory burst (Brickson, Ji *et al*. 2003).

Rats injured and receiving treatment, as opposed to rats not receiving any treatment did not dramatically differ at either the 4 or 24 hour time points (Figure 3.10), although the treated groups demonstrated lower antioxidant status. This was not significant. It may be possible, by increasing the number of animals studied, or by altering the dose to obtain significant differences. This was however, not possible during this study due to both financial and time constraints.

4.2.3 Tissue ORAC results

Illustrated in Figure 3.11, samples were reasonably uniformly rated in terms of antioxidant status, with the exception of the 4 hour irradiated group (showing an increase in antioxidant status). With the exception of the 4 hour groups, all samples had a lower antioxidant status, which, in the context of an inflammatory response, can be explained in terms of the increased ROS production in the respiratory burst (Brickson, Ji *et al*. 2003). Despite these trends, no significant differences were observed. As above this could be down to the low number of animals or inadequate penetration of the laser to reach the tissue. This is currently under investigation in conjunction with the National Laser Centre.

Interestingly, a trend is observed when comparing the injured groups to the laser treated groups (Figure 3.12.). The irradiated groups demonstrate an

increased antioxidant response over both 4 and 24 hours. It has been shown that LLLT can increase antioxidant levels in skeletal muscle (Avni, Levkovitz *et al*. 2005). Though not significant, increasing sample size in future studies might provide significant results.

Interpreting the results in a physiological context is extremely difficult, in that the difficulty does not lie with the lack of an explanation - it is in fact because of the overabundance thereof! Many of the arguments produced to interpret the results are in fact synonymous with shooting an arrow into a wooden plank and painting the bull's-eye around it. In the light of this sentiment, it is worth evaluating the assay and explaining some critical pitfalls and problems with the assay to cultivate a better understanding of the results.

4.2.4 The ORAC assay

The ORAC assay is a hydrogen atom transfer (HAT) assay that determines antioxidant capacity by measuring competitive reaction kinetics. It consists of 3 basic components: a fluorescent probe, a radical donor and a fixed amount of antioxidant to compare the sample antioxidant capacity against ("a standard"). Basically, as the radical donor increases (i.e. the free radical concentration increases), the fixed amount of fluorescent agent present in the reaction mixture will progressively become quenched (by the radical species). Any antioxidant present in the system would scavenge the radicals, effectively "out competing" the fluorescent probe as substrate. This ultimately results in the extended viability of the fluorescent probe and would thus increase the total area under the curve (AUC) generated. By measuring florescent intensities over time, a kinetic curved can be drawn from which the antioxidant capacity in any given sample can be deduced (from the total AUC).

Since it conception is 1993 (Cao, Alessio *et al*. 1993) the ORAC assay has seen a number of modifications. A great leap in terms of both application and economic consideration was the replacement of β-phycoerythrin with fluorescein (FL) (Ou, Hampsch-Woodill *et al*. 2001). In the assay applied in our study, a fluorescein probe was adopted. Furthermore, 2'-azobis(2-

aminodinopropane)-dihydrochloride (AAPH) was used as the radical (more specifically, H_2O_2) donor and 6-Hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic Acid (Trolox) was utilised as the antioxidant standard.

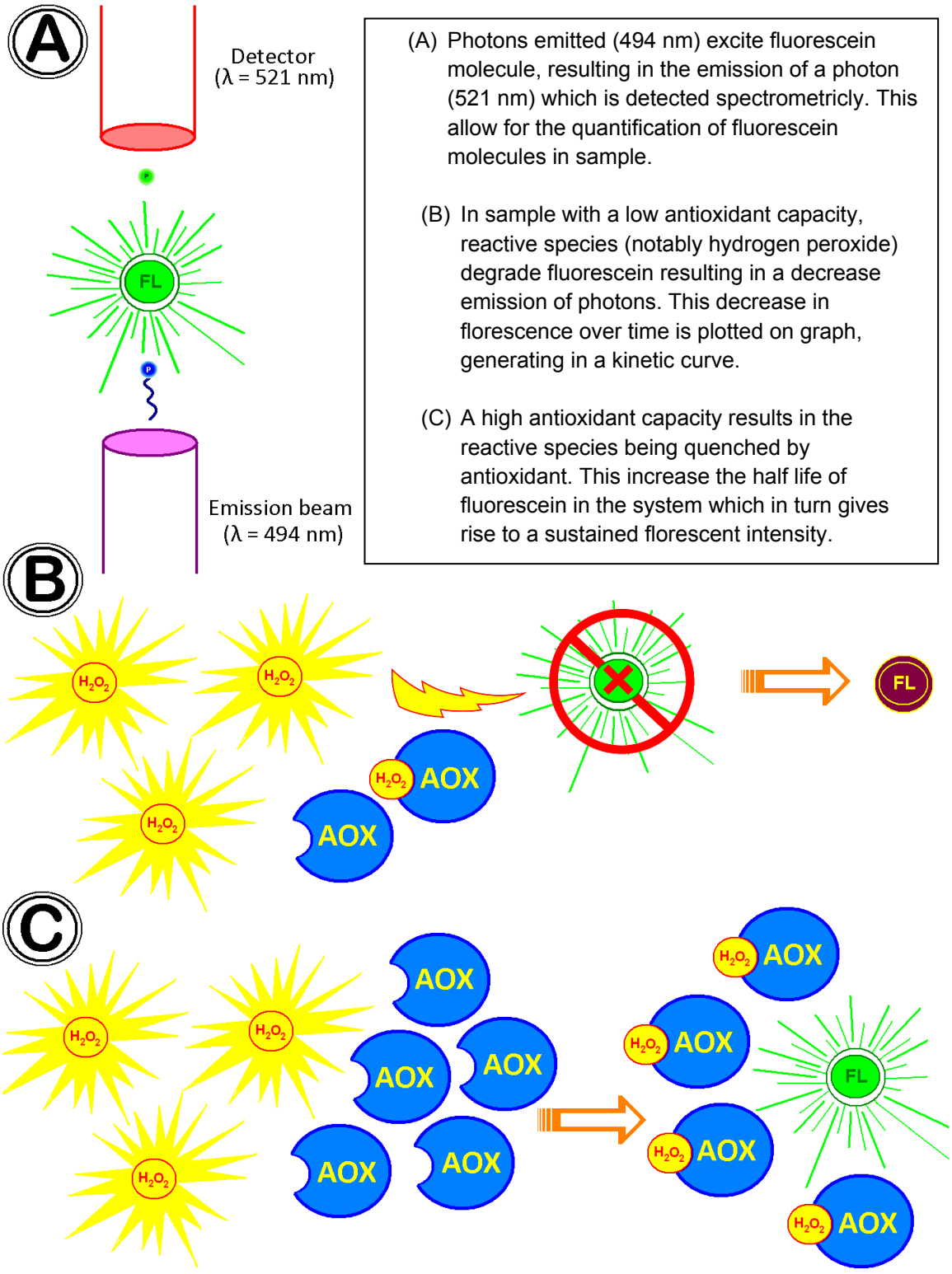


Figure 4.1 Brief summary of the ORAC assay.

The biological “antioxidant- arsenal” at an organism’s disposal can be divided as enzymatic and non-enzymatic. Enzymatic antioxidant elements include the selenocysteine containing glutathione peroxidase, catalase and members of the SOD family. Non-enzymatic antioxidants typically include “sacrificial scavengers” (such s vitamin C and E) as well as chemicals that prevent oxidative damage, but do not in themselves scavenge radical species. As an example of such a chemical, one can consider co-factors of enzymatic scavengers and chelating agents capable of removing transition metals which can form radical species *via* so called Fenton's reaction to be examples (Afanas'ev, Dorozhko *et al*. 1989; Lloyd, Hanna *et al*. 1997). It is also important to note that cells posses no enzymes to scavenge radicals such as singlet oxygen, hydroxyl radicals or peroxynitrite (Huang, Ou *et al*. 2005). As a result the so called “sacrificial scavengers” have evolved.

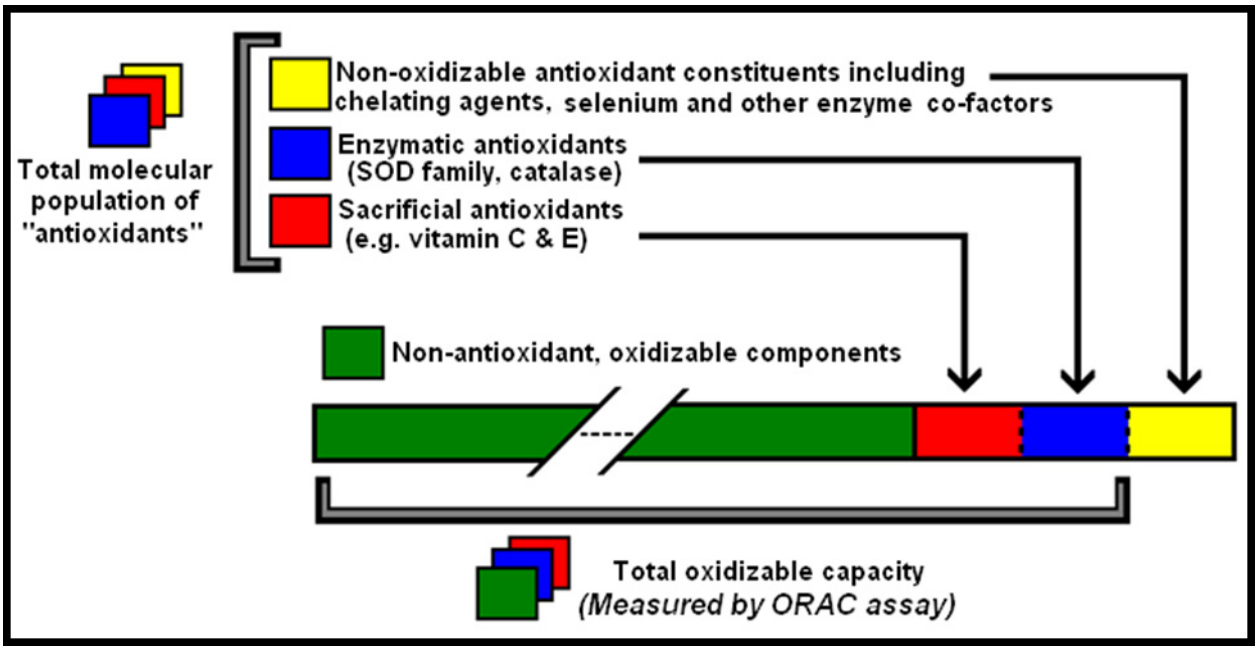


Figure 4.2 Representation of the relationship between cellular oxidizable substrate and molecules considered as antioxidants. Note, the diagram is only representative and not according to scale as this relationship will be tissue specific. Although the total oxidizable capacity of a cell is in general much larger than the population of antioxidants, antioxidants (by definition) have a much larger affinity towards reactive species and are thus able to protect cells from oxidative stress.

Obviously, not all the possible constituents of the broader term “antioxidant” are evaluated by a single assay such as ORAC. But, in regarding H_2O_2 as the major oxidant produced (Fleury, Mignotte *et al*. 2002), those antioxidants capable of its scavenging would give a biologically relevant approximation of the antioxidant capacity of the tissue being analysed. Specifically in our study, the fact that not all antioxidants are water soluble also acts as a compounding factor in our analysis. Nevertheless, modifications to the ORAC assay does exist which allows for the quantification of lipophilic antioxidant capacity (Prior, Hoang *et al*. 2003). Thus, despite the lack of an absolute golden standard for antioxidant capacity determination, the ORAC assay has been shown in the literature to be a rather robust and trusted technique (Prior and Cao 1999).

In the literature some authors use plasma while others prefer serum for measuring antioxidant capacity. In this regard, Ghiselli (Ghiselli, Serafini *et al*. 2000) has pointed out that, during platelet aggregation (necessary for serum extraction) ROS is produced. Also, in the context of radical chemistry, antioxidants are easily oxidized by exposure to atmospheric oxygen and degraded by exposure to light. In addition both radicals and their scavengers are more liable to react with each other over time (if not frozen) (Ghiselli, Serafini *et al*. 2000). There are also growing concerns about inconsistent results possibly arising from experimental artefacts during the preparation of samples (Mayne 2003). In light of these concerns, the use of plasma, instead of serum, was decided upon.

The ORAC assay has not only been used on serum/plasma, but it has also been successfully used to measure antioxidant capacity in mammalian tissue samples (Cao and Prior, 1999; Munkeby *et al*., 2004; Sofic *et al*., 2006), and with special reference in our contexts [muscle], (Kinnunen, Hyyppa *et al*. 2005). In order to investigate any possible change induced by LLLT on the irradiated muscle, the ORAC assay was performed on samples of the *gastrocnemius* muscle. Since experimental rats were already to be sacrificed, it was decided to also extract blood *via* cardiac puncture during the biopsy procedure. This allowed another dimension of analysis by allowing us to evaluate “systemic” antioxidant capacity and so recognize possible anomalies in data as well as look for unexpected correlations.

Most of the molecules partaking in “radical-scavenging” reactions (and thus contributing to the AUC) are not considered antioxidants. In fact, it is by the very nature of the chemical reaction that takes place between reactive species and other metabolites, proteins, lipids etc. (which are potential oxidizable substrates) that reactive species are deemed hazardous to biological systems. In plasma, Cao *et all* found components not usually classified as antioxidants, such as ascorbate, glutathione, α-tocopherol and uric acid contribute to the major “scavengers” of reactive species in an ORAC assay (Cao, Alessio *et al*. 1993). This unfortunately increases ORAC running time (the assay needs to run until completion to generate the AUC). Since reagents are, at least to some extent, heat liable or prone to photo-bleaching, and by reason of practical limitations on instrumentation and feasible time constraints, a balanced compromise between dilution and running time had to be made.

In our ORAC protocol, the amount of runs, and therefore, by implication, the overall running time was much longer (180 min) then applied by most authors (35 min). To insure spontaneous photo bleaching does not compound results, a run in which AAPH substituted with PBS (thus, no radicals to quench the florescent probe) was included to observe “background” photobleaching. Over 180 mins the fluorescent intensity decreased by about 3.7%. Though notable, this contribution is negligible if compared to the impact of AAPH on the rate of decay (which can deplete unprotected fluorescein by about 95% within about 20 mins).

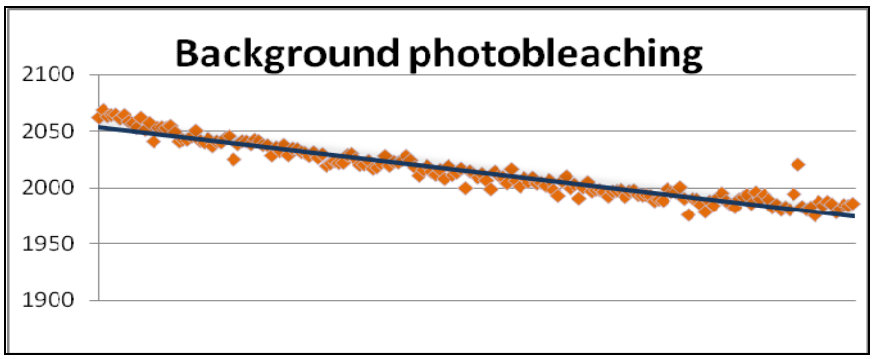


Figure 4.3 Curve demonstrating background photobleaching over 180 minutes.

In order to prevent the runs being too long, samples can be diluted to reduce the antioxidant capacity. This was prohibited by the rationale behind our application of the assay: most authors in the literature were interested in antioxidant capacity per se, whilst, in this study, we were using the antioxidant status as a “derived instrument” to deduce a relative increase in reactive species following laser irradiation. This is a critical difference in our approach as to “classical” implementation of the ORAC assay: an immediate 10% increase in antioxidant capacity would lead to an immediate 10% increase in antioxidant capacity. But the inverse need not necessarily hold true: a 10% immediate increase in ROS production does not result in an immediate 10% decrees in antioxidant capacity. The reason for this is simply because much of the antioxidant capacity of a cell is enzymatic in nature and thus not linearly depletable (as would be the case if cellular antioxidant capacity were 100% based on “sacrificial’ antioxidants).

In effect then dilutions were kept as low as possible to increase the “signal-to-noise” ratio. If pronounced dilution schedules were to be followed the small change in antioxidant capacity could be diluted to such an extent as to be “lost” in experimental “noise” (the change in antioxidant status would be less than the background variance introduced by other factors). By employing longer runs, dilution factors need not be as pronounced, which lead to an increase in the “experimental resolution” of the data generated. Having longer running times with less diluted sample aliquots was also a more favourable strategy from a purely practical point of view: less pipetting (in diluting samples) means less opportunity for potential experimental error to slip in. One other advantage of having longer runs in the assay relates to the fact that the ORAC assay was performed manually. As one pipettes into the wells across the plate (with a multi-channel pipette from top to bottom) there is a marginal time delay between the sample-wells at the top of the plate and the samples-wells at the bottom of the plate (roughly 20-30 seconds). This time delay is miniscule: 30 seconds on 180 min experiment equalling only a 0.28% deviation. One problem with this though is that the ORAC assay is a kinetic assay with the reaction demonstrating an exponential decay curve. Early in the reaction, the antioxidants/oxidizable-substrate are in abundance, promoting an increased reaction kinetic between the reactive species and any

antioxidants in the system (the reactive oxygen formation by AAPH is basically constant for the first couple of hours (Niki 1990)). Simply stated, the rate at which the antioxidants scavenge reactive species slows down over time. Thus the reaction will, by implication, take place at its fastest from the moment the reagents are added.

This “time gradient” was also taken into account with “plate architecture” (when designating the allocated position of samples and standards to specific wells on the plate).

