

1999

Investigation of the contractile and regulatory proteins in malignant hyperthermia and normal skeletal muscle

Louise C. Norbury
Edith Cowan University

Follow this and additional works at: https://ro.ecu.edu.au/theses_hons



Part of the [Anesthesiology Commons](#), and the [Critical Care Commons](#)

Recommended Citation

Norbury, L. C. (1999). *Investigation of the contractile and regulatory proteins in malignant hyperthermia and normal skeletal muscle*. Edith Cowan University. https://ro.ecu.edu.au/theses_hons/505

This Thesis is posted at Research Online.
https://ro.ecu.edu.au/theses_hons/505

Edith Cowan University

Copyright Warning

You may print or download ONE copy of this document for the purpose of your own research or study.

The University does not authorize you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site.

You are reminded of the following:

- Copyright owners are entitled to take legal action against persons who infringe their copyright.
- A reproduction of material that is protected by copyright may be a copyright infringement. Where the reproduction of such material is done without attribution of authorship, with false attribution of authorship or the authorship is treated in a derogatory manner, this may be a breach of the author's moral rights contained in Part IX of the Copyright Act 1968 (Cth).
- Courts have the power to impose a wide range of civil and criminal sanctions for infringement of copyright, infringement of moral rights and other offences under the Copyright Act 1968 (Cth). Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.

**Investigation of the contractile and regulatory proteins in Malignant
Hyperthermia and normal skeletal muscle**

by

Louise C. Norbury (BSc)

**A Thesis Submitted in Partial Fulfilment of the
Requirements for the Award of**

Bachelor of Science (Human Biology) Honours

**At the Faculty of Communication, Health and Science, Edith Cowan
University, Joondalup**

Date of submission: November 12 1999

USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.

ABSTRACT

Malignant hyperthermia/hyperpyrexia (MH) is a serious human skeletal muscle disorder that manifests itself during general anaesthesia. Currently, the most reliable method of diagnosing this disease is the in-vitro contracture test (IVCT). This procedure is both highly invasive for the patient and expensive to perform. The skinned muscle fibre technique is less invasive, and could possibly be used as an alternative diagnostic test for MH.

In this study, skeletal muscle samples were obtained from 8 adults (age 23-54 years) undergoing elective surgery at Royal Perth Hospital. Six patients were diagnosed as normal (controls) and two equivocal for MH (MHE). Using the skinned muscle fibre technique (with exogenous Ca^{2+} and Sr^{2+}), individual muscle fibre segments (10 from each biopsy) were tested for differences in their contractile and regulatory proteins, and the generated force per cross-sectional area (F/CSA). The results indicated that the contractile and regulatory proteins of the MHE fibres displayed altered functionality in comparison to the control fibres. The Type I MHE fibres displayed greater sensitivity to Sr^{2+} (82%), whereas the Mixed MHE fibres displayed greater sensitivity to Ca^{2+} (90%). However, the F/CSA generated by the MHE fibres were overall less than that of the control fibres (43%), denoting statistical insignificance.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education; and that to the best of my knowledge and belief it does not contain any material previously written by another person except where due reference is made in the text.

Signature _____

Date 25/11/00

ACKNOWLEDGMENTS

Firstly, I would like to thank my supervisor Dr Angus Stewart for guiding me through the joys of preparing and completing a thesis. Dr Peter Roberts (co-supervisor) and Dr Richard Brightwell for helping me with my writing and giving general support. To Mr Phillip Morling (from the Department of Neuropathology, Royal Perth Hospital) for supplying me with muscle samples.

To Eric, Bernie, Jane and Marie for helping me out in the lab and giving me tips on how to go about things.

To Chris and Rhonda from the medical suite for doing my autoclaving, and to Hugh, Helen and Bernie for also doing my autoclaving and just helping out.

And finally, to my family for giving me ample support and encouragement for when I needed it the most.

Thanks guys!

LIST OF TABLES

Table 2.1	Current chromosomal locations of human MH	29
Table 3.1	Parameters of normal human skeletal muscle	40
Table 4.1	Contractile parameters of control and MHE skeletal muscle fibre types	47
Table A1.1	Kreb's-Ringer Solution	62
Table A1.2	Relaxing Solution I	62
Table A1.3	Relaxing Solution III	62
Table A1.4	Calcium Solution	63
Table A1.5	Strontium Solution	63

LIST OF FIGURES

Figure 2.1	Two dimensional structure of a sarcomere	13
Figure 2.2	Structure of the actin filament	14
Figure 2.3	Structure of the myosin filament	14
Figure 2.4	Top view of the ryanodine receptor	18
Figure 2.5	Schematic model of the ryanodine receptor	18
Figure 3.1	Equipment setup for the skinned muscle fibre method	35
Figure 3.2	Individual muscle fibre tied with silk sutures	37
Figure 3.3	Individual muscle fibre mounted on stainless steel forceps and pin of the transducer	37
Figure 3.4	Location of the stainless steel forceps and transducer (and pin) in a multiple well solution holder (carousel)	38
Figure 4.1	Force-pCa & -pSr curves for a typical Type I normal (control) muscle fibre	48
Figure 4.2	Force-pCa & -pSr curves for a typical Type I MHE muscle fibre	48
Figure 4.3	Force-pCa & -pSr curves for a typical Type II normal (control) muscle fibre	49
Figure 4.4	Force-pCa & -pSr curves for a typical Type II MHE muscle fibre	49
Figure 4.5	Force-pCa & -pSr curves for a Mixed normal (control) muscle fibre	50
Figure 4.6	Force-pCa & -pSr curves for a Mixed MHE muscle fibre	50

TABLE OF CONTENTS

Abstract	ii
Declaration	iii
Acknowledgments	iv
List of Tables	v
List of Figures	vi
CHAPTER 1: INTRODUCTION	1
1.1 Background to the Study	1
1.2 Significance of the Study	3
1.3 Purpose of the Study	3
1.4 Research Questions	4
1.5 Hypotheses	4
1.6 Definition of Terms/Abbreviations	5
CHAPTER 2: LITERATURE REVIEW	9
2.1 Introduction	9
2.2 History	10
2.3 Incidence	11
2.4 Trigger Agents	11
2.5 Skeletal Muscle Contractile Apparatus	13
2.6 Skeletal Muscle Fibre Types	16
2.7 Ryanodine Receptor	17

2.8 Signs & Symptoms	19
2.9 Treatment	21
2.10 Diagnosis	23
2.11 Genetics	28
2.12 Prognosis	31
CHAPTER 3: MATERIALS & METHODS	32
3.1 Design	32
3.2 Muscle Biopsies	33
3.3 Ethical Considerations	34
3.4 Equipment	34
3.5 Procedure	35
3.6 Data Analysis	39
3.7 Limitations	41
CHAPTER 4: RESULTS	43
4.1 Results	43
4.2 Discussion	51
4.3 Conclusion	54
4.4 Further Developments	55
REFERENCES	56
APPENDIX 1	62
APPENDIX 2	64

CHAPTER 1

INTRODUCTION

1.1 Background to the study

Malignant Hyperthermia is a pharmacogenetic disorder affecting human skeletal muscle under the influence of the inhalant general anaesthetic halothane, and the depolarising muscle relaxant suxamethonium. The disease was first recognised in Australia in 1960 by Denborough and Lovell, who discovered a family in which ten members died spontaneously whilst under general anaesthesia (Joffe, Savage & Silove, 1992; McComas, 1996).

Studies by Walter (1981) and Joffe et al. (1992) determined that there was a defect in the ryanodine receptors (calcium release channel) of the sarcoplasmic reticulum (SR) within skeletal muscle. On exposure to halothane or suxamethonium, these receptors release excess calcium into the muscle fibres, resulting in skeletal muscle contracture. This prolonged contraction of skeletal muscle results in hyperthermia, tachycardia and cardiac arrest (Joffe et al., 1992).

Currently the most reliable method of diagnosing malignant hyperthermia (MH) is the in-vitro contracture test (IVCT): it is highly invasive for the

patient and relatively expensive. The IVCT involves the removal of muscle from the patient's thigh, and exposing it to controlled levels of halothane and caffeine while continuously monitoring tension (Britt, 1979; Joffe et al., 1992; Serfas, Bose, Patel, Wrogemann, Phillips, MacLennan & Greenburg, 1996). Research is currently underway to find a less invasive technique than the IVCT of diagnosing the disease without compromising accuracy (Takagi, Sunohara, Ishihara, Nonaka & Sugita, 1983).

The skinned muscle fibre technique involves the chemical (or mechanical) removal of the cell membrane of the muscle fibre. The sarcoplasmic reticulum (SR) is also disrupted ensuring that all the internal calcium is removed. This therefore allows control over the environment within the muscle cell (Britt, Frodis, Scott, Clements & Endrenyi, 1982; Fink, Stephenson & Williams, 1990). The technique (which was used in this study) has been applied to the testing of muscle biopsies, where caffeine is added to the muscle tissue. This is because caffeine has been found to induce extreme skeletal muscle contraction (Takagi et al., 1983). This will be discussed in detail later. However, in this skinned muscle fibre technique, calcium and strontium ions are normally used to activate the muscle fibre. The technique has been applied to many questions in muscle physiology, notably Duchenne Muscular Dystrophy (Fink et al., 1990).

1.2 Significance of the Study

Current diagnostic techniques for MH are highly invasive, requiring a large skeletal muscle biopsy to be taken from the patient's thigh in the operating theatre, which is both painful to the patient and expensive to the community (Allen, Larach & Kunselman, 1998). Skinned fibre studies require a much smaller biopsy (even needle biopsies have proven reliable) and do not necessitate theatre time (Takagi et al., 1983). It is hoped that this study will validate the use of a modified skinned muscle fibre technique as a possible diagnostic test for MH. In addition, greater detail will be revealed about the pathophysiology of MH, such as the effects of calcium and strontium ions on the contractile apparatus.

1.3 Purpose of the Study

The purpose of this project is to examine the contractile responses/kinetics of MH muscle to exogenous calcium and strontium ion activation in comparison to that of normal human muscle. If significant differences are identified then this technique may be able to be employed diagnostically with significant advantages to the patient and to hospital budgets. The results may also aid in the understanding of the contractile and regulatory proteins of MH muscle and possibly determine why the MH muscle reacts differently to certain pharmacological agents than does normal muscle.

1.4 Research Questions

- Are the functions of the contractile proteins, (myosin S1 subfragments) affected by MH?
- Are the functions of the regulatory proteins, (tropomyosin and troponin) affected by MH?
- Is the skinned muscle fibre method (employed in this study) a suitable candidate for testing for MH?

1.5 Hypotheses

- Individual MH muscle fibres display differing kinetics of contraction to exogenous calcium and strontium ion activation than individual muscle fibres in normal (control) human muscle.
- The force per cross-sectional area generated by MH muscle fibres is different to that of normal (control) muscle.

