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EXTRA-CORPOREAL *IN-VITRO* PERFUSION OF ISOLATED SKELETAL MUSCLE FLAPS IMPROVES ISCHAEMIC SURVIVAL

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Medicine in the branch of Plastic and Reconstructive Surgery.

Johannesburg 2004

ABSTRACT

The field of organ and tissue transplantation has necessitated an improved understanding of their associated pathophysiological pathways. Specific areas of interest involve the changes that follow ischaemia and derangement's that accompany organ and tissue storage, reperfusion injury and the "no-reflow" phenomenon. Strategies have been devised to manipulate and modify these processes, improving tissue and organ survival and function. These have involved the use of preservation solutions. Although most research involves organ transplantation, these principles have been translated and applied to various tissues, surgical flaps and microvascular replantations. These studies have generally used the skin flap as their model with little knowledge regarding muscle flaps, the most vulnerable to the ischaemic process. This study targets the use of one such preservation system and uses skeletal muscle as its tissue model.

The vascular anatomy of the *rectus femoris* muscle in the New Zealand white rabbit was studied anatomically and radiologically and thus described. The isolated *rectus femoris* muscle flap was harvested and perfused *in-vitro* with cooled, oxygenated University of Wisconsin solution (UWS) using a pulsatile renal perfusion pump. UWS was selected as it contains vital additives important in cryopreservation of organs. Monitoring of various physiological parameters was performed. The muscle was examined at 0, 4, 8, 12, 18 and 24 hours of extra-corporeal perfusion using warm and cold, non-perfused controls. The contralateral muscle served as the control. End-points were the percentage of muscle survival, as determined by a new grading system of muscle ischaemia, based on 3 light and 7 electron microscopic criteria.

The overall percentage of muscle survival (combined light and electron microscopy scores) resulted in approximately 58% survival at 24 hours for the perfused muscle versus 31% for the cold stored muscle. The stored muscle had the same survival rate at 12 hours as did the perfused muscle at 24 hours. For all time periods beyond 4 to 8 hours, perfused muscle showed statistically improved survival rates compared to the stored muscle. Eight hours appears to be a crucial point beyond which survival in muscle deteriorates to a much greater degree without perfusion.

Questions remain as to which method of preservation yields the best survival benefit and, as yet, there is no "ideal" perfusate. The future involves manipulating perfusion solutions and trying to arrest or reverse established warm ischaemia. Success of free tissue transfers and replantations of muscle-containing body parts may be enhanced. These techniques may also allow us to effectively store previously harvested flaps and eventually, to enter the realm of "banked" allograft tissue flaps.

DECLARATION

I declare that this thesis is my own, unaided work.
It is being submitted for the degree of Master of Medicine to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg.
It has not been submitted before for any degree or examination in any other University.

GAVIN DE AGUIAR

……day of……., 2004

Johannesburg, South Africa

DEDICATION

This work is dedicated to my greatest teachers: my wife Kim, for teaching me how to love and my daughters Gabriella and Sabrina, for teaching me why one should love.

PRESENTATIONS

Data from this research study was presented [in part] at the International Plastic Surgical Congress, Stockholm, Sweden, 1993 and at the Association of Plastic and Reconstructive Surgery of South Africa (APRSSA) Congress, 1993.

TABLE OF CONTENTS

PAGE

Title Page	I
Abstract	II
Declaration	IV
Dedication	V
Presentations	VI
Table of Contents	VII
List of Illustrations	XI
Preface	XV

CHAPTER 1 - INTRODUCTION

1.1. Scope and objectives of the study	1
1.2. Potential significance	2
1.3. Anatomical studies	3
1.3.1. Anatomy of the *rectus femoris* muscle flap in the rabbit and its vascular supply	3
1.3.2. Angiographic studies	5
1.4. Analysis and background of the problem	6
1.4.1. Ischaemic changes and reperfusion injury	6
1.4.2. Tissue cooling - hypothermic storage and injury during preservation	12
1.4.3. Role of free radical scavengers	16

PAGE

1.4.4. Manipulation of flap models: the role of perfusion and additional strategies designed to enhance tissue preservation 17
1.4.5. Continuous vs. pulsatile perfusion 29
1.4.6. The perfusate 31
1.4.7. Quantification of skeletal muscle necrosis – a grading system of ischaemia 39

CHAPTER 2 - MATERIALS AND METHODS **PAGE**

2.1. Animal model and surgical protocol 44
2.2. Radiographic study 45
2.3. Dissection of the muscle flap and its vascular anatomy 46
2.4. Manipulation of the muscle flap 46
2.5. Perfusion apparatus 47
2.5.1. The pump - transport module and console unit 48
2.5.2. The cassette 50
2.6. Perfusion technique 52
2.7. Monitoring 54
2.7.1. Temperature 54
2.7.2. Gas analysis 55
2.7.3. Perfusion pressures 55
2.7.4. Flow 55
2.8. Fluoresceine study 56

CHAPTER 2 - MATERIALS AND METHODS **PAGE**

2.9. Tissue sampling 57
2.10. Analysis and experimental observations of perfused and stored muscle 58
2.10.1. Light microscopic assessment 58
2.10.2. Electron microscopic assessment 60
2.10.3. Combined assessment 62
2.11. Statistical analysis 62
2.12. Ethical considerations 63

CHAPTER 3 – RESULTS

3.1. Anatomy of the *rectus femoris* muscle flap and its vascular supply 65
3.2. Radiographic study 68
3.3. Perfusion Studies 71
3.3.1. Gas analysis 71
3.3.2. Flow 71
3.4. Fluoresceine study 71
3.5. Demonstration of ischaemic muscle grading system and muscle flap studies 73
3.5.1. Gross pathology 73
3.5.2. Light microscopy 75
3.5.3. Electron microscopy 64
3.5.4. Combined light and electron microscopy 78
3.5.5. Contrasts and interactions between GROUPS and TIMES 80

CHAPTER 4 – DISCUSSION **PAGE**

4.1. Anatomy of the rectus femoris muscle flap and its vascular supply 96

4.2. Current study on ischaemic survival in muscle 98

4.3. Group-time interaction analysis 100

4.4. Clinical applications and future considerations 103

4.5. Concept of the "ideal" perfusion solution 107

CHAPTER 5 – APPENDIX

5.1. List of abbreviations 111

5.2. Acknowledgements 112

5.3. Supervisor / associates / contributors 113

CHAPTER 6 - LIST OF REFERENCES 114

LIST OF ILLUSTRATIONS PAGE

1. LIST OF TABLES

Table 1: Composition of preservation solutions 38
Table 2: Sampling protocol for perfused and stored muscle 56
Table 3: Grading system for muscle ischaemia on light microscopy 59
Table 4: Grading system for muscle ischaemia on electron microscopy 61
Table 5: Percentage of muscle survival for TEST vs. CONTROL muscle on light microscopy 75
Table 6: Percentage of muscle survival for TEST vs. CONTROL muscle on electron microscopy 81
Table 7: Percentage of muscle survival for TEST vs. CONTORL muscle on combined light and electron microscopy 92
Table 8: Contrasts between groups and times for combined light and electron microscopic assessment 95

2. LIST OF PLATES PAGE

Plate 2.5.1: Renal preservation system – Waters MOX-100DCM perfusion pump (and cassette) 49

Plate 2.5.2: Renal preservation system – Waters MOX-100DCM perfusion cassette 51

Plate 2.6 (A): Cannulation of *rectus femoris* muscle flap after harvesting 52

Plate 2.6 (B): Single flap perfusion technique – perfusion of the muscle flap and topical cooling 53

Plate 2.6 (C): Simultaneous double flap perfusion technique – perfusion of the muscle flap and topical cooling 54

Plate 3.1(A): Dissection of *rectus femoris* muscle flap 65

Plate 3.1(B): Femoral vessels and vascular pedicle of the muscle flap 67

Plate 3.1 (C): Axial vascular system of *rectus femoris* muscle 67

Plate 3.2 (A): Radiographic study of arterial anatomy to lower limbs – ligation of right femoral artery distal to inguinal ligament 70

Plate 3.2 (B): Radiographic study of arterial anatomy to lower limbs – isolated right-sided ligation of pedicle to muscle flap 70

Plate 3.4 (A): Fluoresceine study –*rectus femoris* muscle flap: intact 72

Plate 3.4 (B): Fluoresceine study – *rectus femoris* muscle flap: sectioned 72

2. LIST OF PLATES - CONTD. PAGE

Plate 3.5.1: Gross macroscopic appearance of the perfused, cold-stored and warm-stored *rectus femoris* muscle after 24 hours 74

Plate 3.5.2 (A): Photomicrograph of normal *rectus femoris* muscle fibres 77

Plate 3.5.2 (B): Photomicrograph of muscle stored at room temperature (at 25^0C) for 8 hours (warm control) 78

Plate 3.5.2 (C): Photomicrograph of muscle subjected to 24 hours of cold-storage (at 4^0C) 79

Plate 3.5.2 (D): Photomicrograph of muscle subjected to 24 hours of cold perfusion with UWS at 4^0C 80

Plate 3.5.3 (A): Electron micrograph of a normal *rectus femoris* muscle 85

Plate 3.5.3 (B): Electron micrograph of normal muscle (pre-ischaemia) 86

Plate 3.5.3 (C): Electron micrograph of muscle subjected to 8 hours of cold perfusion (4^0C) 87

Plate 3.5.3 (D): Electron micrograph of muscle subjected to 8 hours of cold storage (4^0C) 88

Plate 3.5.3 (F): Electron micrograph of muscle after 24 hours of cold storage 90

Plate 3.5.3 (G): Electron micrograph of muscle after 24 hours of cold perfusion (TEST) 91

3. LIST OF FIGURES PAGE

Figure 1: The biochemical pathway for production of oxygen free radicals during reperfusion of ischaemic tissue (after Granger *et al*, 1986) 7

Figure 2: Animal ethics clearance certificate 64

Figure 3: Percentage of muscle survival for TEST vs. CONTROL muscle on light microscopy

Figure 4: Percentage of muscle survival for TEST vs. CONTROL muscle on electron microscopy 82

Figure 5: Overall arrangement of myofibrils and myofilaments of normal skeletal muscle (after Wheater *et al,* 1979) 84

Figure 6: Architecture and conducting system of the myofibril of skeletal muscle (after Wheater *et al*, 1979) 85

Figure 7: Overall percentage of muscle survival TEST vs. CONTROL muscle on combined light and electron microscopy 93

Figure 8: Patterns of Vascular Anatomy (after Mathes & Nahai, 1981) 97

PREFACE

The advent of microsurgery has revolutionised reconstructive plastic surgery. The microsurgical approach to clinical problems no longer lies at the top of the "reconstructive ladder" - in many instances, these methods provide the best first-line option. In recent years, reconstructive microsurgery has evolved to "state-of-the-art" in solving a variety of reconstructive problems. These innovative procedures have been fraught by such problems of ischaemia and the reconstructive surgeon has become increasingly concerned with their pathophysiology and its management.

Other areas of interest include the changes that occur with hypothermia and storage, reperfusion injury and the “no-reflow” phenomenon. Flap failure due to extrinsic or mechanical causes has become less common, yet intrinsic biochemical causes continue to be problematic (Mellow *et al*,1990).
The elucidation of various methods of intervention to prolong the survival of skin, muscle flaps or muscle-containing amputated parts after an ischaemic event has and continues to be a challenging area of research. This may save such tissue from ischaemia-reperfusion injury and prolong ischaemic survival and ultimately, function of the surviving replanted part / flap.

The death of cells is a complicated process which may proceed in a reasonably ordered fashion, depending on the cause. The early histopathological changes accompanying the necrosis of ischaemic skeletal muscle needs to be accurately defined. The correlation of these changes with the times to loss of viability is of critical importance.

If one can accurately and quantitatively assess these alterations during the early, reversible phases before the onset of the "no-reflow" phenomenon (May *et al,* 1978; Chait, 1978), one could intervene either mechanically or pharmacologically to prevent progression to irreversible cell death (Harishima & Buncke, 1975; Gould, 1985; Rosen *et al*,1985; Pang,1989; Knight *et al,*1991; Kriedstein *et al*,1991; Mellow *et al*,1990; Gordon *et al*,1992; Morris *et al*,1993). Perhaps, by carefully identifying the critical point of transition from reversibility to an irreversible situation, one may be able to intervene so as to salvage otherwise irreparably damaged tissues. The results of such interventions are at times, difficult to interpret. This is due to varying animal models and the protean pharmacological and other therapeutic interventions.

Although many reports appear in the literature concerning creation and maintenance of microvascular anastomoses and extensive research into the pharmacological manipulation of transplanted tissues, a **uniform, standardised and quantitative grading system of ischaemic muscle** still appears to be lacking. Further objectives of this study include an attempt to provide an overall, simple and quantitative grading system which unifies well- known histopathological and ultrastructural features of ischaemic damage to skeletal muscle. It also provides for early detection of potentially reversible changes.

In an attempt to find an "ideal" perfusate solution that could be used in this study, a review of the literature showed that most work in this field was performed on **organ preservation**. Preservation of any organ (or tissue) therefore hinges on two essential strategies (Southard & Belzer, 1993): (1) cooling of the structure and (2) the use of preservative solutions.

The latter may be used as a simple immersion cryopreservative or as more complex perfusion systems. Therefore organ and tissue preservation and more especially using cold preservative solutions (cryopreservation), is an important area of study.

With developing expertise however, it has become clear that different tissues have different metabolic requirements and ischaemic profiles and that solutions of different compositions are required for different tissues (Belzer & Southard, 1988; Kohout *et al*, 1995). A solution suitable for liver preservation may quite different from that required by kidney, skin or muscle. The current literature shows a paucity in the study of isolated **muscle** ischaemia and preservation of **muscle** flaps. Considering that this tissue is uniquely and exquisitely sensitive to ischaemia and its effects (Toutas & Bergman, 1977; Sjostrom *et al*, 1982; Chachques *et al*, 1985; Harris *et al*,1986; Gregory & Mars, 1992; Hickey *et al*, 1992) and the commonplace use of muscle in tissue transplantation, this tissue was therefore a logical choice in a study involving cryopreservation of ischaemic tissue.

Having decided on a perfusion solution (in this case, using a standard organ preservation solution known to limit ischaemic cellular damage - University of Wisconsin solution or UWS) and an appropriately ischaemically-prone tissue (in this case, skeletal muscle), one had to consider the method of delivery of UWS to the muscle. The methods now used for organ preservation prior to transplantation are simple flush, cold storage immersion or pulsatile perfusion by means of a mechanical pump.

Waugh *et al* (2000) studied the effects of pre-ischaemic perfusion of rat skeletal muscle flaps with perfusion solutions delivered by means of mechanical flush with uncontrolled pressures.
Yokoyama *et al* (1996) compared rat whole –limb viability after cold immersion in UWS or Euro-Collins solution. Hickey *et al* (1995) investigated the effects of simple flush of phosphoenolpyruvate / ATP solution of rabbit muscle flaps. Li *et al* (1993) decreased the ischaemia / reperfusion injury of rat muscle flaps with anticoagulated blood, again delivered by simple flush.

A review of the current literature (English language) appears to show that there are no available studies regarding the use of perfusion of an isolated skeletal muscle flap delivered in a **pulsatile** fashion. This method of delivery was chosen as it was felt it is the most physiological method of delivery of perfusate. The experimental design in this study therefore involves extra-corporeal perfusion isolated skeletal muscle in a pulsatile fashion with cooled oxygenated University of Wisconsin solution over a period of 24 hours, using a mechanical pump, in this case a "Waters Instruments Mox 100" renal perfusion pump. End-points of ischaemic damage using the proposed ischaemic grading system are characterised and an analysis made of the critical "point of no return" after which irreversible ischaemic changes occur.

The **HYPOTHESIS** that this study will attempt to address is therefore as follows:
"perfusion of an isolated skeletal muscle flap with cooled, oxygenated University of Wisconsin solution prolongs ischaemic survival."

As our knowledge continues to improve, the possibility of intervention by mechanical or pharmacological means may have tremendous clinical impetus in terms of overall survival and improved muscle function, especially post-replantation of muscle-containing amputated body parts.
Even in the most experienced of hands, this procedure is enshrouded with the danger of failure of the replanted part to survive or to function adequately post-replantation due to muscle necrosis and subsequent replacement fibrosis.
In attempting to improve and prolong muscle flap ischaemic survival time, this study may be extrapolated to the clinical situation outlined above.

During the inevitable delay prior to replantation, it is hoped that, by attaching amputated body parts to a mechanical pump and perfusing it with a similar solution used in this study, beneficial effects regarding survival of the part will be achieved. Further applicability may, in the future, be translated to the potential field of tissue storage using “banked” allograft tissue flaps.

CHAPTER 1 - INTRODUCTION

1.1. SCOPE AND OBJECTIVES OF THE STUDY

1. To determine the vascular anatomy of the *rectus femoris* muscle in the New Zealand White rabbit. This will be performed by means of anatomical dissection and radiographic studies.
2. To review the literature and formulate a new grading system of muscle ischaemia.
3. Following the description of the *rectus femoris* muscle flap, perform various manipulations in order to investigate various questions relating to muscle ischaemia and organ preservation solutions as follows:

- create a statistical control group by storage of the flap under normothermic (25 ^{0}C) and hypothermic (4 ^{0}C) conditions.
- confirm that cold storage of muscle delays the ischaemic process.
- determine if perfusion of the muscle flap can prolong ischaemic survival time using cooled, oxygenated University of Wisconsin solution (UWS).
- determine if ischaemic events occurring in the muscle flap can be influenced.

4. To add to the body of knowledge of ischaemic-reperfusion injury in skeletal muscle and the effects of cryopreservation solutions on ischaemic damage.
5. To gain experience with current practices regarding organ preservation solutions.
6. To add to the body of knowledge in the field of tissue preservation, muscle flaps and microsurgery.

1.2. POTENTIAL SIGNIFICANCE

1. The development of a working animal model that provides a relatively simple and reliable skeletal muscle flap. This may then be employed for further study regarding various manipulations of the flap and their effects on muscle.
2. The establishment of an "ideal" muscle perfusate which may significantly improve ischaemic skeletal muscle survival.
3. If perfusion of muscle flaps can be shown to prolong ischaemic survival time, then perfusion of amputated parts containing muscle (which have a shorter critical ischaemic time than skin), may result in improved survival of such parts post-replantation and transplantation.
4. The development of an effective, readily transportable and simple method of perfusion of muscle-containing amputated parts. This may be used in the clinical situation so that such parts may be perfused during inevitable delays prior to replantation.
5. To reduce the effects of established ischaemic damage and reperfusion injury so as to improve overall survival of myocytes.

1.3. ANATOMICAL STUDIES

1.3.1. Anatomy of the *Rectus femoris* Muscle Flap in the Rabbit and its Vascular Supply

A primary aim of this study is to elucidate the effects of an organ perfusion solution on isolated skeletal muscle subjected to various intervals of ischaemia. Before this aspect of the study could be addressed, it was essential to have an available animal model of a skeletal muscle flap.

The criteria of this flap were to be as follows:

1. easily available small laboratory animal – New Zealand white rabbit
2. isolated skeletal muscle, available bilaterally – *rectus femoris* muscle
3. established vascular anatomy /pattern
4. reliable and documented vascular pedicle to muscle
5. vessels of calibre compatible for microvascular surgery (if necessary)
6. easy dissection

Limb surgery, particularly that involving the use of surgical flaps, demands an understanding of vascular anatomy. Knowledge of its vascular pedicle allows a flap to be raised as an isolated pedicled or even a free flap, so that further research manipulation may take place. This is important for a number of reasons:

1. blood flow in the pedicle can then be controlled and the artery may be canulated for measurement of intra-arterial pressures.
2. the vascular pedicle also provides a convenient entry point for perfusion of the flap with pharmacological solutions.

3. during studies on ischaemia-reperfusion injury, the isolated pedicle may be clamped thus avoiding the crush injury produced by traditional hindlimb tourniquet methods.
4. as a free flap, a muscle may manipulated as required then elevated and micro-anastomosed to the contra - lateral vessels, allowing for a new vista of research information.

It is therefore pertinent to have an accurate description of the blood supply of the *rectus femoris* muscle in this animal model. A search of available veterinary literature as well as direct communications with veterinarians showed a surprising dearth of adequate descriptions of lower limb vascular anatomy in the rabbit. Descriptions included the gross and overall structural layout of the vascular system, but anatomical dissections were inadequate / unavailable for a detailed appraisal of the regional vascularity. A review of the medical literature only provided general descriptions of rat, feline or canine hindlimb animal models (Kuzon *et al*, 1986).

The *rectus femoris* **muscle** flap model in the rabbit has been described previously and has proven to be technically feasible, safe and reliable (Guelinckx *et al*, 1988; Guelinckx & Faulkner, 1992). Hickey *et al* (1992) also describe the basic surgical procedure of elevating the rectus femoris flap in this animal. These authors however, do not detail the vascular or gross anatomy of the flap.

1.3.2. Angiographic Studies

An extensive and detailed angiographic study of the rabbit lower limb has been described by McNally *et al* (1992). They performed high quality arteriographies in normal, un-operated rabbits with a view to understanding variations in vascular anatomy.
No specific reference was made as to the vascular anatomy of skeletal including that of the *rectus femoris* muscles.

A further aim of this study will be to document the regional vascular anatomy in the groin and lower limb region in the rabbit as seen on contrast angiogram and to demonstrate the vascular pedicle to the rectus femoris muscle. It describes in detail the gross anatomy of the *rectus femoris* flap and correlates these findings with that of angiographic studies.

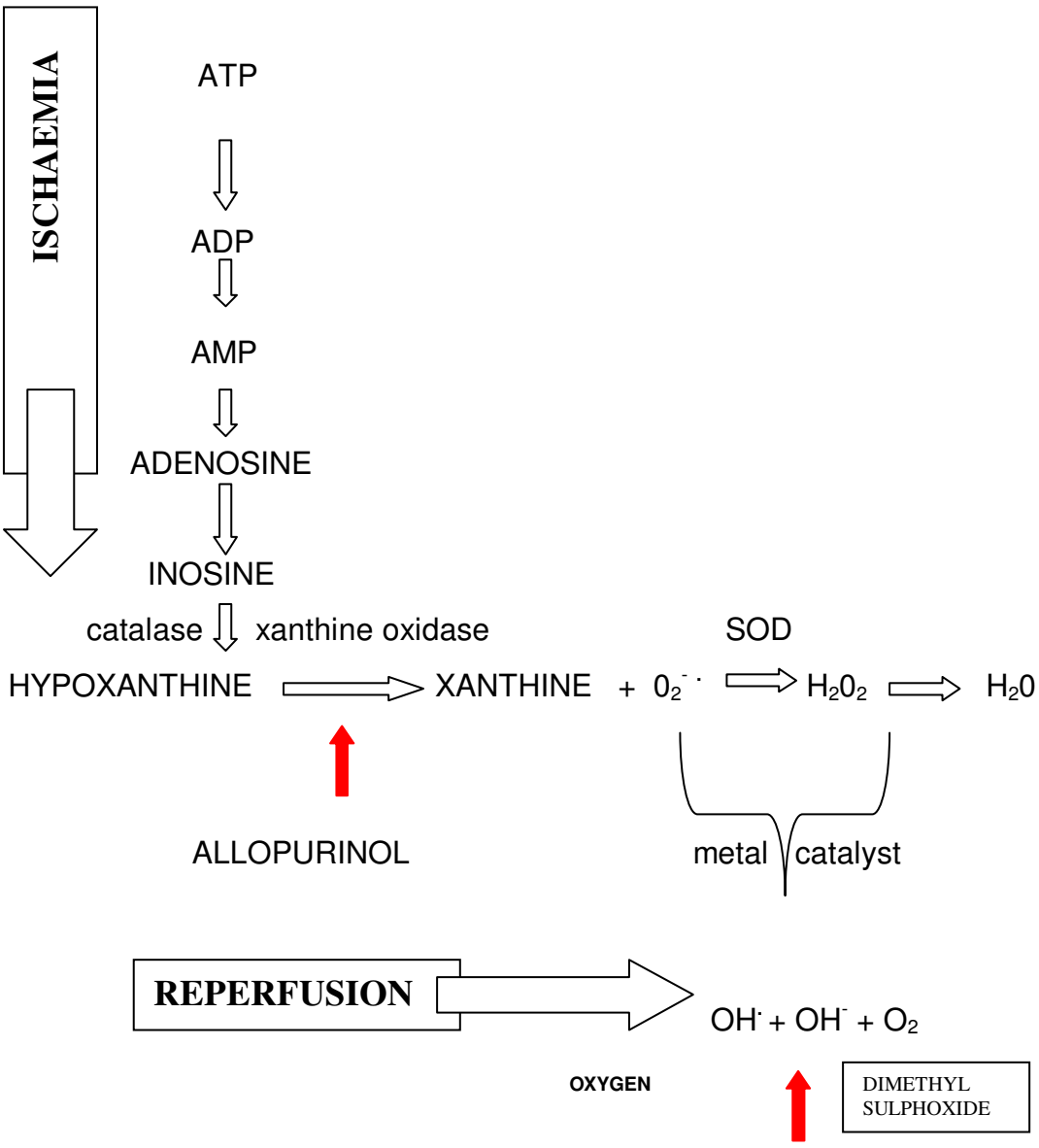
1.4. ANALYSIS AND BACKGROUND OF THE STUDY

1.4.1. Ischaemic Changes and Reperfusion Injury

Historically, the problem of Volkmann's ischaemic contracture lead many of the earlier investigators to devote their attention to the chronic ischaemic changes in skeletal muscle, with little attention to the initial changes that occur in the first 24 hours (Brooks,1922; Jepston,1926; Griffiths,1940; Clarke,1946).

Ischaemia may be defined as a blood supply insufficient to meet a tissue's metabolic demands, resulting in hypoxia and accumulation of toxic metabolites. This results in compromised cellular integrity and ultimately in cell death. During ischaemia, oxygen supply is reduced thus forcing the cell to convert to the less efficient anaerobic glycolytic pathway. This results in the production of lactic acid thereby lowering the intracellular pH (Slater,1984; Swartz *et al*,1978 ; Chaudry,1983). If hypoxia persists, the transmembrane sodium - potassium ATPase pump fails and sodium and calcium cations enters the cell. This increases the osmotic pressure and produces cellular oedema. ADP is catabolised to adenosine, then progressively to inosine, hypoxanthine and xanthine (**Figure 1**).

Upon reperfusion and the return of oxygen to the tissues the accumulated hypoxanthine is acted upon by xanthine oxidase, converting it to uric acid and the toxic superoxide free radical. This is subsequently converted to hydrogen peroxide and the highly reactive hydroxyl ion. Other toxic free radicals have also been demonstrated. The free radicals thus produced are highly unstable and react violently with stable molecules, producing more free radicals, thus propagating their effects. Oxygen free radicals in ischaemic tissues are generated from a number of differing sources. The relative importance of these mechanisms is not known (Kohout *et al*, 1995).