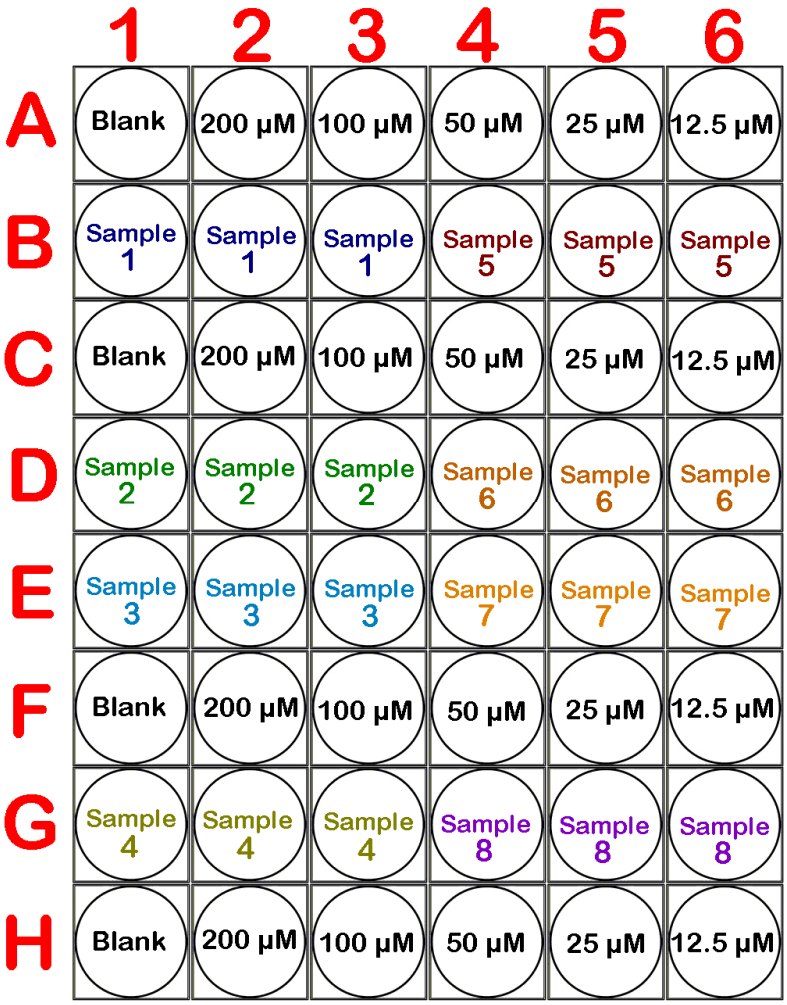


Figure 4.4 Typical plate architecture. Only half the wells on a 96 well plate were used each run. Samples were loaded as to spread entire group (4 rats/Petri dishes in a group = 4 samples in group) according to a loading “time-gradient”. This way, the entire group has a representative sample (1-4 and 5-8) across the plate, promoting consistency.

As mentioned, the ORAC assay does not measure antioxidant capacity, but oxidizable capacity (which includes tissue constituents like proteins), and it is possible to extract proteins from the samples before performing an ORAC assay. However, in this study, it was decided against doing this on the basis that treating samples increase their exposure to atmospheric oxygen which can oxidise samples and therefore influence accuracy. Instead, sample values were divided by their protein concentration (in order to normalise the values) and expressed as µmol Trolox per liter of sample per µg protein.

This turned out to be an error, and, probably the major cause of the variability seen in the results. The effect of protein in samples on the ORAC results is not necessarily corrected for by simply dividing by it. Technically, the part of the ORAC value that originates from the protein's oxidizable capacity should be subtracted from the total ORAC value. But since the relationship between protein concentration and sample oxidizable capacity is not known, it is not possible to do this.

As mentioned in materials and methods section, PBS instead of RIPA buffer was used during the scraping of cells. Since RIPA buffer contains detergents which lyses cells, the use of RIPA buffer greatly assists in the detachment and harvesting of cells in culture. The use of PBS led to the non-uniform scraping of samples which, we believe, ultimately resulted in the amplification of sample-to-sample variability in harvest fractions. Protein content would vary enormously, and, as such, dividing by the protein concentration skewed results even more.

The reasons for not using RIPA buffer to harvest the cells were numerous, and included: the chelating agents present in the buffer quench metal ions that might influence radical formation (notably copper and iron), which, through the Fenton reaction might form highly reactive radicals like the hydroxyl radical; compounds in the RIPA buffer are potentially highly oxidizable substrate for radicals - detergents such as Tween compounds (which are polysorbates) and NP-40 (nonyl phenoxylpolyethoxylethanol) are complex organic compounds with many molecular centres suitable for interactions with reactive species.

A major advantage of the ORAC assay is that is economical. Not only are the reagents inexpensive, but the instrumentation required is routinely available. Secondly, because of the simplicity of the assay (only 3 reagents), it has potential for automation. Due to this the ORAC assay (along with the numerous modifications) has become the gold standard in the food and agricultural industry as an indicator of nutritional antioxidant capacity.

But the assay does have some disadvantages. For one thing, the assay is not able to detect lipophilic antioxidants, and thus it ignores an important contributor to cellular antioxidant capacity. In addition the reagents used in the assay are extremely liable to environmental degradation, for example, fluorescein, like most fluorescent molecules, undergoes photo-bleaching on exposure to light, and the antioxidant standard, Trolox, is unstable and prone to oxidation. Therefore slight changes in experimental conditions and the "reactivity" of the reagents used can contribute towards experiment to experiment variability.

Some precautionary steps were taken and specific experimental "safeguards" were installed in the set up of the protocol to minimize experimental error. For one thing, reagents are relatively stable if stored properly (with the exception of the AAPH which has to be made up freshly for each experiment). In order to minimise variability stocks of the reagents were made, aliquotted and stored appropriately. In addition each sample was run in triplicate and the results inspected in Excel by overlaying the fluorescein decay curve (for example see Figure 4.5). Curves of each triplicate batch were inspected in order to ensure accurate and consistent sample loading. Runs with deviating curves were omitted from data processing and repeated.

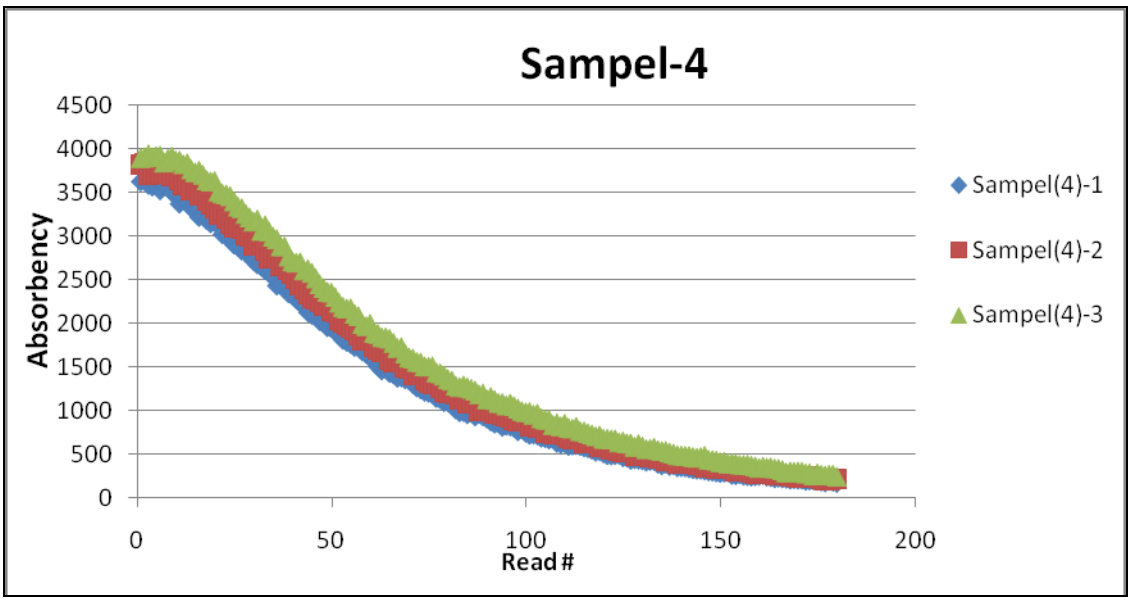


Figure 4.5 Representative of triplicate values of same sample overlaid. Graphs were visually inspected to insure sample loading was accurate.

During the course of this study several pitfalls of the ORAC assay were highlighted. As a result it is the opinion of the researcher that the ORAC assay is neither sensitive enough nor accurate/consistent enough to be routinely employed in applications such as performed in this study.

The ORAC assay, *per se*, is not criticised, rather, the application of the assay in our context is questioned. For our application, other alternative methods are available to allow measurement of both ROS production and oxidative stress in both systemic (Couillard *et al*., 2005; Kadiiska *et al*., 2005; Krieter *et al*., 2006; Stephens *et al*., 2006) as well as local (Alessio, 1993; Cherubini *et al*., 2005) settings. There are also live cell imaging techniques that allow the formation of radicals to be monitored real-time both *in-vitro* (Robinson *et al*., 2006) and *in-vivo* (Flors, Fryer *et al*. 2006).

It is thus the author's opinion that no confident conclusion can be drawn from the ORAC results. Further studies should look at alternative markers (as mentioned).

4.3 Western blot

As mentioned earlier, LLLT have been implicated in ROS production (Thannickal and Fanburg 2000; Hancock, Desikan *et al*. 2001; Pantano, Reynaert *et al*. 2006) and in turn, ROS have been found to modulate phosphorylation states of global cellular signaling components throug its effects on PTPs (Kamata, Shibukawa *et al*. 2000; Lee and Esselman 2002). Both AMPK (Figure 3.6.), being a key player in cellular energy homeostasis (Yamauchi, Kamon *et al*. 2002) as well as Akt (Figure 3.5.) which demonstrate strong anti-apoptotic, signaling molecule as well as promoting protein synthesis (Bodine, Stitt *et al*. 2001) were shown to demonstrate an increase in activation (phosphorylated). An increase in STAT 3 transcription (Figure 3.7) was observed 60 minutes post irradiation. STAT have been implicated in satellite cell activation/ proliferation and promoting cell survival (Kami and Senba 2002), making this molecule an appealing target for our investigation.

4.4 Satellite cells staining

As previously mentioned, there is enough evidence in the literature to support the use of CD34 (Beauchamp, Heslop *et al*. 2000; Hwang, Yuk *et al*. 2004; Shi and Garry 2006) and N-CAM (CD56) (Crameri, Langberg *et al*. 2004; Sinanan, Hunt *et al*. 2004; Olsen, Aagaard *et al*. 2006) as markers for satellite cells. It was decided to co-stain in order to maximize sensitivity and prevent false positive counting of non-satellite cells which also express these markers.

As expected, injury did cause an increase in satellite cell activation, as illustrated in Figure 3.14. At each advancing time point, the number of satellite cells decreased, probably as a result of satellite cells differentiating as they started to fuse and form myotubes.

Laser irradiation itself seemed to have no effect on satellite cell number (Figure 3.15). It might be that laser irradiation acts in concert to the numerous growth factors/cytokines released during an inflammatory response (Smith, Kruger *et al*. 2008). Irradiation also seems to have little effect on injured rats as compared to untreated groups (Figure 3.16.). Again over time, a decrease in the number of satellite cells seen post injury was observed.

One possibly interesting trend observe is that control groups (non-irradiated), demonstrate a sudden peak at 72 hours post injury in satellite cell number (see Figure 3.13 and 3.15). In contrast, irradiated cells do not demonstrate this sudden increase (see Figure 3.15). This could be interpreted in the context of a more advanced healing stage in laser treated groups, where a "second round" of satellite cell activation might not be necessary. But again, such conclusions remain to be pure speculative.

4.4.1 Critical analysis of satellite cell staining/counting procedure

During microscopy a photo taken in any general field of view with a higher number of fast twitch fibers will have less myofibres visible due to their larger cross-sectional area than a muscle containing slow twitch fibres. In order to compensate for this, satellite cell numbers are normalized to fibre number.

Another confounding factor in calculating satellite cell numbers in muscle is that satellite cells might not be equally distributed across fast and slow twitch muscle groups. It is generally accepted that satellite cells are more prevalent at capillaries (Schmalbruch and Hellhammer 1977) and at the site of motor neuron junctions (Wokke, Van den Oord *et al*. 1989). A recent study investigating the anatomical structure of satellite cell niches again affirmed the belief that satellite cells reside in close proximity to capillaries (Christov, Chretien *et al*. 2007). As previously mentioned, slow twitch oxidative muscles are more highly vascularized then fast twitch muscles which imply that more satellite cells reside in this fibre type.

From a practical point of view, the asymmetric accumulation of satellite cells in slow twitch muscles could have an important physiological function: fast twitch muscle produce the majority of its energy by the metabolism of glucose. Under excessive contractile stress, these muscle ferment glucose, forming lactate. Interestingly, muscle is able to not only delay, but partially sidestep the so called “oxygen debt” accrued during high activity periods by shifting some of its respiratory burden to the liver. This “metabolic stunt” (known as the Cori cycle) is performed by exporting lactate into the circulation which is then, in the liver, converted back to into glucose *via* gluconeogenesis (which is ATP dependant, implying oxidative respiration). This glucose is released into circulation and can thus be re-utilized in glycolysis.

It should be noted that the Cori cycle is not sustainable. Anaerobic glycolysis has a total output of two ATP for each glucose, whilst glyconeogenesis require six ATP molecules to salvage one glucose molecule from lactate formed. Glycolysis and gluconeogenesis metabolically run in opposite directions, and as such, the simultaneous activation of this cycle in the body makes this cycle a “futile cycle” (but since to two processes takes place in different organs, it is still useful). Indeed, cancer cells also make use of this cycle, contributing to cachexia by metabolically “wasting” ATP (Tisdale 1997).

Furthermore, the heart (which is principally driven by oxidative respiration) also takes up lactate and feed it into the Krebs cycle (thus utilizing circulating lactate for local oxidative phosphorylation)(Gertz, Wisneski *et al*. 1988). In a way, the resulting effect is as if “oxidative” glycolysis started in the skeletal muscle, but ended in the oxidative muscle of the heart.

In effect, the turnover of energy in fast twitch muscles is much higher than the amount of oxygen the muscle itself consumes. Since cellular respiration (oxidation in the mitochondria) is a major source of ROS, fast twitch fibres will not be subjected to such high levels of oxidative damage as slow twitch, oxidative fibres (which produce the bulk of their energy by local oxidative phosphorylation). Thus, the rate of myonuclei turnover is expected to be higher in oxidative than glycolytic muscle fibers. The increased number of local resident satellite cells in slow twitch muscle is therefore not only as a

result of increased capillary density but may also be as a result of the physiological requirement of the tissue itself.

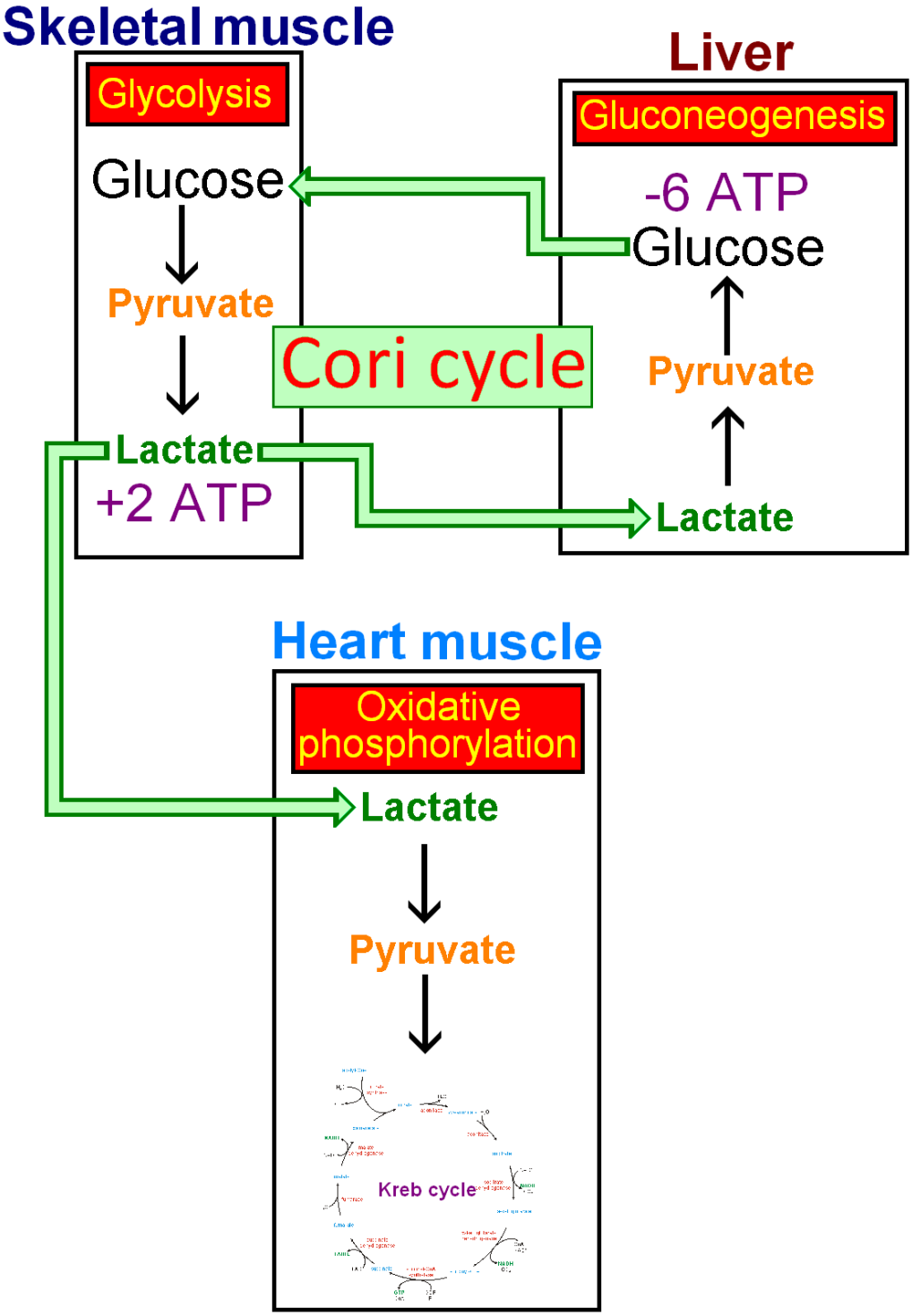


Figure 4.6 How skeletal muscle utilise a glycolytic energy generating strategy to delay (and partial circumvent) the oxygen debt. Note, the skeletal muscle exporting lactate have a dead weight loss of this energy rich molecule to the heart, but avoid a accumulation of local lactate. Also, the glucose supply via the Cori cycle is at a net loss to the body: every two moles of ATP generated in glycolysis require 6 mole of ATP as input to salvage glucose from lactate. But in doing so, the muscle is able to generate energy without any obligation to respiration.

