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Preconditioning in skeletal muscle

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Abstract

Ischaemia reperfusion injury of skeletal muscle is a major cause of morbidity and mortality in various surgical specialities. Developing a protective method or pharmacological agent that will limit this damage will be of considerable benefit to both patients and doctors.

I have used potassium channel openers and calcium as preconditioning agents. The results show that potassium channel openers are a viable option whereas the use of calcium can exacerbate muscle damage.

I looked at various protocols of ischaemic and pharmacological preconditioning. The results from both ischaemic and pharmacological preconditioning have shown a comparable decrease with some pharmacological agents in the extent of skeletal muscle infarction both in the early and late period of reperfusion.

This decrease in the extent of muscle infarction is associated with changes in the levels of nitric oxide in the circulation. There was preservation of skeletal muscle oxygenation in preconditioned muscle. I have shown that preconditioning of skeletal muscle is a viable option in trying to reduce the amount of damage caused by ischaemia reperfusion injury.

Aims

The aims of this thesis were to study the various ischaemic preconditioning (IPC) protocols that have been used to date and determine the most effective one. In addition to study beneficial effects of ischaemic preconditioning on skeletal muscles in ischaemic conditions and attempt to find a pharmacological agent that will mimic the beneficial effects of IPC.

Dedication

To my parents whose support has made this work possible.

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I am also indebted to Mr. John Muddle for his help in writing the programme for skeletal muscle analysis.

Finally the work would not have been possible without the help and continuous support provided by Karen Cheetham.

Note

I was responsible for the majority of the work carried out including setting up the experimental model, acquisition of samples, their processing and analysis, the statistical analysis of the results in addition to the preparation of all the reagents used in the experiments. All the experiments carried out were in this study were conducted in accordance with the Animals Scientific Procedures Act (1986) and with licence from the Home Office. I would like to acknowledge the help of Mr John Muddle in writing up the software for the computerised reading of the skeletal muscle samples. I wrote the manuscript and any remaining errors are solely my responsibility.

List of abbreviation

ARDS:	Acute respiratory distress syndrome
AMG:	Aminoguanidine
ANOVA:	Analysis of variance
ATP:	Adenosine tri- phosphate
Ca^{2+}	Calcium
cAMP:	Cyclic AMP
cGMP:	Cyclic GMP
cNOS:	Constitutive NO synthase
$CaCl_2$:	Calcium Chloride
CPC:	Calcium preconditioning
CytOx:	Cytochrome oxidase
DPL:	Differential path length factor
ECP:	Energy charge potential
5-HD:	5-hydroxydecanoate
HbO2:	Oxyhaemoglobin
Hb:	Deoxyhaemoglobin
HbT:	Total haemoglobin volume
HSP:	Heat shock proteins
IMP:	Inosine 5'-monophosphate
IVFM:	Intravital flow microscope
IPC:	Ischaemic preconditioning
IRI:	Ischaemia reperfusion injury
K_{ATP}:	ATP-sensitive potassium channels
LD:	Laser diodes

LDF:	Laser doppler flowmeter
L-NA:	N^{ω}-nitro-l-arginine
L-NMMA:	*N*G-monomethylyl-L-arginine
MMC	Muscle microcirculation
MMIA	Mean muscle infarction area
NADPH:	Nicotinamide-adenine-dinucleotide phosphate
NIRS:	Near infrared spectroscopy
NO:	Nitric oxide
iNOS:	inducable Nitric oxide synthase
nNOS:	neuronal Nitric oxide synthase
NOS:	Nitric oxide synthase
O_2:	Oxygen
OAG:	1-Oleoyl-2-acetyl glycerol
ODFR:	Oxygen derived free radicals
HbO_2:	Oxyhaemoglobin
PKC:	Protein kinase C
PMA:	Phorbol 12-myristate 13-acetate
PMT:	Photomultiplier tube
POAD:	Peripheral obliterative arterial disease
PVD:	Peripheral vascular disease
Ras/Erk:	Extra cellular signal related kinase
RBC's:	Red blood cells
ROS:	Reactive oxygen species
R-PIA:	R-phenylisopropyl adenosine
SM:	Skeletal muscle