= INHIBITORY

Figure 1: The biochemical pathway for production of oxygen free radicals during reperfusion of ischaemic tissue (after Granger *et al*, 1986)

Regardless of the source, free radicals are toxic to all biological substances, including collagen, proteins, polysaccharides and nucleic acids. Polyunsaturated fatty acids, components of cell membrane phospholipids, are particularly sensitive. Lipid peroxidation of cellular and subcellular membranes is postulated to be a direct cause of cell death (Del Maestro, 1980; Pang, 1990). Free radical injury of the vascular endothelium increases vessel wall permeability and causes tissue oedema (McCord,1985). Free radicals also affect arachidonic acid cascade and shifts the normal prostacycline: thromboxane balance towards production of deleterious vasoconstrictor, thromboxane. Neutrophil infiltration and adhesion to the endothelium, as well as the release of inflammatory mediators such as platelet-activating factor and cytokines, are believed to play major roles in the ischaemia-reperfusion injury (Kerrigan & Stotland, 1993). The resultant damaging chain reactions cause thrombosis in macro- and micro-vessels, necrosis of endothelial and parenchymal cells and vasoconstriction. This results in flap failure or loss of replanted digits or limbs.

Ischaemia and reperfusion in skeletal muscle is unavoidable in many cases of vascular disease, musculoskeletal trauma and reconstructive surgery. Familiar examples include limb amputation with subsequent replantation, revascularisation in cases of peripheral vascular disease after cross clamping and in free tissue transfers of muscle flaps. Although skeletal muscle has a higher tolerance to ischaemia than some organ systems, it remains highly sensitive to cessation of blood supply especially under normothermic conditions. Human leg ischaemia of 1 to 2,5 hours duration due to aortic cross clamping during reconstructive vascular surgery or tourniquet during orthopaedic knee surgery, resulted in significant decline in muscle glycogen and phosphocreatine pools.

However, this biological damage normalised after 5 minutes following reperfusion (Toutas & Bergman, 1977; Sjostrom *et al,* 1982). Some morphological damage , primarily attributable to membrane disturbances, was found after 30 minutes of reperfusion, although no histopathological or ultrastructural evidence of necrosis was present 5 days post-operatively (Sjostrom *et al*, 1982).

Harris *et al* (1986), using the canine gracilis muscle model, demonstrated some muscle necrosis at 4 hours of warm ischaemia (parameters being both biochemical and ultrastructural) and severe injury and necrosis at 7 hours.

Hickey *et al* (1992) examined the consequences of increasing duration of ischaemia to the *rectus femoris* muscle in the rabbit. Post-ischaemic survival, as measured by Nitro blue tetrazolium (NBT) staining 24 hours after normothermic ischaemia showed 91% after 2 hours, 77% after 3 hours, 42% after 3,5 hours, 11% after 4 hours and 0,6% after 6 hours.

Chachques *et al* (1985) subjected rats to hindlimb normothermic tourniquet ischaemia of varying intervals as well as increasing periods of reperfusion. They documented histological evidence of ischaemic damage after 3 – 6 hours ischaemia following reperfusion but recovery of the mitochondrial respiratory chain after 14 days. This group sustained a reversible injury. The second group, subjected to 6 - 9 hours of ischaemia followed by reperfusion underwent a transitional course whereas those suffering ischaemia greater than 9 hours showed irreversible damage.

A critical point in time exists when, despite restoration of adequate blood flow, irreversible damage has occurred and the transferred tissue fails to survive.

This is known as reperfusion injury or the "**no-reflow**" phenomenon, with muscle being especially susceptible (May *et al*,1978). Reperfusion injury thus results in endothelial swelling, vasoconstriction, intravascular sludging and microvascular microthrombi formation. These pathophysiologic events all contribute to the “no-reflow” phenomenon (Chait *et al*, 1978 ; May *et al*, 1978; Weinberg & Song, 1984 ; Douglas *et al*,1987).

The addition of oxygen is controversial and, in fact, may be deleterious (Belzer *et al*, 1982). From the previous discussion regarding generation of free radicals (refer **Section 1.4.1.**) introduction of oxygen may result the generation of toxic 0_2 free radicals. Some studies suggest that perfusion solutions do not require oxygenation because oxygen levels achieved by air equilibration are sufficient for the low metabolic rates during hypothermia (Johnson, 1983). Their study was performed on the kidney, an organ which has a different metabolic rate to muscle, the latter being a highly aerobic tissue.

Various investigators have shown that substrate metabolism (Petterssen *et al*, 1974), ATP production (Garvin *et al*, 1981; Southard, 1984) and active cation transport (Burg & Orloff, 1964) continue during hypothermia. The provision of oxygen may be beneficial in these scenarios. Other studies have demonstrated improved function of kidneys by retrograde persufflation with oxygen via the renal vein (Pegg *et al*, 1989).

The addition of oxygen therefore, remains debatable. Considering the highly aerobic nature of skeletal muscle, it was felt that the addition of oxygen was important in this muscle flap model.

Much of the emphasis in organ and tissue transplantation and replantation has recently shifted from the problem of surgical technique, now largely mastered, to one of **preservation**. Preservation confers 4 major advantages in transplantation (D'Alessandro, 1994):

(1) time to transport the organ or tissue from the donor to the recipient hospital
(2) time to allow tissue matching to be performed
(3) time to prepare the recipient and surgical team and in the case of replantation, to adequately resuscitate the patient
(4) improved organ or tissue survival and function post-transplantation

Recovery of function depends upon preservation of cellular and organ integrity as well as the ability to restore blood flow. Clearly, our ability to alter the progressive effects of reperfusion damage and permit salvage of ischaemic tissue, must be based on an understanding of their biochemical processes.

Preservation of any tissue hinges on 2 major strategies:

1. tissue cooling (refer **Section 1.4.2**)
2. the use of perfusion solutions which, by nature of their composition, minimises the effects of ischaemia and reperfusion injury (refer **Section 1.4.4)**

1.4.2. Tissue Cooling - Hypothermic Storage and Injury During Preservation

The beneficial effects of hypothermia on preserving the viability of anoxic tissue has long been recognised. As early as 1908, Carrel demonstrated the capability of preserving arterial grafts under hypothermic conditions for periods of up to 10 days (Carrell, 1908).

A similar understanding applies to muscle-containing free tissue transfers and amputated parts. Lapchinsky (1960) presented a long-term series of replanted hind limbs of dogs, and described details of limb cooling to 4 ^{o}C. Usui *et al* (1978), documented the beneficial effects of hypothermia in limb replantation in the human model. Longer periods may be obtained with hypothermic preservation. Previous experimental studies of free groin flaps in rabbits, supported by upwards of 72 hours of hypothermia, have demonstrated evidence of tissue survival (Donski *et al*,1980) while successful experimental limb replantation has been achieved after 108 hours of supplemental hypothermia (Lapchinsky, 1960; Tsu-Min Tsai *et al*, 1982).

It is generally accepted that tissue cooling universally improves the viability of explanted tissue by slowing down the metabolic processes and is therefore used in all the currently employed protocols (Kohout *et al*, 1995).
Belzer calculated that lowering the temperature from 37 ^{o}C to 0 ^{o}C will slow down enzymatic processes 12 to 13 fold (Belzer & Southard, 1988).
A number of studies have shown improved preservation at lower-than-body temperatures of solid organs (Belzer & Southard, 1988; Kasiske *et al*, 1990; Bonventre, 1992) and experimental free flaps (Donski *et al*, 1980; Gould *et al*, 1985).

With regards **organ preservation** by means of hypothermic storage, it is generally believed that the organ remains viable only for approximately 24 to 48 hours. By decreasing the parenchymal cell's metabolic activity and oxygen demand, hypothermia can retard the normal catabolic processes from causing damage to the ischaemic tissue . In a temperature range between 0 and 4 ^{o}C Levy (1959) found the oxygen requirement of the canine kidney to be less than 5 % of normal.

At body temperature, 50% survival time of rabbit muscle is 3,5 hours (Hickey *et al*, 1995) and 9 hours for **skin flaps** (May *et al*, 1978). Cooling to 4 - 6 ^{o}C during ischaemia increases 50% survival time of skin to about 3 – 4 days (Donski *et al*, 1980) and skeletal muscle to approximately 16 hours (Waugh *et al*, 2000). In studies on ischaemic rat **muscle** (*cutaneous maximus*), Li *et al* (1993) demonstrated that the muscle displayed normal viability without remarkable histologic abnormality after exposure of up to 6 hours of normothermic ischaemia. After 10 hours at room temperature, profound injury was noted. Chachques *et al* (1985) reported similar findings.

Tsu-Min Tsai *et al* (1982) showed that hypothermic support of dog muscle flaps, particularly if preceded by initial flush with Collins solution, extended viability up to 96 hours.

Yet hypothermia can itself be injurious to basic cellular function by depressing the parenchymal-cell ATPase activity, resulting in the inactivation of the sodium-potassium membrane "pump" and subsequent cellular swelling and metabolic imbalance (Belzer et al 1978).

Potential complications exist as well with tissue perfusion. Although generally accepted that a hypertonic intracellular solution such as Collins

will prolong the ischaemic tolerance of tissues in both experimental and clinical settings, the perfusate, if too hyperosmolar or if administered under excessive pressure, can lead to the development of interstitial oedema and more pronounced tissue anoxia (Flores *et al*, 1972; Hicks *et al*, 1980).

The factors involved in hypothermic damage (i.e. cold storage without perfusion) remains unknown, although this is a subject of extensive review (Bonventre & Weinberg, 1992; Bresticker *et al*, 1992).
During cold storage, structural alterations in cell membranes occur and are characterised by a phase change of the membrane phospholipids from a fluid state to a more crystalline state (Bonventre & Weinberg, 1992; Southard & Belzer, 1993; D'Alessandro *et al*, 1994; Fuller, 1991). The rate at which this phase change occurs is not known, but equilibration of the tissue with the ambient temperature is quite rapid. The phase transition appears to be fully reversible for organs cooled for 10 or less hours and function post-transplantation is rapidly regained (Bonventre & Weinberg, 1992; Clavien *et al*, 1992).

It is generally believed that 4 phenomena contribute to hypothermic injury:

1. Loss of energy-generating capabilities following mitochondrial damage may play a role. Simple cold storage results in loss of energy-generating abilities as a result of mitochondrial damage or the loss of precursors for ATP generation. If tissues cannot restore energy homeostasis, irreversible cellular injury results.
2. Accumulation of toxic free radicals may also be involved in preservation injury (Hoshino *et al*, 1988; Risby *et al*, 1994).
3. The effects of toxic free radicals and reperfusion injury / "no-reflow" in cold- stored tissues and organs have been implicated in preservation injury (Hoshino *et al*, 1988; Risby *et al*, 1994). Suppression of toxic radical

generation and various free radical scavengers by pharmacological means, have had some success in improving clinical tissue and organ preservation (Douglas *et al*, 1987; Fuller *et al*, 1988; Winchell & Halasz, 1989; Pang, 1990; Knight *et al*, 1991).

4. Other proposed mechanisms include activation of lysosomal catabolic enzymes, proteases and phospholipases (Pavlock *et al*, 1984; McAnulty *et al*, 1991; Nicols *et al*, 1994). Active metabolites from arachidonic acids (thromboxane, leukotrienes) have also been implicated in reperfusion injury of preserved organs (Keppler *et al*, 1985; Post *et al*, 1993).

Hypothermia has also been shown to result in cell swelling and thus organ dysfunction. Current cryopreservative solutions therefore contain additives designed to suppress hypothermically induced swelling (Southard & Belzer, 1993). Despite these additives, considerable damage occurs during storage at 4 ^{o}C without continuous perfusion (i.e. cold ischaemia or simple cold storage). The addition of perfusion, although controversial in some studies, attempts to create a more physiological milieu in which the nutrients can reach the microcirculation.

In summary, it is well-known that hypothermic storage of organs and tissues offers unquestionable improvement in survival. Prolonged cooling (without perfusion) results in cellular damage. The use of preservative solutions are designed not only to provide some homeostatic environment, but also to reduce hypothermic damage. The delivery of the cooled preservative solution by either simple flush or pulsatile perfusion is an area of debate, but the latter delivery technique may have advantages.

This study will also aim to confirm the above proposals. Muscle flaps will be harvested and stored and a comparison made regarding quantitative muscle survival at normothermic (25 ^{o}C) versus hypothermic conditions (4 ^{o}C). The

potential benefits of cold perfusion will be explored and compared to the effects of simple cold storage without perfusion. In each case regardless, hypothermic conditions will be carefully controlled to 4 ^{o}C and perfusion pressures will be maintained at similar mean arterial blood pressures as exist in normal rabbit physiology (refer **Chapter 2 - "MATERIALS AND METHODS"**).

1.4.3. Role of Free Radical Scavengers

Following an ischaemic-reperfusion injury and the ensuing free radical cascade (**Figure 1**), it can be seen that the superoxide radical and its derivative the hydroxyl anion, are capable of producing extensive cellular and subcellular damage. It would follow that the addition of so-called "free radical scavengers" should minimise these pathophysiological changes and result in improved survival of the reperfused tissue or organ.

The prevention of the action of free radicals produced by all the pathways would be more effective in preventing tissue damage than by blocking those produced by a single pathway. By inhibiting xanthine oxidase (**Figure 1**), allopurinol has been used to reduce superoxide production *(*Im *et al, 1984;* Angel *et al*, 1987; Suzuki *et al*, 1991; Suarez Nieto *et al*, 1992).

Superoxide dismutase (SOD) and catalase has also been used to scavenge superoxide and hydroxyl radicals in the rat (Crikit *et al*, 1984; Sagi *et al*, 1986; Smith *et al*, 1990).

The iron chelator and free radical scavenger, desferrioxamine has also shown to improve skin flap survival (Sinaceur *et al*, 1984; Angel *et al*, 1989; Kohout *et al*, 1995).

Amongst other crucial ingredients, the perfusion solution used in this study, University of Wisconsin solution, contains the free radical scavengers allopurinol (1 mmol/L) and glutathione (3 mmol/L).

1.4.4. Manipulation of Flap Models: the Role of Perfusion and Additional Strategies Designed to Enhance Tissue Preservation

Many pathophysiological mechanisms operate during the insults of ischaemia, tissue preservation and reperfusion. Additional strategies have been postulated which may ameliorate these insults. These may involve improving the physiological milieu or providing pharmacological additive/s which may target specific derangement's. Given that there is no single agent which can counteract all the pathophysiological changes, the best possibility at present is a mixture of pharmacological agents added to the perfusate.

Most involve either perfusion washout before (and possibly after) the ischaemic insult (Chait *et al*, 1978; Sawhney, 1980; Gould *et al*, 1985; Rosen *et al*, 1985; Rosen *et al*, 1987) or intra-arterial (Sagi *et al*, 1986; Zimmerman *et al*, 1987) or systemic infusion of free radical scavengers before or concomitant with reflow (Manson *et al*, 1986). The latter has been discussed above.

The literature shows a general trend towards **washing out** the microcirculation prior to the ischaemic (Kohout, 1995). This has been achieved by using simple flushing or active perfusion techniques and helps by removing blood and its toxic metabolites from the perfused tissue or organ. However, the subject remains controversial.

Harashina & Buncke (1975) concluded that, in experimental rat leg amputations and replantations, no perfusion gives better results than heparinised saline solution or an intracellular (Downes) solution.

Although they could offer no explanation for their unexpected findings, a possible reason is that perfusion pressures were undocumented and uncontrolled. This may lead to marked and deleterious cellular and inter-cellular oedema.

Chait *et al* (1978), perfused rabbit epigastric skin flaps with various solutions containing mannitol, high molecular weight dextran, prednisolone and heparin and concluded that none of the perfusates had a beneficial effect on skin flap survival.

Similarly, Gould *et al* (1985), concluded that early perfusion with Ross' solution is detrimental to the ultimate fate of the cold ischaemic flap, while late perfusion with the same solution offered no advantage over cold storage (6-7 ^{o}C) alone.

In direct contrast to the above studies, Rosen *et al* (1985) increased flap tolerance to ischaemia by using a complex perfusate simulating rat plasma. The presumed aetiologic basis for this enhanced tissue survival is a delay in the onset of capillary and venular hyperpermeability in perfused flaps as compared to flaps in which stagnant blood remains. However, the complexity of the perfusate makes it difficult to assess which of the components are instrumental in ischaemia tolerance.

Douglas *et al* (1989) perfused rat epigastric flaps with a simple Ringer lactate solution and showed a dramatic increase in flap tolerance to ischaemia.
Hicks *et al* (1980) perfused 6 amputated rat hindquarter models with Ringers lactate, Collins hypertonic renal perfusate and dextran-dextrose ("Perfudex"). Their results indicated that Collins perfusate was less

damaging than Perfudex which, in turn, was less damaging than Ringers lactate. However, no comparison was made between perfusion with these solutions versus no perfusion at all.

In a study using composite tissue, Gordon *et al* (1992) compared perfusion of amputated limbs with University of Wisconsin (UW) solution to topical cooling. Their results showed that pH and tissue levels of ATP declined three times more slowly in the perfused limbs.

Kohout *et al* (1995) assessed the performance of several perfusion solutions on rabbit skin flaps in terms of flap viability and flap biochemistry. The solutions used were heparinised blood, UW and modified UW solution, Euro-Collins solution and a mixture of pharmacological agents targeting specific aspects of ischaemia / reperfusion injury. This mixture included phosphoenolpyruvate, desferrioxamine, nitrendipine, dextran 70 and a platelet-aggregating factor receptor antagonist.
The viability of the skin flaps perfused with this mixture (81%) was significantly higher than that of any other of the groups (39% for controls, 38% for Euro-Collins and 13% for UW solution). Biochemical performance of the skin flaps were also better when perfused with the mixture.

Some of the more recognised (and sometimes controversial) manipulations are as follows:

(a) Modification of pH:

Maintenance of a "normal" or neutral pH has been considered optimal for preservation (Kallerhoff *et al*, 1985; Southard, 1990), yet a mild acidosis can be protective (Bonventre & Cheung, 1985). Given the proven beneficial effects in reducing the pH from 7,4 to 6,9 - 7,0 in kidney (Shanley *et al*, 1988; Weinberg, 1985) and liver (Bonventre & Cheung, 1985), the question arises as to whether preservation would be improved by maintaining pH at 6,9 - 7,0 during hypothermia. This may be of particular benefit in a metabolically active aerobic tissue such as muscle. In the case of organ preservation, maintaining pH in the lowered ranges mentioned above has also shown to improve the efficiency of the solution during normothermia (Ferwana *et al*, 1989; Currin *et al*,1991; Gao *et al*, 1991). Whether or not this may enhance the survival and efficiency of muscle post-replantation is not known, but raises interesting questions.

Although the UWS used in this experimental study contains a phosphate buffer system, a lower pH value was found in the perfusate solution after 24 hours (mean of 7,18). It remains possible that this mild acidosis has a role to play in enhancing survival in muscle as well as in those organs quoted in the above studies.

(b) Desferrioxamine:

This has been shown to be a potent, non-selective scavenger of free radicals (Sinaceur *et al*, 1984).
Also, by chelating iron, desferroxamine reduces the availability of this catalyst and hence the total load of free radicals in the reperfused tissues. This has been directly confirmed by measuring tissue levels of end-products of lipid peroxidation (Green *et al*, 1989). Again, the use of this pharmacologic agent is controversial.

Nishikawa *et al* (1991) suggested that desferroxamine had no effect on the reperfusion damage of vascular endothelium as seen on electron microscopy, microangiography and ultimate flap survival. Different animals, different drug dosages and different experimental protocols were used in each study. It may also be hypothesised that this inconsistency may be explained by different amounts of free radicals generated in each experimental scenario.

Despite these inconsistencies, Kohout *et al* (1995) were convinced of the performance of desferroxamine and used it as a key agent in their pharmacologic mixture for cool perfusion of skin flaps.

Furthermore, in their study on secondary ischaemia due to venous obstruction in rat epigastric skin flaps, Angel *et al* (1989) found that desferrioxamine given prior to reperfusion improved flap survival.

(c) Superoxide dismutase or catalase:

Infusion of these agents just before perfusion of cold-preserved porcine kidneys improved renal function and morphology (Bosco & Schweizer, 1988). Treatment of rabbit donor kidneys with indomethacin, to reduce cyclo-oxygenase mediated reactive oxygen species production prior to harvest, reduced lipid peroxidation products and tended to improve function after transplantation (Gower *et al*, 1989). The literature regarding the use of these agents in skin flaps, reports variable results (Pang, 1989).

(d) Heparinised blood:

Cooley *et al* (1990), reported a significant improvement in survival of free flaps during ischaemic storage using perfusion with heparinised whole blood.

Kohout *et al* (1995), perfused rabbit epigastric skin flaps with heparinised whole blood and showed increased flap viability compared to non-perfused controls, but this difference just failed to reach statistical significance.

Both of these studies suggest that heparin can regulate, by a mechanism yet to be elucidated, the release of inflammatory mediators by blood cells and platelets retained in the microvasculature. Heparin may also have some influence on the hypoxic damage and cell swelling of the endothelial and parenchymal cells, which are other sources of inflammatory mediators. The use of heparin as an antiplatelet drug and its effects on antithrombin III is well established.

Rosen *et al* (1985), used a synthetic plasma substitute and pointed to the possibility of improved results with whole blood washout being independent of its cellular component. They suggest that perfusion washout improves the ischaemic tolerance and prevents the "no-reflow phenomenon in rat epigastric skin flaps. The postulated mechanism is due to the prevention of capillary endothelial damage secondary to the presence of formed blood cells or their products of haemolysis. They also speculated that washout may also prevent sludge formation and therefore thrombus. Intuitively, the benefits of the "acellular" components of blood seem to be a reasonable conclusion, as plasma is a complex solution of ions, macromolecules and buffers (Kohout *et al*, 1995).

The role of the cellular component of blood (i.e. red and white blood cells and platelets), is controversial. It is likely that red cell rigidity and sludging,

polymorph margination and degranulation and platelet aggregation, outweigh their potential benefits.

Oxygenated plasma pumped on a continuous basis has been found to be effective in preserving the kidney for more than 24 hours (Belzer *et al*, 1967; Belzer,1973; Magnussen *et al*,1976).This expensive and complex means of preserving organs has worked clinically over the past two decades because plasma with dissolved oxygen is extremely physiological. A solution that is anything but physiological would best be used on a washout basis, rather than delivered in a pulsatile fashion.

(e) Calcium channel blockers:

There has been considerable interest regarding the role of calcium as a mediator of muscle necrosis during reperfusion (Katz & Reuter,1979; Poole-Wilson *et al*, 1984; Walker, 1986).
It has long been known that there is an accumulation of calcium associated with necrotic tissue but the question remains as to the potential role of intracellular accumulation of calcium in precipitating pathological changes in the cell.

Calcium overload can occur during the ischaemic period because of a redistribution of tissue stores and cause damage (Poole-Wilson *et al*, 1984). However, the most significant calcium entry occurs when blood, laden with calcium, is reintroduced and the cellular membranes are vulnerable to calcium influx due to defects in the membrane itself or alterations in the membrane calcium channels (Crake & Poole-Wilson,1986).

Smith *et al* (1990), confirmed that total tissue calcium levels heralds the onset of catabolic cascades and are associated with significant necrosis in skeletal muscles.

Improved survival was shown by decreasing extracellular calcium (using radical scavengers, controlled oxygen delivery and low ionised calcium concentrations).The early ability to extrude intracellular calcium from the reperfused cell was associated with significant salvage of muscle. This ability may be intimately associated with adequate intracellular energy levels, again confirming the importance of providing energy substrates in the perfusate.

It would therefore seem appropriate that any perfusion solution used should attempt to decrease intracellular calcium levels and, at the same time, ensure an adequate supply of energy.
Calcium channel blockers have been shown to be protective against the effects of ischaemia in certain circumstances in organ (Elkadi *et al*, 1989; Currin *et al*, 1991) and in tissue models (Stein *et al*, 1989).

Stein *et al* (1989) showed that pre-treatment of rodent skin flaps with the calcium channel blocker, verapamil, markedly improved ischaemic survival. These effects were mediated by verapamil's ability to protect against oxygen free radicals. Calcium channel blockers also have an important vasodilatory function (Malis *et al*, 1983).

Using Carolina rinse solution, which contains a calcium channel blocker, Currin *et al* (1991) were able to prolong cell viability compared to University of Wisconsin solution.

(f) Prostanoid modifiers:

Much of the reperfusion injury and the "no-reflow " phenomenon (Chait *et al*, 1978) can be attributed to endothelial cell damage caused by accumulated free radicals. Endothelial damage exposes the subendothelial collagen and provides thrombogenic sites where platelets aggregate and give rise to large increases in plasma levels of thromboxane (TXA_2).

Thromboxane has a direct vasoconstrictor effect. Prostacyclin (PGI_2) is a vasodilatory arachidonic acid metabolite which, in non-ischaemic tissues, is known to increase blood flow in both major vessels and the microcirculation (Higgs *et al*, 1979), including that of skin flaps (Knight *et al*, 1985). PGI_2 also inhibits platelet aggregation and at higher concentrations, platelet adhesion and platelet thromboxane release (Knight *et al*, 1991).
Knight *et al* (1991) demonstrated improved ischaemic skin flap survival in the rabbit with pre-treatment of prostacyclin and prostanoid modifiers such as dipyridamole. This was attributed to the vasodilatory and antithrombotic properties of these drugs.

(g) Other agents:

Anti-PAF – Platelet activating factor (PAF) is thought to be important in the pathogenesis of the reperfusion injury. PAF is thought to affect this injury through its action on neutrophil and platelet activation (Heuer *et al*, 1990). WEB 2170 is a platelet-activating factor antagonist which may reduce migration of neutrophils and decrease levels of thromboxane (Kohout *et al*, 1995).

Macromolecules - The inclusion of macromolecules such as mannitol or hydroxyethyl starch in UWS and in some studies, Dextran (Marshall *et al*, 1991; Kohout *et al*, 1995), helps prevent post-perfusion increase in tissue water, probably through their osmotic action across cell membranes. These effects may only be temporary and, after a time, increased tissue water may be found. Kohout *et al* (1995) reported on "rebound oedema" in flaps perfused with solutions containing macromolecules.
These effects may be due to local metabolites and disruption of cellular ion exchange mechanisms subsequent to the washout of free radical scavengers and osmotically active macromolecules.
Dextran, in addition to its oncotic effects, has rheological properties and decrease Rouleaux formation of red cells and therefore improves flow in the microcirculation.

Purine Supplementation and Glutathione – the benefits of added adenosine and glutathione will be discussed (refer **section 1.4.6.**)

Cyclosporin A – the role of the cellular components of blood, particularly neutrophils and platelets, is a very complex one and beyond the scope of this study. It is therefore not discussed in any great detail here.
Suffice it to say that methods which affect neutrophil chemotaxis, migration and degranulation are likely to result in further improvements in flap survival.