In the light of the above, it might have been more effective to count the total number of myonuclei for comparison to satellite cell nuclei. As the rat *gastrocnemius* muscle is a muscle of mixed fiber type some samples would have had differences in the numbers of fast (glycolytic) and slow (oxidative) fibres in a field of view, thus complicating the determination and interpretation of satellite cell numbers. In addition at more advanced time points, satellite cells could have already contributed to the pool of myonuclei. But since differentiating/differentiated cell nuclei would not be counted as satellite cells (by not expressing CD34 or CD56) this might also lead to the development of an incomplete picture of what was happening in the tissue. Having the total number of myonuclei per fibre might at the very least "flag" a general increase in myonuclei.

Another drawback of this technique is that the spatial orientation and position of the satellite cell (relative to the basal lamina) cannot be utilized as an additional indicator of satellite cell identity, since the basal lamina can not be identified without immunostaining. Also, since both markers stain cells in a particular part of the cell cycle, they might not identify other satellite cells which are more committed to differentiation or wich are totally quiescent. Nevertheless, considering there is not a true universal indicator for cell cycle specific identification of satellite cells (Montarras, Morgan *et al*. 2005), CD34 and N-CAM could be considered to be reasonable markers.

On that note though, another possible shortcoming with using the CD34/N-CAM co-staining approach is the possibility that CD34/N-CAM might not be expressed together on all of the cells, thus leading to an under-counting of satellite cells.

In future it will be possible to use desmin in conjunction with another satellite cell markers. Desmin stains the basal lamina and thus allows for the identification of satellite cells by their position relative to the basal lamina. Used in conjunction with another satellite marker, possibly Pax 7, Pax 3 or even N-CAM it may be possible to have a higher degree of confidence in

counting satellite cells in sections of muscle tissue (Halevy, Piestun *et al*. 2004; Horst, Ustanina *et al*. 2006; Relaix, Montarras *et al*. 2006).

Staining and subsequent counting of satellite cells it is a tedious and labour intensive task and this coupled to the limitations placed on markers one can use by the excitation and emission filters available on any give microscope means that one may want to develop alternative techniques for assessing satellite cell numbers *in-vivo*.

Chapter 5
Concluding remarks

The *in-vitro* results have revealed interesting trends and significant effects in response to laser irradiation. Though the assays used make elucidation of the exact characteristics of these response somewhat ambiguous, the observed results do indicate a positive physiological response to photo-biostimulation. Future studies should aim at identifying the responses in greater detail as well as elucidating the exact mechanisms invoked in these metabolic/cell-signaling systems. This is especially true for Western blotting studies where trends were observed, but no significant result observed.

While the ORAC assay did show more variation between groups, the satellite cell counts between groups did not show much variation other than what one would have expected to be induced by the injury itself. It is thus possible that the ORAC results were merely artifacts of a procedure which inherently produces variable results. It could also be possible, in the *in-vivo* setting that laser did not penetrate the skin, or that the dose used was too low. The diode laser beam profile as well as the degree of tissue penetration of the laser light used in this study has not been categorized, either by ourselves or by anyone in the literature. Future studies should investigate the optical properties of both tissue as well as reagents/instruments used. As an example even media containing no phenol red would still have optical properties.

In conclusion the results of this study suggest that whilst laser irradiation has effects that are easily discernable when using a single cell type in an *in-vitro* setting it is incredibly difficult to transfer these effects and results to an *in-vivo* setting. Further work is required before conclusions can be made about the effectiveness of 638 nm laser irradiation on satellite cells *in-vivo*.

Chapter 6

Addendum

6.1 MTT Colorimetric assay

Reagents

Stock solvent

1% isopropanol

- Isopropanol: 99 ml
- HAL: 1ml

0.1% Triton

- Triton-x: 0.1 ml
- dH_2O: 99.9 ml

MTT assay Solvent (“50/51”)

- 1% isopropanol: 50/51
- 0.1% Triton: 1/51

1% MTT reagent

- MTT: 0.01g
- PBS: 1ml

NOTE:

- Foil wrap container to avoid exposure to light
- Keep on ice
- Vortex solution
- Filter (20µm) to get rid of excess MTT micro-clumps

Method

- Decant media (don't wash)
- Add to each Petri dish (slowly):
  - 1.5 ml PBS (preheated to 37 ºC)
  - 0.5 ml 1% MTT solution

    - Cover in tinfoil
    - Incubate for 2 hr in incubator

	If cells loosened	*If cells remained adhered*
1	Transfer content to opaque/light-proof 2 ml tube	Decant content of Petri dish
2	Spin down : 2min/1000 rpm	Add 1.5 ml Solvent ("50/51")
3	Decant supernatant	
4	Add 1.5 ml Solvent ("50/51") to Petri dish	
5	Add cell-solvent suspension back to original petrel dish.	

    - Cover in tin foil
    - Place on shaker for 5 min
    - Transfer content to "light-prove" (opaque) 2 ml tube
    - Centrifuge 2 min/1400 rpm
  - *Hint: switch on spectrometer wills waiting for centrifuge*
    - Decant supernatant into clean light prove 2 ml micro tube
    - Read absorbance at 540 nm: blank with Solvent ("50/51")

6.2 Deparaffinization-rehydration & tissue permeabilization.

Reagents

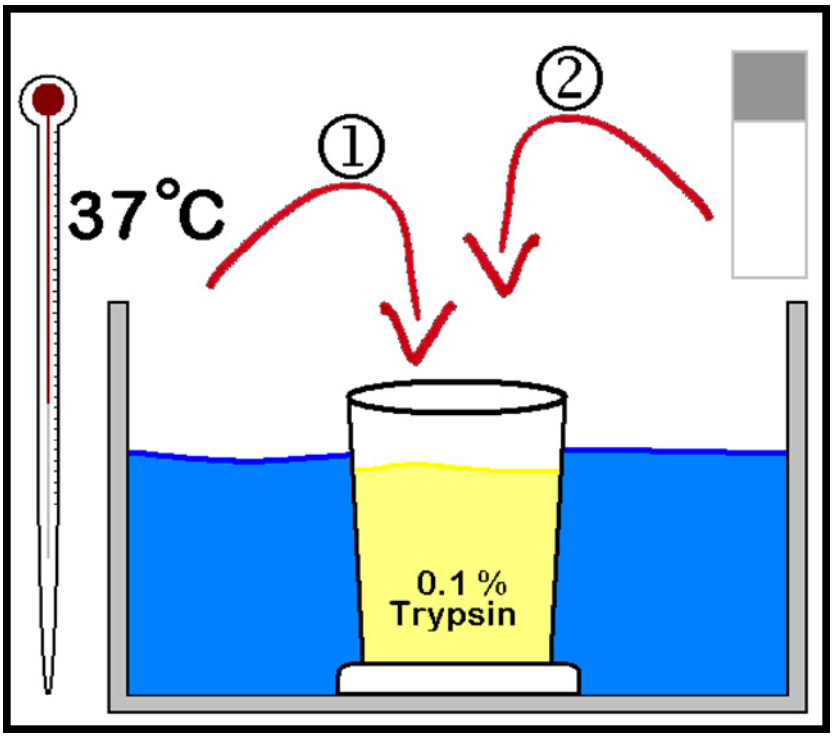
- Xylene— Sigma-Aldric
- Ethanol: 100%, 95%, 80% 50%
- 0.1% Trypsin

Method

Hint: carefully plan possible staggering steps! Prepare 10 Coplin jars in series filed $^2/_3$ with the above mentioned reagents, according to following schedule for each sample incubation step:

1) Xylene (5 min)
2) Xylene (5 min)
3) 100% ethanol (3 min)
4) 100% ethanol (3 min)
5) 95% ethanol (3 min)
6) 95% ethanol (3 min)
7) 80% ethanol (3 min)
8) 50% ethanol (3 min)
9) dH_2O (3 min)
10) dH_2O (3 min)

- Pre-warmed Coplin jars filled $^2/_3$ with 0.1% Trypsin in water baht at 37ºC.
- Place slide in preheated 0.1% Trypsin and incubate 20 min.
- Immediately proceed with immunohistochemistry after deparaffinization-rehydration.



Make sure Trypsin is 37ºC (don't measure the temperature of the water bath- though the water might be 37ºC, the Trypsin might still be cold). Remove thermometer and place processed slide into trypsin bath. Incubate for 20 min.

6.3 Haematoxylin and eosin progressive staining

Reagents

- Ethanol: 100%, 95%, 70%
- Xylene
- "10% Acetic alcohol" (named by convention not actual concentration)
  - (10 ml 1% HCl in 1ℓ 70 % ethanol)
- Eosin
  - Stock: 10 g Eosin dissolved into 1ℓ dH_2O
  - Working solution
    - Prepare fresh: 10 ml Eosin stock in 90 ml dH_2O. Filter solution.
    - Add 2~3 drops glacial acetic acid in to 100 ml work-solution
- Mayer's Haematoxylin

Add the following reagents to 1ℓ dH_2O

- aluminium potassium 50g
- dissolve in dH2O
- 5 g Haematoxylin powder
- dissolve in dH2O
- 0.4g sodium iodate
- 20ml glacial acetic acid

➢ Heat solution up to boiling point. Remove from heat and rapidly cool down in fridge

➢ Add 4 ml glacial acetic acid to 100 ml Haematoxylin solution before staining. Filter before use

- Scott's tap water
  - Dissolve 3.5 g $NaHCO_3$ in tap water
  - Add 20 mg $MgSO_4$

- Add 10 ml 37% formalin

Method

Prepare 21 Coplin jars in series flied $^{2}/_{3}$ with the above mentioned reagents, according to following schedule for each sample incubation step:

1)Xylene (10 min)
2)100% ethanol (10 dips)
3)100% ethanol (10 dips)
4)95% ethanol (10 dips)
5)95% ethanol (10 dips)
6)70% ethanol (10 dips)
7)dH_2O (10 dips)
8)Haematoxylin (10 min)
9)dH_2O (10 dips)
10) Acetic alcohol (10 dips)
11) dH_2O (10 dips)
12) Scott's tap water (10 dips)
13) dH_2O (10 dips)
14) Eosin (2 min)
15) dH_2O (10 dips)
16) 70% ethanol (10 dips)
17) 95% ethanol (10 dips)
18) 95% ethanol (10 dips)
19) 100% ethanol (10 dips)
20) 100% ethanol (10 dips)
21) Xylene (10 dips)

Gently tap of execs xylene and mount sample wit mounting media and coverslip.

6.4 Automatic tissue processing

Reagents

Paraffin wax (Histosec, Merk)
Xylene (Sigma-Aldric) ~2ℓ
Ethanol: 100%, 95%, 90% 70% (2.5, 1.5, 0.8 and1.5 ℓ respectively)

Method

Tissue sample previously soaked in formaldehyde need to be: (**a**) dehydrated, (**b**) "cleared" or be saturated with a medium which accommodate (**c**) impregnation with paraffin.

After preparing reagents, pour ~700 ml of each reagent into glass flask on tissue processor in the order (as indicated below). Tissue processor should be pre-programmed according to following time-schedule per sample incubation step:

(A) Dehydration			
	(1) 70%	ethanol	90 min
	(2) 70%	ethanol	90 min
	(3) 90%	ethanol	90 min
	(4) 95%	ethanol	90 min
	(5) 95%	ethanol	90 min
	(6) 100%	ethanol	90 min
	(7) 100%	ethanol	90 min
	(8) 100%	ethanol	120 min
(B) Clearing			
	(9) Xylene		90 min
	(10) Xylene		120 min
(C) Impregnation			
	(11) paraffin wax		120 min
	(12) paraffin wax		120 min
			=20 hours

6.5 Immunohistochemical staining: double-labelled probing

Notation

1) Fist Primary antibody	$1^{st}1^{o}$	
2) First Secondary antibody	$1^{st}2^{o}$	("anti-1^{st} 1^{o} ")
3) Second primary antibody	$2^{nd}1^{o}$	
4) Second secondary antibody	$2^{nd}2^{o}$	("anti-2^{nd} 2^{o} ")

Suppliers of antibodies

Monoclonal mouse anti-human CD34 (ICO 115) —sc-7324, Santa Cruz
Polyclonal rabbit anti-human NCAM (H-94) — sc-8305, Santa Cruz
Donkey serum, donkey anti-rabbit, donkey anti-mouse — Jackson immunoresearch Inc.

Reagents

Method

All volumes added to slide is 40 µl

- After completing deparaffinization protocol
- Gently flick of excess PBS before encircling with wax pen
- Block for 20 min in serum at room temperature
  - *NOTE:* Use serum from same animal in which 2º antibodies was raised!

- Flick of excess serum
- Incubate section with 1st1º antibody for 4hr at room temperature:
  - Rabbit anti-N-CAM (1:100 dilution)
- Rinse slide in PBS
- Incubate section with 1st2º antibody for 40 min at room temperature
  - Donkey anti-rabbit--Texas Red conjugated (1:1000 dilution)
- Incubate over night at 4ºC
- Rinse slide in PBS
- Incubate section with 2nd1º antibody for 40 min at room temperature
  - Mouse anti-CD34 (1:100 dilution)
- Rinse slide in PBS
- Incubate section with 2nd2º antibody for 30 min at room temperature
  - Donkey anti-mouse--FITC conjugated (1:1000 dilution)
- After 15 min, add Hoechst (1:500) on slide (wile incubating 2nd2º antibody)
- Rinse slide in PBS
- Mount cover slide with DAKO™ fluorescent mounting media

Useful hints

➢ Be extremely careful to avoid cross-recognition of the antigens when selecting antibodies. Also make sure not to mix-up species serum/antibodies combinations. Also, if possible, pick your secondary antibody to be from the same specie. This will ease serum-blocking step.

➢ Flick of excess PBS as this will dilute reagents and give rise to inconstant staining between samples.

➢ Calculate antibody volume carefully- antibodies are expensive! About 40 µl per section should be sufficient. Apply following formula for final volume:

(# Sections **×** # Slides) × 40 µl = final volume antibody (µl) (add extra 40 µl to final volume)

6.6 The ORAC Assay Protocol

Reagents

TROLOX: Vitamin E analogue with potent antioxidant properties. Water soluble (up to 0.5 mg/ml) and cell membrane permeable.

Fluorescein: Fluorescing agent with an absorption maximum at 494 nm and emission maximum of 521 nm (in water). Susceptible to degradation by reactive species.

AAPH: An azo-initiator producing peroxyl free radical (which degrade fluorescein).

Phosphate buffer: Buffered at pH 7.4

Stock solutions

6.6.1.1 Phosphate buffer

- 6.5g K_2HPO_4 in 500ml H_2O (**1**)
- 5.1g KH_2PO_4 in 500ml H_2O (**2**)
- 300 ML (**1**: K_2HPO_4) in 1 beaker
- Add (**2**: KH_2PO_4) until pH 7.4

Trolox

Trolox is made up in a two-step dilution

Trolox *pre-stock (9.988mM)*

- Place 15 ml Falcon tube (wrapped in tin foil) on scale
- Zero scale
- 0.025 g Trolox into tube
- Expose volume indicators and fill up to 10 ml with ice cold phosphate buffer by slowly pipetting
- Gently mix by inverted pipetting gently (avoid rigorous shaking, as this expose the Trolox to O_2 which will readily oxidize the Trolox)

Trolox (400µM Stock)

- Add 21.12 ml Phosphate buffer (cold) into 25 ml brown glass vial
- Add 0.88 ml pre-Stock (9.988mM)
- Gently mix by inverted pipetting gently
- Aliquot 250 µl into 40 pre-marked tubes
- Freeze down in -20 or -80 ◦C depending on estimate time of storage

Only 0.88 ml of the pre-stock was used. If desired, more aliquots of stocks can be made.

Fluorescein (5×10^{-3} mM stock)

- Place 25 ml glass vial on scale
- Place 2 ml light prove tube into the vial as to have the tube rested on lip of the vial cap-screw
- Zero scale
- Add 1.5 mg fluorescein
- Full up with 1.5 ml phosphate buffer
- Gently mix by inverted pipetting
- Pour 50 ml phosphate buffer into light prove vial
- Aliquot 94.33 µl in the vial containing 50 ml phosphate buffer
- Store: 4 °C, keep in dark to avoid photobleaching

Working solutions

Fluorescein (8.16×10^{-5} mM working solution)

- Pour 19.7 ml phosphate buffer into flask wrapped in tin foil
- Add 326 µl Stock in 19.7 ml phosphate buffer
- Keep on ice

AAPH (153 mM)

- Add 414 g into 10 ml phosphate buffer

- Keep on ice, make fresh

Trolox (working solution): standard curve

Make a dilation range as follow:

- Place 200 µl PB in 5 tubes (each) pre-labelled 200, 100, 50, 25, 12.5
- Add 200 µl Trolox stock (400µM) into tube-1 and mix by gently invert pipetting
- Add 200 µl of tube-1 (thus, 100 µM) into tube-2 and mix by gently invert pipetting
- Repeat procedure for tube-3 and tube-4

Preparing samples and reagents

- If phosphate buffer were not stored in fridge, place in fridge to allow phosphate to cool down to ~4 °C

NOTE: *AAPH donate H_2O_2 by thermal decay- something to be postpone until the initiation of the assay in plate reader! Keep cold at all time.*

- Containers in which AAPH and fluorescein are to be made up in flask pre-wrapped in tin foil.
- Defrost Plasma/cells on ice (tissue sample should be ORACE immediately after sonication).
- Defrost Trolox stock solution on ice

While samples are defrosting:

- Mark micro tubes in which samples are to be diluted: each sample is done in triplicate, thus, 3-tubes/sample
- Mark tubes for Trolox dilution curve
- Prepare multi-pipettes: p-200 set at 150 µl ::: p-100 set at 25 µl
- Switch on plate reader, insert black plate and select run-protocol

- Make up Trolox working solution (as explained)
- Dilute plasma (×75) or tissue (× 200) in phosphate buffer (*not* in RIPA buffer!)