1.6 Definition of Terms/Abbreviations

- *Actin*: a "thin" protein filament responsible for the contraction of skeletal and cardiac muscle
- *Ala*: alanine, an amino acid
- *Arg*: arginine, an amino acid
- *Contracture*: permanent muscle contraction in the absence of nervous stimulation
- *Cys*: cysteine, an amino acid
- *Cytoskeleton*: the internal skeleton in the cytosol of a cell
- *Depolarise*: to make a cell's resting membrane potential more positive (or less negative) as measured inside with respect to the outside
- *DHPR*: dihydropyridine receptor, a protein molecule involved in the link between the sarcoplasmic reticulum and transverse tubules in the muscle
- *Dystrophin*: a protein found in skeletal and cardiac muscle
- *Gly*: glycine, an amino acid
- *Halothane*: an inhalant anaesthetic widely used in general anaesthesia
- *His*: histidine, an amino acid
- *Hyperthermia/hyperpyrexia*: an exceedingly high body temperature, defined as being over 41°C
- *IV*: intravenous administration
- *IVCT*: in-vitro contracture test - exposing muscle biopsies to caffeine and halothane to diagnose MH by monitoring the resultant contracture

- ***Kinetic***: pertaining to or producing motion
- ***Malignant***: to become progressively worse and to result in death
- ***Malignant Hyperthermia/Hyperpyrexia (MH)***: a serious skeletal muscle disorder arising during general anaesthesia that results in hyperthermia and death
- ***MHE***: Malignant Hyperthermia Equivocal – a patient who tests positive to either caffeine or halothane (borderline cases) but are clinically treated as MHS
- ***MHN***: Malignant Hyperthermia Normal - a patient who tests negative to caffeine and halothane to the IVCT
- ***MHS***: Malignant Hyperthermia Susceptible - a patient who tests positive to caffeine and halothane to the IVCT
- ***Myofibril***: a subdivision of a muscle cell consisting of a series of sarcomeres (contractile units)
- ***Myosin***: a "thick" protein filament responsible for the contraction of skeletal and cardiac muscle
- ***Pharmacogenetic***: a genetic abnormality that is triggered by the administration of a particular drug or a combination of drugs
- ***PSS***: Porcine Stress Syndrome - the MH equivalent in certain breeds of pig
- ***RCT***: ryanodine contracture test - using high purity ryanodine (98%) on muscle biopsies to diagnose MH
- ***Ryanodine***: a nitrogenous substance (alkaloid) found in plants

- ***Ryanodine receptor***: a receptor mainly found in skeletal and cardiac muscle (on the SR) that releases calcium into the contractile unit
- ***RYR1***: ryanodine receptor 1 gene - one of the possible genes located on chromosome 19 that has been linked to some MH sufferers
- ***S1 subfragment***: another name for the globular heads of the myosin filament necessary for the contraction of muscle
- ***Sarcomere***: the contractile unit of skeletal and cardiac muscle, consisting of sliding filaments of actin and myosin
- ***Sarcoplasm***: the cytoplasm (cytosol "fluid portion" and organelles) of a muscle cell - also called myoplasm
- ***Sarcoplasmic Reticulum (SR)***: a membrane network similar to endoplasmic reticulum surrounding each myofibril that contains supplies of calcium ions for normal muscle contraction
- ***Suxamethonium***: a neuromuscular blocking agent causing muscle relaxation that is commonly used in abdominal surgery – also called succinylcholine chloride
- ***Tropomyosin***: a protein strand that covers the active site of an actin molecule during relaxation
- ***Troponin***: three globular proteins arranged over the tropomyosin and actin molecule and contains the calcium receptor needed for contraction
- ***Type I fibre (slow-twitch)***: red coloured skeletal muscle that primarily uses aerobic metabolism as an energy supply during contraction

- ***Type II fibre (fast-twitch):*** white coloured skeletal muscle that primarily uses anaerobic metabolism as an energy supply during contraction
- ***Vastus medialis:*** one of the four heads of the quadriceps muscle located on the anteromedial aspect of the human femur

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Malignant hyperthermia/hyperpyrexia (MH) is a pharmacogenetic disorder affecting human skeletal muscle fibres (Joffe et al., 1992). Inhalant general anaesthetics such as halothane and depolarising muscle relaxants such as suxamethonium, and their derivatives, are the agents that may trigger an MH attack in malignant hyperthermia susceptible (MHS) and malignant hyperthermia equivocal (MHE) individuals (McCarthy, Tong & MacLennan, 1998). Malignant hyperthermia is also present in certain breeds of swine (*Pietran* and *Landrace*), where an attack can be induced by both stress and trigger agents. This is generally called Porcine Stress Syndrome (PSS) (Joffe et al., 1992). In humans, MH is one of the major causes of general anaesthetic related deaths, (approximately 4% of cases) (Lane, 1996). However, with the intravenous (IV) administration of the muscle relaxant dantrolene, the death rate is significantly reduced (Healy, Quane, Keating, Lehane, Heffron & McCarthy, 1996).

2.2 History

Malignant hyperthermia was first recognised in Australia in 1960 by Denborough and Lovell, who discovered a family of which ten members died after been given a triggering agent during general anaesthesia (Joffe et al., 1992; McComas, 1996). The disease was subsequently incorporated into the International Classification of Diseases in 1983 (Merz, 1986).

In 1971, the in-vitro contracture test (IVCT) using caffeine and halothane was described at the First International Symposium on MH (Toronto, Canada) in humans, and in 1975 the effects of dantrolene (a drug known to reverse the symptoms), in pigs at the Second International Symposium on MH (Katz, 1992). Dantrolene was approved by the US Food and Drug Administration (FDA) in 1979 for use in humans and by 1983 the IVCT was standardised to utilise caffeine and halothane on a biopsy removed from a quadricep muscle. The patient was then diagnosed as either malignant hyperthermia normal (see Section 2.10 p. 23) (MHN), MHS, or MHE (European Malignant Hyperpyrexia Group, 1984; Katz, 1992).

2.3 Incidence

MH occurs in 1 in 12,000 to 15,000 paediatric general surgical cases, and 1 in 40,000 to 100,000 adult general surgical cases (Joffe et al., 1992; Sorrentino & Volpe, 1993; Pennington & Joeris, 1996; Dunn, 1997; Loke & MacLennan, 1998). The most frequent age group for this disease is from 3 years of age through to 30 years of age, with males more commonly affected (Lane, 1996). Porcine stress syndrome (PSS) occurs in up to 90% of stress susceptible pigs (Joffe et al., 1992). Sporadic cases of MH have occurred in thoroughbred racehorses and in greyhounds, cats, cattle and even giraffes (Lane, 1996).

2.4 Trigger Agents

In humans, MH is precipitated by exposure to inhalant anaesthetics, such as halothane, and depolarising muscle relaxants, such as suxamethonium (McCarthy et al., 1998).

Halothane (Fluothane, ICI Pty Ltd) is a fluorinated hydrocarbon that has widespread use in general anaesthesia, due to the fact that it causes little irritation to the respiratory tract, and usually does not initiate the cough reflex. Halothane is usually combined with another inhalant anaesthetic, nitrous oxide to maintain anaesthesia following induction (Galbraith,

Bullock & Manias, 1994). Halothane is the most common and strongest trigger in MHS individuals (Lane, 1996). The other fluorinated hydrocarbons related to halothane, which also trigger MH reactions are isoflurane, sevoflurane, enflurane, desflurane, and methoxyflurane (Galbraith et al., 1994; Malignant Hyperthermia Association of the United States, 1997; *MIMS*, 1997).

Suxamethonium (Scoline, David Bull Pty Ltd) is a depolarising muscle relaxant commonly used in abdominal surgery, as most general anaesthetics do not relax the abdominal muscles sufficiently (Galbraith et al., 1994; *MIMS*, 1997; McCarthy et al., 1998). This drug is structurally similar to the neurotransmitter acetylcholine. It acts as an acetylcholine agonist on the nicotinic receptors of the motor end plate, by binding to the motor end plate, inducing prolonged depolarisation and preventing transmission of any neural impulses. This results in total muscle paralysis (Galbraith et al., 1994). Normally after IV administration, the skeletal muscles go into total spasm, then after a second or so the body should go totally flaccid. However, in MH individuals, the skeletal muscles remain rigid (Galbraith et al., 1994), which strongly indicates that the pathophysiology does not involve the continual depolarisation of the muscle fibres.

In both humans and pigs, halothane and suxamethonium (and their derivatives) cause the release of calcium into the sarcoplasm, resulting in

increased metabolic activity and contracture of the skeletal muscles (Galbraith et al., 1994). The defect lies in the ryanodine receptor (see Section 2.6 p. 16) of the sarcoplasmic reticulum (SR) surrounding individual myofibrils.

2.5 Skeletal Muscle Contractile Apparatus

Skeletal muscle is composed of many contractile units (sarcomeres) that are necessary for muscle function. Sarcomeres contain two major contractile proteins called actin (thin filaments) and myosin (thick filaments). Other proteins involved are found associated with the Z line (intermediate filament), the M line. The Z line is made up of the protein connectin and it interconnects the actin filaments between each adjacent sarcomere. The M line stabilises the myosin filaments in the centre of the sarcomere. Another protein titin, stabilises this structure by linking the myosin filaments to the Z line (Figure 2.1) (McComas, 1996; Martini, 1998).

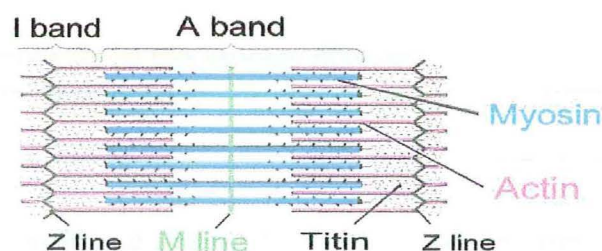


Figure 2.1. Two dimensional structure of a sarcomere
(Source: Martini, 1998)

Actin filaments measure approximately 6-8nm wide and 1 μ m long. The filament consists of tropomyosin (3 subunits), troponin and between 300-400 actin molecules (Figure 2.2). The three troponin subunits consists of: troponin *T* that attaches to the tropomyosin; troponin *I* that binds to the actin molecule, and troponin *C* that binds to calcium (McComas, 1996). Myosin filaments measure approximately 12nm wide and between 1.5-1.6 μ m long. This filament comprises of a single “tail” and two globular cross-bridge “heads” (also called the S1 subfragments) (Figure 2.3) (Martini, 1998).

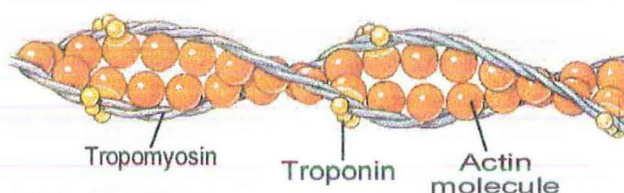


Figure 2.2. Structure of the actin filament
(Source: Martini, 1998)

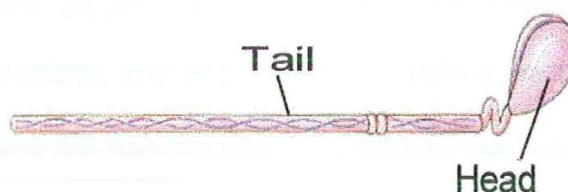


Figure 2.3. Structure of the myosin filament
(Source: Martini, 1998)

In a resting state, a sarcomere (defined as the functional unit of contraction between two Z lines) measures approximately 2.6 μ m long, and when viewed using optical microscopy, comprise of light and dark “bands”

consisting of the above filaments. The A band contains the zone of overlap between the actin and myosin, the I band contains only actin, and the H band contains only myosin and the M line. The naming of the A and I bands were derived from the appearance viewed under polarised light. Anisotropic (A band), light absorbing, and isotropic (I band), light transmitting, were the terms given (Martini, 1998).

The rationale of skeletal muscle contraction is still referred to as the Sliding Filament Theory, devised by Huxley in the early 1950s (McComas, 1996). During contraction, the myosin cross-bridge heads attach themselves to specific binding sites on the actin molecules (active site), causing the heads to pivot backwards, sliding the actin filaments towards the M line, and then releasing. This is called "cross-bridge cycling". For contraction to be initiated, calcium ions have to be released from the SR through the ryanodine receptors, increasing the intracellular concentration from 10^{-8}M to around 10^{-5}M . Calcium binds to the troponin (troponin C) which causes the troponin and tropomyosin to reflect away from the actin binding site and allow the myosin heads to attach, permitting cross-bridge cycling (McComas, 1996; Martini, 1998).

2.6 Skeletal Muscle Fibre Types

In general, there are three distinct categories of muscle fibre types that have varying properties. There are slow fibres (Type I), fast fibres (Type IIB) and intermediate fibres (Type IIA). Slow or red fibres have a slow contraction speed (50-100msec) and a relatively small cross sectional diameter (10-50 μ m) (Martini, 1998). Its colouring is due to a high myoglobin (muscle haemoglobin) content that has plentiful oxygen reserves (Peachey, Adrian & Geiger, 1983). These fibres are primarily used for prolonged contractions such as postural maintenance, therefore they have many mitochondria that use aerobic metabolism (Peachey et al., 1983). Fast or white fibres have a fast contraction speed of 10msec or less and have a large cross sectional diameter (50-100 μ m). These fibres have a low myoglobin content and very few mitochondria, therefore they rely on large glycogen reserves (anaerobic metabolism) for energy (Peachey et al., 1983; Martini, 1998). These fibres produce powerful contractions for activities such as sprinting and lifting, but fatigue rapidly (Martini, 1998). Intermediate fibres are similar to fast fibres in appearance and metabolism, however they do not fatigue as easily (Peachey et al., 1983; Martini, 1998).

2.7 Ryanodine Receptor

The ryanodine receptor is the principal channel through which calcium is released from the sarcoplasmic reticulum (SR) into the sarcoplasm. The naming of the receptor comes from the plant alkaloid ryanodine, as it was found experimentally that this protein has a high affinity to these receptors (Joffe et al., 1992).