The use of agents which block these processes have been investigated and Kucukcelebi & Ozcan (1992), report that cyclosporin A (CsA) offers significant advantages in increasing perfusion flap viability. CsA has multiple actions, including producing a general decrease in leukocyte responsiveness.

CsA also inhibits synthesis and action of many inflammatory mediators, such as cytokines. It may thus block the amplification mechanisms inherent in the inflammatory response.

Haemoglobin solutions – some investigators have reported on the use of perfusion of amputated limbs using synthetic haemoglobin solutions viz. fluorocarbon solutions ("Perfudix").
Usui *et al* (1985), found that continuous gravity perfusion with a fluorocarbon solution was more effective in the prevention of systemic acidosis associated with reperfusion than either intermittent perfusion or topical cooling.

Pharmacologic Mixtures –the pathways involved in the ischaemia /storage / reperfusion injury are extremely complex in nature. It is therefore very likely that a pharmacological mixture or "cocktail" comprising individual components which target separate mechanisms in these injury processes, will have the greatest efficacy. In fact, all modern preservation solutions comprise mixtures of various solutes, solvents and pharmacologic agents, differing in their nature or quantity.

Kohout *et al* (1995) showed an 81% survival of rabbit skin flaps perfused with a pharmacological mixture compared to 39% survival in non-perfused controls. The mixture they used consisted of heparinised blood, mixed with phosphoenolpyruvate, the calcium channel blocker nitrendipine, desferroxamine, WEB 2170 and Dextran 70.
Other sporadic reports, mainly in the field of organ transplantation, have shown some survival benefits with the use of other agents, such as protease inhibitors, phenothiazines, atrial natriuretic peptide and polyethylene glycol (Bonventre & Weinberg, 1992).

The literature abounds with vast numbers of agents that have been tried on various organ and tissue models and this, in itself, is testimony to the fact that there is no single agent which has undisputed benefits in all cases. With further insight into the pathophysiology involved with ischaemia / storage / reperfusion scenarios, we can expect future literature to document a plethora of agents and concoctions all aimed at manipulating these events.

(h) Ischaemic Preconditioning:

Ischaemic preconditioning, defined as brief episodes of ischaemia and reperfusion, has been thought to offer some protection from reperfusion injury that follows prolonged ischaemia. The protection has been described as having 2 windows of opportunity: a transient acute phase lasting approximately 3 hours (Parratt, 1994; Lee & Linweaweaver, 1996) and a delayed second window which begins after several hours and can last for more than 24 hours ((Parratt, 1994; Yellon et al, 1998).

In cardiac muscle, following a primary stress such as ischaemic preconditioning, has frequently been associated with protection from ischaemia-reperfusion (I-R) injury (Currie *et al*, 1993; Tanake *et al*, 1998). This is shown by the expression of heat shock protein 70 (Hsp 70) in the delayed phase. In skeletal muscles, studies of delayed protection by this method are not as common. Although improved perfusion of capillaries has been reported after prolonged ischaemia followed by short-term reperfusion in rat hind limbs (Pudupakkam *et al*, 1998), a long-term reperfusion study in the rat latissimus dorsi, showed only a marginal decrease in muscle necrosis (Carroll *et al*, 1997).

In order to further delineate this question, Lepore & Morrison (2000) investigated the ability of ischaemic preconditioning to induce expression of Hsp 70 and also to improve muscle survival after ischaemia-reperfusion injury using the rat gastrocnemius muscle. They concluded that preconditioning (in this case by means of tourniquet induced ischaemia) did not produce delayed protection from ischaemia-reperfusion injury.

The available literature to date does not provide sufficient evidence to support this modality of treatment as having a delayed protective effect against skeletal muscle necrosis after I-R injury, at least in clinical practice.

1.4.5. Continuous vs. Pulsatile Perfusion

Theoretically, continuous organ (and possibly tissue) perfusion would be the most effective method of maintaining intra- and extra-cellular homeostasis, providing the solution was physiologic (Hicks *et al*, 1980). In the field of preservation and transplantation, studies tend to show that pulsatile perfusion produces superior results, particularly for prolonged storage. However, it must be borne in mind that other centres commonly employ flush topical cooling (a single flush of the organ's vasculature with a preservation solution, usually UWS, followed by topical cooling). This intercellular "washout" solution is effective in avoiding endothelial injury and oedema, while removing metabolites and intravascular elements and creating an environment for the cell to continue its metabolism (Belzer,1973 ; Halasz *et al*,1976 ; Magnussen *et al*,1976).

The field of tissue preservation, specifically limb and flap preservation, is an entirely different arena from organ preservation.

Although the end result of ischaemia is the same for all cells, different organs and tissues have different metabolic profiles and ischaemic tolerances. The early data on tissue preservation was indeed conflicting. Regardless of which method of perfusion or flushing is chosen, most studies show that beneficial effects are generally obtained using physiologic colloid containing solutions.

Whilst perfusion may well be more physiological (flow in the microcirculation is, at least, oscillatory), early studies suggested that it may in fact, be harmful. Hicks (1980), perfused rat hindlimbs by means of an intermittent perfusion pump (pressure un-stated) and abandoned this in favour of continuous perfusion, due to the gross oedema that resulted. More recent studies of skin flap and amputated limbs (composite tissues) have generally shown improved results with perfusion. Kohout *et al* (1995) showed good results on skin flaps using machine perfusion with a peristaltic pump. In their study, careful attention was paid to maintaining intraluminal pressure below 150 mmHg. Similarly, Gordon *et al* (1992) showed improved results with pulsatile perfusion on composite tissues. The particular perfusion delivery technique (continuous versus pulsatile) appears to be of great importance, certainly in the tissue models, such as skin flaps and amputated limbs.

Although flap perfusion offers attractive theoretical survival benefits, there is still lack of agreement on whether the vascular bed of the replanted or transplanted tissue should be perfused by active mechanical pumping or "washed out" by simple flushing. It would seem rational to deliver the perfusate by the most physiological means. This may be especially important in a highly vascular structure such as muscle, where flow in the microcirculation is intermittent, albeit oscillatory, rather than continuous.

Controlled intermittent pulsatile delivery, as used in this study, seems the most logical choice, yet this parameter warrants further investigation.

1.4.6. The Perfusate

The simple exposition of Starlings law is that blood circulation through a capillary bed loses fluid to the interstitial space when the hydrostatic pressure within the capillary exceeds the sum of the external tissue pressure and the colloid osmotic pressure within the lumen of the vessel. In perfusion experiments, the artificial medium and perfusion pressure should be selected to achieve a similar balance, so that net fluid loss from the vascular compartment and cellular and interstitial oedema does not occur. Since perfusion pressure can be controlled, it is the **composition** of the artificial medium or perfusate that is of vital importance in maintaining homeostasis, preserving organ and tissue function and minimising ischaemia / reperfusion injury.

In an attempt to find an "ideal" perfusate solution that could be used in this study, a review of the literature showed that most work in this field was performed on **organ preservation**. With developing expertise, however, it has become clear that different tissues have different metabolic requirements and ischaemic profiles and that solutions of different compositions are required for different tissues (Belzer & Southard, 1988). A solution suitable for hepatocyte preservation may quite different from that required by kidney, skin or muscle.

Collins *et al* (1969), described a solution (**Table 1**) that they used successfully to preserve dog kidneys on ice for up to 30 hours. This solution was high in [K^+] and [Mg^{2+}]; it included glucose, heparin, procaine and

phenoxybenzamine and was phosphate buffered to a pH of 7,0 at 25^{o}C. The kidneys were flushed with 100 –150 ml of this ice cold (0 - 4^{o}C) solution and remained on iced saline for the remainder of the storage time. It was quickly found that heparin (although this is controversial), procaine and phenoxybenzamine were unnecessary and that the latter could be toxic. The rationale for the salt composition of the solution was that it mimicked intracellular fluid, although the [Mg^{2+}] is considerably higher.

The glucose and the impermeant anion, SO_4^{2-} in this solution may have been the primary reason for the success achieved with renal preservation, as these 2 substances limit fluid uptake and therefore suppress cell swelling (Green & Pegg, 1979).

The high [K^+], if present only with a highly permeable anion such as Cl^-, may potentiate cell swelling associated with depletion of energy stores. This is because the ATPase pump is designed to export Na^+ and not K^+ from the cell. Therefore, solutions high in potassium may in fact be suboptimally protective or even toxic at normothermia (Von Oppell *et al*, 1990). For this reason, high [K^+] preservation solutions potentially pose some risk if maximal cooling is not maintained throughout preservation. On the other hand, high [K^+] solutions have several theoretical advantages that may account for the extensive practical success with them. “Preservation of intracellular composition” is a frequently cited rationale for using high [K^+] levels, but the actual beneficial mechanisms are more complex (Bonventre & Weinberg, 1992). Maintaining cell K^+ during preservation avoids the work load required to pump out accumulated Na^+ during the initial stage of reimplantation, at a time when the metabolic status of the cell may be particularly precarious (Brezis *et al*, 1984).

Furthermore, high [K^+] maintains a slightly acidic intracellular pH that appears to be most favourable for resistance to injury (Bonventre & Cheung, 1985; see **Section 1.4.4 (a)**).

The inclusion of high [$MgSO_4$] in Collins solution was another controversial additive and its beneficial effects were doubtful.
When the Eurotransplant Organisation agreed on a standardised preservation solution in 1976, it chose a modified Collins solution that omitted $MgSO_4$ but had a higher concentration of dextrose and total osmolarity (see **Table 1**). This solution was named Euro-Collins and has been widely used for simple flush preservation (Squifflet *et al*, 1981). The literature, therefore, has shown several solutions to have been successful for solid organ preservation with Collins and Euro-Collins solutions being especially successful in preserving kidneys (Moukarzel *et al*, 1990; Bonventre, 1992).

Many other investigators attempted to improve on these preservation solutions, but none conferred any practical advantages over Collins, Euro-Collins (and more recently Belzer) solutions. Although these solutions were satisfactory for the kidney, none were particularly good for preservation of other organs.

With the advent of cyclosporine in the 1980's, successful transplantation of other organs necessitated the development of preservative solutions that could be used for all organs. This was accomplished in the late 1980's by the Belzer and Southard group at the University of Wisconsin. This has become the standard cold storage preservative for most organs and there has been growing evidence that is superior to other solutions (e.g. Euro-Collins), in terms of preservation times and quality (Belzer,1988; Buhren *et al*, 1991; Ferguson *et al*, 1991).

Belzer UW solution (ViaSpan, DuPont Merck Pharmaceuticals, Wilmington, Delaware 19880) contains crucial ingredients thought to be essential for an effective cold storage solution (Southard *et al*, 1990). "UWS" attracted wide interest as it was designed to address many of the major patho-physiologic events thought to contribute to both cold and warm ischaemic injury (Belzer & Southard, 1988).
It has been successfully used in preservation of a variety of organs, such as kidneys (Ploeg *et al*, 1988), heart (Swanson *et al*, 1988) and liver (Jamieson *et al*, 1988). Of more interest to the reconstructive surgeon, it has been used with good results in skin flaps (Turk *et al*, 1991) and in composite tissue models (Gordon, 1992).

The success of **University of Wisconsin Solution** (UWS) it attributable to the simulation of a highly favourable micro-environment for (normal) and ischaemic cells and to certain substrates and additives which limit ischaemic and reperfusion damage. These components (refer **Table 1**), which enhance its preservation performance include:

1. Colloids such as raffinose and lactobionic acid which act as osmotically active, non-metabolisable impermeants to suppress hypothermic cell swelling. This may be advantageous in preventing oedema of a perfused tissue, such as a muscle flap (Belzer,1973 ; Drummond & Shiel,1974 ; Halasz *et al*,1976).
2. Pentafraction or hydroxyethyl starch (HES), a modified pentastarch, provides sufficient colloid osmotic pressure to prevent expansion of the interstitial space either during organ flush or perfusion. Marshall *et al* (1991) modified this component and reported success by replacement of HES with Dextran 40. In addition to its importance as a colloid, dextran has rheological properties and may improve flow in the microcirculation.

3. Glutathione and allopurinol (free radical scavengers), to prevent cytotoxic injury from free radicals.
4. Potassium phosphate for effective hydrogen ion buffering to prevent intracellular acidosis. The high potassium level may also be advantageous in conserving energy by preventing muscle from contracting, akin to induced asystole of cardiac muscle during open heart procedures
5. Adenosine for regenerating high energy phosphate compounds, important in reperfusion and may play an important role as high energy substrates necessary for subsequent muscle function.

Furthermore, UW solution like the original Collins solution contains a high concentration of potassium, which is thought to suppress efflux of potassium from cold stored cells and thus conserve energy needed on reperfusion to restore potassium in the cell.
Belzer UW cold storage solution (UW-CSS) has an approximate calculated osmolarity of 320 mOsM and a pH of 7,4 at 25 ^{O}C.

The development of this solution has, in many studies, clarified some rudimentary concepts about organ and tissue preservation. The first is that hypothermia is a key factor as it retards the rate of tissue metabolism and therefore degradation. The second is that the physical environment of organs or the tissue created by the components should be as physiological as possible. Appropriate additives such as colloids help suppress hypothermia-induced cellular swelling. Thirdly, certain biochemical agents help the cell to restore normal metabolism when reperfused, such as glutathione and allopurinol, which are involved in scavenging free radicals (refer **Section 1.4.1**).

Two of these pharmacological additives have been thought to make a definite difference (Southard *et al*, 1990; Biguzas *et al*, 1990) and warrant further discussion.

Adenosine is added to provide purine substrate for resynthesis of ATP. Adenosine is initially converted to AMP by adenosine kinase (AK), an ATP-requiring enzyme. Since AK shows substrate-dependent inhibition, low concentrations of adenosine are more efficiently metabolised.
It is becoming increasingly appreciated that both ATP and adenosine activate multiple signalling mechanisms. This occurs via cell surface receptors in both tubular and vascular cells, so that effects on haemodynamics and cell work loads, as well as effects on repair processes, may play a role in protection provided by purine supplementation (Paller *et al*, 1990).
In a study of liver preservation, adenosine was the only clearly beneficial pharmacological additive to UWS for cold storage (Marshall *et al*, 1990). Adenosine, which regenerates ATP, also contributes towards energy homeostasis and may be very important for muscle metabolism and energy repletion following ischaemia. Indeed, Vary *et al* (1979) have suggested that depletion of adenosine is responsible for the inadequate energy supply for the metabolic processes in the cell and that it preceded mitochondrial damage as the indicator of cell death.

Glutathione is depleted during both cold and warm ischaemia. It is felt that reduced glutathione (GSH) or one of its component amino acids, glycine, are protective against lytic cell damage occurring during ischaemia i.e. provide a degree of membrane stabilisation. In addition to these direct cytoprotective effects, GSH or glycine may enhance endothelial cell activity (Bonventre & Weinberg, 1992).

In one study however, a UW variant containing adenosine but not glutathione provided as much protection as the complete formulation, which suggests that glutathione was not necessary (Marshall *et al*, 1990).

The remaining components of the original UWS, insulin, dexamethasone and penicillin, do not appear to be necessary for preservation of kidney or liver and, presumably other tissues (Biguzas *et al*, 1990).

As generally true for studies on tissue preservation, successful clinical application has outpaced study of all mechanistic issues, but sufficient data is available to draw reasonable conclusions about the components responsible for the success of UWS. It is not only a theoretical choice for a perfusion study but has been shown in previous work to be of benefit.

Angel *et al* (1993) showed improved skin flap survival following pre-ischaemic perfusion with UWS at room temperature. Although Gordon *et al* (1992) showed beneficial effects of UWS by perfusing **composite tissue**. Its use in muscle flaps has been discussed in **Sections 1.4.2** and **1.4.4.**

An analysis of this data suggested that this solution may be the “best” currently available “off-the-shelf” preservation solution. UWS was therefore considered as the solution of choice for the muscle flap model used in this current study.

Table 1: Composition of preservation solutions

COMPOSITION	COLLINS *	EURO-COLLINS**	UWS ***
COLLOIDS (g/l)			
hydroxyethyl starch			50
FINAL ELECTROLYTE CONCENTRATION (mmol/l)			
	115	115	125
potassium	10	10	30
sodium	15	15	
chloride	30		5
calcium	30		5
magnesium			
IMPERMEANTS (mmol/l)			
lactobionate			100
raffinose			30
osmolality (mOsm/l)	320	355	320
BUFFER			
KH_2PO_4 [HPO_4]	57,5	57,5	25
HCO_3^-	10	10	
pH (at 25°C)	7,0 – 7,3	7,0 – 7,3	7,4
OXYGEN-FREE RADICAL SCAVENGERS (mmol/l)			
glutathione			3
allopurinol			1
ENERGY SUBSTRATES (mmol/l)			
adenosine			5
glucose	126	194	
ADDITIVES			
insulin (u/l)			40
dexamethsone (g/l)			8
penicillin (u/l)			200 000

* - from Collins, 1969; ** - from Johnson, 1983; *** - from Southard *et al*, 1990

1.4.7. Quantification of Skeletal Muscle Necrosis – a Grading System of Ischaemia

A review of the current literature has shown mixed and conflicting data regarding perfusion of experimental flap models. Results have been mixed, partly due to differences in flap model, animals tested, temperature of ischaemic storage, method of perfusion and type of perfusate.

Other important reasons for such incomparability of results may be incomparable experimental models and a lack of a uniform grading system of ischaemia. A review of the literature has shown that very few accurate quantification methods of muscle necrosis exist.
Chachques *et al* (1985) described the quantification of muscle ischaemia based on the histochemical enzyme stain, nitroblue tetrazolium (NBT), which stains viable muscle.
Labbe *et al* (1988) further describe techniques of quantifying post-ischaemic muscle necrosis based on the nitroblue tetrazolium (NBT) and ^{99m}technetium pyrophosphate staining of viable muscle. The NBT test has been used in other muscle studies (Hickey *et al*, 1992; Waugh *et al*, 2000).

Although popular, the NBT test has certain inherent problems. This test relies on reduction of tetrazolium salts by NAD/H, NADP/H, cytochromes or flavoproteins through the action of tissue diaphorase or dehydrogenase enzymes. Absent staining occurs when these enzymes become inactive following cell death. Areas of staining were either traced out freehand (Morris *et al*,1993) or subjected to computerised planimetry (Blebea *et al*,1987).
False positive nitroblue staining can occur if adequate reperfusion does not take place (Factor *et al*, 1981).
Calcium-mediated reperfusion injury destroys dehydrogenase activity and washes out free dehydrogenase from living cells. False negative staining

could occur if living myocytes have active dehydrogenase but depleted substrates (NAD, NADP) (Labbe *et al*, 1988). These events may be particularly applicable in this current experimental model as an ex-vivo situation exists without reperfusion of circulating blood and where the effects of perfusion – reperfusion of a preservation solution are in question. Although this technique is relatively simple and inexpensive, we felt that there are inherent inaccuracies as only a gross appreciation of ischaemic damage is obtained, which demonstrates established ischaemia or cell necrosis. The critical ultrastructural features of early ischaemia are, of course, not demonstrated.

We have investigated quantitative enzyme histochemistry using a variety of propriety enzyme systems and have abandoned these techniques in favour of the more accurate combined light and electron microscopic features described below. In our hands, enzyme histochemistry had inherent problems of variable staining of type 1 and 2 muscle fibres (even in normal muscle), difficulties in quantifying early mitochondrial changes and inaccuracies even with computer analysis of varying reflectance patterns.

Light microscopy has long been used in studying the effects of ischaemic on skeletal muscle. Clarke (1946), applied tourniquets to the legs of rabbits and rats and documented the corresponding histologic findings with time. After only 2 hours of ischaemia, the transverse striations of many fibres were accentuated and after 4 hours, some fibres were beginning to fracture into discs at the Z line, with preservation of striation.

Later changes showed reduced stainability of Q and Z bands with progressive loss of the banding pattern or cross striations, which was reduced in proportion to the disorder of chemistry. Waxy or hyaline change

represented a further, delayed breakdown of the fundamental architecture of the myofibrils. Evidence suggests that granular change, in the absence of waxy or hyaline degeneration, may characterise milder and reversible degrees of damage. If, in the course of the disease process, the ischaemia is progressive with massive necrosis, there is a period when the destroyed fibres have not yet been exposed to autolysis and phagocytosis. This process is known as Zenker's hyaline degeneration, seen as a homogeneous, structureless, faintly acidophilic change in the sarcoplasm. The muscle nuclei appear pyknotic and the striations disappear. Death of the muscle fibre is not established until structural collapse occurs. These changes, indicative of progressive ischaemia, are well described and accepted as histopathologic criteria of ischaemic damage.

In their study, Gordon *et al* (1992) evaluated muscle ischaemia based on similar features reported by Harman (1947). This system is one of the few quantitative light microscopic grading systems. It includes cross striations, non-homogeneous eosinophilic staining of the cytoplasm and alteration of the nuclear-to-cytoplasm ratio.

We have modified this system and have included a broader spectrum of cytoplasmic changes. Furthermore, we have abandoned nuclear-to-cytoplasmic ratios, as even under high power, we feel it is impossible to accurately measure this relationship. We therefore include nuclear pyknosis as features of nuclear damage, as this is easy to perform and correlates with the degree of progressive nuclear insult.
Using light microscopic criteria on ischaemic porcine muscle, Morris *et al* (1993) documented swollen nuclei, separation of myocytes by intercellular oedema, myofibrillar disruption and occasional fragmentation. These changes were only reported qualitatively and no precise grading system

mentioned. Furthermore, we feel that these changes are extremely difficult to accurately assess on light microscopy and we reserve these features for quantitative electron microscopic evaluation.

Ultrastructural changes of ischaemic damage represents the "gold-standard" and allows both quantification of the degree of change and detection of early features of ischaemic damage before an irreversible situation is reached.

Gregory & Mars (1992) assessed sequential morphological changes on an ultrastructural level after an ischaemic insult. They reported glycogen loss, intermyofibrillar oedema, mitochondrial damage and myofibrillar disorganisation. These changes were documented only as a qualitative assessment, without applying strict quantitative criteria.

On the other hand, Morris *et al* (1993) used the EM grading system as proposed by Ashford Gatley & Wilson (1988). Grading of ischaemia was either reported as grade 1 (normal) through to grade 4 (lethal).

This system represents one of the few quantitative EM grading systems. However, this system is entirely based on EM changes in cardiac muscle which has several distinct ultrastructural differences (e.g. intercalated discs) as compared with skeletal muscle. Although a reasonable extrapolation to skeletal muscle can be made, inherent differences do exist.
Furthermore, this grading system does not specifically count and quantify each ultrastructural change as occurs at each grade of injury.

We have therefore proposed a new ischaemic grading system based on 10 overall parameters - a combination of 3 histopathologic and 7 ultrastructural

features of ischaemic damage. These features are well accepted criteria of varying degrees of ischaemic insult, which have been extensively used in many experimental models. We believe that our system offers certain important advances.

Firstly, by combining light and electron microscopic changes, a more complete assessment of the effects of ischaemia is achieved. The electron microscopic features are therefore weighted as these changes are more important than light microscopic changes. This system offers a quantitative analysis applicable specifically to skeletal muscle, which is easily reproducible, simple and comprehensive.
Furthermore, it offers the opportunity for assessing early, potentially reversible ischaemic damage.
The grading system of skeletal muscle ischaemia is described in detail in **CHAPTER 2 - MATERIALS AND METHODS**.

CHAPTER 2 - MATERIALS AND METHODS

2.1. ANIMAL MODEL AND SURGICAL PROTOCOL

The *rectus femoris* muscle in the New Zealand White rabbit was used as the skeletal muscle flap model. A total of 10 flaps were raised using 5 healthy adult New Zealand White rabbits of mixed sexes. Weights ranged from 4,15 kg to 5,0 kg with an average weight of 4,44 kg.

All surgical procedures were carried out using aseptic techniques in the Central Animal Service operating theatres at the University of the Witwatersrand Medical School, Johannesburg, South Africa. Anaesthetic procedures and general care of the animals was conducted under the supervision of a qualified veterinarian. The study was approved by the University of the Witwatersrand Ethics Committee (refer **Section 2.12**.)

The experimental animal was anaesthetised using 60 mg/kg ketamine ("Ketalar" - Parke Davis Pharmaceuticals) and 4 mg/kg zylazine HCl ("Rompun" 2% - Bayer Pharmaceuticals). Anaesthesia was maintained for the duration of surgery using repeated bolus doses of these anaesthetic agents as necessary. The animal was placed in a suitable restraint and the limb to be operated on was shaved, cleaned and draped. Bilateral longitudinal incisions were made in the groins and dissection performed to expose the *rectus femoris* muscles, the femoral vessels and the vascular pedicle to the muscle.

For each experimental animal, the *rectus femoris* muscle to be perfused (labelled **"TEST MUSCLE")** was chosen randomly and the corresponding contralateral *rectus femoris* muscle served as the control (labelled **"CONTROL MUSCLE**"). Each rabbit therefore served as its own control.

2.2. RADIOGRAPHIC STUDY

A radiographic study was performed in order to study the vascular anatomy of the femoral artery and the branches to the *rectus femoris* muscle flap as seen on angiography.

Two New Zealand White rabbits were used in this study. Using a similar anaesthetic technique and surgical protocol as above, the *rectus femoris* muscle flap was dissected and isolated on its vascular pedicle as an island flap. In the first animal ("Rabbit 1"), the femoral vessels were ligated (using stainless steel "Liga" clips) just proximal to the vascular pedicle of the *rectus femoris* muscle. In the second animal ("Rabbit 2"), only the pedicle to the muscle flap was ligated, with the femoral vessels remaining uninterrupted.

A midline laparotomy was then performed in each animal and the aorta was identified and canulated. A distal aortagram was then performed by flushing with 25ml "Conray" contrast medium under screening control. X-ray films of the groin were then taken approximately 2-3 seconds later, to include the femoral vessels and the vascular pedicle of the muscle flap. The animal was then sacrificed with an intravenous bolus of sodium pentobarbitone ("Euthanaze", Centaur Laboratory) using a dosage of 100mg/kg.

The angiographic findings are described in **Chapter 3 – RESULTS, Section 3.2.** and are illustrated in **Plates 3.2 A** and **B**.