Executing assay

- Before commencing with the assay, decant fluorescein and AAPH into separate marked crypts which will accommodate multi-pipetting.

- Using a p-100 pipette:

  - Pipette 25 µl PB into wells designated as blanks
  - Pipette 25 µl Trolox into wells designated for standard curve
  - Pipette 25 µl sample into designated well

- Using a p-200 multi-pipette, add150 µl fluorescein working solution (8.16 $\times 10^{-5}$ mM) into every well
- Incubate plate in plate reader for 10 minutes at 37 $^{\circ}$C (*very important!*)
- Using a p-100 multi-pipette, add 25 µl AAPH (153 mM) into every well
- The reaction commence when the cold AAPH added to the warmed up reaction media- work speedily!

Analysing results

Data can be exported into Excel format. These results should be cut and passed into a pre-programmed Excel spreadsheet to calculate the AUC. Using values obtained for each Trolox dilution (Seen diagram on following page).

Step # 1

Values obtained from plate reader- processed.

SUM	AUC	NET AUC	
15.11368	15.61368		BLANK
71.07933	71.57933	55.96565	Sampl-1
65.35422	65.85422	50.24054	Sample-2
61.76459	62.26459	46.65091	Sample-3
66.42573	66.92573	51.31205	Sample-4
59.66151	60.16151	44.54784	Sample-5
61.82391	62.32391	46.71023	Sample-6
63.58877	64.08877	48.47509	Sample-7
66.85512	67.35512	51.74144	Sample-8
89.83096	90.33096	74.71728	200
55.70509	56.20509	40.59142	100
37.63846	38.13846	22.52478	50
30.23567	30.73567	15.12199	25
24.34946	24.84946	9.235788	12.5

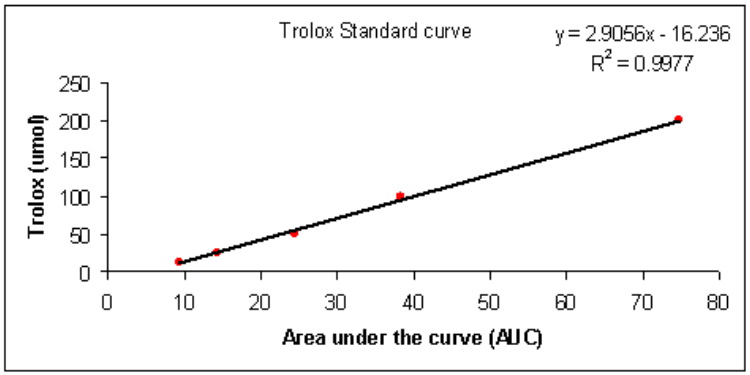
Step # 2

Values obtained for each standard dilution point

AUC	µM Trolox
74.71728	200
38.37367	100
24.55201	50
14.42443	25
9.235788	12.5

Step # 3

Trolox standards potted on graph.



Step # 4

y =(2.9056x - 16.236)×dilution factor

y = ORAC value as µmol Trolox equivalent per liter

x = Net AUC

Substituting net AUC value into X produce Trolox equivalent value expressed as µmol Trolox per liter of sample.(Homogenized muscle was expressed as Trolox per liter of sample per µg protein).

Result!

Sampl-1	21809	T4-1	19678
Sampl-2	19331	T4-2	
Sampl-3	17777	T4-3	
Sampl-4	19794	T4-4	
Sampl-5	16867	Ir48-1	18304
Sampl-6	17803	Ir48-2	
Sampl-7	18566	Ir48-3	
Sampl-8	19980	Ir48-4	

6.7 Bradford: protein quantification

The Bradford method is a cheap, quick and easy method for quantifying protein content in samples. It is sensitive down to the down to 20 µg/ml range and are less liable to interference by other non-protein compounds (with the noteworthy exception of SDS). The method relies on the binding of Coomassie Brilliant Blue to protein, more specifically, basic amino acids like lysine and arginine. This makes the assay a relative one as the binding varies with protein amino acid sequence.

Unbound under acidic conditions, the dye has an absorbance maximum at 470 nm and 650 nm (given rise to a red/pink-brown colour). When bound, the dye's absorbance maximum shift towards 595 nm (bluish colour). A standard curve is generated by measuring the absorbance of control samples over a known dilution range. By measuring the absorbance spectrum of an unknown sample and comparing it to the absorbance on the standard curve, the protein concentration of an unknown sample can be deduced.

Bradford reagent Stock (6 X)

- 500 mg Coomassie Brilliant Blue in 250 ml 95% EtOH
- 500 ml Phosphoric acid (add under a fume hood)
- Full up to 1L with dH_2O
- Store at 4 °C

Bradford working solution

- Dilute with dH_2O in 1:5 ration
- Filter using 2 filter papers dubbed folded.
- Filter procedure might need to be repeated until solution is light brown in colour

Bradford protocol

6.7.1.1 Standard curve

- BSA stocks (1 mg/ml) must be thawed at room temperature. Keep on ice

Hint: mark 7 Eppendorf tube tubes for standard curve as well as tubes for samples will BSA thaw

- Dilute with dH_2O in 1:5 ration for a working solution (200 µg/ml).
- Aliquot samples into marked tubes according to following schedule table:

BSA Standard Curve

200 µg/ml BSA	µg Protein	dH_2O	Bradford Reagents
100 µl	20	0 µl	900µl
80 µl	16	20 µl	900µl
60 µl	12	40 µl	900µl
40 µl	8	60 µl	900µl
20 µl	4	80 µl	900µl
10 µl	2	90 µl	900µl
0 µl	0	100 µl	900µl

NOTE: Tube without any BSA (bottom of table) will be used as blank

- Vortex each tube
- Let samples stand at room temperature for about 5 min
  - *Hint: spectrophotometer can be started up in the mean time*
  - *Take reading within one hour after mixing sample with reagent*
- Aliquot into appropriate covet, zero with blank, read absorbance at 595 nm
- Use Excel to plot a linier standard curve

Samples

Prepare samples and standard together to avoid variation on results

- 900 µl Bradford Reagents
- 95 µl dH_2O
- 5 µl Protein sample
- Vortex each tube
- Let samples stand at room temperature for about 5 min
- Aliquot into appropriate covet, zero with blank, read absorbance at 595 nm

Note:

If sample concentrations are too high (above highest value in your standard curve), samples can be dilute with RIPA buffer.

6.8 Western-blot Analysis

Casting gel

- Clean cover plates with methanol
- Place glass slides back-to-back and clamp securely
- Place clamped plates in stand and add water (check for leakage).

Stacking gel	Volume (µl)	
	2 Gels (1 mm plate)	4 Gels (1 mm plate)
dH2O	3050	6100
1.5M Tris (pH 8.8)	1250	2500
10 % SDS	50	100
40% acrylamide	500	1000
10% APS	50	100
TEMED	10	20

Resolving gel	Volume (µl)	
	2 Gels (1 mm plate)	4 Gels (1 mm plate)
dH2O	7700	15400
1.5M Tris (pH 8.8)	5000	10000
10 % SDS	200	400
40% acrylamide	5000	10000
10% APS	100	200
TEMED	10	20

Executing Electrotransfer (semi-dry system)

Buffers for Electrotransfer system

Anode buffer 1

- 0.3 M Tris-base pH 10.4
- 20 % methanol

Anode buffer 2

- 25 mM Tris-base pH 10.4
- 20 % methanol

Cathode buffer

- 25 mM Tris-base
- 40 mM ε-aminohexanoic acid
- pH 9.4
- Full up with methanol until 20 % of total volume

Preparation

For each gel, gut 8 Whatman 3MM papers and one PVDF membrane to required size: the Whatman slightly smaller than the gel itself, the PVDF membrane slightly smaller than the Whatman paper. Handle the membrane with tweezers. Make sure the transfer apparatus is clean and clear of any debris.

- Soak 3 Whatman papers in anode 1 buffer
- Soak 1 Whatman papers in anode 2 buffer
- Soak 4 Whatman papers in cathode buffer
- On the plate of the semi-dry apparatus, place 3 Whatman papers soaked in anode 1 buffer.
- Wet a glass tube in anode buffer 1 and gently role over Whatman papers to remove any bubbles
- Soak Whatman papers in methanol for about 15 seconds, moving it gently to remove air bubbles
- Place PVDF membrane on Whatman papers
- Wet a glass tube with methanol and gently role over PVDF membrane to remove any bubbles
- Take gel with proteins of interest and wet in anode buffer 2: this can be done when removing the gel from the back plate by gently swirling the gel attached to the plate in anode buffer 2 until it fold of.
- Wet a glass tube in anode buffer 2 and gently role over gels to remove any bubbles
- Place 4 Whatman papers soaked in cathode buffer on top of gel

- Wet a glass tube in cathode buffer and gently role over Whatman papers to remove any bubbles
- Gently wet apparatus plates and clad semi-dry
- Close lid, making sure the electrodes are secure in position. Run for 1 hour at 0.5 A current at 15 V
- After electrophoresis, gently lift lid and remove Whatman papers
- Fix protein to membrane by placing the membrane in 100% methanol for few seconds (long enough to soak the membrane).

Immunoprobing of protein: Akt, Akt-p, AMPK, AMPK-p, STAT-3 and β-actin.

Blocking

- Prepare Tris Bufferd Saline-Tween 20 (TBS-T, 0.05%)
- Use TBS-T20 as solvent: add Elite© non-fat milk to final concentration of 5% (w/v)
- Incubate for at least 2 hours room temperature (preferably over night at 4ºC).

Probing: primary antibody

- Wash membrane in TBS-T thoroughly: 5 washes, 5 minutes each on the "belly-dancer".
- Prepare a 1:1000 dilution of antibody in TBS-T (5µl 1˚ antibody in 5 ml TBS-T)
- Incubating with primary antibodies (diluted in TBS-T) overnight at 4 ˚C.

Probing: secondary antibody

- Wash membrane in TBS-T thoroughly: 5 washes, 5 minutes each on "belly-dancer".
- Prepare a 1:1000 dilution of antibody in TBS-T (5µl 2˚ antibody: 5 ml TBS-T)
- Incubate 1 hour at room temperature
- Wash membrane in TBS-T thoroughly: 5 washes, 5 minutes each on "belly-dancer".

- Add 0.5 ml ECL (enhanced chemiluminescence kit Amersham Bioscience, UK limited) reagent 1 and 2 (each 0.05 ml) to membrane. Ensure reagent is evenly distributed across membrane. Washing membrane with ECL (by gently revolving container) for one minute at room temperature.
- Transfer membrane to plastic membrane protector
- Place protected membrane in film cassette with protein side facing up

- Expose membranes on Hyperfilm (Hyperfilm, Amersham Bioscience, UK limited) in dark room for ± 5 minutes
- Place in developer for about 30 seconds.
- Wash and fix film
- Quantified by densitometry with UN-SCAN-IT© PROGRAM (Silk Scientific corporation, Utah, USA).

6.9 RIPA buffer

The buffer constituents can be divided between “stable” and “unstable” constitutes: it is advise to first proceed adding the “stable” reagents before proceeding with the application of the liable reagents (specifically, protein inhibitors). Some of the reagents are light sensitive- cover beaker (in which 50 mM Tris is to be made up) in tin foil.

Also, if phosphatase assays are to be done, phosphatase inhibitors should be avoided in RIPA buffer (e.g Na_3VO_4 and NaF). Similarly some of the ionic detergents denature some proteins which might result in a decrease in activity. Many companies sell kits and buffers tailored for specific assays and might be consider.

Bearing in mind the amount of reagents required in preparation of RIPA buffer, it is useful to first familiarize oneself with the precise location of the reagents in the lab. Also, give thought to how the RIPA buffer is to be used as this has implication for volume to be aliquot per stored units (eg. 15 ml falcon tube, or 1.5 ml Eppendorf tube).

Final concentrations

- Tris-HCl: 50 mM
- Na-deoxycholate: 0.25%
- EDTA: 1mM
- PMSF: mM
- Leupeptin: 1µg/ml
- SBT1: 4µg/ml
- Benzamidine: 1mM
- Na_3VO_4: 1mM
- NaF: 1mM

Overview of reagents

NaF: (Sodium fluoride): Preserve the protein phosphorylation state in proteins by inhibiting phosphoseryl and phosphothreonyl phosphatases (PSPs) TOXIC (can cause fluoride poisoning).

Na_3VO_4: (Sodium orthovanadate) Inhibit phosphatases and ATPasas

Na-deoxycholate: a biological detergent that lyses cells and solubilise cellular membrane components.

EDTA: Chelating agent that can sequester di- and trivalent metal ions Mn(II), Cu(II), Fe(III), Co(III)

PMSF: (phenylmethanesulphonylfluoride) : block serine proteases (e.g trypsin and chymotrypsin). Short $t_{1/2}$ (+/- 40 min).Toxic!

Leupeptin: inhibitor of protease, including calpain.

SBT1: (Soybean trypsin inhibitor): inhibits trypsin and some enzymes with simile protease mechanisms.

Benzamidine: Reversibly inhibit trypsin/ trypsin-like enzymes as well as serine proteases in a competitive manner.

Triton X-100: Non-ionic surfactant. Solubilise membrane proteins, cause membrane permeability

NP-40: Tergitol-type NP-40. Detergent

The following guideline is for 100 ml RIPA buffer at final concentration as mentioned. Stock solutions of some reagents were made up in advance (stock concentrations is mentioned were applicable.)

Constituents

1) 50 mm Tris-HCl

- 790 mg Tris + 900mg Cl + 75 ml dH_2O
- Solution is alkaline: Calibrate pH Meter range. pH 7.4 using diluted HCl

2) 10 ml 10% NP-40

3) Na-deoxycholate: 0.25%

4) 1 ml 100mM EDTA pH7.4

5) 50 µl PMSF 200 mM

6) 100 µl leupeptin 1mg/ml

7) 80 µl SBT1 5mg/ml

8) 100 µl Benzamidine 1M

9) 1 ml Triton X-100. *(Very viscous- gently warming up before helps with pipetting!)*

6.10 PBS buffer

- 800 ml dH_2O
- 0.2 g KCl
- 8 g NaCl
- 1.44 g Na_2HPO_4
- 0.24 g KH_2PO_4
- Adjust pH to 7.4 with HCl.
- Fill up with H_2O to 1ℓ

References

Afanas'ev, I. B., A. I. Dorozhko, *et al*. (1989). "Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation." Biochem Pharmacol **38**(11): 1763-9.

Afonin, B., M. Ho, *et al*. (2006). "Cell behaviors associated with somite segmentation and rotation in Xenopus laevis." Dev Dyn **235**(12): 3268-79.

Akira, S., T. Taga, *et al*. (1993). "Interleukin-6 in biology and medicine." Adv Immunol **54**: 1-78.

Alameddine, H. S., J. P. Louboutin, *et al*. (1994). "Functional recovery induced by satellite cell grafts in irreversibly injured muscles." Cell Transplant **3**(1): 3-14.

Alexandratou, E., D. Yova, *et al*. (2002). "Human fibroblast alterations induced by low power laser irradiation at the single cell level using confocal microscopy." Photochem Photobiol Sci **1**(8): 547-52.

Alexandratou, E., D. Yova, *et al*. (2005). "A confocal microscopy study of the very early cellular response to oxidative stress induced by zinc phthalocyanine sensitization." Free Radic Biol Med **39**(9): 1119-27.

Allen, D. L., S. R. Monke, *et al*. (1995). "Plasticity of myonuclear number in hypertrophied and atrophied mammalian skeletal muscle fibers." J Appl Physiol **78**(5): 1969-76.

Allenbach, C., C. Zufferey, *et al*. (2006). "Macrophages induce neutrophil apoptosis through membrane TNF, a process amplified by Leishmania major." J Immunol **176**(11): 6656-64.

Alvarez, B., L. S. Quinn, *et al*. (2002). "TNF-alpha modulates cytokine and cytokine receptors in C2C12 myotubes." Cancer Lett **175**(2): 181-5.

Andersen, J. L. (2003). "Muscle fibre type adaptation in the elderly human muscle." Scand J Med Sci Sports **13**(1): 40-7.

Anderson, J. and O. Pilipowicz (2002). "Activation of muscle satellite cells in single-fiber cultures." Nitric Oxide **7**(1): 36-41.

Anderson, J. E. (2000). "A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells." Mol Biol Cell **11**(5): 1859-74.

Anderson, L. V. and K. Davison (1999). "Multiplex Western blotting system for the analysis of muscular dystrophy proteins." Am J Pathol **154**(4): 1017-22.

Ashkenazi, H., Z. Malik, *et al*. (2003). "Eradication of Propionibacterium acnes by its endogenic porphyrins after illumination with high intensity blue light." FEMS Immunol Med Microbiol **35**(1): 17-24.

Auerbach, H. S., M. Williams, *et al*. (1985). "Alternate-day prednisone reduces morbidity and improves pulmonary function in cystic fibrosis." Lancet **2**(8457): 686-8.