The receptor is divided into four domains or radial channels that are connected to a 2nm central channel, and has a four leaf clover appearance (see Figure 2.4) (Joffe et al., 1992; Lane, 1996). The base or foot of the receptor protrudes into the junctional membrane of the SR (where the dihydropyridine (DHP) receptors are located). The N-terminal is hydrophilic ("water loving") and protrudes into the sarcoplasm (see Figure 2.5). The central channel is the calcium release channel essential for the contraction of skeletal muscle (Joffe et al., 1992; Lane, 1996). In MH the ryanodine receptor stays open, subsequently causing prolonged release of calcium into the muscle fibre (Galbraith et al., 1994). Why the receptor stays open is as yet unknown.

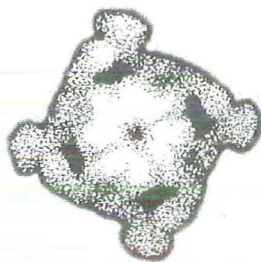


Figure 2.4. Top view of the ryanodine receptor as viewed by an electron microscope
 (Source: Joffe et al., 1992)

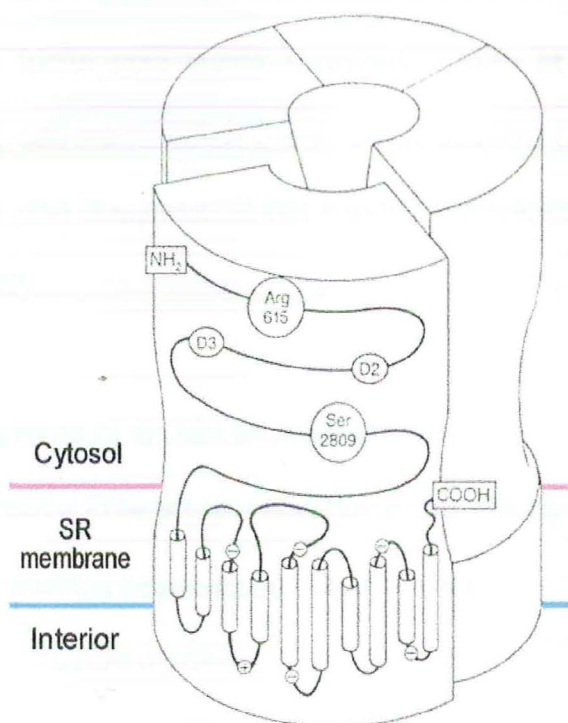


Figure 2.5. Schematic model of the ryanodine receptor
 (Source: Sorrentino & Volpe, 1993)

2.8 Signs & Symptoms

When an MHS individual is exposed to halothane or suxamethonium, a defect in the ryanodine receptor of the internal membrane of the muscle fibre (sarcoplasmic reticulum) causes the release of calcium into the skeletal muscle fibres, resulting in prolonged muscle contraction (Joffe et al., 1992). This continual contraction of the skeletal muscles leads to adenosine triphosphate (ATP) depletion. To overcome this, the body compensates by rephosphorylating ATP from adenosine diphosphate (ADP), thus resulting in excess heat production. Subsequently, aerobic and anaerobic metabolism are accelerated in the skeletal muscles leading to excess lactic acid levels (acidosis) (Joffe et al., 1992; Katz, 1992). Eventually cell membranes break down causing the release of myoglobin, potassium and enzymes. If not treated, irreversible cell death can occur (Katz, 1992).

Early symptoms of an MH attack involve:

- An increase in heart rate and pulse rate (tachycardia).
- Rapid, shallow respirations (tachypnoea).
- Skeletal muscle rigidity.
- Increase in blood pressure (hypertension).
- Initially flushed skin, then pale (cyanotic) and unusually warm.
- Abnormal brain wave pattern (dysrhythmia).
- Increase in end-tidal carbon dioxide (ETCO₂) levels.

The time scale of these events depends on the severity of the disease.

(Merz, 1986; Katz, 1992; MacLennan, 1992)

As an attack progresses, impaired coagulation and subsequent bleeding from body orifices occurs, with pulmonary oedema, acidosis, high serum myoglobin levels, hyperthermia (over 44°C), and masseter muscle spasm (MMS) (Merz, 1986; Katz, 1992; MacLennan, 1992).

Finally, if treatment is delayed or unavailable, there is an increase in blood potassium levels (hyperkalaemia) and a decrease in blood pressure (hypotension), heart rate and pulse rate (bradycardia) with subsequent cardiac arrest (Merz, 1986; Katz, 1992). Death can occur within minutes from ventricular failure and within hours from pulmonary oedema or the inability to clot blood (coagulopathy). In the longer term, the disorder can be fatal days later from neurological damage or myoglobin in the urine (myoglobinurea), leading to renal failure (MacLennan, 1992; Ball & Johnson, 1993). However, with the development of end-tidal carbon dioxide (ETCO₂) analysers and other monitoring systems, an attack can be detected earlier, and dantrolene subsequently administered (see Section 2.9 p. 19) (MacLennan, 1992; Lane, 1996).

MH has been described in species other than humans and is common in pigs (MacLennan, 1992). Porcine stress syndrome (PSS) is associated with lean heavily muscled breeds, notably *Pietran* and *Landrace*. It was

noted that when these breeds were subjected to enormous stress, such as prior to slaughtering, they spontaneously died. Other causes of stress included overheating, exercise, mating and transportation. This is a major economic problem for the pork industry, as the meat produced is pale, soft and pliable (MacLennan, 1992; Loke & MacLennan, 1998). The symptoms of PSS are almost identical to that of human MH and include muscle rigidity, acidosis, increased body temperature, hyperkalaemia, and cardiac arrest (Britt, 1979; Jones & Round, 1990; McComas, 1996). However, unlike humans, stress is the main trigger of an MH crisis in pigs.

2.9 Treatment

Dantrolene sodium (Dantrium, Pharmacia & Upjohn) is the most effective treatment for an MH attack if it is administered early intravenously (Merz, 1986). Dantrolene is a muscle relaxant that reverses the effects of excess calcium in the skeletal muscle fibres by paralysing SR function and closing the calcium channels of the ryanodine receptor. Dantrolene does not interfere with nervous transmission to the muscle, nor with the depolarisation of the muscle membrane itself (Galbraith et al., 1994).

Should an attack occur during surgery, the following procedures must be undertaken to ensure the safety of the patient:

- Halt surgery.

- Discontinue triggering agents.
- Administer intravenous (IV) dantrolene (within a 5-minute window period) - Dose: 1mg/kg up to maximum of 10mg/kg over a 15-minute period.
- Turn off vaporiser and change rubber tubing on anaesthesia machine.
- Hyperventilate patient with 100% oxygen (O₂).
- Reduce body temperature at several different sites: surface, intravenously, intragastrically, intrarectally, and at the site of surgical opening. If the body temperature is still high, then additional doses of dantrolene can be administered.
- IV potassium chloride to reduce the effects of hyperkalaemia.
- IV mannitol to reverse any cerebral or muscle oedema, and to help dislodge myoglobin from the nephrons.
- If the surgery has to continue, then a trigger-free agent must be used, such as nitrous oxide and oxygen (N₂O:O₂), narcotics, tranquilisers, barbituates or non-depolarising muscle relaxants.

(Merz, 1986; Lane, 1996)

After the patient has been stabilised, a flare-up can occur on return to the recovery room or in the ward. Therefore, the patient has to be closely monitored for at least 24 hours in the intensive care unit (ICU) (Merz, 1986). Patient monitoring involves the following:

- Body temperature, especially axillary temperature
- Electrocardiogram (ECG)

- End-tidal CO₂ (ETCO₂)
- Arterial oxygen saturation levels (pulse oximetry)
- Blood pressure
- Skin circulation and appearance
- Blood gas analysis
- Urine output
- Serum electrolytes, especially potassium
- Blood pH
- Myoglobin levels

And the administration of oral dantrolene - Dose: 4mg/kg per day.

(Merz, 1986; Lane, 1996)

2.10 Diagnosis

The most commonly used test to diagnose MH in humans, is the in-vitro contracture test (IVCT) or caffeine-halothane contracture test (CHCT). The abbreviation IVCT will be used from now on. Both caffeine and halothane have been shown to induce constant skeletal muscle contractions in muscle biopsies in the laboratory (Joffe et al., 1992; Abraham, Adnet, Glauber & Perel, 1998; Allen et al., 1998; Wappler et al., 1998). There are two methods of the IVCT - deemed the North American method and the European method respectively.

The European method of diagnosing MH was standardised in 1983 (European Malignant Hyperpyrexia Group, 1984). The protocol involves taking a biopsy from one of the muscles of the quadriceps group including a motor end point. The biopsy measures approximately 40mm long and 30mm wide. The muscle is then placed in Kreb's-Ringer solution (see Appendix 1 Table A1.1 p.62) where it is to be transported to the diagnostic laboratory. This solution mimics the extracellular ionic environment, nonetheless the muscle has to be tested within a 5 hour period or it will deteriorate beyond usefulness. When the muscle has been dissected into smaller strips (measuring 25mm x 3mm), it is placed into an organ bath at 37°C and perfused with Kreb's-Ringer solution and equilibrated with carbogen (mixture of 95% oxygen and 5% carbon dioxide), and stimulated at a frequency of 0.2Hz. Three separate tests are performed.

1) Static caffeine

This involves increasing the concentration of caffeine in the perfusate from 0.25 millimol per litre (mmol L^{-1}) to 4.0 mmol L^{-1} over a 3 minute period.

2) Static halothane

This involves increasing the concentration of halothane in the perfusate from 0.5%v/v to 3.0%v/v over a 3 minute period.

3) Dynamic halothane

This involves stretching the muscle at a constant rate for 1 minute, then increasing the halothane concentration from 0.5%v/v to 3.0%v/v, until muscle tension is sustained at 0.2g-wt.

These three tests help to distinguish the MH status of the individual in the following three categories:

1) Malignant hyperthermia susceptible (MHS)

This is where the start of contracture (threshold) is less than or equal to 1.5mmol of caffeine, and is less than or equal to 2.2%v/v of halothane.

2) Malignant hyperthermia normal (MHN)

This is where the start of contracture is greater than or equal to 3mmol of caffeine without the contracture threshold for halothane being greater than or equal to 3%v/v.

3) Malignant hyperthermia equivocal (MHE)

This is where only the caffeine threshold (MHEc) or the halothane threshold (MHEh) is reached, as per the MHS concentrations. However, these people are clinically regarded as MHS.

(European Malignant Hyperpyrexia Group, 1984)

The North American method was standardised in 1987 (Larach, 1989) and is similar to the European method. The main biopsy sites are the quadriceps or the rectus abdominus, however a large mixed type I/II muscle (such as biceps brachii) can also be used. The biopsies measure approximately 10-20mm long and 1-5mm wide. The organ bath and solutions are the same as the European method, however the testing protocols are different.

1) Halothane

The concentration of halothane in the perfusate increases from 2.7% to 3.3% over a period of 10 minutes.

2) Caffeine

The concentration of caffeine in the perfusate increases from 0.5 millimolar (mM) to 32mM over a period of 10 minutes.

3) Joint caffeine-halothane

This is an optional test performed by some laboratories and involves the muscle being exposed to 1% halothane for 10 minutes then increasing the concentration of caffeine from 0.25mM to 32mM.

The diagnosis for the North American method is also different as it does not use equivocal (MHE) as an MH status. A positive result can be one of the following:

- 1) If the start of contracture (threshold) for halothane is greater than 0.2g-wt.
- 2) If the start of contracture (threshold) for 2mM caffeine is greater than or equal to 0.2g-wt.
- 3) If the start of contracture (threshold) occurs at a caffeine concentration of 1mM or less in the presence of 1% halothane.

(Larach, 1989; Lane, 1996).

The IVCT is a highly invasive technique as it involves the removal of a substantial mass of skeletal muscle from either a quadriceps or abdominal

muscle. This procedure is performed under a trigger-free general anaesthetic for children (and pigs), as it is less traumatic, and under a local anaesthetic for adults (Serfas et al., 1996). The testing procedure is also expensive. False-positive and false-negative results have been known to occur, especially with the European method, as this protocol uses the MHE category. As the North American method only distinguishes people as either normal or susceptible, there is the less likelihood of producing false results (MacLennan, 1992).

There is another form of the IVCT using the plant alkaloid, ryanodine. The ryanodine contracture test (RCT) has been used by the European Malignant Hyperthermia Group since 1993 (Hopkins, Hartung, Wappler & EMHG, 1998). The biopsy (measuring 15-30mm x 2-3mm x 2-3mm) is removed from one muscle of the quadriceps group, usually the vastus lateralis. Like the IVCT the muscle is transported in Kreb's-Ringer, equilibrated with carbogen and testing performed within 5 hours. Ryanodine (98-99% pure) is then added in 1 micromol (μmol) and 2 μmol concentrations, where three phases of contracture are observed.