2.3. DISSECTION OF THE RECTUS FEMORIS MUSCLE FLAP AND ITS VASCULAR ANATOMY

Via a longitudinal incision extending from the inguinal region in the groin to the knee, the *rectus femoris* muscle was identified and dissected free. Following division of its origin on the pelvic bone and its insertion into the patella, the muscle flap was mobilised as an island pedicle flap on its vascular pedicle. This part of the dissection was performed under magnification and included meticulous identification of the proximal and distal superficial femoral artery and veins and their respective branches and tributaries. This is described and illustrated in detail in **Chapter 3 – RESULTS, Section 3.1, Plates 3.1 A – C.**

2.4. MANIPULATION OF THE MUSCLE FLAP

Prior to division of the proximal vessels, the femoral artery was canulated (see **Plate 2.4**) and baseline recordings of the mean systemic arterial pressures was obtained using a pressure transducer ("Stathan Medical") with strain gauge and AC coupler connected to a recorder ("Bioscience"). The subsequent perfusion pressures of the perfusion pump (see **Sections 2.5**. and **2.6.)** were continually adjusted throughout the experiment so as to approximate these readings.

The proximal and distal femoral vessels were divided between "Liga" clips, thus harvesting the free muscle flap on its vascular pedicle (**Plates 3.1A** to **C**). This muscle flap (TEST) was then manipulated as described in **Sections 2.5., 2.8.** and **2.9.**

A similar dissection was performed in the opposite groin and the contralateral *rectus femoris* muscle harvested as a CONTROL. The muscle was wrapped in a saline soaked-gauze swab and placed in a container and stored at 4 ^{o}C in a thermostatically controlled refrigerator. The temperature of the refrigerator was monitored using an electronic thermocouple and was maintained at the same temperature as that of the perfusate solution.

In one set of experiments, the CONTROL muscle was divided - one half was stored at 4 ^{o}C as above and the other half was stored using the same method but at 25 ^{o}C in a thermostatically controlled water bath. This served to document the effects of warm ischaemia on the muscle.

After harvesting of the muscle flaps, the animal was sacrificed using intravenous sodium pentobarbitone, 100mg/kg ("Eutha-naze", Centaur Laboratory).

2.5. PERFUSION APPARATUS (Plates 2.5.1 and 2.5.2)

A renal perfusion pump ("Waters MOX - 100DCM" ,Waters Instruments, Inc., Rochester, Mn., USA) was used for pulsatile perfusion of the muscle (TEST) flaps which were perfused with oxygenated, cooled "University of Wisconsin" solution (UWS) for up to 24 hours (**Plates 2.5.1** and **2.5.2**).

The “Waters MOX - 100” renal preservation system is a gravity-flow system in which circulating perfusate returns by gravity from the perfused tissue or organ through a venous reservoir, flow meter, enclosed membrane envelope, arterial reservoir and to the inlet of the pulsatile pump.

The perfusate is then pumped through a heat exchanger and bubble-trap and returned to the perfused tissue or organ. The entire perfusion apparatus consists of 2 units:

2.5.1. The Pump - Transport module and console unit (Plate 2.5.1):

This unit delivers the perfusate at a variable rate (strokes / minute – see **Plate 2.5.1** pulse rate control knob, PRC), which was adjusted to simulate the rabbits own heart rate at rest (75 to 80 pulsations / min.). The perfusion pressure is controlled by " stroke volume" controller (labelled SVC) and displayed on a dial registering in mmHg. This was adjusted according to the mean arterial pressures measured by canulating the femoral artery as described earlier. The perfusion pressures were continuously recorded as discussed in **Section 2.6**. below. Therefore, perfusion pressures and rate were delivered to the flap at physiological values.

The pump has a refrigeration unit (labelled RU) which was thermostatically controlled (labelled TC) and delivers cooled perfusate at a desired temperature (in this case, 4 ^{o}C). This was also monitored continuously using a thermocouple (refer **Section 2.7**.).

Further features of the pump included attachments for inlets so that perfusate may be delivered at controlled partial oxygen pressure. A controlled flow of oxygen (O_2) and carbon dioxide (CO_2) [see **Plates 2.5.1** and **2.5.2**] enters the membrane oxygenator which is surrounded by a sealed membrane oxygenation chamber (labelled MOC) on the cassette. By regulating the flow of CO_2 and O_2, the system's pH and $p0_2$ can be controlled . These were also monitored at regular intervals (refer **Section 2.7**.).

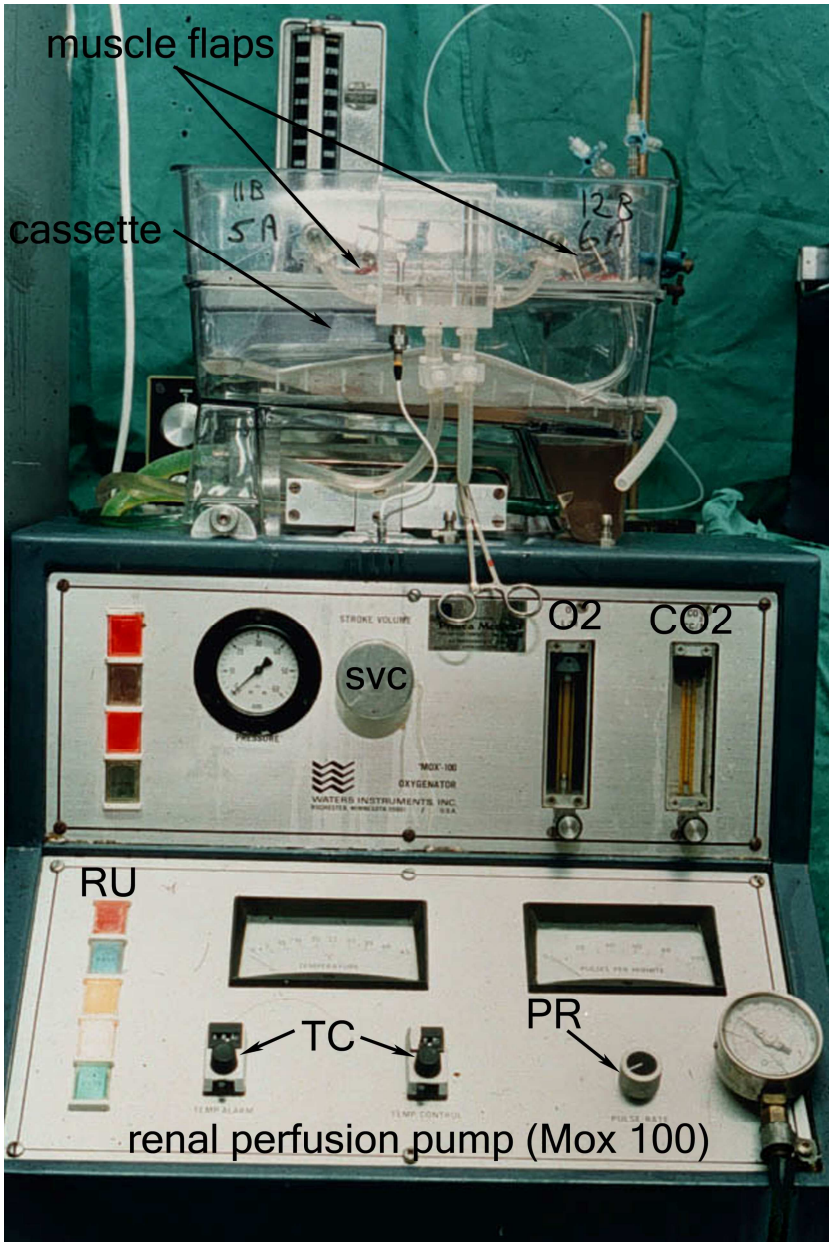


Plate 2.5.1: Renal preservation system – Waters MOX-100DCM perfusion pump (and cassette)

SVC – stroke volume control knob; PR – pulse rate control knob;

TC – temperature control knobs (upper and lower limits); O_2 – oxygen flowmeter (l/min); CO_2 – carbon dioxide flowmeter (l/min)

2.5.2. The cassette (Plate 2.5.2):

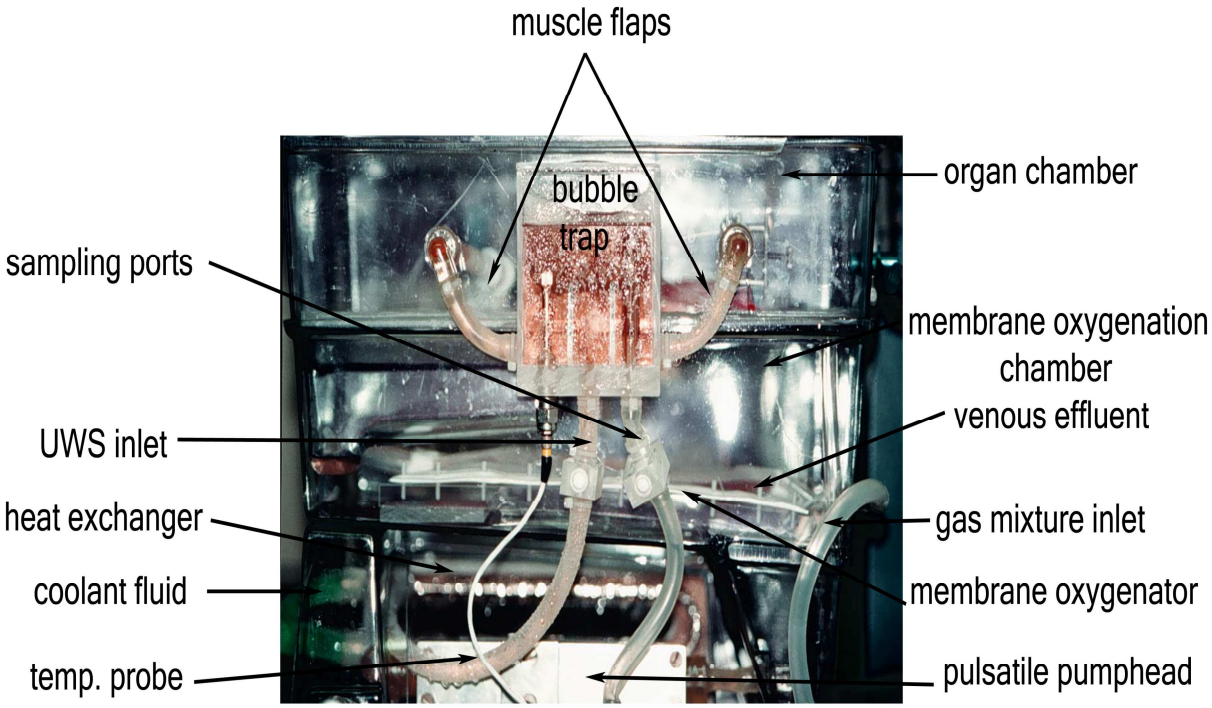
This pre-sterilised disposable plastic cassette contains a complete circulatory system and mounts onto the pump unit.

A bubble-trap ensures separation of all trapped air bubbles from the perfusate before they reach the perfused tissue or organ. Special fittings are provided in the bubble trap and flow lines to permit sampling of the perfusate.

Pulsatile flow is provided by a pair of occlusive non-traumatic vinyl plastic valves placed in series within a vinyl tube. The chamber between the valves is alternatively compressed and released by a cam-driven arm. The pulsatile pump is provided with a variable pulse rate control and mechanical control for adjusting the stroke volume.

The cassette was primed with approximately 300 ml UWS perfusion solution which was allowed to circulate for several hours for equilibration.
Prior to connecting the muscle flap in the cassette, baseline readings of perfusate temperature, pH, PO_2, PO_2 were obtained.

The organ chamber contains a mesh tray on which the muscle flap was placed and 2 inlet ports, which provided the cooled, oxygenated perfusate.



Waters Instruments Mox 100 Cassette Module

Plate 2.5.2: Renal preservation system – Waters MOX-100DCM perfusion cassette

2.6. PERFUSION TECHNIQUE (Plates 2.6 A to C)

The arterial and venous ends of the vascular pedicle of the muscle flap was cannulated with silicone tubing, using magnification so that endothelial damage was minimised. A “Portex” silicone tube (size No. 140) was used on the artery and a No. 200 for the venous end.

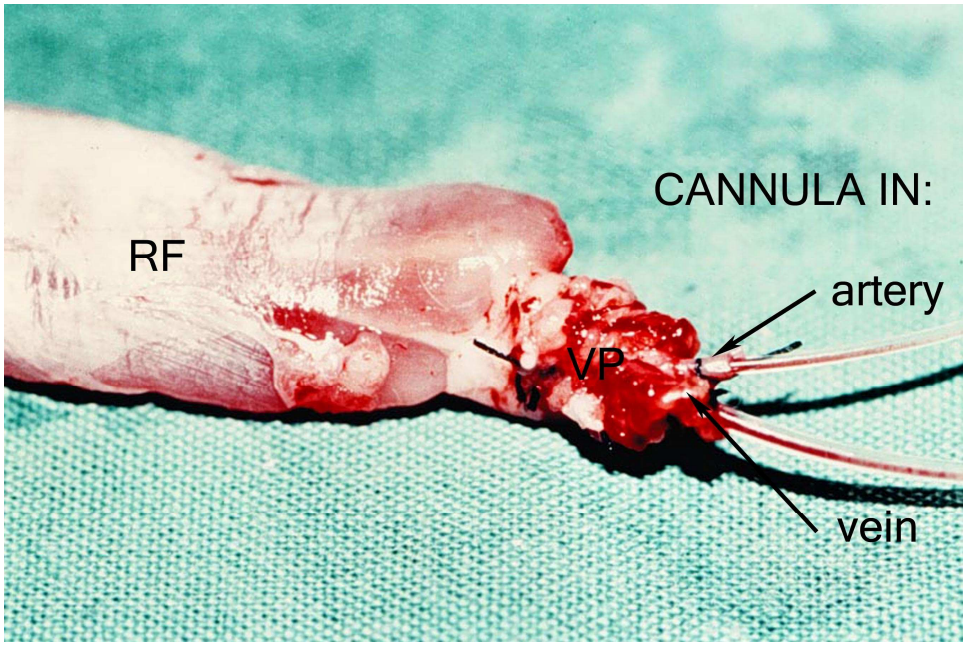


Plate 2.6 (A): cannulation of *rectus femoris* muscle flap after harvesting
RF = *rectus femoris* muscle flap; VP = vascular pedicle

The cannulated arterial end of the isolated muscle flap (RF) was connected to the perfusion pump and a strain gauge coupler by means of a 3 way tap, so that continuous recording of perfusion pressures could be obtained throughout perfusion (via inlet port - see **Plates 2.6 (B)** and **(C)**). The perfusion pressure was maintained at approximately 100mmHg, as determined by the mean arterial blood pressure recorded in the animal's femoral artery prior to harvesting the muscle.

The effluent from the cannulated venous end of the muscle flap was collected at 2 hourly intervals and the flow rates recorded.

In addition to pulsatile perfusion, the muscle was kept moist and cooled by topical cooling with the same perfusion solution which washed over the muscle from inlet port B (**Plates 2.6 (B)** and **(C)**). The isolated muscle flap can be seen resting on porous membrane within the perfusion cassette. **Plate 2.6 (C)** demonstrates a similar system but utilising a simultaneous double flap perfusion technique.

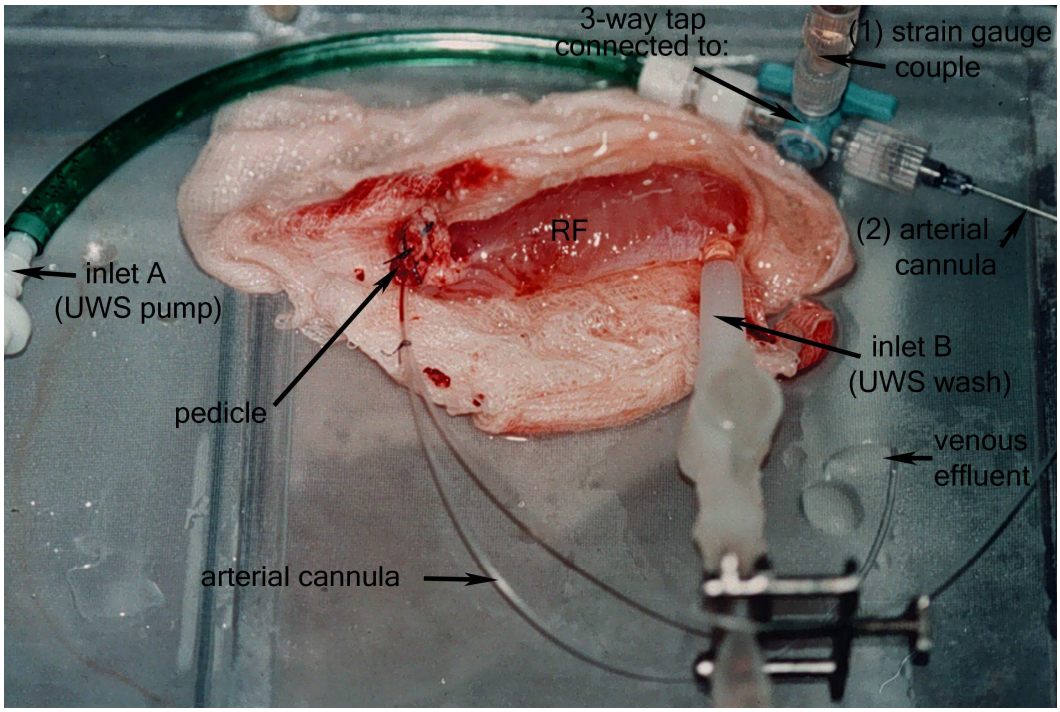


Plate 2.6 (B): Single flap perfusion technique – perfusion of the muscle flap and topical cooling

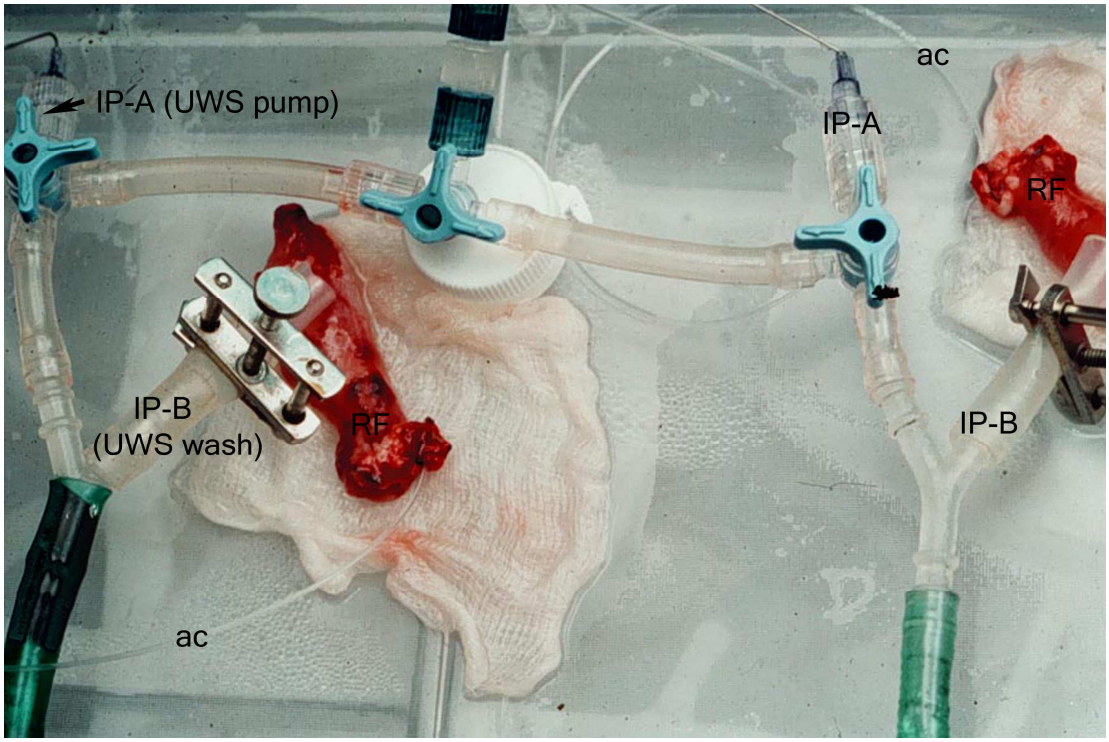


Plate 2.6 (C): Simultaneous double flap perfusion technique– perfusion of the muscle flap and topical cooling
RF – rectus femoris muscle (1 or 2); IP-A – inlet port A; IP-B – inlet port B; ac – arterial cannula (1 or 2)

2.7. MONITORING

The sampling protocol regarding various aspects of flap monitoring is outlined in **Table 2**.

2.7.1. Temperature:

The temperature of the perfusate was monitored using an electronic thermocouple and maintained at approximately 4 ^{o}C. The temperature of the refrigerator in which the control muscle was stored was also monitored and maintained at a similar temperature to that of the perfusate.

2.7.2. Gas Analysis:

The pH, PO_2, PCO_2 of the perfusate were determined initially after equilibration using a blood gas analyser. The average partial pressures of oxygen was maintained at 280 to 300mmHg. The gases were further monitored at regular intervals as per sampling protocol.

2.7.3. Perfusion Pressures:

The perfusion pressures as delivered by the perfusion pump apparatus were monitored continuously throughout the 24 hours of perfusion by means of a pressure transducer ("Stathan Medical") with strain gauge and AC coupler connected to a recorder ("Bioscience Instruments"). Pressures were adjusted so as to approximate the mean arterial blood pressure in the anaesthetised rabbit as discussed above.

2.7.4. Flow:

Flow was monitored from the effluent of the canulated vein of the flap's vascular pedicle ("venous output"). The rate of flow was measured as volumes per unit time, as calculated by the weight of the effluent using a "Sartorius" balance.

Table 2: **Sampling protocol** for perfused and stored muscle

TIME INTERVAL (minutes)	**INTERVENTION**
15	perfusion pressures, adjusted as necessary
30	perfusion pressures flow rates
60	as above plus: temperature control (perfusate and fridge) gas analysis
120	above plus: tissue sampling (refer **Section 2.9**.)

2.8. FLUORESCEINE STUDY

In order to determine whether the perfusate solution had perfused the entire muscle, a fluoresceine dye injection test was performed on one rabbit *rectus* femoris muscle.

1 ml of fluoresceine was added to the perfusate using the same study design as above. After 5 minutes, the perfusion was halted and the muscle was sectioned in both the longitudinal and transverse planes. This was then illuminated with a Wood's lamp and the green-yellow fluorescence of the muscle fibres noted (refer **Plates 3.4 (A)** and **(B)**).

2.9. TISSUE SAMPLING

Sampling of both TEST and CONTROL muscles were performed during perfusion and storage respectively at 0, 4, 8, 12, 18 and 24 hours.
A 1 cm cylinder of muscle was isolated between two silk sutures and then harvested atraumatically by excision on either side of the suture. The muscle was then placed on a wooden spatula and the sutures tied behind the spatula. This technique preserved resting length and thus prevented contraction artefact.
All tissue samples were immediately processed (as described below) and were submitted to Dr. V. Fabian and Mrs. G. Norman at the South African Institute for Medical Research, Johannesburg, South Africa, for histological and electron microscopic analysis.

Biopsy tissue for **light microscopy** were immediately placed in neutral formalin fixative (pH 7,4). Paraffin embedded tissue was sectioned and stained with Haematoxylin and Eosin.

Tissue for **electron microscopy** was fixed in 3% glutaraldehyde and 4% formaldehyde (Karnovsky,1965) in Millonig's phosphate buffer. After rinsing in buffer alone, post-fixation was carried out in 1% osmium tetroxide, buffered in Millonig's solution for 1 hour. Following further rinsing in buffer, the tissue was dehydrated in a graded series of ethanol's prior to infiltration with Spurr resin. Polymerisation took place for 3 hours at 100 ^{o}C. Thin sections (60nm) were stained with alcoholic saturated uranyl acetate and alkaline lead citrate at pH 13,5. Sections were mounted on copper grids and examined using a transmission electron microscope ("Hitachi H600").

2.10. ANALYSIS AND EXPERIMENTAL OBSERVATIONS OF PERFUSED AND STORED MUSCLE

2.10.1. Light Microscopic Assessment (LM)

Each of the prepared histological specimens (TEST and CONTROL) were evaluated blindly and independently by Dr.'s V. Fabian and G. De Aguiar. A modification of the grading system as described by Harman (1947), was used.

One hundred muscle fibres in ten randomly selected high power fields at 400x magnification (i.e. 10 muscle fibres in each high power field), were examined for the presence of changes reflecting ischaemic damage in 3 areas:

1. **cross striations** or overall banding pattern of the muscle fibre - being either preserved (normal), or wavy or absent (abnormal).
2. **cytoplasmic changes** - being either normal or showing the abnormal features of homogenous or granular necrosis.
3. **nuclear changes** - being either normal or showing the pathological features of pyknosis or karyolysis (absent nucleus).

The normal muscle fibre was arbitrarily assigned 1 point and the muscle fibre with ischaemic changes was assigned 0 points. Therefore, for each "ischaemic feature" (1, 2, or 3 above) , a total score of 100 was possible if no features of ischaemic damage were present. If all 3 features were normal, an overall score of 300 was possible. Thus, scores could range from 0 (indicating presence of all 3 categories of ischaemic damage in all of the fibres) to 300 points (indicating no structural changes). This was divided by 3 to give an overall percentage of normal versus abnormal or ischaemic muscle fibres (**Table 3**). Typical examples of these light microscopic criteria are depicted in **Plates 3.5.2 (A) – (D)**.

Table 3: Grading system for muscle ischaemia on **light microscopy**

LIGHT MICROSCOPIC FEATURE	**SCORE** (points/fibre)
1. BANDING PATTERN (cross striations)	
normal (preserved)	1
abnormal (wavy or absent striations)	0
2. CYTOPLASM	
normal (unchanged)	1
abnormal (homogenous or granular necrosis)	0
3. NUCLEUS	
normal (unchanged)	1
abnormal (pyknotic or absent)	0

TOTAL SCORE OBTAINED BY ANALYSING 100 MUSCLE FIBRES

2.10.2. Electron Microscopic Assessment (EM)

Each of the electron micrographs on the prepared samples were examined blindly and independently by 2 electron microscopists and 1 pathologist.

A quantitative electron microscopic grading system was based on 7 changes which reflect of ischaemic damage and /or necrosis. These changes were selected as they provide an overall picture of the effects of ischaemia on skeletal muscle and show a wide spectrum of changes ranging from early (mitochondrial) to late (architectural) changes.