Avni, D., S. Levkovitz, *et al*. (2005). "Protection of skeletal muscles from ischemic injury: low-level laser therapy increases antioxidant activity." Photomed Laser Surg **23**(3): 273-7.

Baldwin, K. M. (1996). "Effects of altered loading states on muscle plasticity: what have we learned from rodents?" Med Sci Sports Exerc **28**(10 Suppl): S101-6.

Baldwin, K. M., D. A. Cooke, *et al*. (1977). "Time course adaptations in cardiac and skeletal muscle to different running programs." J Appl Physiol **42**(2): 267-72.

Barany, M. (1967). "ATPase activity of myosin correlated with speed of muscle shortening." J Gen Physiol **50**(6): Suppl:197-218.

Barbero, A., R. Benelli, *et al*. (2001). "Growth factor supplemented matrigel improves ectopic skeletal muscle formation--a cell therapy approach." J Cell Physiol **186**(2): 183-92.

Beauchamp, J. R., L. Heslop, *et al*. (2000). "Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells." J Cell Biol **151**(6): 1221-34.

Beiner, J. M. and P. Jokl (2001). "Muscle contusion injuries: current treatment options." J Am Acad Orthop Surg **9**(4): 227-37.

Beiting, D. P., S. K. Bliss, *et al*. (2004). "Interleukin-10 limits local and body cavity inflammation during infection with muscle-stage Trichinella spiralis." Infect Immun **72**(6): 3129-37.

Belcastro, A. N., G. D. Arthur, *et al*. (1996). "Heart, liver, and skeletal muscle myeloperoxidase activity during exercise." J Appl Physiol **80**(4): 1331-5.

Belcastro, A. N., L. D. Shewchuk, *et al*. (1998). "Exercise-induced muscle injury: a calpain hypothesis." Mol Cell Biochem **179**(1-2): 135-45.

Berki, T., P. Nemeth, *et al*. (1991). "Effects of photosensitization and low-power helium-neon laser irradiation on liposomes and cell membranes." Scanning Microsc **5**(4): 1157-64.

Bernas, T. and J. Dobrucki (2002). "Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes." Cytometry **47**(4): 236-42.

Bernhard, O. (1926). Light treatment in surgery. London Edward Arnold & Co.

Berridge, M. V. and A. S. Tan (1993). "Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction." Arch Biochem Biophys **303**(2): 474-82.

Berthois, Y., J. A. Katzenellenbogen, *et al*. (1986). "Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture." Proc Natl Acad Sci U S A **83**(8): 2496-500.

Berti, A., S. Rigacci, *et al*. (1994). "Inhibition of cellular response to platelet-derived growth factor by low M(r) phosphotyrosine protein phosphatase overexpression." FEBS Lett **349**(1): 7-12.

Bewick, G. S., P. S. Zammit, *et al*. (1993). "Restoration of motor unit properties and fiber type distribution in reinnervated axolotl skeletal muscle." Dev Biol **158**(2): 288-300.

Bicer, S. and P. J. Reiser (2004). "Myosin light chain isoform expression among single mammalian skeletal muscle fibers: species variations." J Muscle Res Cell Motil **25**(8): 623-33.

Blau, H. M., G. K. Pavlath, *et al*. (1985). "Plasticity of the differentiated state." Science **230**(4727): 758-66.

Bleakley, C., S. McDonough, *et al*. (2004). "The use of ice in the treatment of acute soft-tissue injury: a systematic review of randomized controlled trials." Am J Sports Med **32**(1): 251-61.

Bodine, S. C., T. N. Stitt, *et al*. (2001). "Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo." Nat Cell Biol **3**(11): 1014-9.

Boelens, R., R. Wever, *et al*. (1983). "An EPR study of the photodissociation reactions of oxidised cytochrome c oxidase-nitric oxide complexes." Biochim Biophys Acta **724**(2): 176-83.

Bogoyevitch, M. A., D. C. Ng, *et al*. (2000). "Intact mitochondrial electron transport function is essential for signalling by hydrogen peroxide in cardiac myocytes." J Mol Cell Cardiol **32**(8): 1469-80.

Bondesen, B. A., S. T. Mills, *et al*. (2004). "The COX-2 pathway is essential during early stages of skeletal muscle regeneration." Am J Physiol Cell Physiol **287**(2): C475-83.

Bondesen, B. A., S. T. Mills, *et al*. (2006). "The COX-2 pathway regulates growth of atrophied muscle via multiple mechanisms." Am J Physiol Cell Physiol **290**(6): C1651-9.

Bordet, T., J. C. Lesbordes, *et al*. (2001). "Protective effects of cardiotrophin-1 adenoviral gene transfer on neuromuscular degeneration in transgenic ALS mice." Hum Mol Genet **10**(18): 1925-33.

Borutaite, V., A. Budriunaite, *et al*. (2000). "Reversal of nitric oxide-, peroxynitrite- and S-nitrosothiol-induced inhibition of mitochondrial respiration or complex I activity by light and thiols." Biochim Biophys Acta **1459**(2-3): 405-12.

Bottinelli, R., R. Betto, *et al*. (1994). "Maximum shortening velocity and coexistence of myosin heavy chain isoforms in single skinned fast fibres of rat skeletal muscle." J Muscle Res Cell Motil **15**(4): 413-9.

Bottinelli, R., M. Canepari, *et al*. (1996). "Force-velocity properties of human skeletal muscle fibres: myosin heavy chain isoform and temperature dependence." J Physiol **495 (Pt 2)**: 573-86.

Bottinelli, R. and C. Reggiani (2000). "Human skeletal muscle fibres: molecular and functional diversity." Prog Biophys Mol Biol **73**(2-4): 195-262.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-54.

Breitbart, R. E. and B. Nadal-Ginard (1987). "Developmentally induced, muscle-specific trans factors control the differential splicing of alternative and constitutive troponin T exons." Cell **49**(6): 793-803.

Brenner, B. (1986). "The cross-bridge cycle in muscle. Mechanical, biochemical, and structural studies on single skinned rabbit psoas fibers to characterize cross-bridge kinetics in muscle for correlation with the actomyosin-ATPase in solution." Basic Res Cardiol **81 Suppl 1**: 1-15.

Brenner, I. K., V. M. Natale, *et al*. (1999). "Impact of three different types of exercise on components of the inflammatory response." Eur J Appl Physiol Occup Physiol **80**(5): 452-60.

Brent, L., C. G. Brooks, *et al*. (1976). "Transplantation tolerance." Br Med Bull **32**(2): 101-6.

Brickson, S., J. Hollander, *et al*. (2001). "Oxidant production and immune response after stretch injury in skeletal muscle." Med Sci Sports Exerc **33**(12): 2010-5.

Brickson, S., L. L. Ji, *et al*. (2003). "M1/70 attenuates blood-borne neutrophil oxidants, activation, and myofiber damage following stretch injury." J Appl Physiol **95**(3): 969-76.

Brown, G. C. and V. Borutaite (2002). "Nitric oxide inhibition of mitochondrial respiration and its role in cell death." Free Radic Biol Med **33**(11): 1440-50.

Brown, G. C. and C. E. Cooper (1994). "Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase." FEBS Lett **356**(2-3): 295-8.

Brown, T. A. (2002). Genomes, BIOS. Second Edition.

Brune, B. (2003). "Nitric oxide: NO apoptosis or turning it ON?" Cell Death Differ **10**(8): 864-9.

Bruunsgaard, H., A. Hartkopp, *et al*. (1997). "In vivo cell-mediated immunity and vaccination response following prolonged, intense exercise." Med Sci Sports Exerc **29**(9): 1176-81.

Buckwalter, J. A. (1995). "Should bone, soft-tissue, and joint injuries be treated with rest or activity?" J Orthop Res **13**(2): 155-6.

Buga, G. M., M. E. Gold, *et al*. (1991). "Shear stress-induced release of nitric oxide from endothelial cells grown on beads." Hypertension **17**(2): 187-93.

Buller, A. J., J. C. Eccles, *et al*. (1960). "Interactions between motoneurones and muscles in respect of the characteristic speeds of their responses." J Physiol **150**: 417-39.

Callaghan, G. A., C. Riordan, *et al*. (1996). "Reactive oxygen species inducible by low-intensity laser irradiation alter DNA synthesis in the haemopoietic cell line U937." Lasers Surg Med **19**(2): 201-6.

Cannon, J. G. and B. A. St Pierre (1998). "Cytokines in exertion-induced skeletal muscle injury." Mol Cell Biochem **179**(1-2): 159-67.

Cantini, M., E. Giurisato, *et al*. (2002). "Macrophage-secreted myogenic factors: a promising tool for greatly enhancing the proliferative capacity of myoblasts in vitro and in vivo." Neurol Sci **23**(4): 189-94.

Cantini, M., M. L. Massimino, *et al*. (1995). "Human satellite cell proliferation in vitro is regulated by autocrine secretion of IL-6 stimulated by a soluble factor(s) released by activated monocytes." Biochem Biophys Res Commun **216**(1): 49-53.

Cao, G., H. M. Alessio, *et al*. (1993). "Oxygen-radical absorbance capacity assay for antioxidants." Free Radic Biol Med **14**(3): 303-11.

Capers, C. R. (1960). "Multinucleation of skeletal muscle in vitro." J Biophys Biochem Cytol **7**: 559-66.

Carp, H. (1982). "Mitochondrial N-formylmethionyl proteins as chemoattractants for neutrophils." J Exp Med **155**(1): 264-75.

Carraro, U., L. Dalla Libera, *et al*. (1982). "Chronic denervation of rat diaphragm: selective maintenance of adult fast myosin heavy chains." Muscle Nerve **5**(7): 515-24.

Caselli, A., R. Marzocchini, *et al*. (1998). "The inactivation mechanism of low molecular weight phosphotyrosine-protein phosphatase by H2O2." J Biol Chem **273**(49): 32554-60.

Cassatella, M. A. (1995). "The production of cytokines by polymorphonuclear neutrophils." Immunol Today **16**(1): 21-6.

Chakravarthy, M. V., T. W. Abraha, *et al*. (2000). "Insulin-like growth factor-I extends in vitro replicative life span of skeletal muscle satellite cells by enhancing G1/S cell cycle progression via the activation of phosphatidylinositol 3'-kinase/Akt signaling pathway." J Biol Chem **275**(46): 35942-52.

Charakida, A., E. D. Seaton, *et al*. (2004). "Phototherapy in the treatment of acne vulgaris: what is its role?" Am J Clin Dermatol **5**(4): 211-6.

Cheek, D. B. (1985). "The control of cell mass and replication. The DNA unit--a personal 20-year study." Early Hum Dev **12**(3): 211-39.

Chiarugi, P., P. Cirri, *et al*. (1997). "LMW-PTP is a negative regulator of insulin-mediated mitotic and metabolic signalling." Biochem Biophys Res Commun **238**(2): 676-82.

Christov, C., F. Chretien, *et al*. (2007). "Muscle satellite cells and endothelial cells: close neighbors and privileged partners." Mol Biol Cell **18**(4): 1397-409.

Cleeter, M. W., J. M. Cooper, *et al*. (1994). "Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases." FEBS Lett **345**(1): 50-4.

Clementi, E. and E. Nisoli (2005). "Nitric oxide and mitochondrial biogenesis: a key to long-term regulation of cellular metabolism." Comp Biochem Physiol A Mol Integr Physiol **142**(2): 102-10.

Collazos, J., J. Mayo, *et al*. (1999). "Muscle infections caused by Salmonella species: case report and review." Clin Infect Dis **29**(3): 673-7.

Collins, C. A., I. Olsen, *et al*. (2005). "Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche." Cell **122**(2): 289-301.

Conboy, I. M., M. J. Conboy, *et al*. (2005). "Rejuvenation of aged progenitor cells by exposure to a young systemic environment." Nature **433**(7027): 760-4.

Cornelison, D. D., S. A. Wilcox-Adelman, *et al*. (2004). "Essential and separable roles for Syndecan-3 and Syndecan-4 in skeletal muscle development and regeneration." Genes Dev **18**(18): 2231-6.

Corral-Baques, M. I., T. Rigau, *et al*. (2005). "Effect of 655-nm diode laser on dog sperm motility." Lasers Med Sci **20**(1): 28-34.

Crameri, R. M., H. Langberg, *et al*. (2004). "Changes in satellite cells in human skeletal muscle after a single bout of high intensity exercise." J Physiol **558**(Pt 1): 333-40.

Crisco, J. J., P. Jokl, *et al*. (1994). "A muscle contusion injury model. Biomechanics, physiology, and histology." Am J Sports Med **22**(5): 702-10.

Cuzzocrea, S., C. Thiemermann, *et al*. (2004). "Potential therapeutic effect of antioxidant therapy in shock and inflammation." Curr Med Chem **11**(9): 1147-62.

D'Antona, G., Pellegrino, M.A., Rossi, R., Carlizzi, C.N., Reggiani, C., Bottinelli, R. (2000). "Disuse Induced-Atrophy and Contractile Impairment of Human Skeletal Muscle Fibres." Basic Appl Myol **10**((1&2)): 27-32.

da Cunha, A., N. A. Parizotto, *et al*. (2001). "The effect of therapeutic ultrasound on repair of the achilles tendon (tendo calcaneus) of the rat." Ultrasound Med Biol **27**(12): 1691-6.

De Rossi, M., P. Bernasconi, *et al*. (2000). "Cytokines and chemokines are both expressed by human myoblasts: possible relevance for the immune pathogenesis of muscle inflammation." Int Immunol **12**(9): 1329-35.

Dhawan, J. and T. A. Rando (2005). "Stem cells in postnatal myogenesis: molecular mechanisms of satellite cell quiescence, activation and replenishment." Trends Cell Biol **15**(12): 666-73.

Diffee, G. M., K. Kalfas, *et al*. (2002). "Altered expression of skeletal muscle myosin isoforms in cancer cachexia." Am J Physiol Cell Physiol **283**(5): C1376-82.

Dijkstra, C. D., E. A. Dopp, *et al*. (1985). "The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3." Immunology **54**(3): 589-99.

Drexler, H., U. Riede, *et al*. (1992). "Alterations of skeletal muscle in chronic heart failure." Circulation **85**(5): 1751-9.

Du, M., M. M. Islam, *et al*. (1997). "Promotion of proliferation of murine BALB/C3T3 fibroblasts mediated by nitric oxide at lower concentrations." Biochem Mol Biol Int **41**(3): 625-31.

Eigen, H., B. J. Rosenstein, *et al*. (1995). "A multicenter study of alternate-day prednisone therapy in patients with cystic fibrosis. Cystic Fibrosis Foundation Prednisone Trial Group." J Pediatr **126**(4): 515-23.

Elfering, S. L., T. M. Sarkela, *et al*. (2002). "Biochemistry of mitochondrial nitric-oxide synthase." J Biol Chem **277**(41): 38079-86.

Elman, M. and J. Lebzelter (2004). "Light therapy in the treatment of acne vulgaris." Dermatol Surg **30**(2 Pt 1): 139-46.

Febbraio, M. A. and B. K. Pedersen (2002). "Muscle-derived interleukin-6: mechanisms for activation and possible biological roles." FASEB J **16**(11): 1335-47.

Fernandez-Sola, J., E. Sacanella, *et al*. (1995). "Significance of type II fiber atrophy in chronic alcoholic myopathy." J Neurol Sci **130**(1): 69-76.

Fielding, R. A., T. J. Manfredi, *et al*. (1993). "Acute phase response in exercise. III. Neutrophil and IL-1 beta accumulation in skeletal muscle." Am J Physiol **265**(1 Pt 2): R166-72.

Fillipin, L. I., J. L. Mauriz, *et al*. (2005). "Low-level laser therapy (LLLT) prevents oxidative stress and reduces fibrosis in rat traumatized Achilles tendon." Lasers Surg Med **37**(4): 293-300.

Finkel, T. (2000). "Redox-dependent signal transduction." FEBS Lett **476**(1-2): 52-4.

Fitts, R. H. and J. J. Widrick (1996). "Muscle mechanics: adaptations with exercise-training." Exerc Sport Sci Rev **24**: 427-73.

Fitzpatrick, T. B. and M. A. Pathak (1959). "Historical aspects of methoxsalen and other furocoumarins." J Invest Dermatol **32**(2, Part 2): 229-31.

Fleury, C., B. Mignotte, *et al*. (2002). "Mitochondrial reactive oxygen species in cell death signaling." Biochimie **84**(2-3): 131-41.

Florini, J. R. and K. A. Magri (1989). "Effects of growth factors on myogenic differentiation." Am J Physiol **256**(4 Pt 1): C701-11.

Flors, C., M. J. Fryer, *et al*. (2006). "Imaging the production of singlet oxygen in vivo using a new fluorescent sensor, Singlet Oxygen Sensor Green." J Exp Bot **57**(8): 1725-34.

Fluck, M. and H. Hoppeler (2003). "Molecular basis of skeletal muscle plasticity--from gene to form and function." Rev Physiol Biochem Pharmacol **146**: 159-216.

Forman, H. J., J. M. Fukuto, *et al*. (2004). "Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers." Am J Physiol Cell Physiol **287**(2): C246-56.

Frenette, J., M. St-Pierre, *et al*. (2002). "Muscle impairment occurs rapidly and precedes inflammatory cell accumulation after mechanical loading." Am J Physiol Regul Integr Comp Physiol **282**(2): R351-7.

Froemming, G. R., B. E. Murray, *et al*. (2000). "Comparative analysis of the isoform expression pattern of Ca(2+)-regulatory membrane proteins in fast-twitch, slow-twitch, cardiac, neonatal and chronic low-frequency stimulated muscle fibers." Biochim Biophys Acta **1466**(1-2): 151-68.

Frontera, W. R., C. N. Meredith, *et al*. (1988). "Strength conditioning in older men: skeletal muscle hypertrophy and improved function." J Appl Physiol **64**(3): 1038-44.