- 1) Start of contracture in minutes.
- 2) Time (minutes) when the contracture reaches 0.2g-wt.
- 3) Time (minutes) when the contracture reaches 1.0g-wt.

It has been found that MHS muscle contracts faster in these three phases than MHN muscle. The RCT can also reduce the number of equivocal results to either normal or susceptible, hence this test should be performed in

conjunction with the European IVCT (Wappler, Roewer, Kochling, Scholz, Steinfath & Schulte-am-Esch, 1996; Hopkins et al., 1998).

The main problem with the IVCT is that it cannot be used for screening the general population due to the low prevalence of the disease, the cost of performing the biopsies and the trauma of the procedure (Allen et al., 1998). It is hoped in the future that a genetic test may be available for humans (Abraham et al., 1998), or a modified testing procedure requiring very small amounts of muscle that can be collected by needle biopsy.

2.11 Genetics

Malignant hyperthermia is commonly inherited as an autosomal dominant trait, but sporadic cases of autosomal recessiveness and codominance have been known to occur (Denborough, 1998). Six chromosome localities have been identified in human MH, the most common being expressed on chromosome 19. This chromosome contains the ryanodine receptor 1 gene (RYR1) on its long arm (q), where linkage studies have found a 50% possibility that MH is connected to this gene (Loke, Tong, McCarthy & MacLennan, 1998). At present, 17 point mutations have been located on the RYR1 gene (Lane, 1996; Wallace, Wooldridge, Kingston, Harrison, Ellis & Ford, 1996; Loke & MacLennan, 1998; Loke et al., 1998; Soto &

Sopher, 1998). Table 2.1 shows all the current chromosomal locations and the possible genes and mutations identified to these chromosomes.

Table 2.1. Current chromosomal locations of human MH

Chromosomal Location	Gene	Mutation
19q13.1	RYR1	17 point mutations including: Arg615Cys; Gly2434Arg; Arg614Cys; Gly314Arg; Arg163Cys (wild type)
17q11.2-q24	DHPR-gamma subunits (CACNLB1 & CACNLG)	Unidentified
	Sodium channel alpha-subunit (SCN4A)	Gly1306Ala
7q21.1	DHPR alpha 2/delta-subunit (CACNL2A)	Unidentified
3q13.1	Unidentified	Unidentified
1q32	DHPR alpha 1-subunit (CACNL1A3)	Arg1086His
5p (short arm)	Unidentified	Unidentified

(Source: Censier, Urwyler, Zorzato & Treves, 1998; Denborough, 1998; Loke & MacLennan, 1998; Monsieurs, Van Broeckhoven, Martin, Van Hoof & Heytens, 1998)

In pigs, PSS is an autosomal recessive disease, and is associated with a single mutation in the RYR Arg615Cys gene on chromosome 6q (Joffe et al., 1992; Shomer, Mickelson & Louis, 1995; Lane, 1996; Laver, Owen, Junankar, Taske, Dulhunty & Lamb, 1997). The mutation in the RYR gene contains the amino acid substitution of cystine for arginine. This substitution is enough to alter the protein structure sufficiently to prevent the ryanodine receptor from closing properly (McComas, 1996). However, as this is a single point mutation, a genetic test is now available. The test requires 50µl of whole blood, and can be performed quickly and accurately (MacLennan, 1992).

MH has also been linked to other neuromuscular diseases (NMDs). There is a genetic link between MH and central core disease (CCD), as the gene for CCD is close to the RYR1 gene on chromosome 19q (Denborough, 1998). CCD is an autosomal dominant disease and its clinical features include foot deformities, forward curving of the spine (kyphoscoliosis), congenital hip dislocation and joint contractures. Electron microscopic analysis revealed that the contractile apparatus in CCD muscle showed marked disintegration (Loke & MacLennan, 1998).

MH may be related to two other NMDs, Duchenne muscular dystrophy (DMD) and King-Denborough syndrome (KDS) (Wedel, 1992). DMD is a sex-linked recessive disorder affecting young males, and the gene is located on chromosome Xp21. This disease causes progressive muscle

wasting due to the absence of the muscle protein dystrophin (Cummings, 1994). Those DMD patients who have suffered an MH-type reaction during surgery usually test positive to the disease and account for approximately 4% of all positive IVCTs (Lane, 1996). KDS affects males and its clinical features include a short, stocky stature, chest abnormalities, kyphoscoliosis, a hump-back (thoracic kyphosis) and undescended testes (cryptorchidism). It has been found that those KDS patients who have had an MH crisis test positive to the disease (Lane, 1996).

2.12 Prognosis

The survival rate of MH in the 1990s is extremely high (96-97%). This is due to a better awareness of the disease and the consistent use of monitoring, by such means as blood gas analysis, body temperature and ETCO_2 analysis (Mertz, 1986; Lane, 1996). Familiarity with the MH protocols by surgical staff has led to the earlier cessation of trigger anaesthetics and the administration of IV dantrolene (Lane, 1996). However, the IVCT still remains the only form of diagnosis for the disease in humans. The genetics involved in human MH are complicated compared to PSS, therefore a genetic test for humans is still a long way off (Joffe et al., 1992).

CHAPTER 3

MATERIALS & METHODS

3.1 Design

The design used in this study consisted of the analysis of single skeletal muscle fibres using exogenous calcium and strontium ion activation, and the subsequent mathematical analysis of force-ion concentration relationships. The method involved the chemical removal of the cell membrane from the single muscle fibre, thus leaving only the contractile apparatus. The SR was also disrupted, ensuring that all the internal calcium was removed to prevent any confounding of the results (Britt et al., 1982; Fink et al. 1990). This method was used to examine the dynamics of the contractile and regulatory proteins in both MH and control muscle. It is also hoped that this study will indicate if another less invasive technique could be used to test for MH without compromising accuracy.

3.2 Muscle Biopsies

Skeletal muscle biopsies (measuring 40mm x 30mm x 5mm) were removed from the vastus medialis muscle under a regional anaesthetic by RPH surgical staff. The MH status of the patient was unknown at this stage. The muscle biopsy was transported in Kreb's-Ringer solution to the Department of Neuropathology (Royal Perth Hospital) where it was divided into three portions: one portion for the IVCT (according to the European Malignant Hyperthermia Group protocol); one for histological analysis, and the last portion for this study. Only after the biopsy had undergone testing for this study was the result of the IVCT compared with the results obtained here. Biopsies determined as MHN were used as the controls for this present study.

The biopsies used for the study were then transported in a relaxing solution containing 50% glycerol to prevent damage to the muscle fibres (see Appendix 1 p.62). Muscle testing was delayed for approximately 4 days in order to remove any regional anaesthetic still remaining in the tissue. This did not impinge on the viability of the sample.

3.3 Ethical Considerations

The study was approved by both the Edith Cowan University Ethics Committee and Royal Perth Hospital's Ethics Board. The muscle samples used were obtained after written consent from the patient (see Appendix 2 p.64) on the day of admission.

3.4 Equipment

- microdissecting surgical instruments
- dissecting binocular microscopes – Carl Zeiss Jena Citoval 2, Leica Wild M3Z
- fibre optic 100W cold light source
- micro-force transducer and electrical amplifier/coupler
- 3-D micromanipulator
- multiple well, rotary solution holder
- Deknatel, 10/0 black silk suture
- Macintosh LC 575, MacLab 2E, MacLab GP Amp, MacLab v3.5 ADInstruments (see Figure 3.1)
- Olympus digital camera

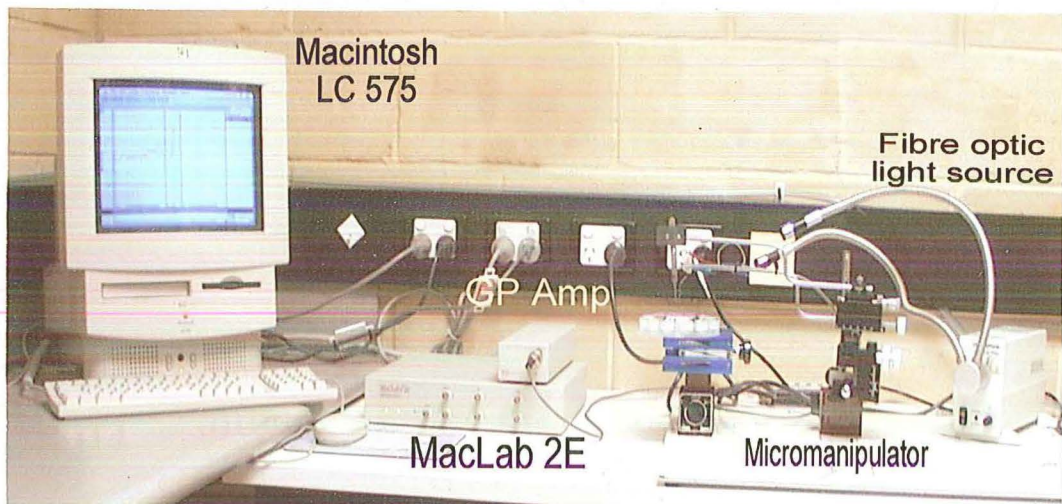


Figure 3.1. Equipment setup for the skinned muscle fibre method

3.5 Procedure

Ten individual muscle fibres were removed from 8 biopsies. The individual muscle fibres were dissected from a muscle strip (measuring 10mm x 2mm x 2mm). In a petri dish, the muscle strip was covered by a layer of paraffin oil at 4°C in order to prevent the muscle from drying out. Microdissecting instruments and a dissecting microscope illuminated by a fibre optic cold light source were used to dissect free the individual muscle fibres. Each fibre was tied with 10/0 black silk suture at each end, forming a loop with the diameter of a 19 gauge needle (see Figure 3.2). The fibre was then mounted onto the stainless steel forceps and pin of the transducer (see Figures 3.3 & 3.4), where it was adjusted to optimal length

for contraction by trial and error, and measured length by width (divisions) using a graticule (8 divisions equated to 1mm).

The fibre was dipped in 2% Triton X-100 (detergent) in relaxing solution I (Appendix 1 Table A1.2 p.62) for 10 minutes to render the membrane permeable, and then into the weaker relaxing solution III (Appendix 1 Table A1.3 p.62) for a further 60 seconds, where the force signal was set to electrical zero. The fibre was subsequently tested at various concentrations of calcium and strontium, from the weakest through to the strongest concentration (Appendix 1 Tables A1.4 & A1.5 p.63). This is the gradual or "stepping" increase in concentration to see where the muscle begins to contract. When the fibre contracted it pulled against the pin of the transducer. This altered the electrical resistance of the transducer, causing a voltage change at the input stage of the GP Amp. The signal was digitised by the MacLab computer system and displayed on the computer screen as a continuous graph of force versus time. Force maxima and minima were logged against ionic concentration for subsequent analysis by fitting a mathematical equation to the data (Fink et al., 1990). Experiments were performed at room temperature (22-24°C).