The features were recorded in each of the micrographs and ranged from an arbitrary number of 10 points (if the feature was entirely normal) to 0 points (indicating the presence of ischaemic damage). An entirely normal muscle fibre could thus obtain a score of 70 points, whereas the totally ischaemic fibre scores zero. The points obtained overall were converted to percentages. The electron microscopic grading system is shown in **Table 4.**

Table 4: Grading system for muscle ischaemia on **electron microscopy**

ELECTRON MICROSCOPIC FEATURE	**SCORE** (points/fibre)
1. OVERALL ARCHITECTURE OF FIBRE	
normal (architecture retained; preserved Z / I bands)	10
abnormal (architecture disrupted)	0
2. SARCOPLASMIC RETICULUM	
tubules undilated	10
tubules dilated	0
3. MYOFIBRILLAR ORGANISATION	
normal organisation	10
disorganisation (focal or general)	0
4. MYOFIBRILLAR LOSS	
no loss	10
focal loss	5
extensive loss	0
5. GLYCOGEN CONTENT	
normal	10
decreased	5
absent	0
6. BASEMENT MEMBRANE	
intact	10
disrupted	0
7. MITOCHONDRIA	
normal appearance	10
slightly swollen, internal cristae intact	7,5
50% of mitochondria intact	5
degenerate	0

2.10.3. Overall Score Combining Light and Electron Microscopy (LM plus EM)

The overall percentage of survival of muscle fibres (and conversely, the overall percentage of ischaemic damaged fibres) were obtained by adding the percentage of the light microscopic scores (based on the 3 LM features) and electron microscopic scores (based on the 7 EM features). An overall global quantification of the degree of ischaemic survival or damage was obtained on the combined 10 criteria (LM plus EM). This, we believe, provides a true representation of ischaemic damage in skeletal muscle.

2.11. STATISTICAL ANALYSIS

Statistical analysis of all light and electron microscopic data (muscle survival or ischaemic damage) were conducted independently by Dr. P. Bekker and Mrs. E. Viljoen of the University of the Witwatersrand Biostatistics Department.

The experiment was conducted and analysed as a two-factor within design, the two within main effects being PERFUSION (with levels perfused and not perfused) and TIME (with levels 0, 4, 8, 12, 18, 24 hours).

The response variable was percentage muscle survival as assessed by light and electron microscopy respectively and as a combination of the two (overall muscle survival). Inter-group quantitative data was analysed on a computer with appropriate analysis of variance (ANOVA), so as to provide an analysis of the total data.

When TIME was found to be significant (i.e. some of the levels are different from one another), contrasts were tested to assess pairwise differences between individual times. Also of interest was to compare the levels of PERFUSION at the specific times. This was done using a Student's t-test.

2.12. ETHICAL CONSIDERATIONS

This study was submitted to and approved by the Animal Ethics Committee of the University of the Witwatersrand, Johannesburg, South Africa (Ethics clearance No. 92/112/2b).

The study was conducted in the University of the Witwatersrand Central Animal Service under the supervision of a qualified veterinarian, Dr. S. Maeder.

All surgery was performed under general anaesthesia. After harvesting the muscle flap, the animals were humanely sacrificed by means of an intravenous bolus of sodium pentobarbitone, 100mg/kg ("Eutha-naze", Centaur Labs).

STRICTLY CONFIDENTIAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:	92	112	2b

APPLICANT: Dr G de Aguiar

DEPARTMENT: Plastic Surgery, Medical School

PROJECT TITLE: Extra-corporeal *in-vitro* perfusion of skeletal muscle flaps improves ischaemic survival

SPECIES	NUMBER	DATE OF EXPIRY
Rabbits	5	September 1994
FOR A PILOT STUDY		

The use of these animals is subject to AESC Guidelines for the use and care of animals, to the procedures specified in the application form, and to:

NIL

SIGNED _____ DATE 8/9/92

(Chairman: Animal Ethics Screening Committee)

Figure 2: Animal Ethics Clearance Certificate

CHAPTER 3 - RESULTS

3.1. ANATOMY OF THE RECTUS FEMORIS MUSCLE FLAP AND ITS VASCULAR SUPPLY (Plates 3.1 A – C)

Plate 3.1(A) demonstrates the typical dissection of the muscle flap in the groin. The inguinal ligament (IL) marks the superior aspect of the groin, above which is the fat pad in the groin. The central area of **Plate 3.1(A)** shows the anterior thigh region and the most inferior aspect is the knee area. The *rectus femoris* muscle (RF) has been mobilised after the insertion at the knee has been divided. The muscle flap has been reflected superiorly (thus showing its posterior aspect) to demonstrate the vascular anatomy. The superficial femoral vessels (FVS) and the vascular pedicle (VP) to the flap are demonstrated by white markers.

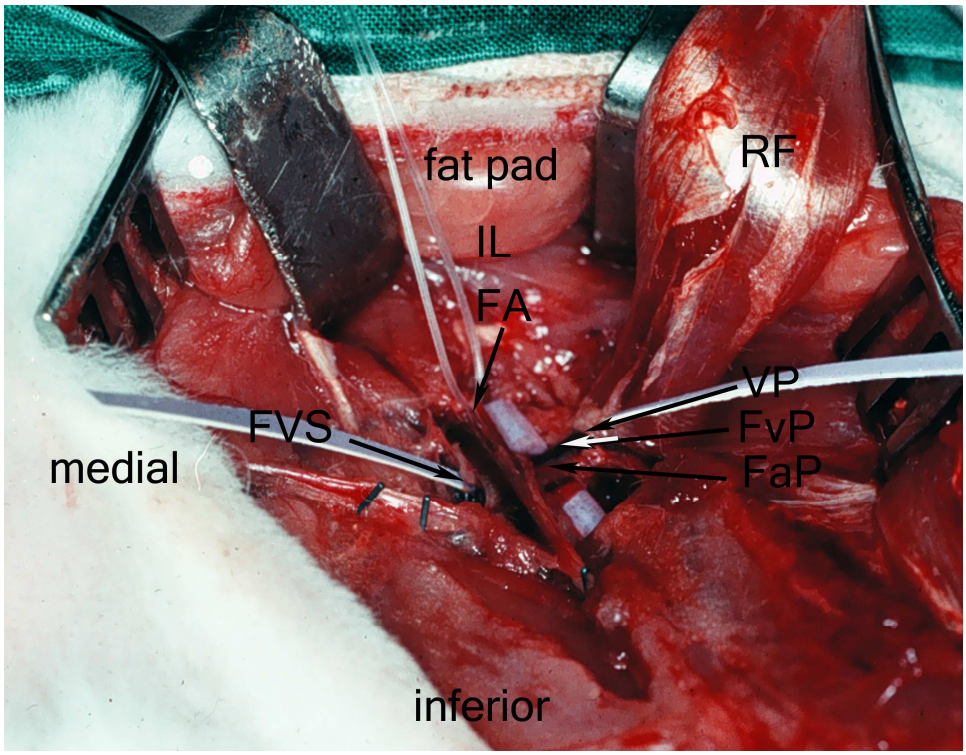


Plate 3.1(A): Dissection of *rectus femoris* muscle flap

The vascular pedicle to the *rectus femoris* muscle was identified and carefully preserved - see **Plates 3.1 (B) and (C)**.

Plate 3.1(B) demonstrates the vascular pedicle to the muscle. A white marker (VP) points to the vascular pedicle to the *rectus femoris* muscle flap (RF). White visibility background material has been placed under the pedicle.

The pedicle to the flap was noted to leave the femoral vessels on their lateral aspect at a variable distance (approximately 3 to 10mm) below the inguinal ligament (IL). A second white marker (FVS) points to the superficial femoral vascular bundle. A clear polyethylene vessel loop has been placed around the femoral artery (FA). The femoral artery and vein then give branches and tributaries respectively to the muscle flap (FaP and FvP respectively), which forms the vascular pedicle to the flap. These vessels then entered the muscle on its medial aspect (see **Plate 3.1(C)**) and continued along the length of the surface of the muscle as an axialised system of vessels (AV), sending perforating branches into the depths of the muscle.

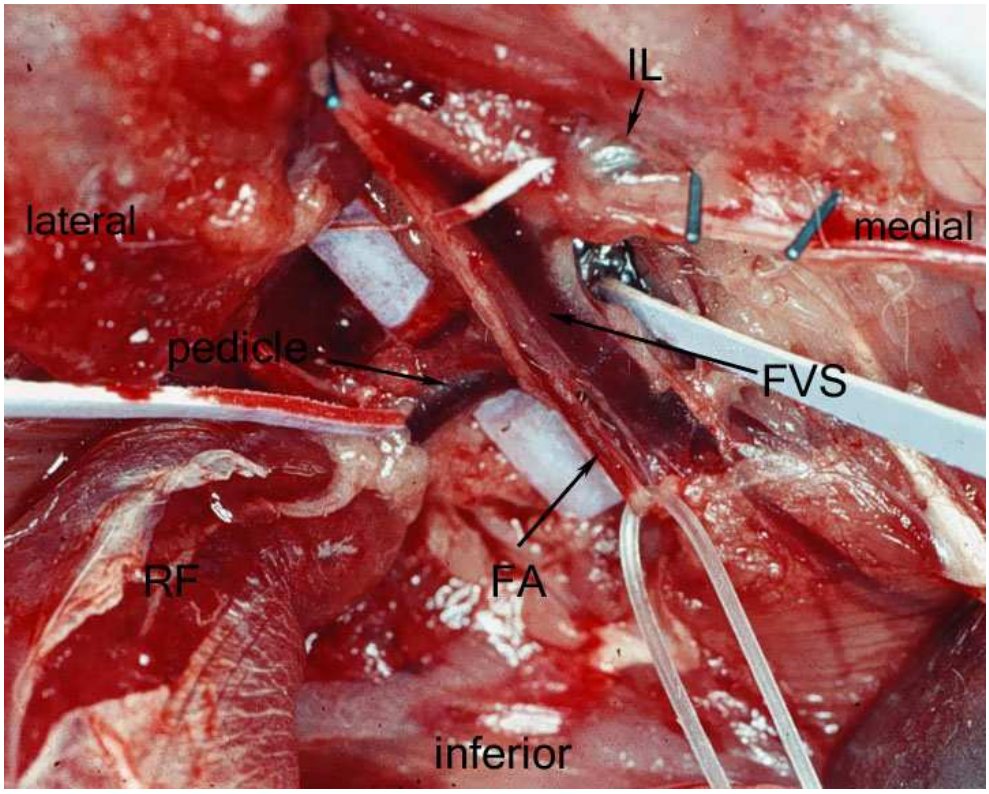


Plate 3.1(B): Femoral vessels and vascular pedicle of the muscle flap

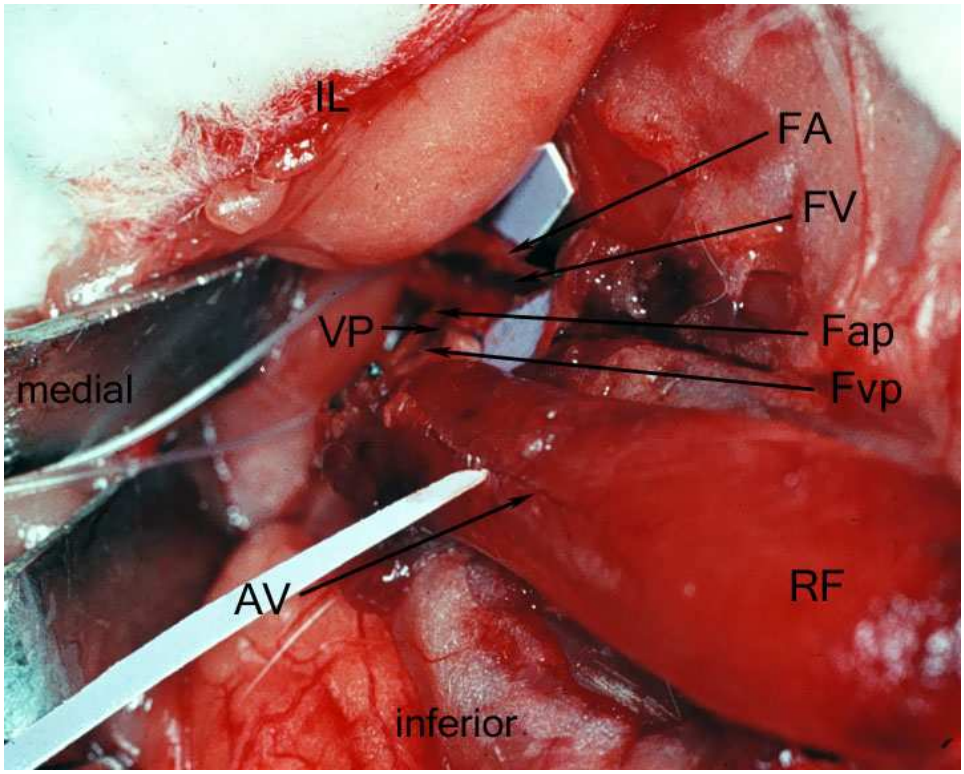


Plate 3.1(C): Axial vascular system of *rectus femoris* muscle

The proximal part of the superficial femoral artery and vein were controlled separately on vessel loops, whilst the distal vessels were ligated 1 to 2 cm below the vascular pedicle to the muscle (refer **Plates 3.1(B)** to **(C)**). Sparing the pedicle, ligation of the various branches of the femoral system was performed using "Liga" clips. These branches included:

1. A supero-lateral branch entering the muscle near its insertion
2. A medial branch entering the adductor muscles, approximately 15 to 20 mm below the inguinal ligament.
3. A lateral branch distal to the pedicle, entering the remainder of the quadriceps group of muscles.
4. A smaller twig leaving the inferior aspect of the pedicle itself and supplying the closely applied adductors.
5. A variable system of vessels around the posterior aspect of the proximal femoral artery and vein, several millimetres distal to the inguinal ligament.

3.2. RADIOGRAPHIC STUDY (Plates 3.2 A and B)

In the first study (**Plate 3.2(A)**), the right femoral vessels were isolated and then ligated just distal to the inguinal ligament. The left femoral vessels were undisturbed and served as the control. The flush aortagram showed no distal flow beyond the ligated femoral artery at this level. Although a single medial branch of the femoral artery remained patent, an adequate collateral circulation is not present and no distal runoff occurs as compared to the control side. If the *rectus femoris* muscle flap was to be isolated by ligation of the femoral vessels at this level, the distal limb would have been rendered ischaemic.

In the second study (**Plate 3.2(B)**), the vascular pedicle to the *rectus femoris* muscle flap was dissected and the muscle flap isolated as an island pedicle flap. The direct vascular pedicle to the muscle was ligated with the distal femoral artery remaining patent. The contrast medium perfused the distal limb with distal runoff remaining essentially the same as the control side. This demonstration has shown that, provided the vascular pedicle to the *rectus femoris* muscle was ligated only, distal vasculature remains undisturbed and the lower limb would not be rendered ischaemic.

This has bearing in future *in-vivo* experimentation and is of relevance if microsurgical transplantation requires the animal not to be sacrificed. Further research could involve re-attaching the perfused muscle into the contralateral groin by microvascular replantation so as to determine the effects after *in-vivo* reperfusion. If the distal limb is to remain viable, the harvest of the muscle flap and/or anastomosis must take place at the level of the vascular pedicle to the muscle flap and not at the femoral vessel level.

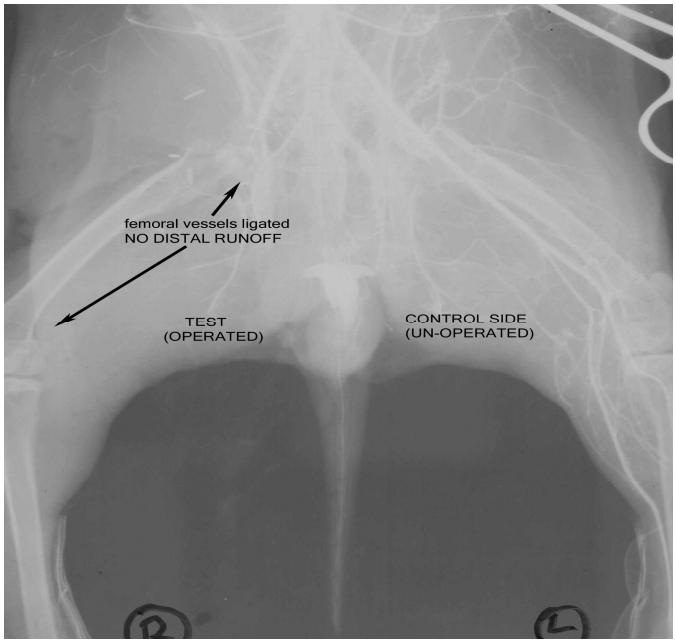


Plate 3.2(A): Radiographic study of arterial anatomy to lower limbs – ligation of right femoral artery distal to inguinal ligament

NOTE: poor distal runoff of right lower limb

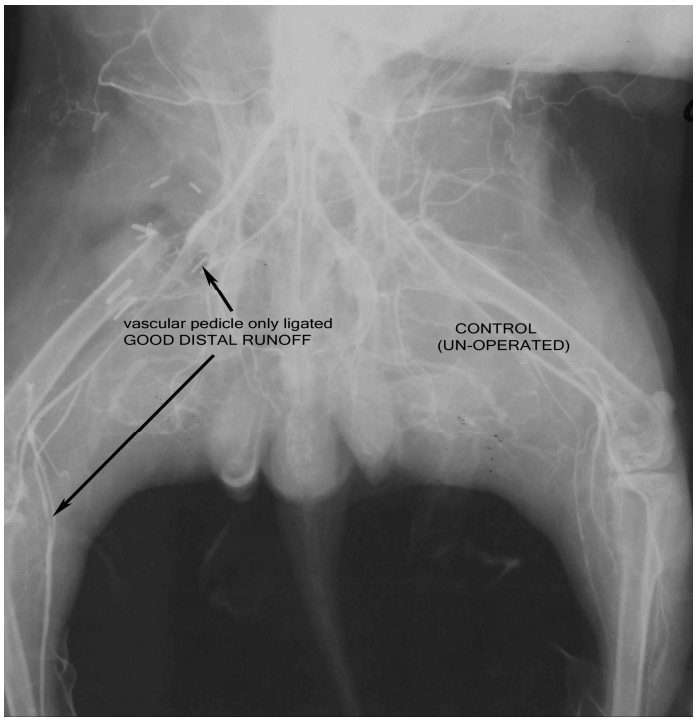


Plate 3.2(B): Radiographic study of arterial anatomy to lower limbs – isolated right-sided ligation of pedicle to muscle flap

NOTE: preserved distal runoff to entire lower limb

3.3. PERFUSION STUDIES

3.3.1. Gas Analysis

It was generally noted that PO_2 and PCO_2 remained stable throughout the 24 hours of perfusion, largely due to the buffering capacity of UWS. The pH values of the 5 perfused flaps at 24 hours, averaged 7,18 with a range of 7,12 - 7,23. The pH of the UW solution is made to 7,40 at 25 ^{o}C and is a phosphate buffered system. In this series of experimental studies conducted with cooled UWS (4 ^{o}C), baseline pH readings of 7,30 were found universally. The possible protective role of mild acidosis has been discussed (**section 1.4.4.**).

3.3.2. Flow

Flow rates were measured over the 24 hour period in the 5 perfused flaps. The average flow rate of perfusate from the flap measured 0,610 ml/minute (range 0,243 - 1,010 ml/min).

3.4. FLUORESCEINE STUDY (Plates 3.4 A and B)

The *rectus femoris* muscle is shown in its entirety prior to sectioning (**Plate 3.4 (A)**) as it appeared under Wood's ultraviolet illumination. The entire surface shows the characteristic yellowish-green colouration, confirming that the perfusate has reached the superficial areas of the muscle. The sectioned muscle (**Plate 3.4 (B)**) confirmed that the solution had perfused the entire muscle. This suggested that all muscle units or fibres are perfused to a similar degree and are therefore susceptible to the effects of any perfusion solution to a similar degree. Furthermore, this study illustrates that muscle biopsies contain a representative sample regardless of the site of tissue biopsy.

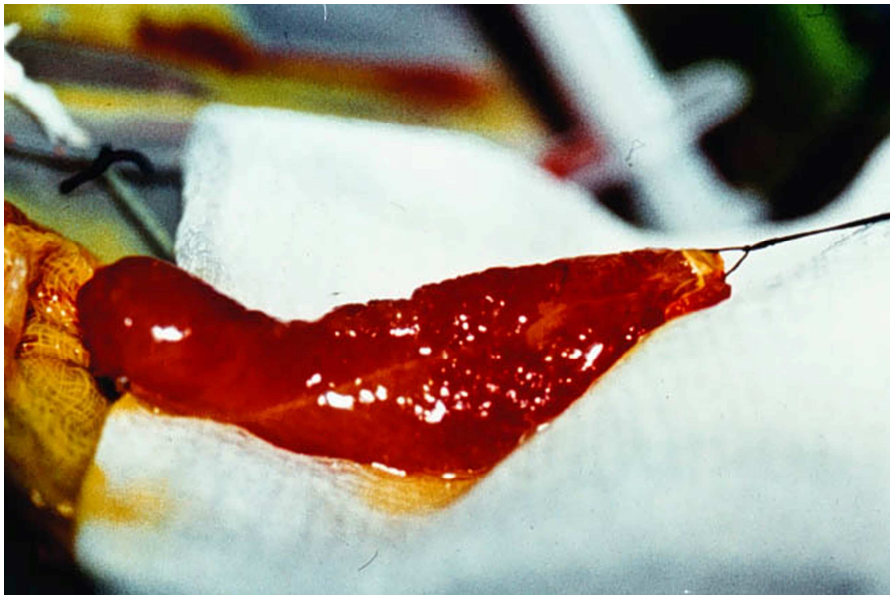


Plate 3.4 (A): Fluoresceine study – intact *rectus femoris* muscle flap

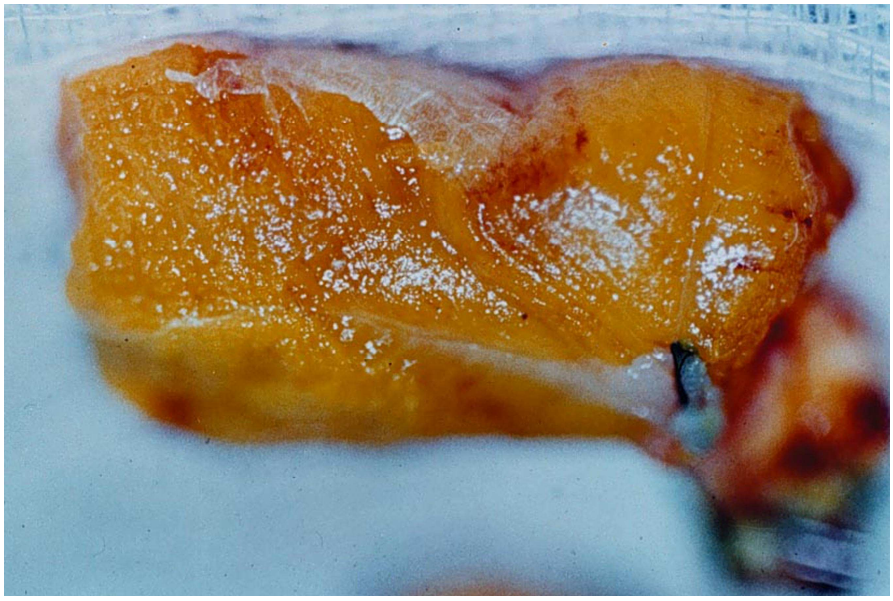


Plate 3.4 (B): Fluoresceine study – *rectus femoris* muscle flap: sectioned

3.5. DEMONSTRATION OF ISCHAEMIC MUSCLE GRADING SYSTEM AND MUSCLE FLAP STUDIES

3.5.1. Gross Pathology

The overall gross appearance of the muscle flaps after 24 hours of cold perfusion with UWS, simple cold storage and storage at 25 ^{o}C (from left to right respectively), are shown in **Plate 3.5.1.**

The perfused flap appeared to be moist and pale pink in colour, with no overt necrosis. The flap subjected to simple cold storage at 4 ^{o}C appeared to be contracted and desiccated with an overall appearance of tissue dehydration. The flap subjected to warm ischaemia showed extreme desiccation and had a grey-white appearance representative of non-viable muscle. Although this plate shows the muscle subjected to 24 hours of warm ischaemia (25 ^{o}C), similar gross macroscopic findings were seen in muscle stored at room temperature for only 8 hours.
Although gross macroscopic appearance does not provide an accurate assessment of tissue viability, it does allow for some appreciation of the clinical overall viability of the muscle flaps.

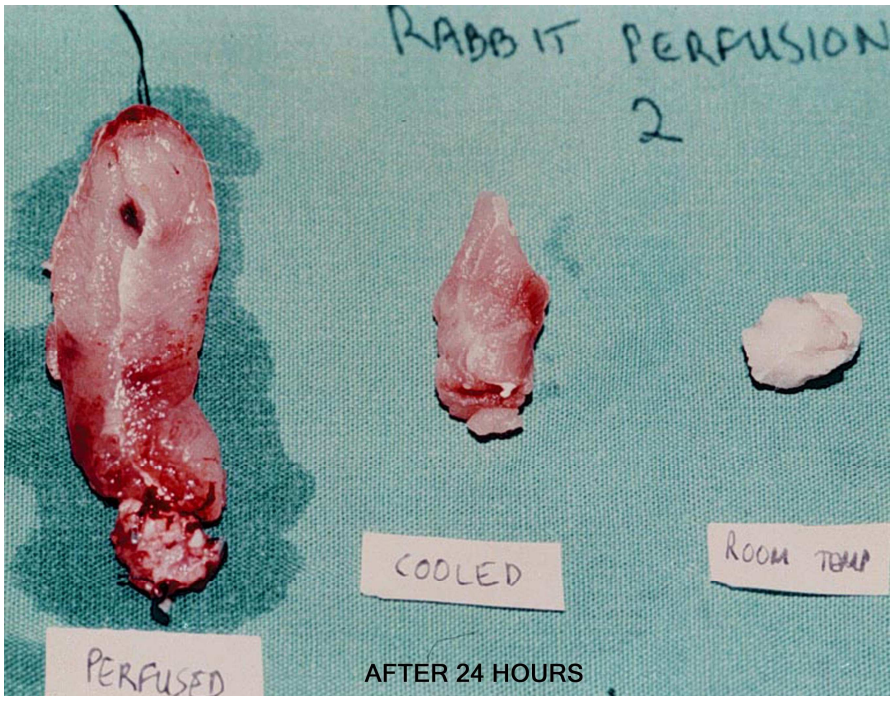


Plate 3.5.1: Gross macroscopic appearance of the perfused, cold-stored and warm-stored *rectus femoris* muscle after 24 hours

3.5.2. Light Microscopy

The percentage of muscle survival as seen on light microscopy (as analysed according to the assessment criteria detailed in **Table 3**), was assessed by means of a pairwise comparison between the TEST (perfused) and CONTROL (stored) muscle. This was performed at TIMES of 0, 4, 8, 12, 18 and 24 hours.

The means, standard deviations and P-values are tabulated in **Table 5** and illustrated in **Figure 3**.