Fry, A. C., C. A. Allemeier, *et al*. (1994). "Correlation between percentage fiber type area and myosin heavy chain content in human skeletal muscle." Eur J Appl Physiol Occup Physiol **68**(3): 246-51.

Fujimaki, Y., T. Shimoyama, *et al*. (2003). "Low-level laser irradiation attenuates production of reactive oxygen species by human neutrophils." J Clin Laser Med Surg **21**(3): 165-70.

Gaboury, J. P., B. Johnston, *et al*. (1995). "Mechanisms underlying acute mast cell-induced leukocyte rolling and adhesion in vivo." J Immunol **154**(2): 804-13.

Galli, S. J. (1993). "New concepts about the mast cell." N Engl J Med **328**(4): 257-65.

Ganote, C. E. (1983). "Contraction band necrosis and irreversible myocardial injury." J Mol Cell Cardiol **15**(2): 67-73.

Garrett, W. E., Jr. (1996). "Muscle strain injuries." Am J Sports Med **24**(6 Suppl): S2-8.

Garwin, L. and T. Lincoln. (2003). "The first laser." from http://www.press.uchicago.edu/Misc/Chicago/284158_townes.html.

Gayeski, T. E. and C. R. Honig (1986). "O2 gradients from sarcolemma to cell interior in red muscle at maximal VO2." Am J Physiol **251**(4 Pt 2): H789-99.

Gayeski, T. E. and C. R. Honig (1988). "Intracellular PO2 in long axis of individual fibers in working dog gracilis muscle." Am J Physiol **254**(6 Pt 2): H1179-86.

Gertz, E. W., J. A. Wisneski, *et al*. (1988). "Myocardial substrate utilization during exercise in humans. Dual carbon-labeled carbohydrate isotope experiments." J Clin Invest **82**(6): 2017-25.

Ghiselli, A., M. Serafini, *et al*. (2000). "Total antioxidant capacity as a tool to assess redox status: critical view and experimental data." Free Radic Biol Med **29**(11): 1106-14.

Gibson, M. C. and E. Schultz (1982). "The distribution of satellite cells and their relationship to specific fiber types in soleus and extensor digitorum longus muscles." Anat Rec **202**(3): 329-37.

Gierer, P., T. Mittlmeier, *et al*. (2005). "Selective cyclooxygenase-2 inhibition reverses microcirculatory and inflammatory sequelae of closed soft-tissue trauma in an animal model." J Bone Joint Surg Am **87**(1): 153-60.

Goldberg, D. J. and B. A. Russell (2006). "Combination blue (415 nm) and red (633 nm) LED phototherapy in the treatment of mild to severe acne vulgaris." J Cosmet Laser Ther **8**(2): 71-5.

Goldfarb, A. H., R. J. Bloomer, *et al*. (2005). "Combined antioxidant treatment effects on blood oxidative stress after eccentric exercise." Med Sci Sports Exerc **37**(2): 234-9.

Gonzalez, R. J. and J. B. Tarloff (2001). "Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity." Toxicol In Vitro **15**(3): 257-9.

Gordon, A. M., E. Homsher, *et al*. (2000). "Regulation of contraction in striated muscle." Physiol Rev **80**(2): 853-924.

Gordon, S. A. and K. Surrey (1960). "Red and far-red action on oxidative phosphorylation." Radiat Res **12**: 325-39.

Greco, M., G. Guida, *et al*. (1989). "Increase in RNA and protein synthesis by mitochondria irradiated with helium-neon laser." Biochem Biophys Res Commun **163**(3): 1428-34.

Grossman, N., N. Schneid, *et al*. (1998). "780 nm low power diode laser irradiation stimulates proliferation of keratinocyte cultures: involvement of reactive oxygen species." Lasers Surg Med **22**(4): 212-8.

Grounds, M. D. and J. K. McGeachie (1989). "A comparison of muscle precursor replication in crush-injured skeletal muscle of Swiss and BALBc mice." Cell Tissue Res **255**(2): 385-91.

Haddad, F., F. Zaldivar, *et al*. (2005). "IL-6-induced skeletal muscle atrophy." J Appl Physiol **98**(3): 911-7.

Halevy, O., Y. Piestun, *et al*. (2004). "Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal." Dev Dyn **231**(3): 489-502.

Hambrecht, R., E. Fiehn, *et al*. (1997). "Effects of endurance training on mitochondrial ultrastructure and fiber type distribution in skeletal muscle of patients with stable chronic heart failure." J Am Coll Cardiol **29**(5): 1067-73.

Hambrecht, R., J. Niebauer, *et al*. (1995). "Physical training in patients with stable chronic heart failure: effects on cardiorespiratory fitness and ultrastructural abnormalities of leg muscles." J Am Coll Cardiol **25**(6): 1239-49.

Hancock, J. T., R. Desikan, *et al*. (2001). "Role of reactive oxygen species in cell signalling pathways." Biochem Soc Trans **29**(Pt 2): 345-50.

Havenith, M. G., R. Visser, *et al*. (1990). "Muscle fiber typing in routinely processed skeletal muscle with monoclonal antibodies." Histochemistry **93**(5): 497-9.

Henriksson, J. and J. S. Reitman (1977). "Time course of changes in human skeletal muscle succinate dehydrogenase and cytochrome oxidase activities and maximal oxygen uptake with physical activity and inactivity." Acta Physiol Scand **99**(1): 91-7.

Hiatt, W. R., J. G. Regensteiner, *et al*. (1996). "Effect of exercise training on skeletal muscle histology and metabolism in peripheral arterial disease." J Appl Physiol **81**(2): 780-8.

Hill, M., A. Wernig, *et al*. (2003). "Muscle satellite (stem) cell activation during local tissue injury and repair." J Anat **203**(1): 89-99.

Holloszy, J. O. and F. W. Booth (1976). "Biochemical adaptations to endurance exercise in muscle." Annu Rev Physiol **38**: 273-91.

Holloszy, J. O. and E. F. Coyle (1984). "Adaptations of skeletal muscle to endurance exercise and their metabolic consequences." J Appl Physiol **56**(4): 831-8.

Holloszy, O. (1982). "Enzymatic adaptations of skeletal muscle to endurance exercise." Curr Probl Clin Biochem **11**: 118-21.

Honda, H., H. Kimura, *et al*. (1990). "Demonstration and phenotypic characterization of resident macrophages in rat skeletal muscle." Immunology **70**(2): 272-7.

Hopkinson-Woolley, J., D. Hughes, *et al*. (1994). "Macrophage recruitment during limb development and wound healing in the embryonic and foetal mouse." J Cell Sci **107 (Pt 5)**: 1159-67.

Horst, D., S. Ustanina, *et al*. (2006). "Comparative expression analysis of Pax3 and Pax7 during mouse myogenesis." Int J Dev Biol **50**(1): 47-54.

Howatson, G., D. Gaze, *et al*. (2005). "The efficacy of ice massage in the treatment of exercise-induced muscle damage." Scand J Med Sci Sports **15**(6): 416-22.

Huang, D., B. Ou, *et al*. (2002). "High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format." J Agric Food Chem **50**(16): 4437-44.

Huang, D., B. Ou, *et al*. (2005). "The chemistry behind antioxidant capacity assays." J Agric Food Chem **53**(6): 1841-56.

Hughes, G., M. P. Murphy, *et al*. (2005). "Mitochondrial reactive oxygen species regulate the temporal activation of nuclear factor kappaB to modulate tumour necrosis factor-induced apoptosis: evidence from mitochondria-targeted antioxidants." Biochem J **389**(Pt 1): 83-9.

Hurme, T., H. Kalimo, *et al*. (1991). "Healing of skeletal muscle injury: an ultrastructural and immunohistochemical study." Med Sci Sports Exerc **23**(7): 801-10.

Hwang, J. H., S. H. Yuk, *et al*. (2004). "Isolation of muscle derived stem cells from rat and its smooth muscle differentiation [corrected]." Mol Cells **17**(1): 57-61.

Irani, K., Y. Xia, *et al*. (1997). "Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts." Science **275**(5306): 1649-52.

Janjic, D. and C. B. Wollheim (1992). "Islet cell metabolism is reflected by the MTT (tetrazolium) colorimetric assay." Diabetologia **35**(5): 482-5.

Jarvinen, M. (1975). "Healing of a crush injury in rat striated muscle. 2. a histological study of the effect of early mobilization and immobilization on the repair processes." Acta Pathol Microbiol Scand [A] **83**(3): 269-82.

Jarvinen, M. (1976). "Healing of a crush injury in rat striated muscle. 4. Effect of early mobilization and immobilization on the tensile properties of gastrocnemius muscle." Acta Chir Scand **142**(1): 47-56.

Jarvinen, M. J. and M. U. Lehto (1993). "The effects of early mobilisation and immobilisation on the healing process following muscle injuries." Sports Med **15**(2): 78-89.

Johnson, S. E. and R. E. Allen (1995). "Activation of skeletal muscle satellite cells and the role of fibroblast growth factor receptors." Exp Cell Res **219**(2): 449-53.

Josephson, R. K. (1975). "Extensive and intensive factors determining the performance of striated muscle." J Exp Zool **194**(1): 135-53.

Jostarndt-Fogen, K., A. Puntschart, *et al*. (1998). "Fibre-type specific expression of fast and slow essential myosin light chain mRNAs in trained human skeletal muscles." Acta Physiol Scand **164**(3): 299-308.

Kahn, B. B., A. S. Rosen, *et al*. (1992). "Expression of GLUT1 and GLUT4 glucose transporters in skeletal muscle of humans with insulin-dependent diabetes mellitus: regulatory effects of metabolic factors." J Clin Endocrinol Metab **74**(5): 1101-9.

Kamata, H., Y. Shibukawa, *et al*. (2000). "Epidermal growth factor receptor is modulated by redox through multiple mechanisms. Effects of reductants and H2O2." Eur J Biochem **267**(7): 1933-44.

Kami, K. and E. Senba (2002). "In vivo activation of STAT3 signaling in satellite cells and myofibers in regenerating rat skeletal muscles." J Histochem Cytochem **50**(12): 1579-89.

Kannus, P., J. Parkkari, *et al*. (2003). "Basic science and clinical studies coincide: active treatment approach is needed after a sports injury." Scand J Med Sci Sports **13**(3): 150-4.

Kaplanski, G., V. Marin, *et al*. (2003). "IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation." Trends Immunol **24**(1): 25-9.

Karnes, J. L. and H. W. Burton (2002). "Continuous therapeutic ultrasound accelerates repair of contraction-induced skeletal muscle damage in rats." Arch Phys Med Rehabil **83**(1): 1-4.

Karu, T. (1989). "Photobiology of low-power laser effects." Health Phys **56**(5): 691-704.

Karu, T. (1999). "Primary and secondary mechanisms of action of visible to near-IR radiation on cells." J Photochem Photobiol B **49**(1): 1-17.

Karu, T., L. Pyatibrat, *et al*. (1995). "Irradiation with He-Ne laser increases ATP level in cells cultivated in vitro." J Photochem Photobiol B **27**(3): 219-23.

Karu, T. I., G. S. Kalendo, *et al*. (1982). "Biostimulation of HeLa cells by low intensity visible light." Il Nuovo Cimento D **1**(6): 828-840.

Karu, T. I., G. S. Kalendo, *et al*. (1984). "Biostimulation of HeLa cells by low-intensity visible light III. Stimulation of nucleic-acid synthesis in plateau phase cells." Il Nuovo Cimento D **3**(2): 319-325.

Karu, T. I., Kalendo, G.S., Ketokhov, V.S. & Lobko, V.V. (1984). "Biostimulation of HeLa cells by low intensity visible light II. Stimulation of DNA and RNA synthesis in a wide spectral

range." Ikvantovaya Elektronika(10): 1138-1144.

Karu, T. I. and S. F. Kolyakov (2005). "Exact action spectra for cellular responses relevant to phototherapy." Photomed Laser Surg **23**(4): 355-61.

Karu, T. I., L. V. Pyatibrat, *et al*. (1987). "Biostimulation of HeLa cells by low-intensity visible light. V. Stimulation of cell proliferation*in vitro* by He−Ne Laser irradiation." Il Nuovo Cimento D **9**(12): 1485-1494.

Karu, T. I., L. V. Pyatibrat, *et al*. (2005). "Absorption measurements of a cell monolayer relevant to phototherapy: reduction of cytochrome c oxidase under near IR radiation." J Photochem Photobiol B **81**(2): 98-106.

Kawada, A., Y. Aragane, *et al*. (2002). "Acne phototherapy with a high-intensity, enhanced, narrow-band, blue light source: an open study and in vitro investigation." J Dermatol Sci **30**(2): 129-35.

Keller, C., A. Steensberg, *et al*. (2001). "Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content." FASEB J **15**(14): 2748-50.

Khan, K. N., H. Masuzaki, *et al*. (2005). "Regulation of hepatocyte growth factor by basal and stimulated macrophages in women with endometriosis." Hum Reprod **20**(1): 49-60.

Kim, Y. G., S. C. Pak, *et al*. (2000). "Hairless mouse epidermal antioxidants and lipid peroxidation assessed by He-Ne laser." Lasers Surg Med **27**(5): 420-6.

Kinnunen, S., S. Hyyppa, *et al*. (2005). "Oxygen radical absorbance capacity (ORAC) and exercise-induced oxidative stress in trotters." Eur J Appl Physiol **95**(5-6): 550-6.

Kjeldstad, B. (1984). "Photoinactivation of Propionibacterium acnes by near-ultraviolet light." Z Naturforsch [C] **39**(3-4): 300-2.

Konstan, M. W., P. J. Byard, *et al*. (1995). "Effect of high-dose ibuprofen in patients with cystic fibrosis." N Engl J Med **332**(13): 848-54.

Korn, E. D. (2000). "Coevolution of head, neck, and tail domains of myosin heavy chains." Proc Natl Acad Sci U S A **97**(23): 12559-64.

Koshland, D. E., Jr. (1993). "Molecule of the year." Science **262**(5142): 1953.

Kraemer, W. J., J. A. Bush, *et al*. (2001). "Influence of compression therapy on symptoms following soft tissue injury from maximal eccentric exercise." J Orthop Sports Phys Ther **31**(6): 282-90.

Krebs, H. A. and W. A. Johnson (1980). "The role of citric acid in intermediate metabolism in animal tissues." FEBS Lett **117 Suppl**: K1-10.

Kreuzberg, M. M., K. Willecke, *et al*. (2006). "Connexin-mediated cardiac impulse propagation: connexin 30.2 slows atrioventricular conduction in mouse heart." Trends Cardiovasc Med **16**(8): 266-72.

Kron, S. J. and J. A. Spudich (1986). "Fluorescent actin filaments move on myosin fixed to a glass surface." Proc Natl Acad Sci U S A **83**(17): 6272-6.

Kubes, P. and J. P. Gaboury (1996). "Rapid mast cell activation causes leukocyte-dependent and -independent permeability alterations." Am J Physiol **271**(6 Pt 2): H2438-46.

Lacerda, L., R. M. Smith, *et al*. (2006). "TNFalpha-induced cytoprotection requires the production of free radicals within mitochondria in C2C12 myotubes." Life Sci **79**(23): 2194-201.

Langen, R. C., A. M. Schols, *et al*. (2001). "Inflammatory cytokines inhibit myogenic differentiation through activation of nuclear factor-kappaB." FASEB J **15**(7): 1169-80.

Lapointe, B. M., J. Frenette, *et al*. (2002). "Lengthening contraction-induced inflammation is linked to secondary damage but devoid of neutrophil invasion." J Appl Physiol **92**(5): 1995-2004.

Larsson, L., X. Li, *et al*. (1996). "Effects of removal of weight-bearing function on contractility and myosin isoform composition in single human skeletal muscle cells." Pflugers Arch **432**(2): 320-8.

Larsson, L. and R. L. Moss (1993). "Maximum velocity of shortening in relation to myosin isoform composition in single fibres from human skeletal muscles." J Physiol **472**: 595-614.

Lauber, K., S. G. Blumenthal, *et al*. (2004). "Clearance of apoptotic cells: getting rid of the corpses." Mol Cell **14**(3): 277-87.

Lee, K. and W. J. Esselman (2002). "Inhibition of PTPs by H(2)O(2) regulates the activation of distinct MAPK pathways." Free Radic Biol Med **33**(8): 1121-32.

Lefaucheur, L., D. Milan, *et al*. (2004). "Myosin heavy chain composition of different skeletal muscles in Large White and Meishan pigs." J Anim Sci **82**(7): 1931-41.

Lescaudron, L., E. Peltekian, *et al*. (1999). "Blood borne macrophages are essential for the triggering of muscle regeneration following muscle transplant." Neuromuscul Disord **9**(2): 72-80.

Letokhov, V. S. (1991). "Effects of transient local heating of spatially and spectrally heterogeneous biotissue by short laser pulses." Il Nuovo Cimento D **13**(7).

Levenberg, S., J. Rouwkema, *et al*. (2005). "Engineering vascularized skeletal muscle tissue." Nat Biotechnol **23**(7): 879-84.

Li, M. O., M. R. Sarkisian, *et al*. (2003). "Phosphatidylserine receptor is required for clearance of apoptotic cells." Science **302**(5650): 1560-3.

Li, Y., J. Cummins, *et al*. (2001). "Muscle injury and repair." Curr Opin Orthop(12): 409-415.

Li, Y. P. (2003). "TNF-alpha is a mitogen in skeletal muscle." Am J Physiol Cell Physiol **285**(2): C370-6.

Li, Y. P. and R. J. Schwartz (2001). "TNF-alpha regulates early differentiation of C2C12 myoblasts in an autocrine fashion." FASEB J **15**(8): 1413-5.

Linke, W. A. and M. C. Leake (2004). "Multiple sources of passive stress relaxation in muscle fibres." Phys Med Biol **49**(16): 3613-27.