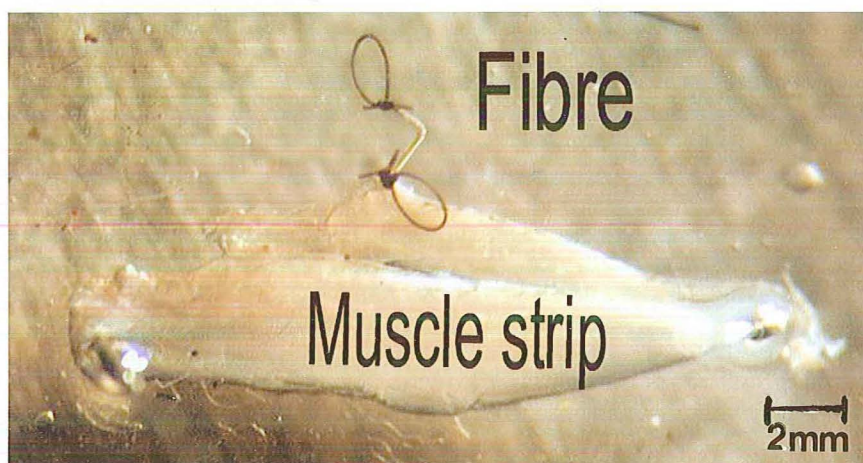


Figure 3.2. Individual muscle fibre (top) tied with silk sutures

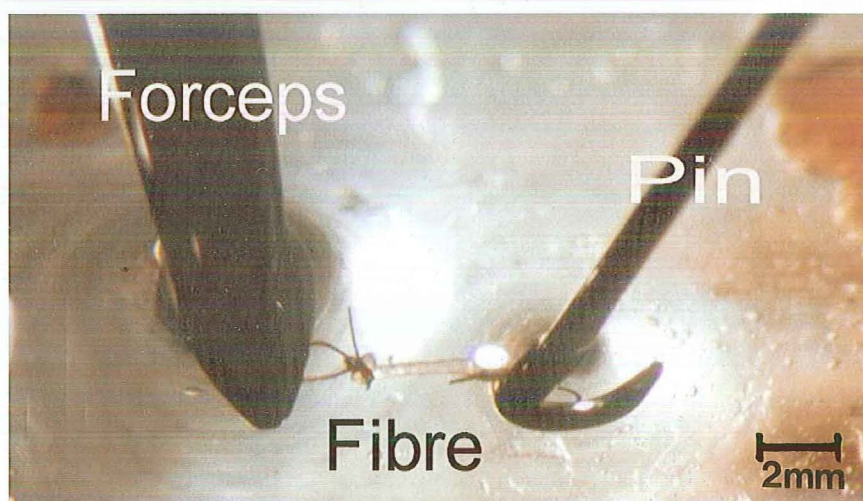


Figure 3.3. Individual muscle fibre mounted on stainless steel forceps (left) and pin of the transducer (right)

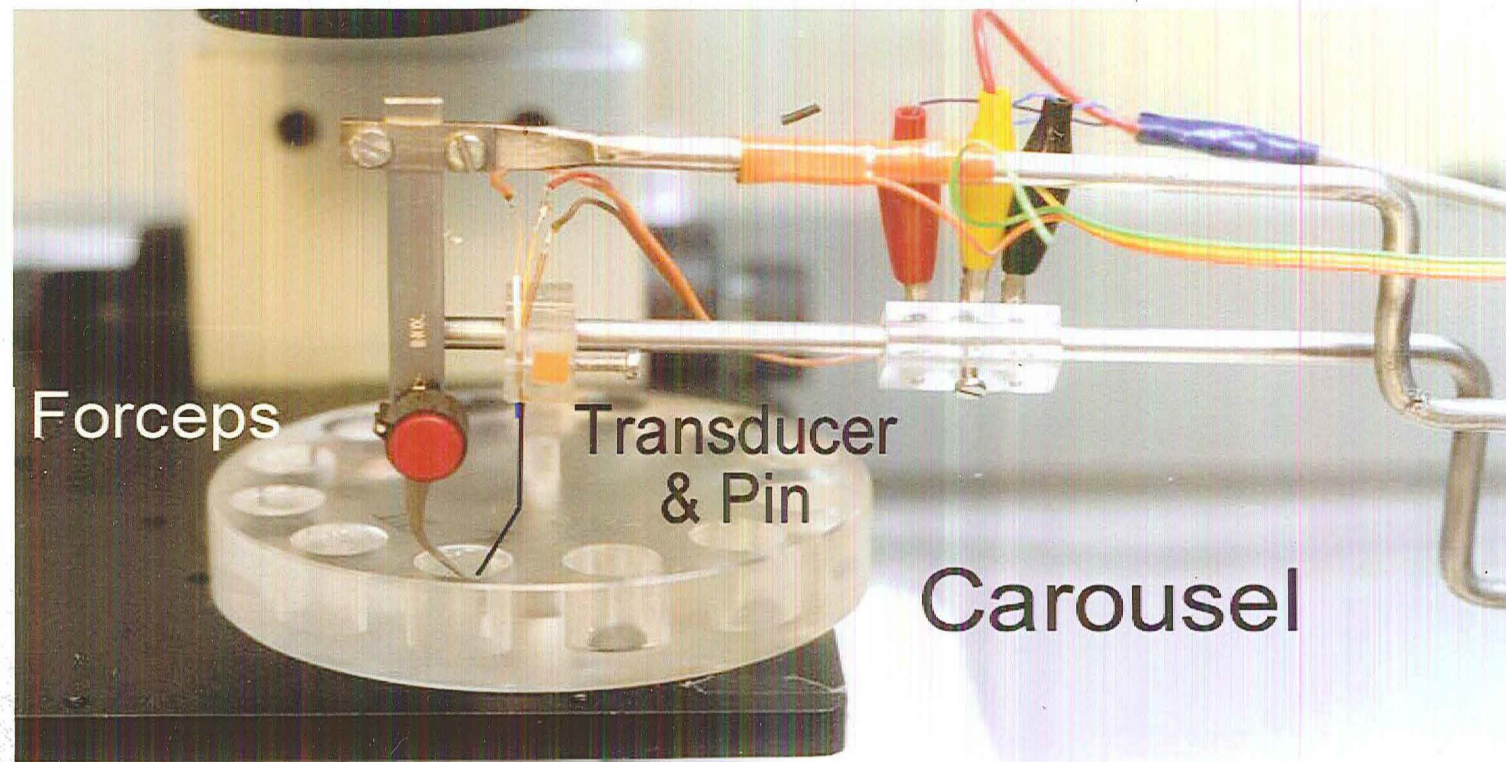


Figure 3.4. Location of the stainless forceps and transducer (and pin) in a multiple well solution holder (carousel)

3.6 Data Analysis

The datum points logged on MacLab v3.5 (Macintosh AD Instruments scientific software) were converted to Microsoft Excel format, which was then reinterpreted in DeltaPoint Delta Graph Pro 3.5 to produce plots of the calcium concentration (pCa)- and strontium concentration (pSr)-force relationships (see below). Curve parameters (affecting slope and lateral shift - see Hill Equation p. 40) between MH equivocal (MHE) and normal (control) data were analysed by paired Student t-test. Differences were considered statistically significant if $P < 0.1$. Values were represented as mean \pm standard error of the mean (S.E.M). Measured fibre width was used to calculate force per cross-sectional area (F/CSA - see p. 41). The data were categorised into two groups: controls and MHE.

pCa- pSr-force Relationships:

- pCa₅₀ and pSr₅₀ equates to 50% of the maximum activated for response. This value indicates the relative sensitivity of the contractile apparatus to each activating ion.
- pCa₁₀ and pSr₁₀ equates to 10% of the maximum activated force response. This value indicates the activation threshold of the contractile apparatus to each activating ion.
- pCa₅₀-pSr₅₀ is the difference in relative sensitivity of the contractile apparatus to the two activating ions.

(Stephenson, O'Callaghan & Stephenson, 1994)

Hill Equation:

The Hill coefficients (n_{Ca} and n_{Sr}) reflect the steepness of the curve (fitted from the datum points) by the following equation:

$$P_r = K[X^{2+}]^n / (1 + K[X^{2+}]^n)$$

where: P_r = relative force
 K = constant (as defined below)
 X^{2+} = concentration of Ca^{2+} or Sr^{2+}

However the above equation was altered in order to calculate these solutions. The calculation is as follows:

$$\log_{10} K = n_{Ca} pCa_{50} (n_{Sr} pSr_{50})$$

Parameters set by Fink et al., 1990 for normal human skeletal muscle (Type I and II) are defined in the following table:

Table 3.1 Parameters of normal human skeletal muscle

	Type I	Type II
n_{Ca}	2.20 (± 0.06)	4.70 (± 0.3)
n_{Sr}	2.30 (± 0.1)	4.30 (± 0.3)
pCa_{50}	6.05 (± 0.05)	6.07 (± 0.02)
pCa_{10}	6.48 (± 0.06)	6.27 (± 0.01)
pSr_{50}	5.61 (± 0.09)	4.76 (± 0.04)
pSr_{10}	6.10 (± 0.07)	4.99 (± 0.02)
$pCa_{50} - pSr_{50}$	0.32 (± 0.04)	1.31 (± 0.04)
(Fink et al., 1990)		

The values in the brackets represent the standard error of the mean (\pm S.E.M.). This is expressed as the following:

$$SEM = \sigma^{n-1} / \sqrt{n}$$

(Fink et al., 1990; Stephenson et al., 1994; Lynch, Hayes, Lam, & Williams, 1993; Lynch, Stephenson & Williams, 1995)

Force per cross-sectional area (F/CSA):

This equates to the maximum activated force of Ca^{2+} and Sr^{2+} divided by the cross-sectional area of the fibre. The calculations are as follows:

$$\text{CSA} = \pi r^2$$

where: $\pi = \text{Pi}$
 $r = \text{radius}$

$$\text{Force} = ma$$

where: $m = \text{mass (g)}$
 $a = \text{acceleration constant (9.8m/sec}^2\text{)}$

Force was expressed in milliNewtons per millimetres (mN/mm^2) for this study.

Parameters set by Fink et al., 1990 (in N/cm^2) for normal human skeletal muscle (Type I and II) are as follows:

Type I	26.1 (± 2.7)
Type II	30.1 (± 9.0)

Brackets represent \pm S.E.M.

(Fink et al., 1990)

3.7 Limitations

The study was limited to the biopsy samples supplied by Royal Perth Hospital.

This had several implications for the study, namely:

- There was no control over the number of MHN, MHS or MHE samples.

- Biopsies from only one muscle (Vastus medialis) were available.
- As muscle biopsies are only removed from patients undergoing elective surgery, there was a risk of the surgery being postponed or cancelled, thus affecting the collection of samples.
- There was no control over the quality and amount of muscle in the received biopsy samples.

CHAPTER 4

4.1 Results

As a consequence of routine MH testing at the Department of Neuropathology, Royal Perth Hospital, 8 biopsy samples were made available for use in this study. Of those, 6 were deemed by the IVCT to be normal (controls), whilst the remaining 2 were categorised as equivocal (MHE). Ten individual muscle fibre segments (approximately 0.5-1mm in length) were dissected from each biopsy, resulting in a total of 80 fibres (60 control, 20 MHE) being studied. Of the 80 fibres only 72 fibres (57 control, 15 MHE) were analysed, as the remaining 8 fibres (3 control, 5 MHE) were unresponsive. This may have been due to fibre damage during dissection and mounting in the apparatus, or during the original biopsy removal.

Normal (Control) Fibres

Of the 57 fibres collated, only 45 could be classified as either Type I (slow-twitch) fibres or Type II (fast-twitch) fibres according to the parameters defined by Fink et al. (1990) (see Section 3.6 p.40). Fifteen fibres were classified as Type I (Table 4.1), where the slope values showed that the force-pCa (n_{Ca}) and force-pSr (n_{Sr}) curves were relatively shallow (n_{Ca} , n_{Sr} < 2) (Figure 4.1). The fibres showed a greater than normal sensitivity ($pCa_{50} - pSr_{50} = 0.92$) compared to the results reported by Fink et al.

(1990) with a value of 0.32 (Table 3.1 p.40). The force per cross-sectional area (F/CSA) generated by strontium (Sr^{2+}) was four times greater than that of calcium (Ca^{2+}). The other thirty fibres were classified as Type II fibres (Table 4.1). The force-pCa and force-pSr curves were relatively steep (>2) (Figure 4.3) and sensitivity to calcium and strontium was less than that of the Type I fibres with a value of -0.62. The F/CSA was greater in Sr^{2+} than it was for Ca^{2+} .

The other 12 fibres could not be categorised as either fast- or slow-twitch fibres, as these fibres displayed unusual or mixed characteristics. Although the mean slope values displayed in Table 4.1 are characteristic of fast twitch fibres, many individual fibres displayed shallow n_{Ca} curves (slope values from 1 to 2) and very steep n_{Sr} curves (slope values as either 1 or 3) (Figure 4.5). The reverse occasionally occurred where the Ca^{2+} curves were steeper than the Sr^{2+} curves. These fibres also showed an average sensitivity of 1.29 ($\text{pCa}_{50} - \text{pSr}_{50}$), and the F/CSA for Sr^{2+} was three times greater than that of Ca^{2+} .

MHE Fibres

Of the 15 fibres analysed only 5 could be categorized as being either fast or slow twitch, of which four fibres were classified as Type I (Table 4.1). The force-pCa (<1.40 ; statistical significance $P<0.1$) and force-pSr curves ($n_{\text{Sr}} <2.13$) were relatively shallow (Figure 4.2), and the fibres were

generally less sensitive than the controls giving a value of $pCa_{50} - pSr_{50} = -3.01$. The F/CSA was far greater in the Sr^{2+} series (63.61 mN/mm^2 ; statistical significance $P < 0.1$) than in the Ca^{2+} series (15.13 mN/mm^2) but overall less than the controls. Only one fibre could be categorised as a fast-twitch fibre (Table 4.1), however the n_{Ca} and n_{Sr} curves were steeper (2.13 and 3.37 respectively) (Figure 4.4). The sensitivity of this fibre was less than that of the control fibres, giving a value of -1.06 ($pCa_{50} - pSr_{50}$). The F/CSA generated was more in Ca^{2+} than Sr^{2+} (24.87 mN/mm^2 and 16.51 mN/mm^2 respectively) but overall less than the control fibres.