Table 5: Percentage of muscle survival of TEST and CONTROL muscle on **light microscopy**

TIME (hour)	**TEST** % survival mean (+/- SD)	**CONTROL** % survival mean (+/- SD)	**p- VALUE**
0	100 (0)	100 (0)	1.0000
4	95,2 (4,76)	76,4 (9,56)	0,0013 **
8	85,4 (9,15)	73,4 (7,60)	0,0044 **
12	83,2 (8,07)	54,0 (8,22)	0,0090 **
18	69,8 (5,40)	43,0 (10,17)	0,0068 **
24	55,8 (5,17)	25,6 (14,59)	0,0103 **

** - highly statistically significant (at a 1% level of significance)

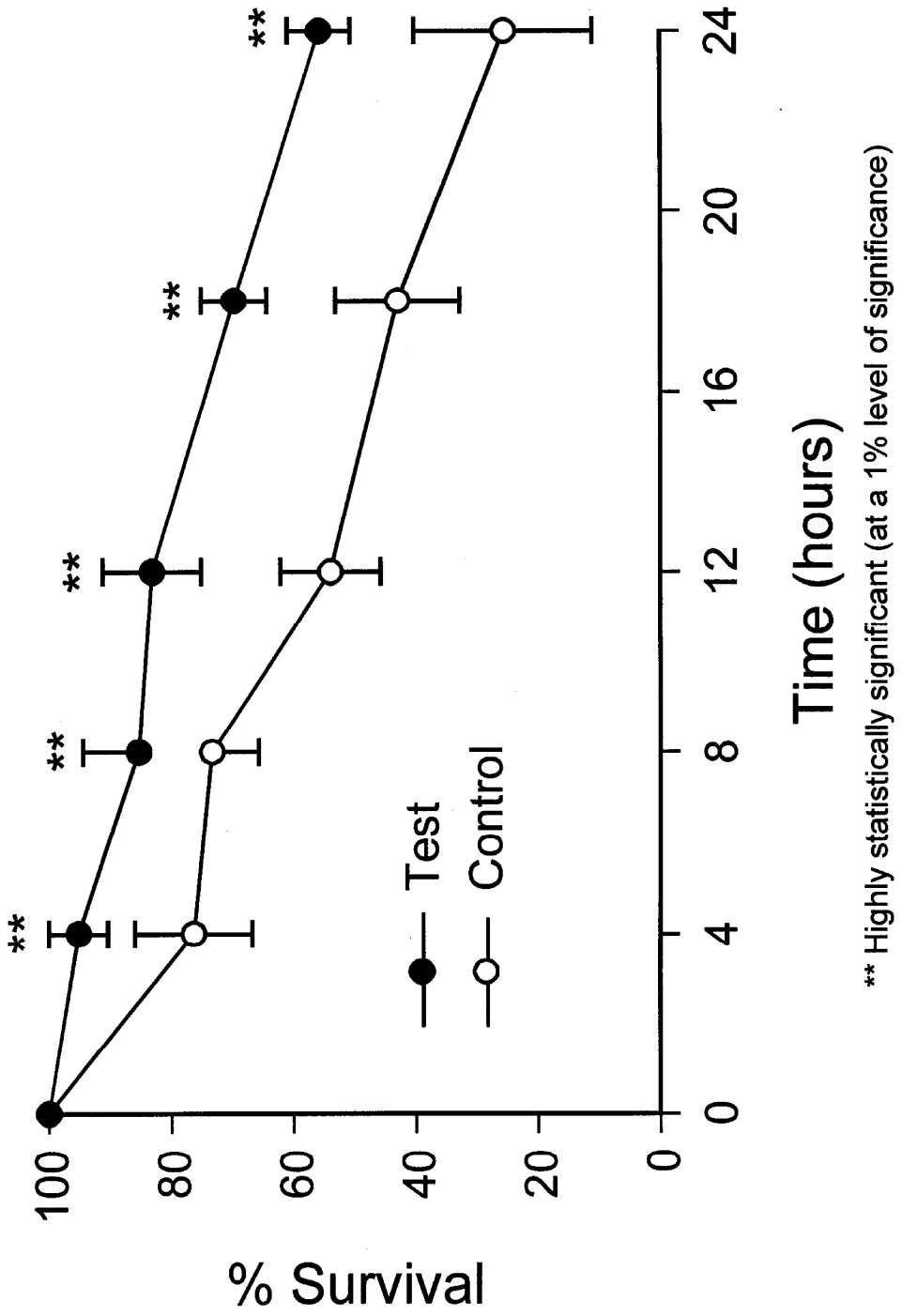


Figure 3: Percentage of muscle survival for TEST vs. CONTROL muscle on **light microscopy**

Plates 3.5.2 A - D are photomicrographs of the *rectus femoris* muscle, illustrating typical light microscopy findings at various time intervals and temperatures (Haematoxylin and Eosin staining technique, 400x).

Plate 3.5.2 (A) shows an example of a NORMAL *rectus femoris* muscle (in this case, a sectioned muscle at time interval 0 hours or pre-ischaemia).
The 3 important light microscopic features are clearly illustrated.
Note the cross striations (CS) or normal banding pattern of the muscle. These are crisp, broad and well defined.
The cytoplasm (CP) is seen to be clear and even, without a granular or diffuse homogenous appearance.
The nuclei (N) are seen to be clear and well defined and the nucleoli are visible in this micrograph.

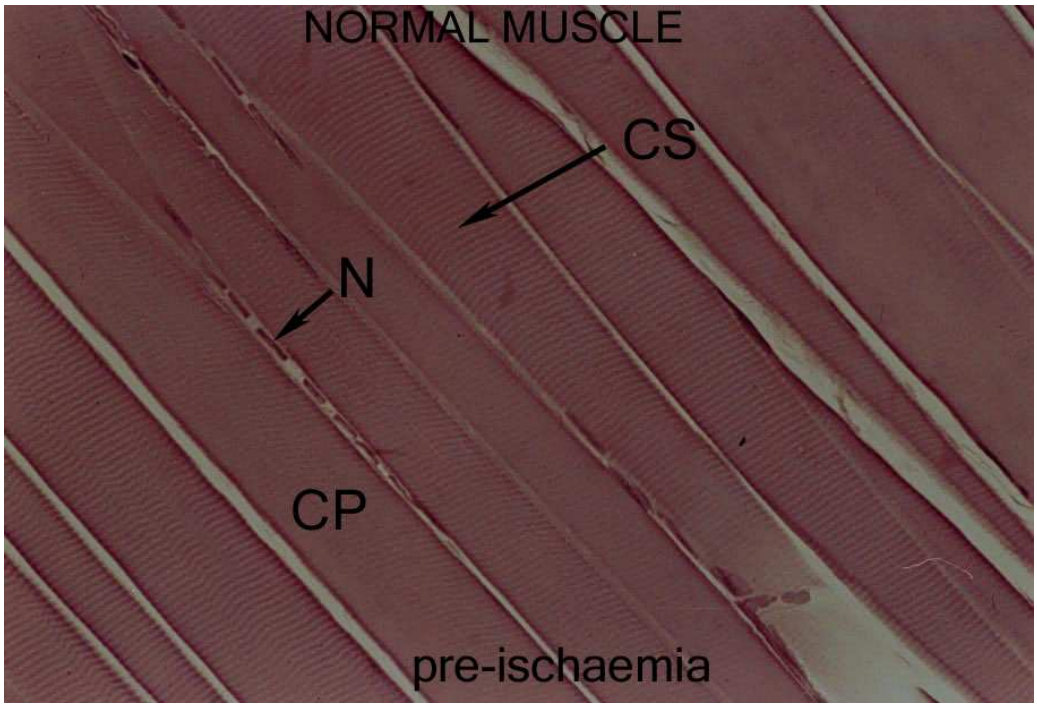


Plate 3.5.2 (A): Photomicrograph of normal *rectus femoris* muscle fibres (pre-ischaemia); H & E stain, 400x
CS – cross striations; CP – cytoplasm; N – nuclei
Plates 3.5.2 (B) and **(C)** provide interesting comparative studies of warm and cold perfused muscles at various time intervals.

Plate (B) demonstrates muscle fibres which have been subjected to 8 hours of warm ischaemia (WARM CONTROL). Although the banding pattern is preserved in some fibres, most take on a homogenous appearance (H) and a distinct granularity (G) of the cytoplasm is present. The nuclei are shrivelled and pyknotic (PN) and are absent from some fibres. This muscle is not viable.

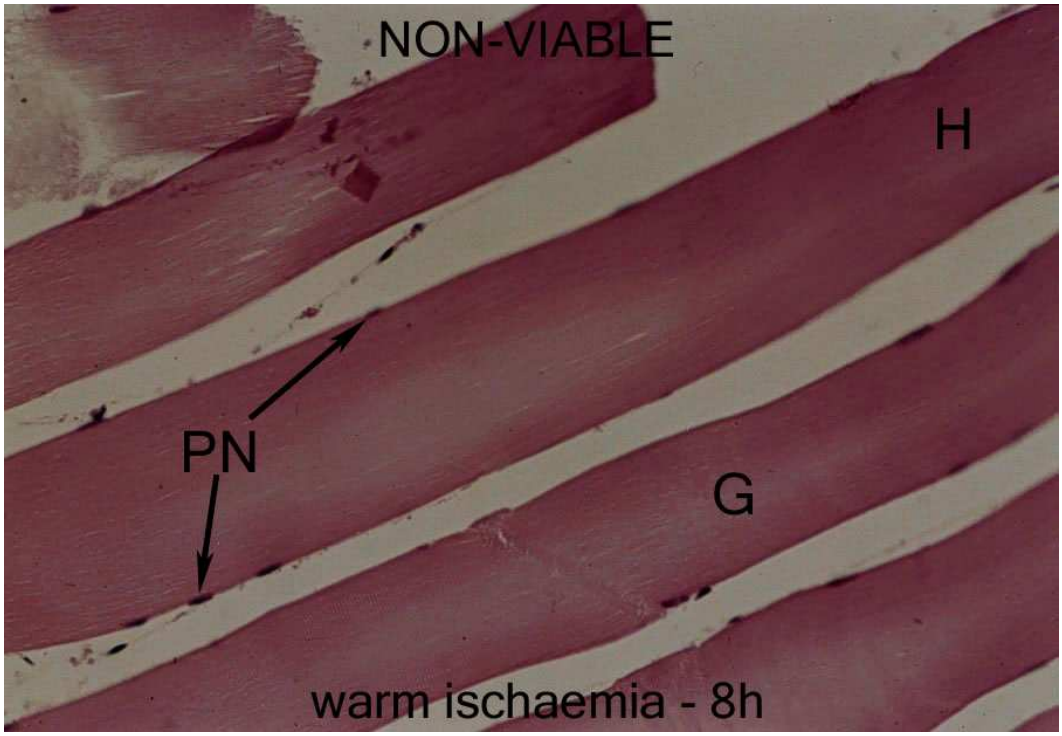


Plate 3.5.2 (B): Photomicrograph of muscle stored at room temperature (at 25^0C) for 8 hours (warm control); H & E stain, 400x

Plates C and **D** demonstrate interesting comparisons, and show muscle fibres at 24 hours for both cold stored (CONTROL)and perfused (TEST) muscles respectively.

Plate 3.5.2 (C) (cold stored, 24 hours) show all the LM features of necrotic muscle with absent or pyknotic nuclei (PN), and granular or homogenous appearance to the cytoplasm (G, H).

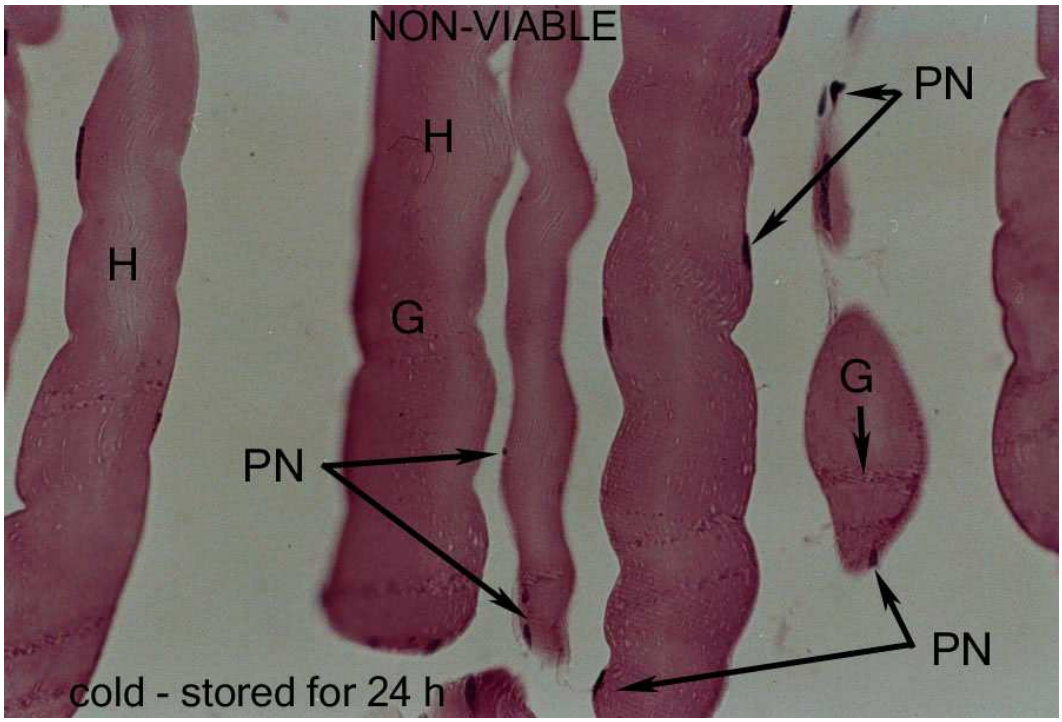


Plate 3.5.2 (C): Photomicrograph of muscle subjected to 24 hours of cold-storage (at 4^0C); H & E, 400x
H – homogenous cytoplasm; G – granular necrosis of cytoplasm; PN – pyknotic nuclei

Plate 3.5.2 (D) demonstrates typical findings of muscle that has been perfused for 24 hours. The cross striations (CS) are preserved in several fibres (60% of fibres in this high power field) with the remainder been replaced by a homogenous appearance to the cytoplasm (H). No granular necrosis (as noted in the cold stored muscle at 24 hours) was present. The nuclei are well preserved, with the nucleoli clearly visible (N). Although this muscle has been damaged by ischaemia, the presence of normal nuclei and patchy preservation of cross striations make this a viable muscle. Despite a more than doubled ischaemic time, the cold perfused muscle survives better than the muscle kept at room temperature.

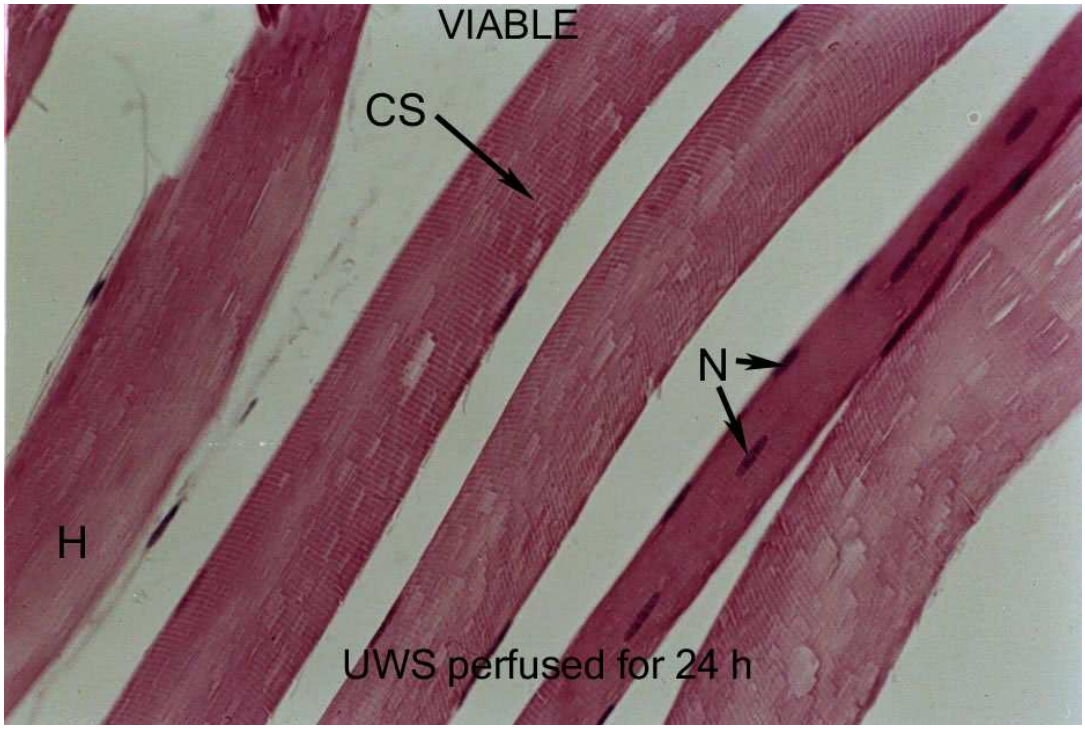


Plate 3.5.2 (D): Photomicrograph of muscle subjected to 24 hours of cold perfusion with UWS at 4^0C; H & E stain, 400x

CS - cross striations; H – homogeneous cytoplasm; N – nuclei

3.5.3. Electron Microscopy:

The percentage of muscle survival as seen on electron microscopy (as analysed according to the assessment criteria detailed in **Table 4**), was assessed by means of pairwise comparison between TEST and CONTROL muscle as for 3.3.2. above.

The results are depicted in **Table 6** and in **Figure 4**.

Table 6: Percentage of muscle survival for TEST vs. CONTROL muscle on **electron microscopy**

TIME (hours)	**TEST** % SURVIVAL mean (+/- SD)	**CONTROL** % SURVIVAL mean (+/- SD)	**P–VALUE**
0	100 (0)	100 (0)	1,0000
4	97,14 (6,40)	90,02 (8,14)	0,0345 *
8	86,44 (12,47)	82,84 (11,96)	0,3703
12	80,72 (3,17)	67,14 (4,71)	0,0374 *
18	76,42 (9,31)	55,70 (7,38)	0,0398 *
24	60,00 (5,87)	37,14 (10,28)	0,0262 *

* - statistically significant (at a 5 % level of significance)

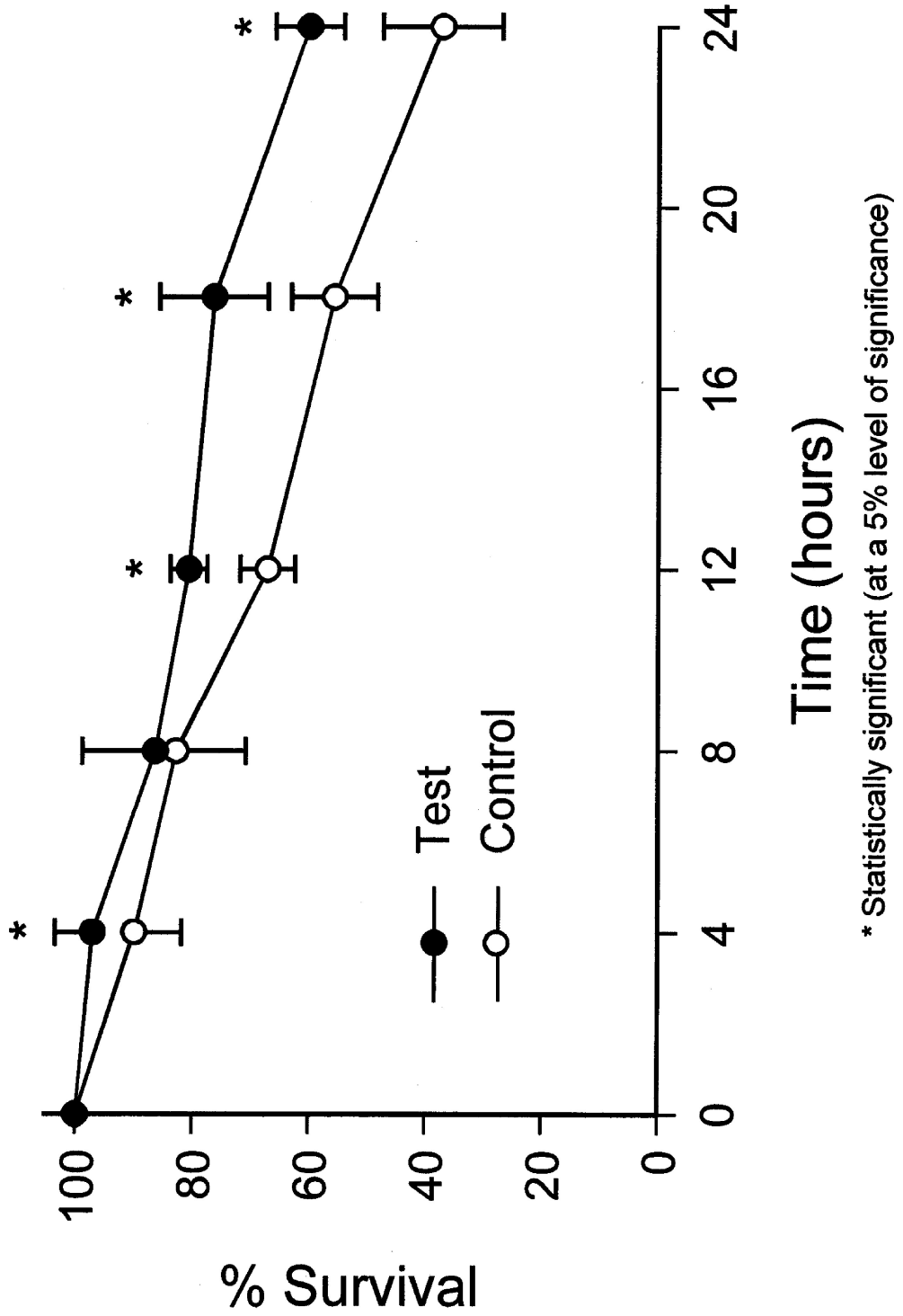


Figure 4: Percentage of muscle survival for TEST vs. CONTROL muscle on **electron microscopy**

In order to understand the ultrastructural derangement's of the myofibril when subjected to an ischaemic insult, one must first examine the normal architecture of muscle as seen on electron microscopy. The following description and diagrams (**Figures 5** and **6**; **Plate 3.5.3 (A)**) allow us to familiarise with the overall arrangement of the myofibrils and the conducting system of normal skeletal muscle.

Under the electron microscope, the arrangement of the contractile proteins and the conducting system may be seen.
The sarcomere consists of two types of myofilaments, thick and thin filaments. Each type remains constant in length regardless of the state of contraction of the muscle. The filaments are arranged in a symmetrical interdigitating manner parallel to the long axis of the myofibril. The thick filaments, which are composed mainly of the protein myosin, are maintained in parallel by their attachment to a disc-like zone represented by the M band. These thick filaments can be seen on EM as dark A bands.

Similarly, the thin filaments, which are composed mainly of the protein actin, are united in a disc-like zone represented by the Z band. These thin filaments can be seen as light I bands under the electron microscope.
The I and H bands, both areas of low electron-density, represent areas where thick and thin filaments do not overlap one another.

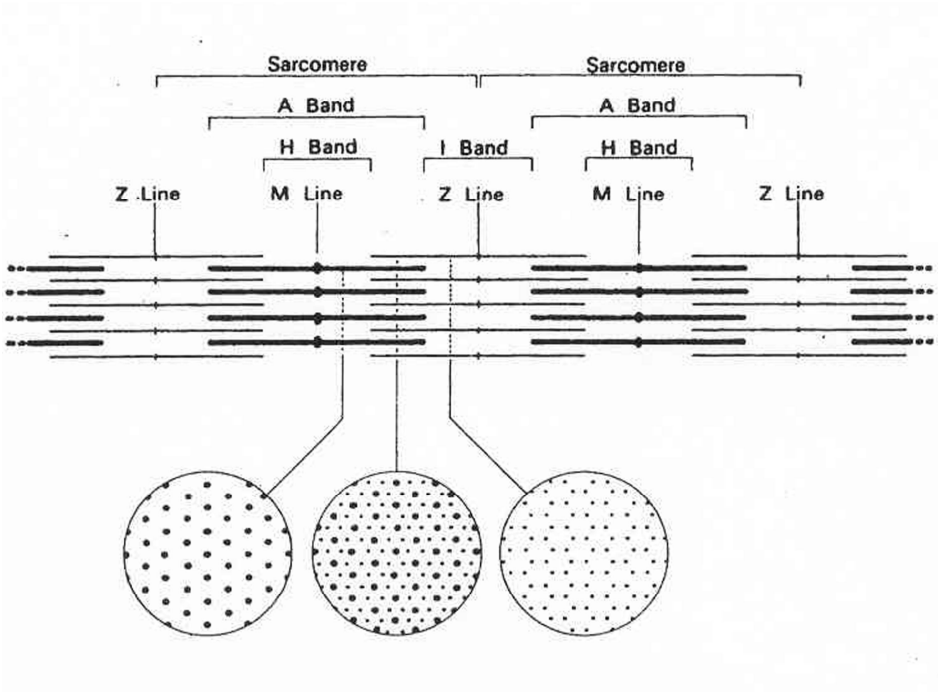


Figure 5: Overall arrangement of myofibrils and myofilaments of normal skeletal muscle (after Wheater *et al*, 1979)

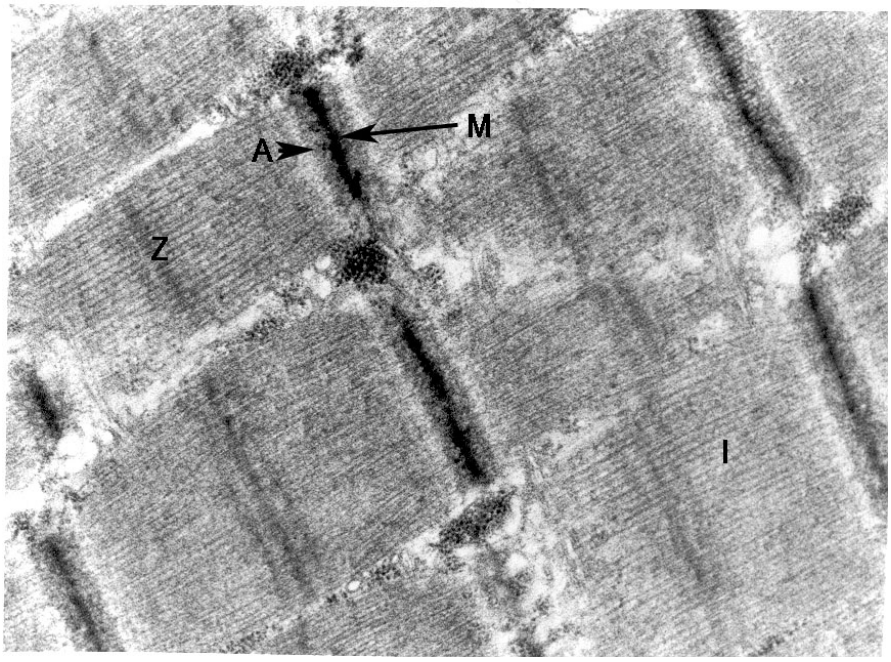


Plate 3.5.3 (A): Electron micrograph of a normal *rectus femoris* muscle; 50 000x A – dark band; I – light band; M – m line; Z – Z line

To permit synchronous contraction of all sarcomeres in the muscle fibre, a system of tubular extensions of the muscle cell membrane or sarcolemma extends transversely into the muscle cell to surround each myofibril in the region of the junction of the A and I bands. Thus, throughout the muscle fibre, there is a tubular system, the T system, the lumen of which is continuous with the extracellular space.