Lleo-Perez, A., A. Ortuno-Soto, *et al*. (2004). "Intraobserver reproducibility of retinal nerve fiber layer measurements using scanning laser polarimetry and optical coherence tomography in normal and ocular hypertensive subjects." Eur J Ophthalmol **14**(6): 523-30.

Lloyd, R. V., P. M. Hanna, *et al*. (1997). "The origin of the hydroxyl radical oxygen in the Fenton reaction." Free Radic Biol Med **22**(5): 885-8.

Lopes-Martins, R. A., R. L. Marcos, *et al*. (2006). "Effect of low-level laser (Ga-Al-As 655 nm) on skeletal muscle fatigue induced by electrical stimulation in rats." J Appl Physiol **101**(1): 283-8.

Lopes, J., D. M. Russell, *et al*. (1982). "Skeletal muscle function in malnutrition." Am J Clin Nutr **36**(4): 602-10.

Lowe, D. A. and S. E. Alway (1999). "Stretch-induced myogenin, MyoD, and MRF4 expression and acute hypertrophy in quail slow-tonic muscle are not dependent upon satellite cell proliferation." Cell Tissue Res **296**(3): 531-9.

Lowe, D. A., G. L. Warren, *et al*. (1995). "Muscle function and protein metabolism after initiation of eccentric contraction-induced injury." J Appl Physiol **79**(4): 1260-70.

Lowey, S., G. S. Waller, *et al*. (1993). "Function of skeletal muscle myosin heavy and light chain isoforms by an in vitro motility assay." J Biol Chem **268**(27): 20414-8.

Lutz, H., H. Weber, *et al*. (1979). "Fast and slow myosin within single skeletal muscle fibres of adult rabbits." Nature **281**(5727): 142-4.

Mach, F., U. Schonbeck, *et al*. (1999). "T lymphocytes induce endothelial cell matrix metalloproteinase expression by a CD40L-dependent mechanism: implications for tubule formation." Am J Pathol **154**(1): 229-38.

Maganaris, C. N. and J. P. Paul (2000). "In vivo human tendinous tissue stretch upon maximum muscle force generation." J Biomech **33**(11): 1453-9.

Maiman, T. H. (1960). "Stimulated optical radiation in ruby." Nature **187** (4736): 493-494.

Majno, G. and I. Joris (1995). "Apoptosis, oncosis, and necrosis. An overview of cell death." Am J Pathol **146**(1): 3-15.

Makela, A. M. (2006). "Influence of laser light on AMPK as a factor in the laser therapy of diabetes

" Proc. SPIE **6140**.

Martin, W. H., 3rd, S. S. Murphree, *et al*. (1989). "Beta-adrenergic receptor distribution among muscle fiber types and resistance arterioles of white, red, and intermediate skeletal muscle." Circ Res **64**(6): 1096-105.

Mason, M. G., P. Nicholls, *et al*. (2006). "Nitric oxide inhibition of respiration involves both competitive (heme) and noncompetitive (copper) binding to cytochrome c oxidase." Proc Natl Acad Sci U S A **103**(3): 708-13.

Massimino, M. L., E. Rapizzi, *et al*. (1997). "ED2+ macrophages increase selectively myoblast proliferation in muscle cultures." Biochem Biophys Res Commun **235**(3): 754-9.

Matsumoto-Ida, M., M. Akao, *et al*. (2006). "Real-time 2-photon imaging of mitochondrial function in perfused rat hearts subjected to ischemia/reperfusion." Circulation **114**(14): 1497-503.

Mauro, A. (1961). "Satellite cell of skeletal muscle fibers." J Biophys Biochem Cytol **9**: 493-5.

Mayne, S. T. (2003). "Antioxidant nutrients and chronic disease: use of biomarkers of exposure and oxidative stress status in epidemiologic research." J Nutr **133 Suppl 3**: 933S-940S.

McCourt, M., J. H. Wang, *et al*. (2001). "Activated human neutrophils release hepatocyte growth factor/scatter factor." Eur J Surg Oncol **27**(4): 396-403.

McLennan, I. S. (1993). "Resident macrophages (ED2- and ED3-positive) do not phagocytose degenerating rat skeletal muscle fibres." Cell Tissue Res **272**(1): 193-6.

Menasche, P. (2003). "Skeletal muscle satellite cell transplantation." Cardiovasc Res **58**(2): 351-7.

Menetrey, J., C. Kasemkijwattana, *et al*. (1999). "Suturing versus immobilization of a muscle laceration. A morphological and functional study in a mouse model." Am J Sports Med **27**(2): 222-9.

Merly, F., L. Lescaudron, *et al*. (1999). "Macrophages enhance muscle satellite cell proliferation and delay their differentiation." Muscle Nerve **22**(6): 724-32.

Mester, E., G. Ludany, *et al*. (1968). "[Studies on the inhibiting and activating effects of laser beams]." Langenbecks Arch Chir **322**: 1022-7.

Mester, E., G. Ludany, *et al*. (1968). "[On the effect of laser beams on bacterial phagocytosis of leukocytes]." Acta Biol Med Ger **21**(3): 317-21.

Mester, E., T. Spiry, *et al*. (1971). "Effect of laser rays on wound healing." Am J Surg **122**(4): 532-5.

Mester, E., B. Szende, *et al*. (1968). "[The effect of laser beams on the growth of hair in mice]." Radiobiol Radiother (Berl) **9**(5): 621-6.

Meszaros, A. J., J. S. Reichner, *et al*. (2000). "Macrophage-induced neutrophil apoptosis." J Immunol **165**(1): 435-41.

Miller, K. J., D. Thaloor, *et al*. (2000). "Hepatocyte growth factor affects satellite cell activation and differentiation in regenerating skeletal muscle." Am J Physiol Cell Physiol **278**(1): C174-81.

Moilanen, E. and H. Vapaatalo (1995). "Nitric oxide in inflammation and immune response." Ann Med **27**(3): 359-67.

Moncada, S. and J. D. Erusalimsky (2002). "Does nitric oxide modulate mitochondrial energy generation and apoptosis?" Nat Rev Mol Cell Biol **3**(3): 214-20.

Mong, F. S. (1988). "Satellite cells in the regenerated and regrafted skeletal muscles of rats." Experientia **44**(7): 601-3.

Montarras, D., J. Morgan, *et al*. (2005). "Direct isolation of satellite cells for skeletal muscle regeneration." Science **309**(5743): 2064-7.

Morimoto, K., H. Amano, *et al*. (2001). "Alveolar macrophages that phagocytose apoptotic neutrophils produce hepatocyte growth factor during bacterial pneumonia in mice." Am J Respir Cell Mol Biol **24**(5): 608-15.

Moriyama, Y., E. H. Moriyama, *et al*. (2005). "In vivo study of the inflammatory modulating effects of low-level laser therapy on iNOS expression using bioluminescence imaging." Photochem Photobiol **81**(6): 1351-5.

Morozov, V. I., P. V. Tsyplenkov, *et al*. (2006). "The effects of high-intensity exercise on skeletal muscle neutrophil myeloperoxidase in untrained and trained rats." Eur J Appl Physiol **97**(6): 716-22.

Mosmann, T. (1983). "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays." J Immunol Methods **65**(1-2): 55-63.

Mulder, K., V. Sakoman, *et al*. (1991). "[Compartment syndrome after femoral contusion. Case report and review of the literature]." Aktuelle Traumatol **21**(4): 139-42.

Nagaraju, K., N. Raben, *et al*. (1998). "A variety of cytokines and immunologically relevant surface molecules are expressed by normal human skeletal muscle cells under proinflammatory stimuli." Clin Exp Immunol **113**(3): 407-14.

Nathan, C. (2002). "Points of control in inflammation." Nature **420**(6917): 846-52.

Navratil, L. and J. Kymplova (2002). "Contraindications in noninvasive laser therapy: truth and fiction." J Clin Laser Med Surg **20**(6): 341-3.

Naya, F. J. and E. Olson (1999). "MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation." Curr Opin Cell Biol **11**(6): 683-8.

Negi, A. S. and S. C. Anand (1985). A Textbook of Physical Chemistry New Age International.

Nguyen, H. X. and J. G. Tidball (2003). "Interactions between neutrophils and macrophages promote macrophage killing of rat muscle cells in vitro." J Physiol **547**(Pt 1): 125-32.

Nielsen, A. R., R. Mounier, *et al*. (2007). "Expression of interleukin-15 in human skeletal muscle effect of exercise and muscle fibre type composition." J Physiol **584**(Pt 1): 305-12.

Niki, E. (1990). "Free radical initiators as source of water- or lipid-soluble peroxyl radicals." Methods Enzymol **186**: 100-8.

Nisoli, E. and M. O. Carruba (2006). "Nitric oxide and mitochondrial biogenesis." J Cell Sci **119**(Pt 14): 2855-62.

Nouri, K. and L. M. Villafradez-Diaz (2005). "Light/laser therapy in the treatment of acne vulgaris." J Cosmet Dermatol **4**(4): 318-20.

Novotova, M., M. Pavlovicova, *et al*. (2006). "Ultrastructural remodeling of fast skeletal muscle fibers induced by invalidation of creatine kinase." Am J Physiol Cell Physiol **291**(6): C1279-85.

Ocana-Quero, J. M., R. Gomez-Villamandos, *et al*. (1997). "Biological effects of helium-neon (He-Ne) laser irradiation on acrosome reaction in bull sperm cells." J Photochem Photobiol B **40**(3): 294-8.

Ohtsuki, I., K. Maruyama, *et al*. (1986). "Regulatory and cytoskeletal proteins of vertebrate skeletal muscle." Adv Protein Chem **38**: 1-67.

Olsen, S., P. Aagaard, *et al*. (2006). "Creatine supplementation augments the increase in satellite cell and myonuclei number in human skeletal muscle induced by strength training." J Physiol **573**(Pt 2): 525-34.

Ordway, G. A. and D. J. Garry (2004). "Myoglobin: an essential hemoprotein in striated muscle." J Exp Biol **207**(Pt 20): 3441-6.

Ostrowski, K., T. Rohde, *et al*. (1999). "Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans." J Physiol **515 (Pt 1)**: 287-91.

Ostrowski, K., T. Rohde, *et al*. (2001). "Chemokines are elevated in plasma after strenuous exercise in humans." Eur J Appl Physiol **84**(3): 244-5.

Otis, J. S., R. R. Roy, *et al*. (2004). "Adaptations in metabolic capacity of rat soleus after paralysis." J Appl Physiol **96**(2): 584-96.

Ou, B., M. Hampsch-Woodill, *et al*. (2001). "Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe." J Agric Food Chem **49**(10): 4619-26.

Pantano, C., N. L. Reynaert, *et al*. (2006). "Redox-sensitive kinases of the nuclear factor-kappaB signaling pathway." Antioxid Redox Signal **8**(9-10): 1791-806.

Papageorgiou, P., A. Katsambas, *et al*. (2000). "Phototherapy with blue (415 nm) and red (660 nm) light in the treatment of acne vulgaris." Br J Dermatol **142**(5): 973-8.

Partridge, T. A. (2003). "Stem cell route to neuromuscular therapies." Muscle Nerve **27**(2): 133-41.

Passarella, S., A. Ostuni, *et al*. (1988). "Increase in the ADP/ATP exchange in rat liver mitochondria irradiated in vitro by helium-neon laser." Biochem Biophys Res Commun **156**(2): 978-86.

Pastore, D., M. Greco, *et al*. (2000). "Specific helium-neon laser sensitivity of the purified cytochrome c oxidase." Int J Radiat Biol **76**(6): 863-70.

Patel, S. R., T. P. Olenginski, *et al*. (1997). "Pyomyositis: clinical features and predisposing conditions." J Rheumatol **24**(9): 1734-8.

Pedersen, B. K. and M. Febbraio (2005). "Muscle-derived interleukin-6--a possible link between skeletal muscle, adipose tissue, liver, and brain." Brain Behav Immun **19**(5): 371-6.

Pereira, A. N., P. Eduardo Cde, *et al*. (2002). "Effect of low-power laser irradiation on cell growth and procollagen synthesis of cultured fibroblasts." Lasers Surg Med **31**(4): 263-7.

Perry, R. L. and M. A. Rudnick (2000). "Molecular mechanisms regulating myogenic determination and differentiation." Front Biosci **5**: D750-67.

Pette, D., H. Peuker, *et al*. (1999). "The impact of biochemical methods for single muscle fibre analysis." Acta Physiol Scand **166**(4): 261-77.

Pette, D. and R. S. Staron (2000). "Myosin isoforms, muscle fiber types, and transitions." Microsc Res Tech **50**(6): 500-9.

Pette, D. and G. Vrbova (1992). "Adaptation of mammalian skeletal muscle fibers to chronic electrical stimulation." Rev Physiol Biochem Pharmacol **120**: 115-202.

Pizza, F. X., T. J. Koh, *et al*. (2002). "Muscle inflammatory cells after passive stretches, isometric contractions, and lengthening contractions." J Appl Physiol **92**(5): 1873-8.

Pizza, F. X., T. J. McLoughlin, *et al*. (2001). "Neutrophils injure cultured skeletal myotubes." Am J Physiol Cell Physiol **281**(1): C335-41.

Pizza, F. X., J. M. Peterson, *et al*. (2005). "Neutrophils contribute to muscle injury and impair its resolution after lengthening contractions in mice." J Physiol **562**(Pt 3): 899-913.

Plant, D. R., F. Beitzel, *et al*. (2005). "Length-tension relationships are altered in regenerating muscles of the rat after bupivacaine injection." J Appl Physiol **98**(6): 1998-2003.

Plomgaard, P., M. Penkowa, *et al*. (2005). "Fiber type specific expression of TNF-alpha, IL-6 and IL-18 in human skeletal muscles." Exerc Immunol Rev **11**: 53-63.

Popper, K. R. (1972). Objective knowledge, Oxford University Press.

Posen, Y., V. Kalchenko, *et al*. (2005). "Manipulation of redox signaling in mammalian cells enabled by controlled photogeneration of reactive oxygen species." J Cell Sci **118**(Pt 9): 1957-69.

Potapov, A. F., V. E. Trepilets, *et al*. (1995). "[Effects of intravasal laser irradiation of blood on lipid peroxidation in patients with abdominal surgery]." Anesteziol Reanimatol(1): 19-22.

Prior, R. L. and G. Cao (1999). "In vivo total antioxidant capacity: comparison of different analytical methods." Free Radic Biol Med **27**(11-12): 1173-81.

Prior, R. L., H. Hoang, *et al*. (2003). "Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL))) of plasma and other biological and food samples." J Agric Food Chem **51**(11): 3273-9.

Proctor, D. N., P. C. O'Brien, *et al*. (1999). "Comparison of techniques to estimate total body skeletal muscle mass in people of different age groups." Am J Physiol **277**(3 Pt 1): E489-95.

Ragoussis, J., G. P. Elvidge, *et al*. (2006). "Matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry in genomics research." PLoS Genet **2**(7): e100.

Rantanen, J., O. Thorsson, *et al*. (1999). "Effects of therapeutic ultrasound on the regeneration of skeletal myofibers after experimental muscle injury." Am J Sports Med **27**(1): 54-9.

Ranvier, L. (1873). "Propriétés et structures differéntes des muscles rouges et des muscles blancs chez les lapins et chez les raies." C R Acad Sci Paris(77): 1030-1034.

Rayment, I., W. R. Rypniewski, *et al*. (1993). "Three-dimensional structure of myosin subfragment-1: a molecular motor." Science **261**(5117): 50-8.

Reddy, G. K., L. Stehno-Bittel, *et al*. (1998). "Laser photostimulation of collagen production in healing rabbit Achilles tendons." Lasers Surg Med **22**(5): 281-7.

Reitsma, W. (1969). "Skeletal muscle hypertrophy after heavy exercise in rats with surgically reduced muscle function." Am J Phys Med **48**(5): 237-58.

Relaix, F., D. Montarras, *et al*. (2006). "Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells." J Cell Biol **172**(1): 91-102.

Reth, M. (2002). "Hydrogen peroxide as second messenger in lymphocyte activation." Nat Immunol **3**(12): 1129-34.

Rizzi, C. F., J. L. Mauriz, *et al*. (2006). "Effects of low-level laser therapy (LLLT) on the nuclear factor (NF)-kappaB signaling pathway in traumatized muscle." Lasers Surg Med **38**(7): 704-13.

Roitti, I. and A. Rabson (2000). Really essential medical immunology, Blackwell Science.

Rome, L. C. and S. L. Lindstedt (1998). "The Quest for Speed: Muscles Built for High-Frequency Contractions." News Physiol Sci **13**: 261-268.

Rubinstein, N. A. and H. Holtzer (1979). "Fast and slow muscles in tissue culture synthesise only fast myosin." Nature **280**(5720): 323-5.

Rucavado, A., T. Escalante, *et al*. (2002). "Increments in cytokines and matrix metalloproteinases in skeletal muscle after injection of tissue-damaging toxins from the venom of the snake Bothrops asper." Mediators Inflamm **11**(2): 121-8.

Russell, B., D. J. Dix, *et al*. (1992). "Repair of injured skeletal muscle: a molecular approach." Med Sci Sports Exerc **24**(2): 189-96.

Salvemini, D., G. de Nucci, *et al*. (1989). "Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor." Proc Natl Acad Sci U S A **86**(16): 6328-32.

Salvemini, D., E. Masini, *et al*. (1990). "Synthesis of a nitric oxide-like factor from L-arginine by rat serosal mast cells: stimulation of guanylate cyclase and inhibition of platelet aggregation." Biochem Biophys Res Commun **169**(2): 596-601.

Sampaolesi, M., S. Blot, *et al*. (2006). "Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs." Nature **444**(7119): 574-9.

Sanders, V. M. and K. J. Jones (2006). "Role of immunity in recovery from a peripheral nerve injury." J Neuroimmune Pharmacol **1**(1): 11-9.