There were 10 fibres of mixed characteristics (Table 4.1) where the force- pCa curves were generally shallow ($n_{Ca} < 2$; statistical significance $P < 0.1$) and the force- pSr curves were generally very steep ($n_{Sr} > 2.5$) (Figure 4.6). These fibres were less sensitive to Ca^{2+} and Sr^{2+} ($pCa_{50} - pSr_{50} = 1.52$) than that of the control mixed fibres, and the F/CSA by Sr^{2+} (68.97 mN/mm^2) was greater than that of the Ca^{2+} (43.06 mN/mm^2).

Comparisons

For Type I fibres (see Figures 4.1 & 4.2), the values of n_{Ca} , pCa_{50} , pCa_{10} and F/CSA (both Ca^{2+} and Sr^{2+}) for control fibres were greater than for the equivocal. The equivocal fibres were more sensitive than the controls for n_{Sr} , pSr_{50} and pSr_{10} . However, the equivocal responded better to the Sr^{2+} solution than the Ca^{2+} , but the controls were overall more sensitive to the two activating ions (Table 4.1). Statistically, there were significant

differences for n_{Ca} , n_{Sr} and F/CSA for Sr^{2+} ($P < 0.1$) between the controls and the MHE fibres.

For Type II fibres (see Figures 4.3 & 4.4), the values of n_{Ca} , F/CSA (Ca^{2+} and Sr^{2+}) and sensitivity (pCa_{50} - pSr_{50}) for control fibres were greater than for the equivocal. The equivocal were greater than the controls for slope (n_{Sr}), activation threshold (pCa_{10} and pSr_{10}) and relative sensitivity (pCa_{50} and pSr_{50}) to each activating ion (Table 4.1). This group could not be statistically analysed due to only one MHE fibre being found.

For the mixed fibre group (see Figures 4.5 & 4.6), the controls were more sensitive to n_{Ca} , pCa_{10} and F/CSA (Sr^{2+}). The equivocal were more sensitive to n_{Sr} , pCa_{10} , and relative sensitivity to each activating ion (pCa_{50} and pSr_{50}). Overall, the MHE fibres were more sensitive (pCa_{50} - pSr_{50}) than the controls (Table 4.1). Statistically, there were significant differences for n_{Ca} , n_{Sr} and pCa_{10} ($P < 0.1$).

Table 4.1. *Contractile parameters of control and MHE skeletal muscle fibre types*

	Type I		Type II		Mixed	
	Control	MHE	Control	MHE	Control	MHE
samples (n)	15	4	30	1	12	10
n_{Ca}	1.75 ±0.09	1.40 ±0.19*	2.82 ±0.97	2.13	2.57 ±0.28	1.57 ±0.13*
pCa₅₀	7.43 ±1.25	4.90 ±0.61	5.10 ±0.19	5.49	5.20 ±0.28	5.74 ±0.33
pCa₁₀	8.00 ±1.26	5.63 ±0.70	5.45 ±0.19	5.93	5.61 ±0.29	6.38 ±0.35*
F/CSA	50.83 ±10.85	15.13 ±3.30	79.20 ±14.94	24.87	41.65 ±12.33	43.06 ±10.30
n_{Sr}	1.82 ±0.12	2.13 ±0.11*	2.78 ±0.10	3.37	2.14 ±0.25	3.16 ±0.40*
pSr₅₀	6.52 ±0.58	7.90 ±0.94	5.73 ±0.38	6.55	3.90 ±0.24	4.22 ±0.42
pSr₁₀	7.08 ±0.56	8.35 ±0.92	6.08 ±0.39	6.83	4.42 ±0.26	4.55 ±0.43
F/CSA	200.53 ±52.41	63.61 ±22.44*	173.31 ±33.97	16.51	127.86 ±44.75	68.97 ±35.41
pCa₅₀-pSr₅₀	0.92 ±1.24	-3.01 ±1.33	-0.62 ±0.43	-1.06	1.29 ±0.43	1.52 ±0.59

Data are represented as mean ±S.E.M.
 *Denotes statistical significance at $P<0.1$

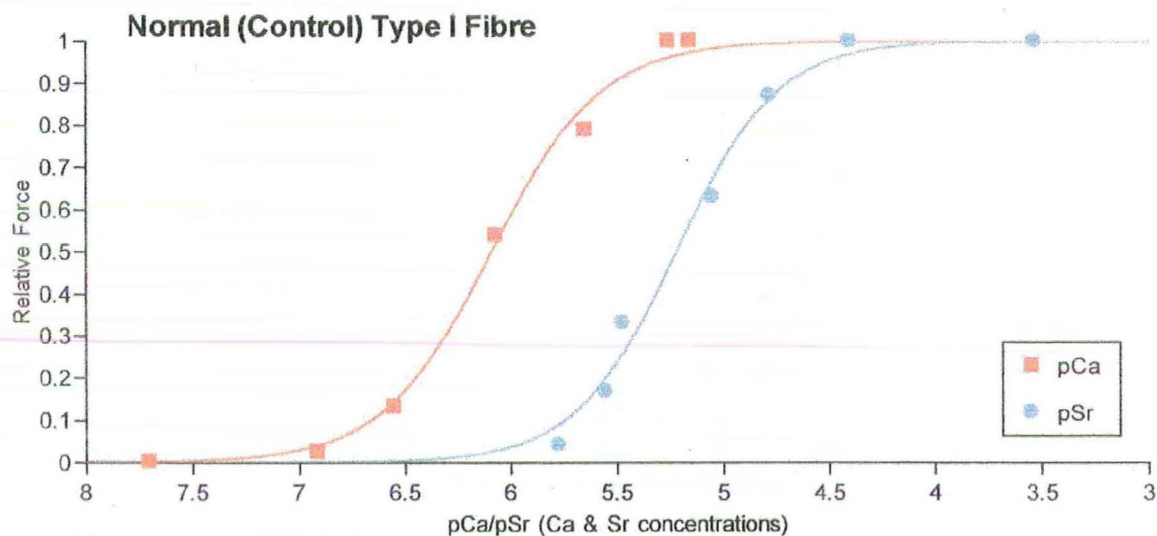


Figure 4.1 Force-pCa and -pSr curves for a typical Type I normal (control) muscle fibre. Scattering of individual points lie within each allocated symbol. Curves drawn from the Hill equation for $n_{Ca} = 1.75$, $n_{Sr} = 1.82$.

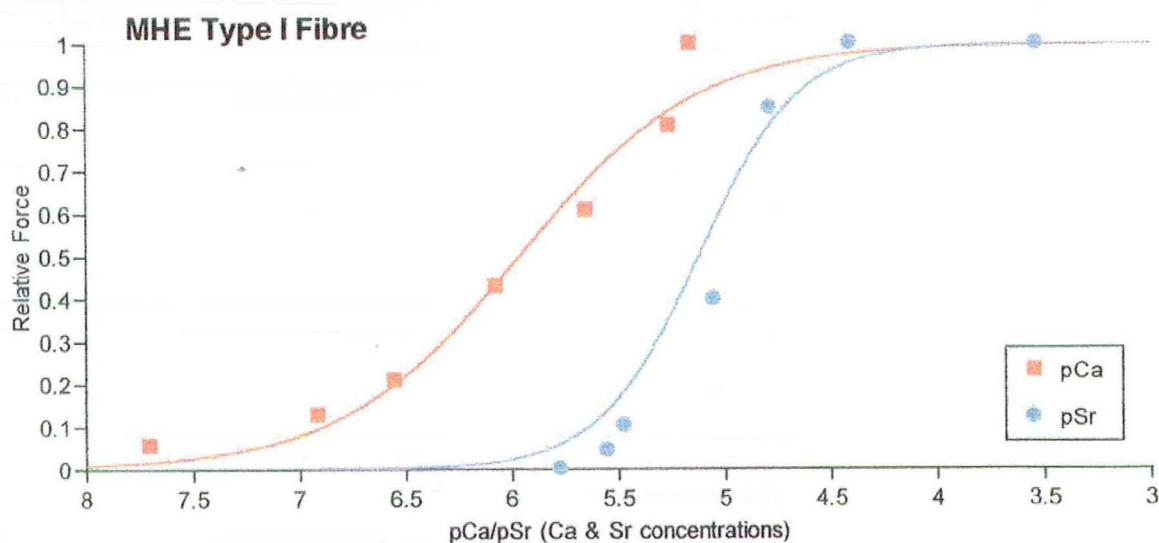


Figure 4.2 Force-pCa and -pSr curves for a typical Type I MHE muscle fibre. Scattering of individual points lie within each allocated symbol. Curves drawn from the Hill equation for $n_{Ca} = 1.03$, $n_{Sr} = 1.85$.

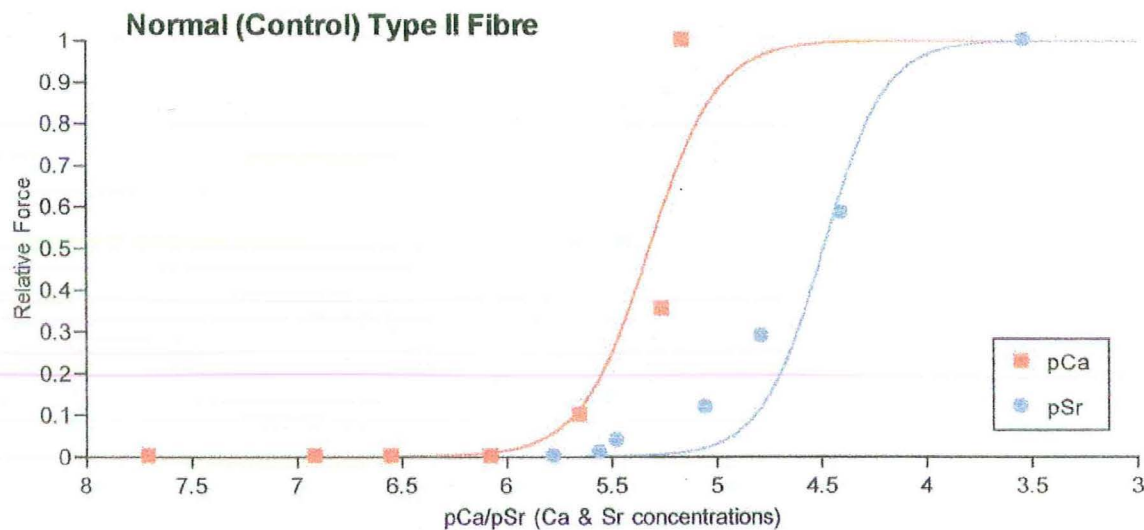


Figure 4.3 Force-pCa and -pSr curves for a typical Type II normal (control) muscle fibre. Scattering of individual points lie within each allocated symbol. Curves drawn from the Hill equation for $n_{Ca} = 2.82$, $n_{Sr} = 2.78$.

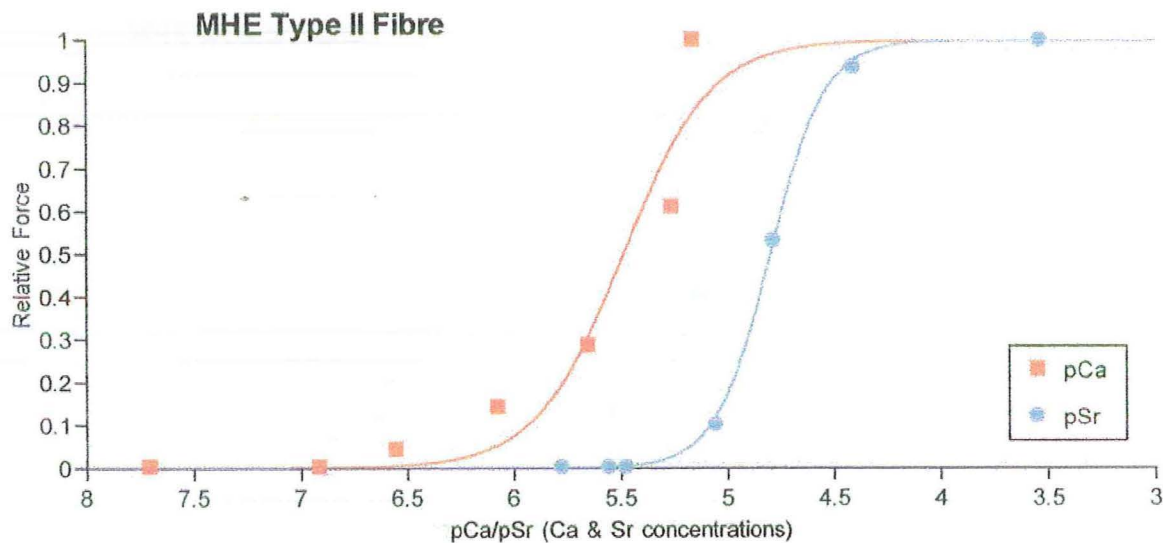


Figure 4.4 Force-pCa and -pSr curves for a typical Type II MHE muscle fibre. Scattering of individual points lie within each allocated symbol. Curves drawn from the Hill equation for $n_{Ca} = 2.13$, $n_{Sr} = 3.37$.