Closely associated, but not connected with each T tubule system, there are two complementary membrane systems derived from smooth endoplasmic reticulum. These are called sarcoplasmic reticulum. They ramify to form a membranous network which embraces each myofibril. Each T tubule, with its pair of associated sarcoplasmic reticulum elements called terminal cisternae, form a triad near the junction of the I and A bands of each sarcomere.

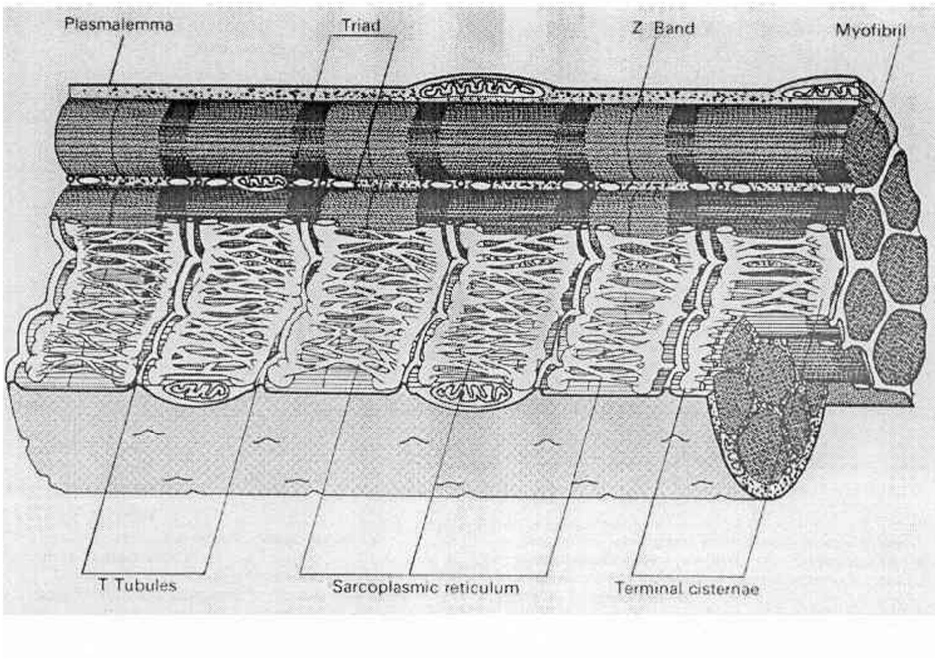


Figure 6: Architecture and conducting system of the myofibril of skeletal muscle (after Wheater *et al*, 1979)

We may now examine typical electron microscopic findings of the *rectus femoris* muscle in this study. **Plates 3.5.3 (B) – (G)** depict electron micrographs of findings of muscle subjected to varying ischaemic intervals and temperatures.

Plate 3.5.3 (B) is an electron micrograph demonstrating the normal *rectus femoris* muscle, prior to any ischaemic insult. The overall architecture is preserved, with organised myofibrils and well-defined Z and I bands.

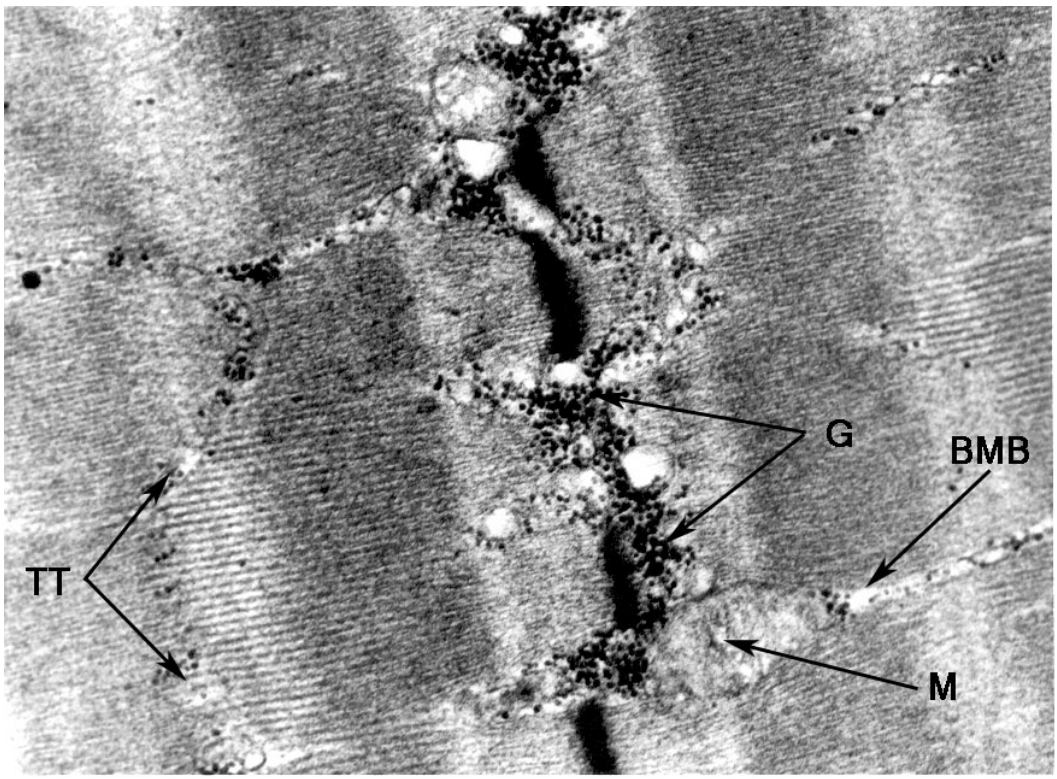


Plate 3.5.3 (B): Electron micrograph of normal muscle (pre-ischaemia); 52 500x

G – glycogen granules; M – mitochondria; BMB - basement membranes; TT – T tubules

Plates 3.5.3 (C) – (E) show the muscle subjected to various interventions but all at 8 hours of ischaemia.

Plate 3.5.3 (C) shows the cold perfused muscle (TEST). Findings are as for normal muscle as shown in **Plates 3.5.3 (A)** and **(B)**. Therefore, for periods up to 8 hours, cold perfusion has a definite preservation effect and no ischaemic damage is seen.

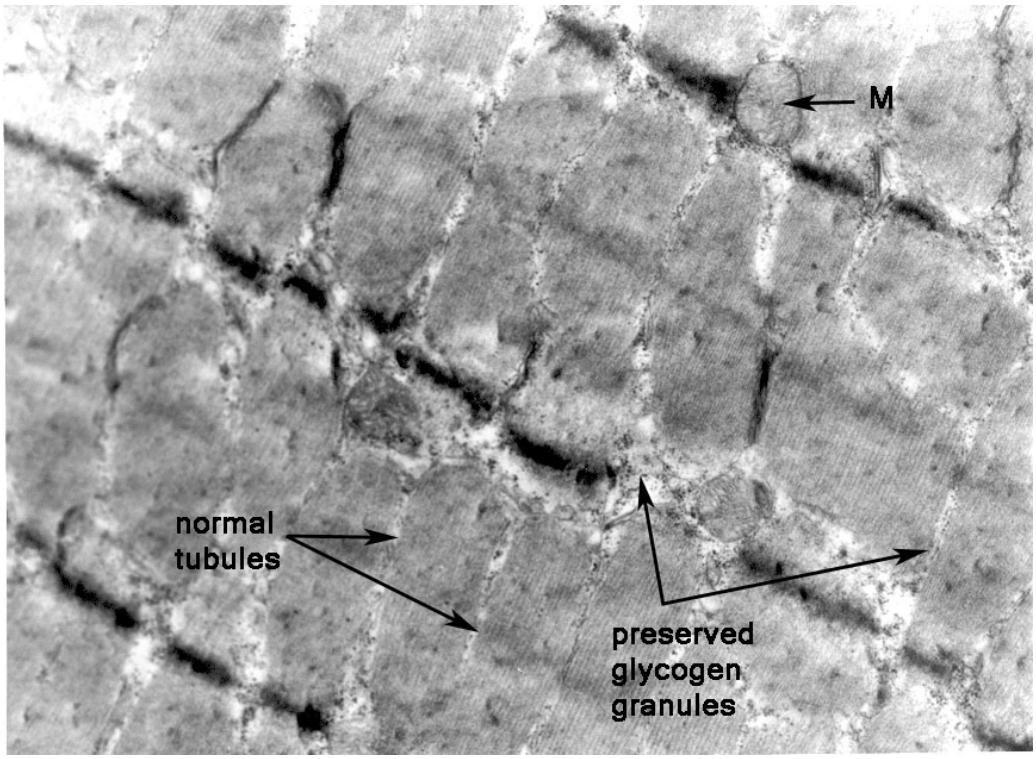


Plate 3.5.3 (C): Electron micrograph of muscle subjected to 8 hours of cold perfusion (4^0C); 50 000x

M - mitochondria

Plate 3.5.3 (D) depicts muscle subjected to 8 hours of simple cold storage (COLD CONTROL). Glycogen content is depleted and the mitochondria are swollen, with disruption of the internal cristae (IC). The overall architecture remains preserved. This muscle, although showing ischaemic changes, remains viable.

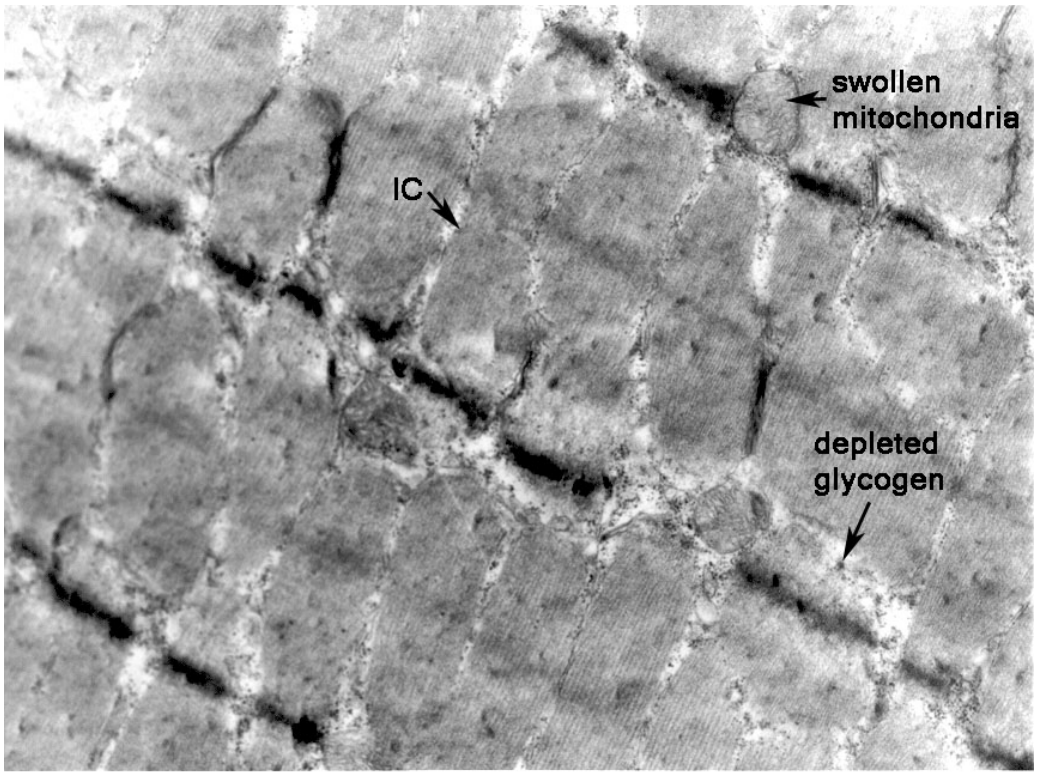


Plate 3.5.3 (D): Electron micrograph of muscle subjected to 8 hours of cold storage (4^0C); 35 000x

Plate 3.5.5 (E) depicts muscle subjected to 8 hours of warm ischaemia. The overall architecture is disrupted with disruption of the basement membrane. Myofibrils are less numerous than the cooled muscles (myofibrillar loss). Glycogen is absent and the tubules (T) of the sarcoplasmic reticulae are dilated. The mitochondria are degenerate and the black dots within them indicate severe hypoxic changes.
This muscle is non-viable.

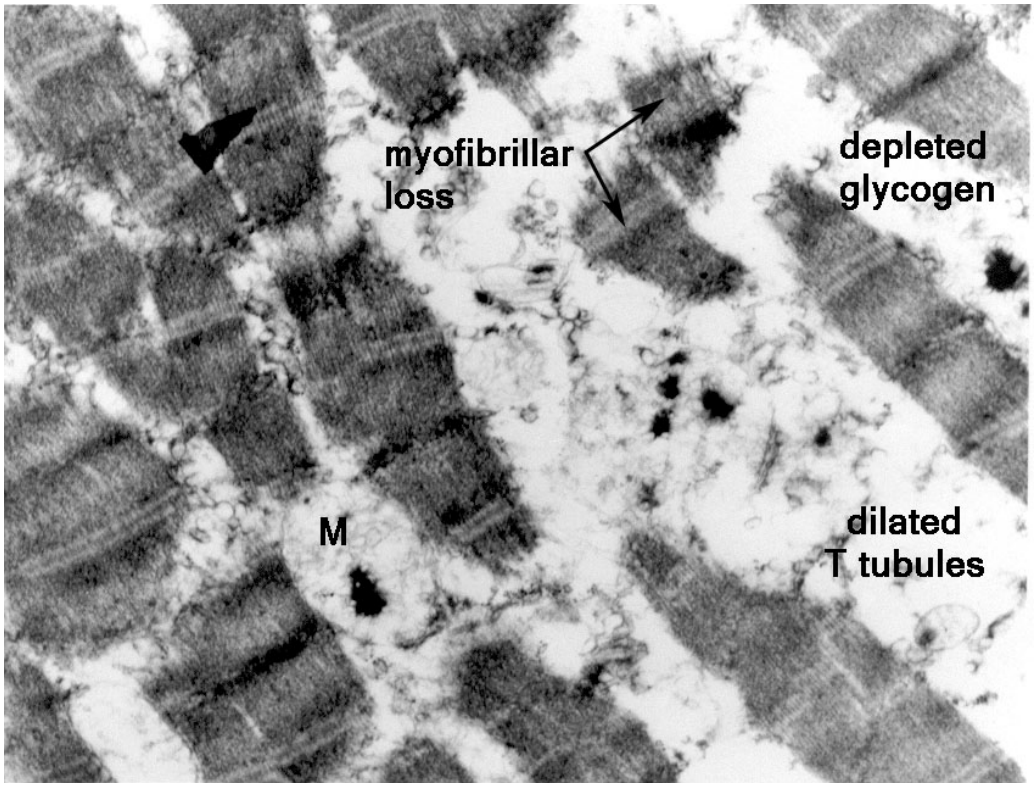


Plate 3.5.3 (E): Electron micrograph of muscle after 8 hours of warm ischaemia (25^0C); 36 000x

Plates 3.5.3 (F) and **(G)** provide interesting contrasts, and show muscle subjected to 24 hours of ischaemia.

Plate 3.5.3 (F) shows the cold stored muscle (CONTROL, 24 hours) and **Plate 3.5.3 (G)** depicts the perfused muscle (TEST, 24 hours).

The cold stored muscle **(F)** is rendered necrotic after 24 hours of cold ischaemia without perfusion with UWS. There is gross disruption of the architecture with a disorganised overall appearance. The basement membranes are shattered, tubules are dilated and the mitochondria are swollen, have no internal cristae and are degenerate. All 7 of the EM features show a non-viable picture.

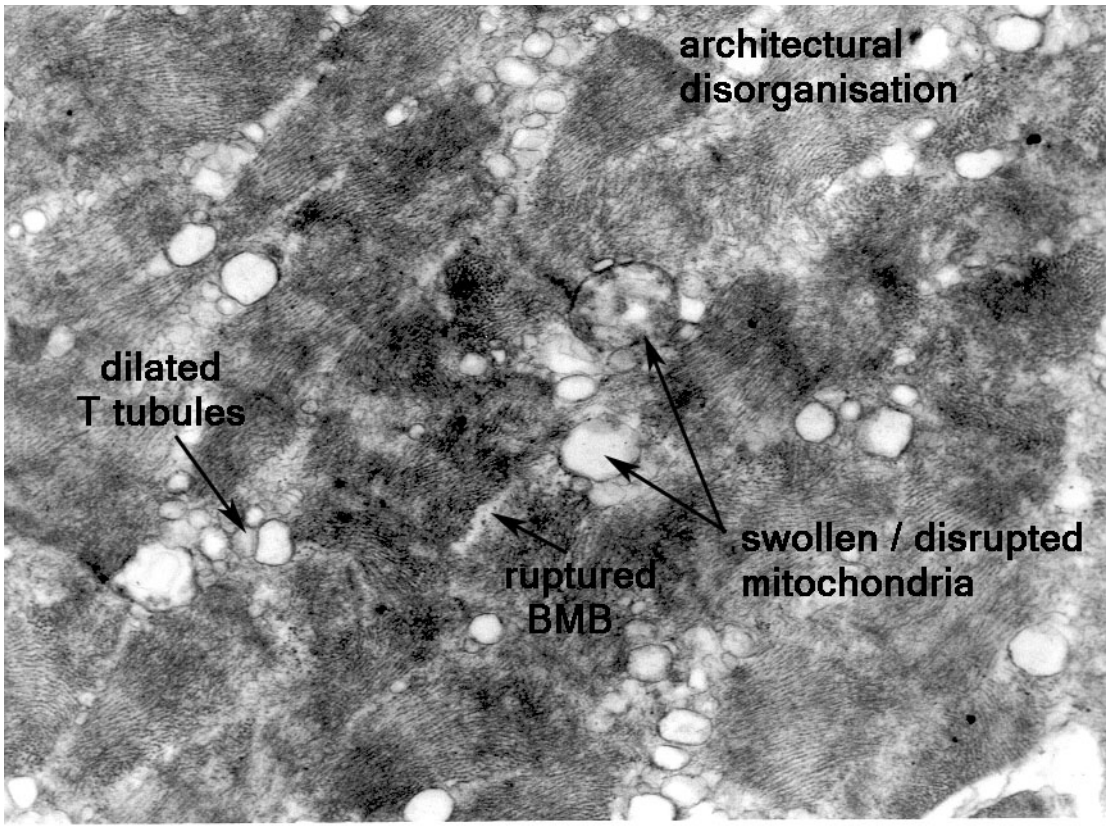


Plate 3.5.3 (F): Electron micrograph of muscle after 24 hours of cold storage (CONTROL); 21 000x

In contrast to this, although the perfused muscle **(G)** has suffered some ischaemic damage, the gross architecture is preserved. There has been some mitochondrial hypoxic damage (as evidenced by mitochondrial swelling), there is but they are not degenerate and their internal cristae are intact. The glycogen granules are depleted, although can be seen in places. The remaining of the EM features are generally intact and the myofibrils and basement membrane remains unaffected. This muscle is viable.
The EM features of muscle after 24 hours of perfusion show similar changes to the muscle that is cold stored for only 12 hours (refer **Table 6**). Perfusion therefore has prolonged ischaemic survival almost twofold.

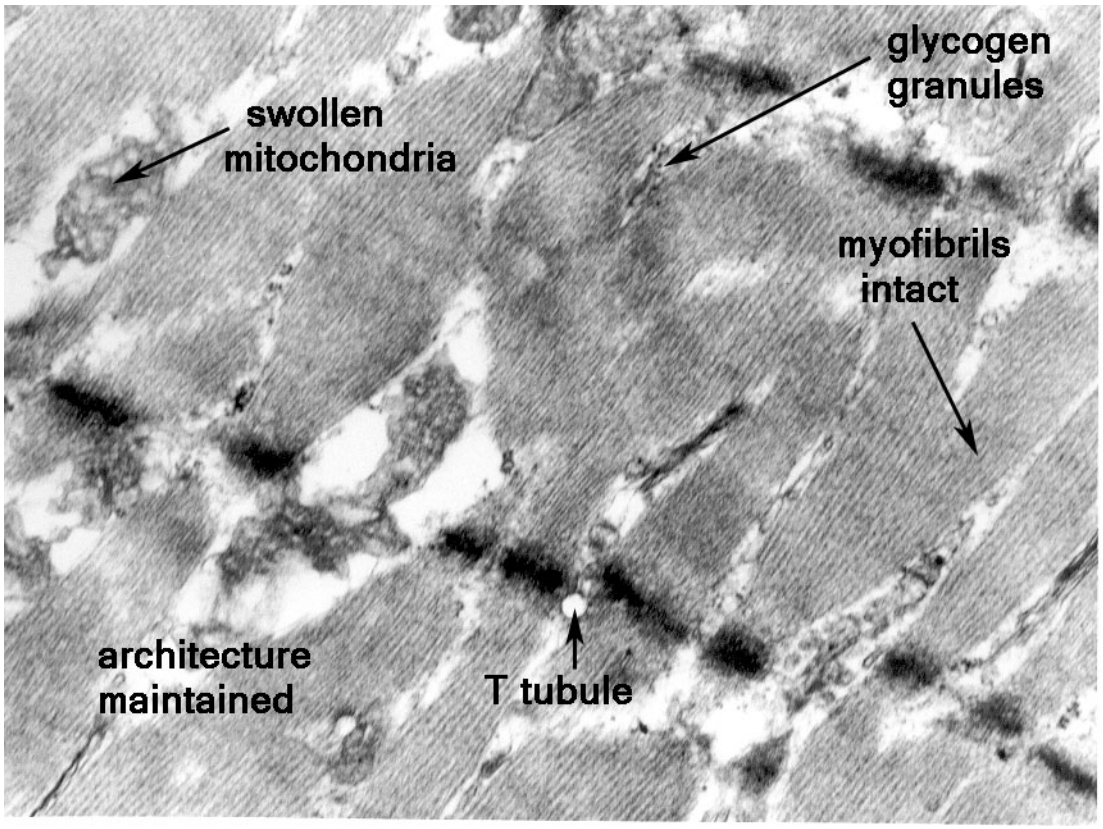


Plate 3.5.3 (G): Electron micrograph of muscle after 24 hours of cold perfusion (TEST); 17 000x

3.5.4. Combined Assessment of Muscle Survival (LM + EM):

An overall assessment of the percentage of muscle survival was performed by combining the results obtained on light and electron microscopy based on the 10 features (3 LM and 7 EM) as described earlier (**Section 2.10.**).

This provides an overall global assessment of ischaemic muscle survival for each time interval and is depicted as pairwise comparisons as means and standard deviations in **Table 7** and **Figure 5**.

Table 7: Percentage of muscle survival for TEST vs. CONTROL muscle on **combined light and electron microscopy**

TIME (hours)	**TEST** % SURVIVAL mean (+/- SD)	**CONTROL** % SURVIVAL mean (+/-SD)	**p–VALUE**
0	100 (0)	100 (0)	1,0000
4	96,17 (3,30)	83,21 (2,35)	0,0006 **
8	85,92 (10,59)	78,12 (7,50)	0,0103 **
12	81,96 (5,60)	60,57 (3,65)	0,0013 **
18	73,11 (2,52)	49,35 (7,53)	0,0025 **
24	57,90 (2,50)	31,37 (11,40)	0,0084 **

** - highly statistically significant (at a 1% level of significance)

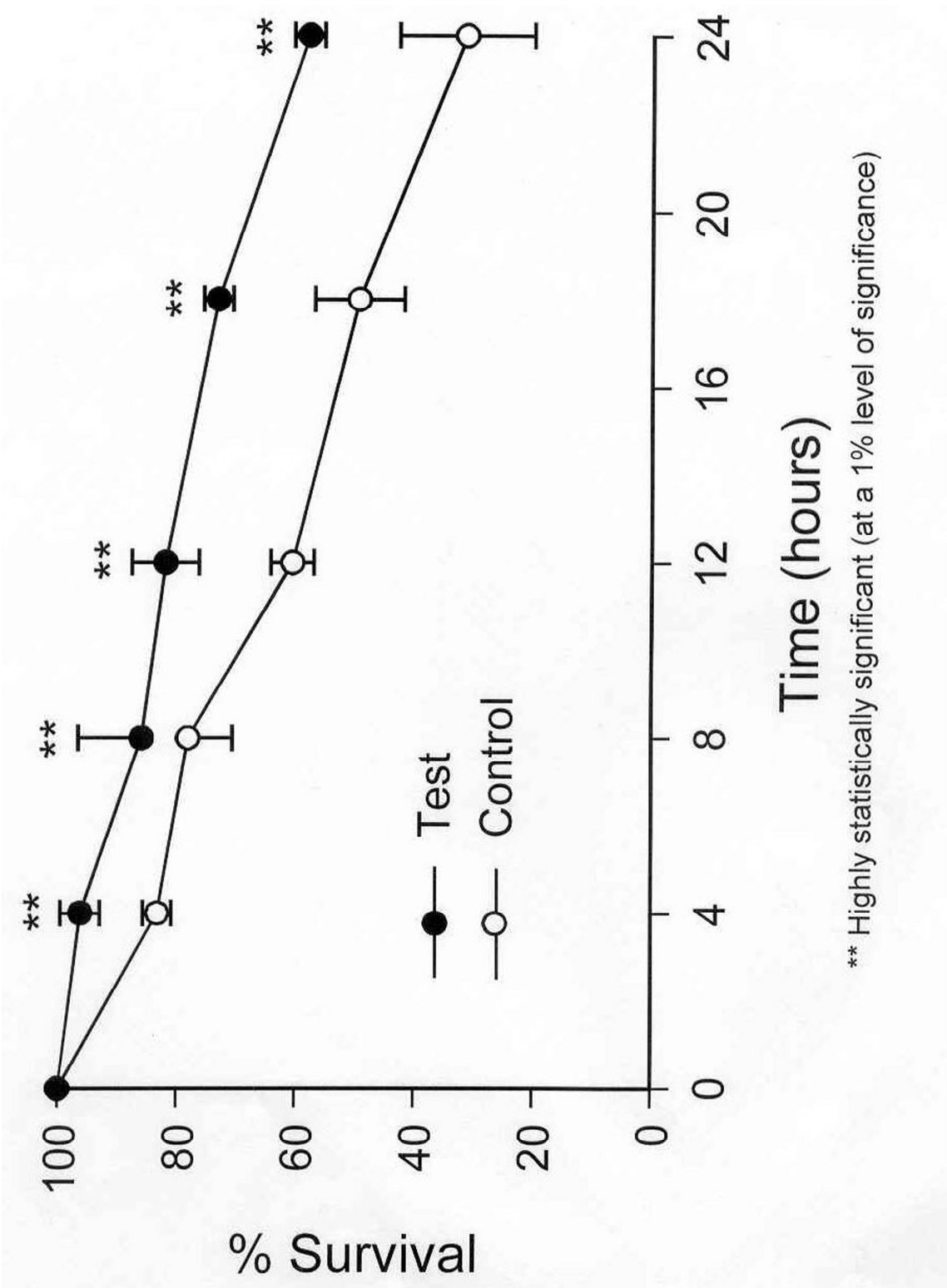

% Survival
100
80
60
40
20
0
0
4
8
12
16
20
24
Time (hours)
Test
Control
**
**
**
**
**
** Highly statistically significant (at a 1% level of significance)

Figure 7: Overall percentage of muscle survival **(combined light and electron microscopy)**

3.5.5 Contrasts and Interactions Between Groups and Times

An ANOVA test was performed to assess whether the interaction between the groups and times were significantly different, as expressed by gt (group-time). This is depicted in **Table 8**.