Sant'ana Pereira, J. A., A. Wessels, *et al*. (1995). "New method for the accurate characterization of single human skeletal muscle fibres demonstrates a relation between mATPase and MyHC expression in pure and hybrid fibre types." J Muscle Res Cell Motil **16**(1): 21-34.

Scaffidi, P., T. Misteli, *et al*. (2002). "Release of chromatin protein HMGB1 by necrotic cells triggers inflammation." Nature **418**(6894): 191-5.

Schaeffer, P., K. Conley, *et al*. (1996). "Structural correlates of speed and endurance in skeletal muscle: the rattlesnake tailshaker muscle." J Exp Biol **199**(Pt 2): 351-8.

Schiaffino, S. and C. Reggiani (1996). "Molecular diversity of myofibrillar proteins: gene regulation and functional significance." Physiol Rev **76**(2): 371-423.

Schiller, J., R. Suss, *et al*. (2004). "Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry in lipid and phospholipid research." Prog Lipid Res **43**(5): 449-88.

Schilling, N., D. Arnold, *et al*. (2005). "Evolutionary aspects and muscular properties of the trunk--implications for human low back pain." Pathophysiology **12**(4): 233-42.

Schmalbruch, H. and U. Hellhammer (1977). "The number of nuclei in adult rat muscles with special reference to satellite cells." Anat Rec **189**(2): 169-75.

Schneider, B. S., H. Sannes, *et al*. (2002). "Desmin characteristics of CD11b-positive fibers after eccentric contractions." Med Sci Sports Exerc **34**(2): 274-81.

Schultz, E., M. C. Gibson, *et al*. (1978). "Satellite cells are mitotically quiescent in mature mouse muscle: an EM and radioautographic study." J Exp Zool **206**(3): 451-6.

Schultz, E., D. L. Jaryszak, *et al*. (1985). "Response of satellite cells to focal skeletal muscle injury." Muscle Nerve **8**(3): 217-22.

Scott, C. D. (2004). "Chemical models for simulating single-walled nanotube production in arc vaporization and laser ablation processes." J Nanosci Nanotechnol **4**(4): 368-76.

Segal, S. S. (1994). ""Nitric oxide release is present from incubated skeletal muscle preparations"." J Appl Physiol **77**(6): 2517-8.

Seidel, J. C. (1967). "Studies on myosin from red and white skeletal muscles of the rabbit. II. Inactivation of myosin from red muscles under mild alkaline conditions." J Biol Chem **242**(23): 5623-9.

Sharpe, M. A. and C. E. Cooper (1998). "Interaction of peroxynitrite with mitochondrial cytochrome oxidase. Catalytic production of nitric oxide and irreversible inhibition of enzyme activity." J Biol Chem **273**(47): 30961-72.

Shen, W., V. Prisk, *et al*. (2006). "Inhibited skeletal muscle healing in cyclooxygenase-2 gene-deficient mice: the role of PGE2 and PGF2alpha." J Appl Physiol **101**(4): 1215-21.

Shi, X. and D. J. Garry (2006). "Muscle stem cells in development, regeneration, and disease." Genes Dev **20**(13): 1692-708.

Shinin, V., B. Gayraud-Morel, *et al*. (2006). "Asymmetric division and cosegregation of template DNA strands in adult muscle satellite cells." Nat Cell Biol **8**(7): 677-87.

Simoneau, J. A. and C. Bouchard (1989). "Human variation in skeletal muscle fiber-type proportion and enzyme activities." Am J Physiol **257**(4 Pt 1): E567-72.

Sinanan, A. C., N. P. Hunt, *et al*. (2004). "Human adult craniofacial muscle-derived cells: neural-cell adhesion-molecule (NCAM; CD56)-expressing cells appear to contain multipotential stem cells." Biotechnol Appl Biochem **40**(Pt 1): 25-34.

Smith, C., M. J. Kruger, *et al*. (2008). "The Inflammatory Response to Skeletal Muscle Injury: Illuminating Complexities." Sports Medicine **38**(11): 947-969.

Snow, M. H. (1978). "An autoradiographic study of satellite cell differentiation into regenerating myotubes following transplantation of muscles in young rats." Cell Tissue Res **186**(3): 535-40.

Snow, M. H. (1983). "A quantitative ultrastructural analysis of satellite cells in denervated fast and slow muscles of the mouse." Anat Rec **207**(4): 593-604.

Solien, J., V. Haynes, *et al*. (2005). "Differential requirements of calcium for oxoglutarate dehydrogenase and mitochondrial nitric-oxide synthase under hypoxia: impact on the regulation of mitochondrial oxygen consumption." Comp Biochem Physiol A Mol Integr Physiol **142**(2): 111-7.

Sommer, J. R. and B. Scherer (1985). "Geometry of cell and bundle appositions in cardiac muscle: light microscopy." Am J Physiol **248**(6 Pt 2): H792-803.

Soteriou, A., M. Gamage, *et al*. (1993). "A survey of interactions made by the giant protein titin." J Cell Sci **104 (Pt 1)**: 119-23.

Spamer, C. and D. Pette (1977). "Activity patterns of phosphofructokinase, glyceraldehydephosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase in microdissected fast and slow fibres from rabbit psoas and soleus muscle." Histochemistry **52**(3): 201-16.

Spangenburg, E. E., M. V. Chakravarthy, *et al*. (2002). "p27Kip1: a key regulator of skeletal muscle satellite cell proliferation." Clin Orthop Relat Res(403 Suppl): S221-7.

Sreter, F. A. (1969). "Temperature, pH and seasonal dependence of Ca-uptake and ATPase activity of white and red muscle microsomes." Arch Biochem Biophys **134**(1): 25-33.

St Pierre, B. A. and J. G. Tidball (1994). "Differential response of macrophage subpopulations to soleus muscle reloading after rat hindlimb suspension." J Appl Physiol **77**(1): 290-7.

St Pierre Schneider, B., L. A. Correia, *et al*. (1999). "Sex differences in leukocyte invasion in injured murine skeletal muscle." Res Nurs Health **22**(3): 243-50.

Stadler, I., R. J. Lanzafame, *et al*. (2001). "830-nm irradiation increases the wound tensile strength in a diabetic murine model." Lasers Surg Med **28**(3): 220-6.

Starkie, R., S. R. Ostrowski, *et al*. (2003). "Exercise and IL-6 infusion inhibit endotoxin-induced TNF-alpha production in humans." FASEB J **17**(8): 884-6.

Staron, R. S. (1997). "Human skeletal muscle fiber types: delineation, development, and distribution." Can J Appl Physiol **22**(4): 307-27.

Steinhoff, M., N. Vergnolle, *et al*. (2000). "Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism." Nat Med **6**(2): 151-8.

Stephenson, G. M. (2001). "Hybrid skeletal muscle fibres: a rare or common phenomenon?" Clin Exp Pharmacol Physiol **28**(8): 692-702.

Stevens, D. L. (1999). "The flesh-eating bacterium: what's next?" J Infect Dis **179 Suppl 2**: S366-74.

Strand, H., O. C. Ingebretsen, *et al*. (2008). "Real-time detection and quantification of mitochondrial mutations with oligonucleotide primers containing locked nucleic acid." Clin Chim Acta **390**(1-2): 126-33.

Suzuki, J., Y. Yamazaki, *et al*. (2000). "Involvement of Ras and Ral in chemotactic migration of skeletal myoblasts." Mol Cell Biol **20**(13): 4658-65.

Suzuki, K., M. Totsuka, *et al*. (1999). "Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics, and muscle damage." J Appl Physiol **87**(4): 1360-7.

Sweeney, H. L., M. J. Kushmerick, *et al*. (1988). "Myosin alkali light chain and heavy chain variations correlate with altered shortening velocity of isolated skeletal muscle fibers." J Biol Chem **263**(18): 9034-9.

Taieb, J., C. Delarche, *et al*. (2002). "Polymorphonuclear neutrophils are a source of hepatocyte growth factor in patients with severe alcoholic hepatitis." J Hepatol **36**(3): 342-8.

Talmadge, R. J. (2000). "Myosin heavy chain isoform expression following reduced neuromuscular activity: potential regulatory mechanisms." Muscle Nerve **23**(5): 661-79.

Talmadge, R. J., R. R. Roy, *et al*. (1993). "Muscle fiber types and function." Curr Opin Rheumatol **5**(6): 695-705.

Talmadge, R. J., R. R. Roy, *et al*. (1995). "Prominence of myosin heavy chain hybrid fibers in soleus muscle of spinal cord-transected rats." J Appl Physiol **78**(4): 1256-65.

Talmadge, R. J., R. R. Roy, *et al*. (1996). "Myosin heavy chain profile of cat soleus following chronic reduced activity or inactivity." Muscle Nerve **19**(8): 980-8.

Talmadge, R. J., R. R. Roy, *et al*. (1999). "Persistence of hybrid fibers in rat soleus after spinal cord transection." Anat Rec **255**(2): 188-201.

Tatsumi, R., J. E. Anderson, *et al*. (1998). "HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells." Dev Biol **194**(1): 114-28.

Tesch, P. A. and J. Karlsson (1985). "Muscle fiber types and size in trained and untrained muscles of elite athletes." J Appl Physiol **59**(6): 1716-20.

Tesch, P. A., A. Thorsson, *et al*. (1984). "Muscle capillary supply and fiber type characteristics in weight and power lifters." J Appl Physiol **56**(1): 35-8.

Thannickal, V. J. and B. L. Fanburg (2000). "Reactive oxygen species in cell signaling." Am J Physiol Lung Cell Mol Physiol **279**(6): L1005-28.

Thomason, D. B., R. E. Herrick, *et al*. (1987). "Time course of soleus muscle myosin expression during hindlimb suspension and recovery." J Appl Physiol **63**(1): 130-7.

Thorsson, O., B. Lilja, *et al*. (1997). "Immediate external compression in the management of an acute muscle injury." Scand J Med Sci Sports **7**(3): 182-90.

Thorsson, O., J. Rantanen, *et al*. (1998). "Effects of nonsteroidal antiinflammatory medication on satellite cell proliferation during muscle regeneration." Am J Sports Med **26**(2): 172-6.

Tidball, J. G. (1995). "Inflammatory cell response to acute muscle injury." Med Sci Sports Exerc **27**(7): 1022-32.

Tidball, J. G. (2005). "Inflammatory processes in muscle injury and repair." Am J Physiol Regul Integr Comp Physiol **288**(2): R345-53.

Tiidus, P. M. (1998). "Radical species in inflammation and overtraining." Can J Physiol Pharmacol **76**(5): 533-8.

Tiidus, P. M., M. Deller, *et al*. (2005). "Oestrogen influence on myogenic satellite cells following downhill running in male rats: a preliminary study." Acta Physiol Scand **184**(1): 67-72.

Timson, D. J. (2003). "Fine tuning the myosin motor: the role of the essential light chain in striated muscle myosin." Biochimie **85**(7): 639-45.

Tisdale, M. J. (1997). "Biology of cachexia." J Natl Cancer Inst **89**(23): 1763-73.

Tisdale, M. J. (1997). "Cancer cachexia: metabolic alterations and clinical manifestations." Nutrition **13**(1): 1-7.

Tomiya, A., T. Aizawa, *et al*. (2004). "Myofibers express IL-6 after eccentric exercise." Am J Sports Med **32**(2): 503-8.

Trelles, M. A. and E. Mayayo (1987). "Bone fracture consolidates faster with low-power laser." Lasers Surg Med **7**(1): 36-45.

Trinick, J. (1994). "Titin and nebulin: protein rulers in muscle?" Trends Biochem Sci **19**(10): 405-9.

Trump, B. F. and I. K. Berezesky (1995). "Calcium-mediated cell injury and cell death." FASEB J **9**(2): 219-28.

Tsivitse, S. K., E. Mylona, *et al*. (2005). "Mechanical loading and injury induce human myotubes to release neutrophil chemoattractants." Am J Physiol Cell Physiol **288**(3): C721-9.

Tsujinaka, T., C. Ebisui, *et al*. (1995). "Muscle undergoes atrophy in association with increase of lysosomal cathepsin activity in interleukin-6 transgenic mouse." Biochem Biophys Res Commun **207**(1): 168-74.

Urso, M. L. and P. M. Clarkson (2003). "Oxidative stress, exercise, and antioxidant supplementation." Toxicology **189**(1-2): 41-54.

van Breugel, H. H. and P. R. Bar (1992). "Power density and exposure time of He-Ne laser irradiation are more important than total energy dose in photo-biomodulation of human fibroblasts in vitro." Lasers Surg Med **12**(5): 528-37.

van Loon, L. J. and B. H. Goodpaster (2006). "Increased intramuscular lipid storage in the insulin-resistant and endurance-trained state." Pflugers Arch **451**(5): 606-16.

Ventura-Clapier, R., A. Garnier, *et al*. (2004). "Energy metabolism in heart failure." J Physiol **555**(Pt 1): 1-13.

Vignaud, A., J. Cebrian, *et al*. (2005). "Effect of anti-inflammatory and antioxidant drugs on the long-term repair of severely injured mouse skeletal muscle." Exp Physiol **90**(4): 487-95.

Vladimirov, Y. A., E. A. Gorbatenkova, *et al*. (1988). "Photoreactivation of superoxide dismutase by intensive red (laser) light." Free Radic Biol Med **5**(5-6): 281-6.

Voet, D. and J. D. Voet (2004). Biochemistry, John Wikey & Sons, Inc.

Wadley, G. D. and G. K. McConell (2007). "Effect of nitric oxide synthase inhibition on mitochondrial biogenesis in rat skeletal muscle." J Appl Physiol **102**(1): 314-20.

Wang, L. J., Y. Y. Lu, *et al*. (2002). "Neuroprotective effects of glial cell line-derived neurotrophic factor mediated by an adeno-associated virus vector in a transgenic animal model of amyotrophic lateral sclerosis." J Neurosci **22**(16): 6920-8.

Wang, N., R. S. Hikida, *et al*. (1993). "Muscle fiber types of women after resistance training--quantitative ultrastructure and enzyme activity." Pflugers Arch **424**(5-6): 494-502.

Wang, X., J. Ge, *et al*. (2006). "Evaluation of MTT assay for measurement of emodin-induced cytotoxicity." Assay Drug Dev Technol **4**(2): 203-7.

Warden, S. J. and J. M. McMeeken (2002). "Ultrasound usage and dosage in sports physiotherapy." Ultrasound Med Biol **28**(8): 1075-80.

Warren, G. L., D. A. Hayes, *et al*. (1993). "Mechanical factors in the initiation of eccentric contraction-induced injury in rat soleus muscle." J Physiol **464**: 457-75.

Weber, M., M. Rau, *et al*. (2005). "Diagnostic utility of new immunoassays for the cardiac markers cTnI, myoglobin and CK-MB mass." Clin Biochem **38**(11): 1027-30.

Wehling, M., M. J. Spencer, *et al*. (2001). "A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice." J Cell Biol **155**(1): 123-31.

Welbourn, C. R., G. Goldman, *et al*. (1991). "Neutrophil elastase and oxygen radicals: synergism in lung injury after hindlimb ischemia." Am J Physiol **260**(6 Pt 2): H1852-6.

Widrick, J. J., S. T. Knuth, *et al*. (1999). "Effect of a 17 day spaceflight on contractile properties of human soleus muscle fibres." J Physiol **516 (Pt 3)**: 915-30.

Widrick, J. J., J. G. Romatowski, *et al*. (1997). "Effect of 17 days of bed rest on peak isometric force and unloaded shortening velocity of human soleus fibers." Am J Physiol **273**(5 Pt 1): C1690-9.

Wilson, J. R., D. M. Mancini, *et al*. (1993). "Exertional fatigue due to skeletal muscle dysfunction in patients with heart failure." Circulation **87**(2): 470-5.

Witte, M. B. and A. Barbul (2002). "Role of nitric oxide in wound repair." Am J Surg **183**(4): 406-12.

Wittenberg, J. B. and B. A. Wittenberg (1990). "Mechanisms of cytoplasmic hemoglobin and myoglobin function." Annu Rev Biophys Biophys Chem **19**: 217-41.

Wokke, J. H., C. J. Van den Oord, *et al*. (1989). "Perisynaptic satellite cells in human external intercostal muscle: a quantitative and qualitative study." Anat Rec **223**(2): 174-80.

Wong-Riley, M. T., H. L. Liang, *et al*. (2005). "Photobiomodulation directly benefits primary neurons functionally inactivated by toxins: role of cytochrome c oxidase." J Biol Chem **280**(6): 4761-71.

Wright, D. C., P. C. Geiger, *et al*. (2004). "Phorbol esters affect skeletal muscle glucose transport in a fiber type-specific manner." Am J Physiol Endocrinol Metab **287**(2): E305-9.

Yamashita, H., S. Sugiura, *et al*. (2003). "Myosin light chain isoforms modify force-generating ability of cardiac myosin by changing the kinetics of actin-myosin interaction." Cardiovasc Res **60**(3): 580-8.

Yamauchi, T., J. Kamon, *et al*. (2002). "Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase." Nat Med **8**(11): 1288-95.

Yanagida, T., T. Arata, *et al*. (1985). "Sliding distance of actin filament induced by a myosin crossbridge during one ATP hydrolysis cycle." Nature **316**(6026): 366-9.

Yguerabide, J., J. A. Schmidt, *et al*. (1982). "Lateral mobility in membranes as detected by fluorescence recovery after photobleaching." Biophys J **40**(1): 69-75.

Zammit, P. and J. Beauchamp (2001). "The skeletal muscle satellite cell: stem cell or son of stem cell?" Differentiation **68**(4-5): 193-204.

Zhang, Y., S. Song, *et al*. (2003). "cDNA microarray analysis of gene expression profiles in human fibroblast cells irradiated with red light." J Invest Dermatol **120**(5): 849-57.

Zong, H., J. M. Ren, *et al*. (2002). "AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation." Proc Natl Acad Sci U S A **99**(25): 15983-7.