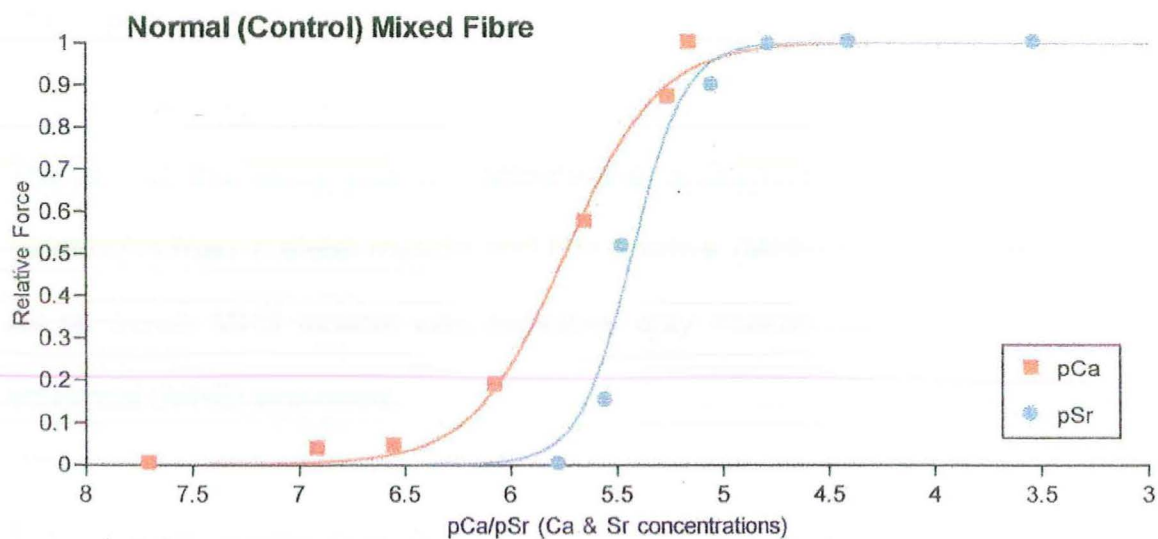


Figure 4.5 Force-pCa and -pSr curves for a Mixed normal (control) muscle fibre. Scattering of individual points lie within each allocated symbol. Curves drawn from the Hill equation for $n_{Ca} = 2.57$, $n_{Sr} = 2.14$.

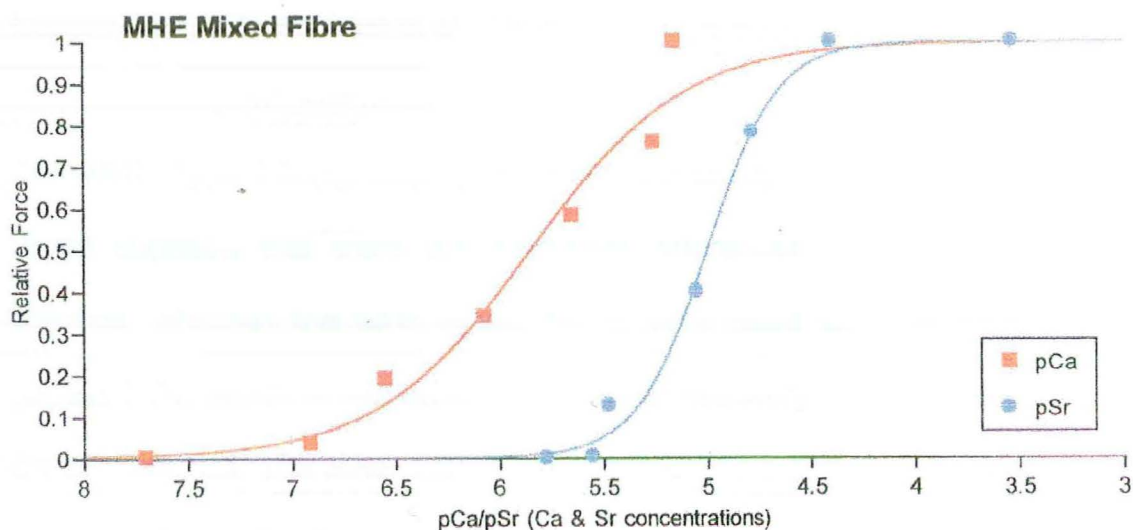


Figure 4.6 Force-pCa and -pSr curves for a Mixed MHE muscle fibre. Scattering of individual points lie within each allocated symbol. Curves drawn from the Hill equation for $n_{Ca} = 1.16$, $n_{Sr} = 2.52$.

4.2 Discussion

The aim of this study was to compare the differences between normal (control) human skeletal muscle and MH positive (MHS) skeletal muscle. As no actual MHS muscle was available, only muscle classified as MH equivocal (MHE) was used.

The skinned muscle fibre technique (used in this study) identifies the contractile kinetics of single skeletal muscle fibres to exogenous calcium and strontium ion activation. Differences in the kinetics of responses to Ca^{2+} can indicate functional differences of the contractile proteins (myosin S1 subfragment), whereas differences in the kinetics of responses to Sr^{2+} can indicate functional differences of the regulatory proteins (troponin and tropomyosin) (Stephenson et al., 1994).

The MHE Type I fibres displayed greater sensitivity to Sr^{2+} (pSr_{50} 7.90) which suggests that there are functional differences in the regulatory proteins, whereas the MHE mixed fibres were more sensitive to Ca^{2+} (pCa_{50} 5.74) which is indicative of a greater reactivity in the contractile proteins as well. The MHE Type II fibre showed no significant differences in sensitivity to either ion as it was almost identical in slope and lateral shift to the Type II control fibres.

The most noticeable differences between the MHE muscle and the control muscle, were observed during the testing phase on the MacLab system. Most of the MHE fibres were more sensitive to the weaker Ca^{2+} and Sr^{2+} solutions resulting in stronger contractions, whereas the normal muscle was unresponsive. However, these effects on the MacLab could not be reproduced in Delta Graph as the calculation of the Relative Force normalised these values.

The F/CSA generated by the control fibres was generally greater than that of the equivocals for both Ca^{2+} and Sr^{2+} . As fibre diameter is usually greater in MHS muscle than that of normal muscle (Lane, 1996), equivocal muscle has not been measured in any published studies, therefore F/CSA is unknown in these cases. However, there was a negative statistical significance (F/CSA $P < 0.1$) between the Type I fibres for MHE and controls during Sr^{2+} activation.

The skinned muscle fibre technique (involving Ca^{2+} and Sr^{2+}) has been used extensively in the research of Duchenne Muscular Dystrophy (DMD) in both humans and rodents. In comparison to the human DMD contractile parameters performed by Fink et al. (1990), both the controls and MHE fibres (fast and slow fibres) were more sensitive to Sr^{2+} than to Ca^{2+} . In comparison to normal mammal skeletal muscle (Wilson & Stephenson, 1990), again both the controls and MHE fibres were more sensitive to Sr^{2+} , notably pSr_{50} and pSr_{10} . Normal rat soleus muscle however was slightly

different (Stephenson et al., 1994). The controls and MHE fibres were more sensitive to pCa_{50} and pSr_{50} (fast and slow fibres) than just one type of ion. On the whole, the slope parameters (n_{Ca} and n_{Sr}) for all these examples (human DMD, mammal and rat) were unique to each case, and rarely did the controls and MHE fibres (from this study) have similar values.

The caffeine skinned muscle fibre technique conducted by Britt et al. (1982), involved only the chemical disruption of the sarcolemma. In this study, they were testing the Ca^{2+} release from the SR on exposure to caffeine. They found that this technique provided more information about the calcium release mechanism of the intact SR than that of the IVCT. Takagi, Sunohara & Nonaka (1980) found in a similar study that the caffeine contracture threshold was lower in MHS muscle fibres than for normal muscle fibres.

Fibre type composition in human MH is rarely tested, however a study compiled by Heiman-Patterson, Fletcher, Rosenberg & Tahmoush (1987) found that fibre type profiles and MH did not correlate. The same also applied in fibre studies conducted in porcine stress syndrome (PSS).

4.3 Conclusion

The contractile and regulatory proteins are all interconnected with non-contractile proteins to form a complex cytoskeleton that holds and stabilises the muscle fibre during relaxation and contraction (Sugita, 1997). The non-contractile protein dystrophin binds to the actin filament (regulatory protein). Dystrophin also binds to the inner and outer membrane, therefore playing an important role in the stabilisation of the muscle cell (Sugita, 1997). In response to the first hypothesis: *Individual MH muscle fibres display differing kinetics of contraction to exogenous calcium and strontium ion activation than individual muscle fibres in normal (control) human muscle*, both the contractile and regulatory proteins appeared to be affected by this disease. Therefore, this would impact on the mechanics of the whole muscle fibre, such as making it unstable during contraction. However, to determine if these two proteins are affected, positive MH muscle needs to be examined to draw a definite conclusion. For the second hypothesis: *The force per cross-sectional area generated by MH muscle fibres is different to that of normal (control) muscle*, the F/CSA generated by the MHE muscle fibres was less than that of the control muscle. However the Type I and mixed fibres did display greater F/CSA to Sr^{2+} rather than to Ca^{2+} , as did the control fibres in all three fibre types.

The main problem with this study was the availability of muscle samples. The majority of the biopsies were performed for other reasons than a family history of MH. For example, biopsies were performed for unusual reactions to a previously administered general anaesthetic such as raised blood carbon dioxide levels. Erring on the side of caution, the hospital had to investigate all unusual reactions if further elective surgery was required. Other problems encountered were the cancellation of surgery six times in the period of muscle testing and the status of every individual not being known on the day of admission. Therefore obtaining positive MH biopsies was unpredictable. Also, accessibility to the genetic information of the individual (if they were from an MH family) was unobtainable for this study.

4.4 Further developments

Although this study provided limited results, the skinned fibre method using caffeine has been previously applied to this disease and has shown some promising results. If this method could be refined (using exogenous Ca^{2+} , Sr^{2+} , caffeine and possibly ryanodine on intact as well as skinned fibres), then the technique could be used as a replacement to the IVCT. If so, the benefits to the patient (in terms of muscle biopsies) and the community at large could be a positive step forward.

REFERENCES

- Abraham, RB., Adnet, P., Glauber, V. & Perel, A. (1998). Malignant hyperthermia - review. *Postgraduate Medical Journal*, 74(867): 11-17.
- Allen, GC., Larach, MG. & Kunselman, AR. (1998). The sensitivity and specificity of the caffeine-halothane contracture test: a report from the North American Malignant Hyperthermia Registry. The North American Malignant Hyperthermia Registry of MHAUS. *Anaesthesiology*, 88(3): 579-88.
- Ball, SP. & Johnson, KJ. (1993). The genetics of malignant hyperthermia. *Journal of Medical Genetics*, 30: 89-93.
- Britt, BA. (1979). *Malignant hyperthermia* (Vol 17, No. 4). Boston, USA: Little, Brown and Company (Inc.), pp. 1-167.
- Britt, BA., Frodis, W., Scott, E., Clements, M. & Endrenyi, L. (1982). Comparison of the caffeine skinned fibre tension (CSFT) test with the caffeine-halothane contracture (CHC) test in the diagnosis of malignant hyperthermia. *Canadian Anaesthetists' Society Journal*, 29(6): 550-562.
- Censier, K., Urwyler, A., Zorzato, F. & Treves, S. (1998). Intracellular calcium homeostasis in human primary muscle cells from malignant hyperthermia-susceptible and normal individuals: effect of overexpression of recombinant wild-type and Arg163Cys mutated ryanodine receptors. *The Journal of Clinical Investigation*, 101(6): 1233-1242.
- Cummings, MR. (1994). *Human heredity principles and issues*, 3rd ed. St. Paul, USA: West Publishing Company, pp. 119.
- Denborough, M. (1998). Malignant hyperthermia. *The Lancet*, 352(9134): 1131-1136.