These data represent the overall combined electron and light microscopic findings of ischaemic survival, thus providing a global illustration of group - time interactions. All results were at 1 degree of freedom.

It is important to note that for all time groups beyond 0 hours, the TEST (perfused) group **survives statistically better** than the CONTROL (cold stored) group. One notable exception is the electron microscopy results at 8 hours where no statistical difference between TEST and CONTROL groups was found. The group – time interaction serves to determine which group deteriorates more rapidly for a particular TIME (refer **Section 4.3.**).

Table 8: Contrasts between groups and times for **combined light and electron microscopic** assessment

GROUPS / TIMES	**P – VALUE**
t4 – t8	0,0579
gt	0,1326
t4 – t12	0,0004 **
gt	0,0650
t4 – t18	0,0006 **
gt	0,0725
t4 – t24	0,0003 **
gt	0,0892
t8 – t12	**0,0251 ***
gt	**0,0010 ** **
t8 – t18	**0,0098 ** **
gt	**0,0018 ** **
t8 – t24	**0,0035 ** **
gt	**0,0118 ***
t12 – t18	0,0026 **
gt	0,5021
t12 – t24	0,0011 **
gt	0,3631
t18 – t24	0,0010 **
gt	0,3429

* - significant (at a 5% level of significance)

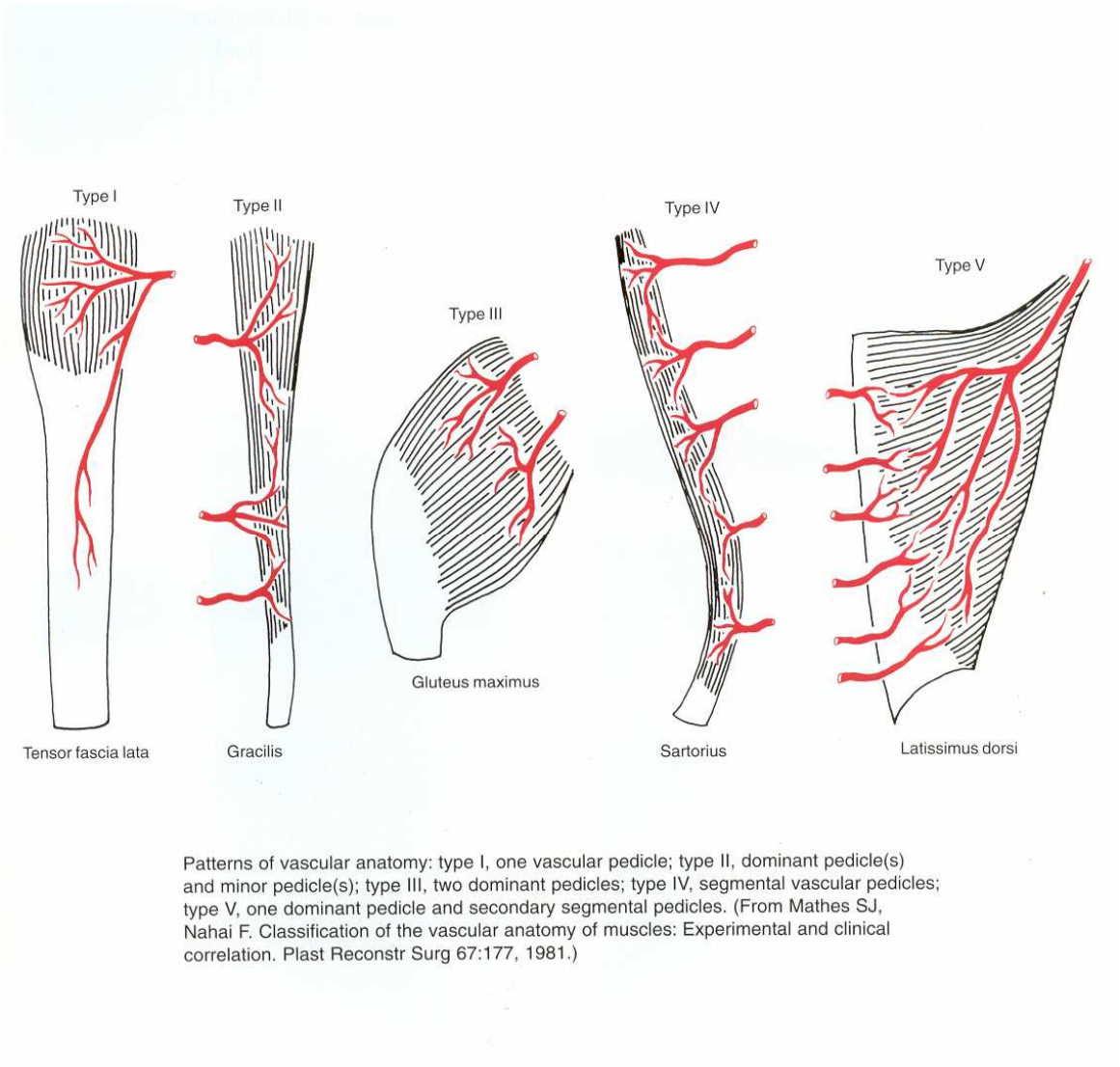
** - highly significant (at a 1% level of significance)

CHAPTER 4 - DISCUSSION

4.1. Anatomy Of The Rectus Femoris Muscle Flap And Its Vascular Supply (refer " MATERIALS AND METHODS", Plates 2.6 A – C; "RESULTS", Plates 3.1 A – C; 3.2 A - B)

In their landmark paper, Mathes & Nahai (1981) classified human skeletal muscle flaps based on their vascular supply. This was important because it allowed the reconstructive surgeon to safely dissect and raise various muscle flaps based on a knowledge of the vascular pedicles. It afforded predictability with regards which vessels could or could not be ligated and on which pedicle/s the muscle largely relied upon. With microsurgical reconstruction, it is imperative to have a knowledge of the major vascular pedicle prior to use of a muscle flap. This classification is depicted in **Figure 4.1**.

The anatomical dissection and angiographic studies (**Plates 2.6 A-C; 3.1 A-C** and **3.2 A - B**) demonstrate that the Mathes & Nahai Classification (1981) of the *rectus femoris* flap in the rabbit would be a Type 1 flap – i.e. a single dominant pedicle. It is therefore a useful flap for microsurgery and for pharmacological studies as the muscle flap can be isolated entirely on this single pedicle and manipulated as required.



Patterns of vascular anatomy: type I, one vascular pedicle; type II, dominant pedicle(s) and minor pedicle(s); type III, two dominant pedicles; type IV, segmental vascular pedicles; type V, one dominant pedicle and secondary segmental pedicles. (From Mathes SJ, Nahai F. Classification of the vascular anatomy of muscles: Experimental and clinical correlation. Plast Reconstr Surg 67:177, 1981.)

Figure 8: Patterns of Vascular Anatomy (after Mathes & Nahai, 1981)

4.2. CURRENT STUDY ON ISCHAEMIC SURVIVAL OF MUSCLE

This study served to demonstrate the effects of cryoperfusion with UWS (TEST) compared to those of simple cold storage (CONTROL). The end-points used reflected ischaemic survival on light and electron microscopy and a combination of both. The parameters of ischaemic damage include those features which were felt to be quintessential. A new grading system of ischaemia is offered (refer **Section 1.4.7.**).

The concept of muscle survival following reperfusion is interesting and merits further work. After a known period of ischaemia, the recovery or the deterioration of muscle after re-anastomosis and reperfusion could provide illuminating data. A critical "cut-off" point of non-survival could be documented. This could be related to this current study, in which the percentage of muscle survival (as reflected on LM + EM) may correlate with overall muscle recovery and thus, survival.

On LIGHT MICROSCOPY (**Table 5, Figure 3**, **Plates 3.5.2 (A)- (D)**), a highly statistically significant difference in percentage survival between the TEST and CONTROL groups was noted for all time intervals beyond 4 hours (at a 1% level of significance). Muscle which was cold stored deteriorated even after 4 hours, with percentage survival of approximately 75 - 70% at 4 and 8 hours. After 8 hours, considerable deterioration was noted in the stored muscle to the point that only half the muscle fibres survived at 12 hours. At 24 hours, only 25% survival was seen in this group, whilst the perfused muscle (TEST) showed a two-fold improvement in survival of muscle cells (56%).

The more sensitive and specific ELECTRON MICROSCOPIC analysis (**Table 6, Figure 4, Plates 3.5.3 (B) - (G)**) demonstrates some interesting findings.

The differences in survival between the 2 groups is again significant at 4 hours (at a 5% level of significance) yet not significant at 8 hours, with 86% survival noted in the perfused group and 83% survival in the stored group. This may suggest that management of ischaemic muscle with less than 8 hours duration of ischaemia could well be managed along traditional lines of cold storage without perfusion (although perfusion does no harm and shows slight improvement of survival). However, the spurious result at 8 hours may well be due to the wide standard deviation of +/- 12 as a result of the small sample size. After 8 hours, a significant difference in survival is noted between the 2 groups (at a 5% level of significance). Even after 24 hours of ischaemia, perfused muscle showed a 60% survival compared to 37% if cold stored.

The most accurate assessment of muscle ischaemia is the combined scoring system. Based on this, a highly significant difference in survival is noted between the perfused and stored groups from 4 hours onwards.

At 24 hours, a two-fold improvement in survival rates is demonstrated (approximately 60% for perfused muscle vs. 31% for stored muscle). Furthermore, stored muscle shows the same survival rate at 12 hours as does the perfused muscle at 24 hours.

This study has demonstrated conclusively that perfusion of isolated skeletal muscle with cooled oxygenated UWS offers significant survival benefits beyond 4 hours. After 8 hours, cold perfusion confers highly significant benefits on ischaemic survival as compared to simple cold storage.

With extended periods of ischaemia (1 day), perfusion can double the ischaemic survival or for the same % survival, double the duration of survival (beyond 12 hours).

4.3. GROUP – TIME INTERACTION ANALYSIS

The analysis of the GROUP – TIME (gt) interaction (**Table 8**) illustrates several interesting facts.

Firstly, with the exception of **times** 4 compared with 8 hours, ischaemic survival is significantly different when each **time** is compared to another regardless of test or control **groups** (total numbers of test and control results are combined respectively and one is only comparing differences at varying times). In this case, 4 hours survives statistically better than 12 hours and so on, regardless of test or control groups. There is no statistically significant difference in survival when 4 is compared with 8 hours (gt = 0,1326 which is greater than a p-value of 0,05 which is the minimum cut-off for significance at 95%confidence limits).

In both TEST and CONTROL groups, a significant difference in survival is seen beyond 8 hours. Therefore regardless of whether one cold perfuses or cold stores muscle, survival deteriorates after 8 hours. It is interesting to note that cold treated muscle (by whatever means) only survives well for up to 8 hours. Muscle stored at room temperature (25 ^{O}C) will be grossly necrotic well short of 8 hours (see **Section 3.5.1.).** Warm ischaemia beyond 2 to 4 hours is likely to show deleterious changes.
This has direct bearing on the clinical setting where amputated muscle-containing parts or free muscle transfers should only be subjected to the minimum periods of warm ischaemia (certainly less that 4 hours).
Topical cooling of an amputated limb or elevated free skeletal muscle flap, therefore, should be employed in all cases.
When cold stored, up to 8 hours is compatible with very little ischaemic damage (refer **Plates 3.5.3 (C) - (E)**).

We have therefore discussed important cut-off TIMES for muscle subjected to cold storage, by whatever means (simple cold storage or cold perfusion). Eight hours appears to be a critical time event.
This is particularly seen on electron microscopy, where no statistical difference could be demonstrated at 8 hours for both TEST and CONTROL groups (p = 0,3703). When the light microscopic criteria and 10 overall combined criteria (LM + EM) are used, a significant difference between the 2 groups was noted at all time periods beyond 0 hours. However, 8 hours appears to a "cut-off" time period after which deterioration is greater in the cold stored group.

It is now prudent to examine whether, for a given time, one method of cryopreservation yields superior results compared with the other. The performance of simple cold storage (CONTROL) is compared to that of cold perfusion with UWS (TEST). This is analysed by using the ANOVA group-time (gt) interaction. When the **groups** (test or control) are compared to **times** (4, 8, 12, 18, 24 hours), as shown by gt in the table, the performance between each group for any given time may be assessed. If the interaction (*g*) is statistically significant, this means that one group (e.g. control) had a significant change (deterioration) in survival when compared at two different times (e.g. 8 vs. 12 hours), whilst the other group (e.g. test) did not have a significant alteration in survival, when compared at these times. This enables us to specifically assess the performance of those muscles perfused with UWS compared to those without perfusion.
As shown in **Table 8** and highlighted in bold, the interaction (*gt*) between times and groups were statistically significant for 8 vs. 12 hours, 8 vs. 18 hours and 8 vs. 24 hours.

In each of these (when 8 hours was compared with 12, 18 and 24 hours respectively), the TEST survived better than the CONTROL group viz. there were no significant differences in ischaemic survival in the test groups whilst there was a difference in survival in the control groups.
This means that with times beyond 8 hours of cold ischaemia, the stored muscle shows statistically significant more deterioration than does the perfused muscle, which shows statistically less deterioration at 8, 12, 18 and 24 hours.

It is crucial to note that although deterioration occurs in both test and control groups at the time intervals above, the deterioration is significantly more in the control groups for every time group compared except 4 and 8 hours. For time periods less than 8 hours, cold perfusion potentially offers more benefits than simple cold storage (as shown on light microscopy).

It would therefore appear that muscle which is simply placed in cold storage beyond a cut-off point of 8 hours deteriorates more rapidly than muscle which is perfused with cold storage solution and that perfusion of muscle has benefit beyond 8 hours.

4.4. CLINICAL APPLICATIONS AND FUTURE CONSIDERATIONS

The development of a solution suitable for reliable and possibly even long-term preservation of explanted tissues has occupied investigators since the advent of tissue transplantation. In the field of reconstructive microsurgery particularly, a successful protocol for preservation of skin, muscle and composite flaps, would be highly desirable.

In the rapidly advancing field of tissue transplantation surgery, it is imperative that we explore ways to minimise the deleterious effects of ischaemia, storage and reperfusion. Such ways and means would also improve the results of limb and digit replantation and may, in fact, allow for storage of previously harvested flaps and pave the way for the eventual establishment of banks of allograft tissue flaps for use in reconstructive surgery.

This study has shown that a significant improvement in survival of ischaemic skeletal muscle can be obtained by cold perfusion with oxygenated UW solution. Even at 24 hours of ischaemia, this method has resulted in an almost two-fold improvement in survival.

In addition to simple “laboratory relevance”, this study, I believe, has important practical and clinical applications to the reconstructive microsurgeon. Certain protocols can therefore be advanced.

With regard to all muscle containing parts (muscle- containing amputated parts or free tissue transplants), the recommendations are as follows:

1. all tissue, particularly highly aerobic and vulnerable tissues such as **muscle**, must be subjected to the minimum periods of warm

ischaemia, preferably less than 2 hours and definitely less than 4 hours. In 1946, Clark documented early histologic changes in muscles subjected to tourniquet ischaemia after only 2 hours of ischaemia. In this study, 8 hours of cold ischaemia seems to be a crucial "cut-off" point after which muscle deteriorates rapidly.

2. for ischaemic intervals less than 8 hours, cold storage is imperative i.e. all muscle rendered ischaemic should be cooled. For time periods less than 8 hours, is not clear as to which method of storage offers more advantages (be it simple cold storage in refrigerator at 4 ^{O}C or perfusion with cryopreservative solution). On electron microscopy, no significant differences in survival were seen between the 2 groups after less than 8 hours, yet there is a trend in favour of perfusion. When one analyses the overall features of ischaemia (on light and electron microscopy combined), perfused muscle performs significantly better after ischaemic periods as brief as 4 hours.
3. Beyond 8 hours, a highly significant difference between the 2 methods has been demonstrated, regardless of which assessment is used (LM, EM, or combined).Cryopreservation with cooled, oxygenated UWS performed much better than simple cold storage and confers distinct survival advantages. This is especially true of prolonged periods of ischaemia of up to 24 hours. It is recommended that muscle rendered ischaemic for more than 4 hours and definitely for periods beyond 8 hours, should be cryoperfused with UWS.

The clinical implications are obvious. Free tissue transfers using muscle are commonplace and transfers usually have warm ischaemic time of 2 to 3 hours depending on various technical factors.

The microsurgeon should limit ischaemic time as far as possible and should ensure that recipient vessels are well prepared so that the transplanted muscle may be re-anastomosed and the muscle reperfused with minimal delay. Furthermore, whilst the muscle is explanted, one should limit the warm ischaemic process by topical cooling of the muscle flap. This common-sense practice is often overlooked in the clinical setting. As we have demonstrated from the studies on warm vs. cold storage, muscle performs much better if cooled even if ischaemic time periods are short.

The decrease in metabolic rate may have a direct bearing on the functional performance of the muscle once transplanted. Although it was beyond the scope of this study to analyse the function of the muscle following its storage/ perfusion, it is likely that muscle with improved cellular survival would function better after replantation.

Topical cooling continues the overall beneficial effects of hypothermia on ischaemic tissue. Traditionally, topical physiological solutions such as normal saline or Ringer lactate have been used. In this study, UWS was used both topically and as the perfusate with statistical benefit. Again, it was beyond the scope of the study to compare the effects of different topical cooling agents. Suffice it to say that the crucial ingredients of UWS make it a logical choice as a topical cooling agent for muscles subjected to ischaemia less than 8 hours or for intra-operative cooling.

With regard replantation surgery of amputated muscle containing parts, clinical practice tells us that it is very difficult to establish reperfusion of these parts within 8 hours of injury. Delays in accessing the injured victim, transporting the patient, admitting, resuscitating and mobilising a microsurgical team are generally the norm. This is particularly true in patients who have been injured some distance away from a major referral centre with microsurgical facilities and expertise.

If delays beyond 8 hours are anticipated, this study has conclusively demonstrated that cryoperfusion of the amputated part may be prudent. In practical terms, the major artery of the amputated part can be rapidly and easily cannulated and perfused with UWS. Portable perfusion pumps are available and may have a place on long-distance patient transportation, be they by road or air. If such pumps are unavailable, perhaps a simple flush with UWS may be prudent.

This study has provided important information on several levels, but as with any research, unanswered questions remain. A few of these issues are as follows and hopefully may be answered by future studies:

1. Would simple flushing achieve similar results as compared to perfusion ?
2. What is the best method of perfusion – continuous versus pulsatile?
3. What would the effects be using a more simple solution e.g. Ringer lactate or saline?
4. How would our results perform on a larger series of flaps?
5. How would other tissue models perform, most importantly, muscle-containing amputated parts in the human?
6. How could we relate percentage of muscle damage / overall survival to muscle performance and function after replantation?
7. Can we actually reverse documented ischaemic damage by means of perfusion techniques and therefore salvage the “dying” cell, preventing it from becoming irreversible damaged?
8. What are the effects of perfusion on the muscle after it has been replanted and reperfused – i.e. does prior perfusion affect reperfusion injury?
9. what is the role of oxygenation?

4.5. CONCEPT OF THE "IDEAL" PERFUSION SOLUTION

Prolonged ischaemia, hypothermic storage and subsequent reperfusion represent conditions and stresses which neither tissues nor organs are designed to cope with. "Simple" crystalloid and even more complex colloids such as plasma, may also not be able to cope with these stresses. The addition of certain pharmacological agents designed specifically to target certain pathways that develop in pathophysiological conditions (ischaemia, storage or reperfusion), seem to be very necessary.

The development of advanced tissue preservation solutions needs to be supplemented by strategies targeting perfusion phenomena, i.e. oxygen free radical generation, neutrophil chemotaxis, migration and degranulation and platelet activation.

Several mechanisms of how perfusion preserves tissues generally and skeletal muscle specifically, can be postulated.
First, perfusion may dilute and washout toxic metabolites, prevent sludging and thrombus formation. The importance of this, highlighted by the studies of Rosen *et al* (1987) and Marshall *et al* (1988), has already been discussed in **Section 1.4.**

Second, the perfusate may deliver those substrates that are essential for muscle metabolism, particularly after the stress of ischaemia and reperfusion. This includes an adequate supply of glucose, oxygen and adenine to support the oxidative synthesis of ATP. Finally, the components of the perfusate may help to minimise ischaemic damage to cells and target specific pathophysiological pathways.

There is an inherent difficulty in proposing an “ideal” solution due to the undoubtedly elaborate nature of these pathways whose complexity if far from being understood. The likelihood of “maximal preservation” is also both organ and model-specific and what may be beneficial in one tissue type or animal model, may in fact be detrimental in another.

The rationale for including certain additives has to be based, at present, on application of known principles (e.g. the inclusion of a buffer) and on the inclusion of agents which have, when tested in isolation in past experiments, shown either significant benefit or promise for the preservation of ischaemic tissues. Ultimately, the most suitable preservation strategy will depend on a trial-and-error approach, since there is little doubt that ischaemia / storage / reperfusion / no-reflow, is a complex phenomenon amenable to modulation on many fronts.

The benefit of inclusion of any active ingredient in a perfusion solution is limited to the period of perfusion and storage. Other limitations include the dosage of the active ingredient which differs for any given situation and animal model.

This study on the isolated skeletal muscle flap in the rabbit, I believe, has provided invaluable information regarding one particular preservation protocol in one particular model. It has been shown with statistical validation that perfusion of this flap with cooled oxygenated University of Wisconsin solution has improved ischaemic survival. As with any experimental study, particularly in the field of cryopreservation and transplantation biology, one must appreciate the limitations of the investigation.

This current study has provided some answers, but also provides a platform on which further investigation may be built. Important future research may help extend the applicability of this one.

It is increasingly probable that future preservation protocols will be inseparably linked to reperfusion and transplantation protocols. An important extension of this study would be to investigate those phases which follow that of ischaemia and hypothermic storage. The phases of reperfusion following subsequent transplantation or reimplantation are important to follow, including the impact that prior perfusion has on ultimate survival. This may be performed by subjecting the muscle to a known ischaemic insult, perfusing the muscle ex-vivo and then re-implanting the muscle and assessing muscle damage after a period of reperfusion.

This study points to the need for a new, integrated approach to the field of preservation and transplantation or replantation. The many areas of past research in perfusion solutions, pharmacological additives and the role of normal cells subjected to abnormal environment (e.g. role of neutrophil and platelet activation and red cells), need to be brought together in the development of comprehensive tissue preservation protocols. The development of such protocols will therefore need to reconcile two general approaches to one problem.

On the reductionist level, more investigation is needed regarding the complex pathways that orchestrate the pathophysiological derangements that are at play in the ischaemia/ storage/ reperfusion/ no-reflow scenarios. Amongst others, areas that need further investigation include the microcosm of the inflammatory mediators, including free radicals and other toxic metabolites.

On the holistic level, research needs to concentrate on the dynamic interplay of the various components of the ischaemia/ storage/ reperfusion/ no-reflow scenarios and on how these pathways may be modulated or manipulated by pharmacological intervention. We also need to integrate treatments which may target individual components.

The future may in fact bring the elusive "holy grail" closer towards our grasp, allowing us to minimise the effects of ischaemia and reperfusion injury so as to create a "**reflow phenomenon**" and improve overall survival of organs and tissues.

CHAPTER 5 - APPENDIX

5.1. LIST OF ABBREVIATIONS

[ ] – concentration of

ADP – adenosine diphosphate

AK – adenosine kinease

AMP – adenosine monophosphate

ATP – adenosine triphosphate

C - centigrade

CsA – cyclosporin A

EM – electron microscopy

HES - hydroxyethyl starch

Hsp 70 - heat shock protein 70

I-R – ischaemia reperfusion

LM – light microscopy

mmHg – millimetres of mercury

mmol/l – millimole per litre

mOsm - milliosmoles

NAD, NADP – nicotinamide adenine dinucleide/ phosphate

NBT - nitroblue tetrazolium

PAF – platelet activating factor

PCO_2 – partial pressure of carbon dioxide

PGI_2 - prostacyclin I_2

PO_2 – partial pressure of oxygen

ROS – reactive oxygen species

SOD – superoxide dismutase

TXA_2 - thromboxane A_2

UWS – University of Wisconsin solution

5.2. ACKNOWLEDGEMENTS:

1. To my family and friends whose enduring love and support has made all things possible.
2. Dr. Morris Ritz, whose depth of thought is matched only by its laterality – his directing force allowed this project to develop to fruition.
3. Dr. Vicky Fabian, one of the project supervisors, whose excellent knowledge and superb histopathology skills made this project possible.
4. Prof. L. Chait, for agreeing to supervise this study and for his wisdom and guidance over the years.
5. This work was supported by the South African Institute for Medical Research who generously sponsored all electron and light microscopic material.
6. Mrs. Esther Viljoen – for her excellent endeavours regarding the statistical analysis of this project.
7. Mrs. Ucelia Norval (Department of Histopathology) and Mrs. G. Norman (Department of Electron Microscopy) – their technical advice, continued devotion to this work and highly specialised skills were greatly needed and appreciated.
8. Dr. Rene Botha – for generously providing the use of the renal perfusion apparatus and donating the University of Wisconsin solution.
9. Dr. Gavin Norton – for allowing us the use of the physiology laboratory and all the necessary apparatus and for his excellent advice regarding the physiology of this experimental study.
10. Dr. J. van Westing – for kindly agreeing to take on the mammoth task of commenting on this report and for his excellent input and proof reading skills.
11. Dr. P. Bekker – for his superb advice regarding the statistical planning of this project.

12. To any other person/s who were involved in this project and who may have been unintentionally omitted.

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CHAPTER 6 - LIST OF REFERENCES

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