- Denborough, M. & Lovell, R. (1960). Anaesthetic deaths in a family. *The Lancet*, ii: 45.
- Dunn, D. (1997). Malignant hyperthermia. *AORN Journal*, 65(4): 728-62.
- European Malignant Hyperpyrexia Group. (1984). A protocol for the investigation of malignant hyperpyrexia (MH) susceptibility. *British Journal of Anaesthesia*, 56: 1267-9.
- Fink, RHA., Stephenson, DG. & Williams, DA. (1990). Physiological properties of skinned fibres from normal and dystrophic (Duchenne) human muscle activation by Ca^{2+} and Sr^{2+} . *Journal of Physiology*, 420: 337-53.
- Galbraith, A., Bullock, S. & Manias, E. (1994). *Fundamentals of pharmacology*. Sydney: Addison-Wesley Publishing Group, pp. 327-30.
- Healy, JM., Quane, KA., Keating, KE., Lehane, M., Heffron, JJ. & McCarthy, TV. (1996). Diagnosis of malignant hyperthermia: a comparison of the in vitro contracture test with the molecular genetic diagnosis in a large pedigree. *Journal of Medical Genetics*, 33(1): 18-24.
- Heiman-Patterson, TD., Rosenberg, H., Fletcher, JE. & Tahmoush, AJ. (1987). No relationship between fibre type and halothane contracture test results in malignant hyperthermia. *Anaesthesiology*, 67: 82-4.
- Hopkins, PM., Hartung, E., Wappler, F. & EMHG. (1998). Multicentre evaluation of ryanodine contracture testing in malignant hyperthermia. The European Malignant Hyperthermia Group. *British Journal of Anaesthesia*, 80(3): 389-94.
- Joffe, M., Savage, N., & Silove, M. (1992). The biochemistry of malignant hyperthermia: recent concepts. *International Journal of Biochemistry*, 24(3): 387-98.

Jones, DA. & Round, JM. (1990). *Skeletal muscle in health & disease*. Manchester, UK: Manchester University Press, pp. 213.

Katz, DN. (1992). Current understanding of malignant hyperthermia: genesis, prevention and treatment. *CRNA: The Clinical Forum for Nurse Anaesthetists*, 3(2): 54-63.

Lane, RJM. (1996). *Handbook of muscle disease*. New York, USA: Marcel Dekker, Inc., pp. 451-71, 630-98.

Larach, MG. (1989). Standardisation of the caffeine halothane muscle contracture test. *Anaesthesia & Analgesia*, 69: 511-5.

Laver, DR., Owen, VJ., Junankar, PR., Taske, NL., Dulhunty, AF. & Lamb, GD. (1997). Reduced inhibitory effect of Mg^{2+} on ryanodine receptor- Ca^{2+} release channels in malignant hyperthermia. *Biophysical Journal*, 73(4): 1913-24.

Loke, JCP. & MacLennan, DH. (1998). Malignant hyperthermia and central core disease: disorders of Ca^{2+} release channels. *The American Journal of Medicine*, 104(5): 470-86.

Loke, JCP., Tong, J., McCarthy, TV., & MacLennan, DH. (1998). Aspects of malignant hyperthermia. *Muscle & Nerve, Supplement 7*: S28.

Lynch, GS., Hayes, A., Lam, MHC. & Williams, DA. (1993). The effects of endurance exercise on dystrophic *mdx* mice. II. contractile properties of skinned muscle fibres. *Proc. R. Soc. Lond.*, 253: 27-33.

Lynch, GS., Stephenson, DG. & Williams, DA. (1995). Analysis of Ca^{2+} and Sr^{2+} activation characteristics in skinned muscle fibre preparations with different proportions of myofibrillar isoforms. *Journal of Muscle Research and Cell Motility*, 16: 65-78.

MacLennan, D. (1992). The genetic basis of malignant hyperthermia. *Trends in Pharmacological Sciences*, 13: 330-4.

- Martini, F. (1998). *Fundamentals of anatomy & physiology, 4th ed.* New Jersey, USA: Prentice-Hall Inc., pp. 279-89, 302-3.
- McCarthy, TV., Tong, J., & MacLennan, DH. (1998). Genotype phenotype correlation of ryanodine receptor gene mutations in malignant hyperthermia. *Muscle & Nerve, Supplement 7*: S28.
- McComas, AJ. (1996). *Skeletal muscle form & function.* Illinois, USA: Human Kinetics, pp. 171-82.
- Merz, B. (1986). Malignant hyperthermia: nightmare for anaesthesiologists - and patients. *Journal of the American Medical Association, 255*(6): 709-15.
- Malignant Hyperthermia Association of the United States (MHAUS). (1997). *What is Malignant Hyperthermia?* [on-line]. Available WWW: <http://www.mhaus.org/whatismh.html>.
- MIMS, Vol. 34, No. 2. (1997). NSW: Australia: Promail Printing Group.
- Monsieurs, KG., Van Broeckhoven, C., Martin, J., Van Hoof, V. & Heytens, L. (1998). Gly341Arg mutation indicating malignant hyperthermia susceptibility; specific cause of chronically elevated serum creatine kinase activity. *Journal of Neurological Sciences, 154*: 62-5.
- Peachey, LD., Adrian, RH. & Geiger, SR. (1983). *Handbook of physiology* (ed.). Maryland, USA: American Physiological Society, pp. 73-81.
- Pennington, GP. & Joeris, L. (1996). Malignant hyperthermia in a 3-month-old infant: a case report. *Journal of the Medical Association of Georgia, 85*(3): 162-3.
- Serfas, KD., Bose, D., Patel, L., Wrogemann, K., Phillips, MS., MacLennan, DH. & Greenburg, CR. (1996). Comparison of the segregation of the RYR1 C1840T mutation with the segregation of the caffeine/halothane contracture test results for malignant hyperthermia susceptibility in a large Manitoba Mennonite family. *Anaesthesiology, 84*(2): 322-9.

- Shomer, NH., Mickelson, JR. & Louis, CF. (1995). Ca^{2+} release channels of pigs heterozygous for malignant hyperthermia. *Muscle & Nerve*, 18(10): 1167-76.
- Sorrentino, V. & Volpe, P. (1993). Ryanodine receptors: how many, where and why? *Trends in Pharmacological Sciences*, 141: 98-103.
- Soto, RG. & Sopher, MJ. (1998). *UCLA Department of Anaesthesiology: Different Diagnosis* [on-line]. Available WWW: [http://www.anes.ucla.edu/dept/mh.html#Different Diagnosis](http://www.anes.ucla.edu/dept/mh.html#Different%20Diagnosis).
- Stephenson, GMM., O'Callaghan, A. & Stephenson, DG. (1994). Single-fibre study of contractile and biochemical properties of skeletal muscles in streptozotocin-induced diabetic rats. *Diabetes*, 43: 622-28.
- Sugita, H. (1997). Molecular diagnosis of muscular dystrophies. *Brain Pathology*, 7: 1289-91.
- Takagi, A., Sunohara, N., Ishihara, T., Nonaka, I. & Sugita, H. (1983). Malignant hyperthermia and related neuromuscular diseases: caffeine contracture of the skinned muscle fibres. *Muscle & Nerve*, 6: 510-14.
- Takagi, A., Sunohara, N. & Nonaka, I. (1980). Sensitivity of muscle fibres to halothane or caffeine in vitro. Analyses on calcium loaded single skinned fibres. *Hiroshima Journal of Anaesthesia*, 16 (Suppl.): 187.
- Wallace, AJ., Wooldridge, W., Kingston, HM., Harrison, MJ., Ellis, FR. & Ford, PM. (1996). Malignant hyperthermia - a large kindred linked to the RYR1 gene. *Anaesthesia*, 51(1): 16-23.
- Walter, WG. (1981). *Skeletal muscle pharmacology*. Amsterdam: Excerpta Medica, pp. 284-5.
- Wappler, F., Roewer, N., Kochling, A., Scholz, J., Steinfath, M. & Schulte am Esch, J. (1996). In vitro diagnosis of malignant hyperthermia susceptibility with ryanodine-induced contractures in human skeletal muscles. *Anaesthesia & Analgesia*, 82(6): 1230-6.

Wappler, F., Scholz, J., Von Richthofen, V., Fiege, M., Kochling, A., Matschke, J., Winkler, G., & Schulte-am-Esch, J. (1998). Incidence of deposition for malignant hypothermia in patients with neuromuscular diseases. *Anaesthesiology*, 33(6): 373-80.

Wedel, DJ. (1992). Malignant hyperthermia and neuromuscular disease. *Neuromuscular Disorders*, 2(2): 157-164.

Wilson, GJ. & Stephenson, DG. (1990). Calcium and strontium activation characteristics of skeletal muscle fibres from the small marsupial *Sminthopsis macroura*. *Journal of Muscle Research and Cell Motility*, 10: 12-24.

APPENDIX 1

Tables

Table A1.1 *Kreb's-Ringer Solution*

NaCl	118.1 mmol L ⁻¹
KCl	3.4 mmol L ⁻¹
MgSO ₄	0.8 mmol L ⁻¹
KH ₂ PO ₄	1.2 mmol L ⁻¹
Glucose	11.1 mmol L ⁻¹
NaHCO ₃	25.0 mmol L ⁻¹
CaCl ₂ H ₂ O	2.5 mmol L ⁻¹
pH	7.4

(European Malignant Hyperpyrexia Group, 1984)

Table A1.2 *Relaxing Solution I*

HEPES ^a	60mM
MgCl ₂	1mM
EGTA ^b	50mM
NaN ₃	1mM
Caffeine	10mM
ATP ^c	8mM
pH	7.1

Relaxing Solution I & 50% Glycerol:

Same solution components as Table A1.2 made up to 100mls, plus 100mls of glycerol.

Table A1.3 *Relaxing Solution III*

HEPES ^a	60mM
MgCl ₂	1mM
HDTA ^d	50mM
NaN ₃	1mM
Caffeine	10mM
ATP ^c	8mM
pH	7.1

Table A1.4 Calcium Solution

HEPES ^a	60mM
CaCO ₃	48.5mM
MgCl ₂	1mM
EGTA ^b	50mM
NaAzide	1mM
Caffeine	10mM
ATP ^c	8mM
pH	7.1

NB. Solutions of varying concentration were made by mixing the above with Relaxing Solution I

Table A1.5 Strontium Solution

HEPES ^a	60mM
SrCO ₃	40mM
MgCl ₂	1mM
EGTA ^b	50mM
NaAzide	1mM
Caffeine	10mM
ATP ^c	8mM
pH	7.1

NB. Solutions of varying concentration were made by mixing the above with Relaxing Solution I

Legend:

- ^a HEPES: N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
- ^b EGTA: ethyleneglycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetate
- ^c ATP: adenosine 5-triphosphate
- ^d HDTA: hexamethylenediamine-N,N,N',N'-tetraacetate

APPENDIX 2

Patient Consent Form

SHENTON PARK CAMPUS
8 Stibby Street
SHENTON PARK 6008
WESTERN AUSTRALIA

Telephone: (08) 9382 7171
Facsimile: (08) 9382 7351



Royal Perth Hospital

DEPARTMENT OF NEUROPATHOLOGY

WELLINGTON STREET CAMPUS
Wellington Street Perth, WA 6000
PO Box X2213 GPO
PERTH 6167
WESTERN AUSTRALIA

Telephone: (08) 9224 2244
Facsimile: (08) 9224 3311
T.T.Y. Line: (08) 9224 7016

TEL: (61-8) 9224 2433

FAX: (61-8) 9224 2556

PATIENT CONSENT FORM CONSENT FOR THE USE OF BIOPSY TISSUE FOR DISEASE RESEARCH

I,
of.....
hereby give permission for a section of this muscle biopsy to be used in
disease research.

I understand that only tissue not required for the testing procedures for which
the biopsy was taken will be used for research purposes.

I realise that this permission can be withdrawn at any time.

The purpose of this consent form has been explained to me.

Signature:

Dated:

Witness declaration

I,
have read over and explained to the consenting party the purpose of this
consent and they affixed their signature in my presence.

Witness signature..... On.....