8-SPT:	8-(p-Sulphonyl)-theopylline
SNP:	Sodium nitroprusside
SPT:	Sulfophenyl-theophylline
SR:	Sarcoplasmic reticulum
TNF-α	Tumour necrosis factor α
$TcpO_2/pCO_2$:	Transcutaneous pO_2/pCO_2
VO_2:	Oxygen consumption
UW solution:	University of Wisconsin solution

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Chapter One

Vascular preconditioning and reperfusion injury:

The horns of a clinical dilemma

1.1 Introduction

Ischaemia reperfusion injury (IRI) is a major cause of morbidity and mortality. Acute limb ischaemia has a mean mortality rate of 25%[1] and an amputation rate of 20% for patients after revascularisation[2]. The restoration of blood supply to ischaemic tissues results in both local and systemic sequela.

Acute limb ischaemia occurs in many clinical situations including trauma, vascular disease and prolonged arterial occlusion following surgery. Ischaemia is defined as a reduction in the blood flow to tissues, whereby the oxygen supply to the tissues does not meet the tissue's demands and has been known, for a long time, to cause severe tissue damage. Originally it was thought that most of the tissue damage occurred during the period of ischaemia. However, it has since become apparent that re-establishing blood flow to an ischaemic part is accompanied by an additional injury, often of greater severity. There is an increased leakage from capillaries and venules and the formation of oxygen free radicals resulting in further muscle damage by necrosis, unlike neutrophils and vascular endothelial cells which undergo apoptosis. This is termed IRI[3,4].

Reperfusion of ischaemic tissues has both systemic and local consequences, the severity of which depends on the extent and duration of the ischaemic insult, and the metabolic rate of the ischaemic tissues. Skeletal muscle (SM) usually tolerates two hours of ischaemia after which ischaemic damage will begin[5]. Ischaemia which occurs in limbs following arterial damage has wide ranging metabolic consequences such as Legrain-Cormier[6] or myonephropathic-metabolic syndromes[7]. Other complications such as acute respiratory distress syndrome (ARDS) and renal dysfunction are well

established[7,8]. The generalised release of cytokines and neutrophil infiltration also leads to the development of a systemic inflammatory response (SIRS) and multiple organ dysfunction syndromes (MODS)[9].

1.2 The effects of ischaemia on tissues

The tolerance of the different cells in the body to ischaemia varies according to their metabolic rate as the higher the metabolic rate the higher the demand for oxygen will be. The first effect of hypoxia is on the cell's aerobic respiration which in turn will lead to a marked diminution of the intracellular generation of ATP[5]. This decrease in ATP production leads to an increase in anaerobic glycolysis leading to depletion of glycogen. This process of increased glycolysis can only be maintained for a short period of time (20-30 minutes) in the case of myocardial cells and hepatocytes which are the richest cells in glycogen content. The process of glycolysis also leads to the intra cellular accumulation of lactic acid, inorganic phosphates from the hydrolysis of phosphate esters and protons leading to the overall reduction of intracellular pH[10,11]. Since energy is continuously needed for the cell to function, this loss of ATP production and intracellular acidosis has important consequences for cells, with coiling and clumping of DNA, nuclear pyknosis, and failure of new RNA formation which in turn leads to the failure of protein synthesis[12]. Loss of the ATP driven sodium/potassium pump leads to the accumulation of intracellular sodium and water with loss of potassium to the extra cellular space. The accumulation of sodium and water in the cells leads to cellular swelling and eventually rupture of the intracellular organelles[12].

If oxygen supply is restored before the rupture of intracellular organelles the above changes are reversible with oxidative phosphorylation and ATP production resuming from mitochondria that have not been damaged. In addition the sodium/potassium pump will start to function again and the excess water and sodium that has accumulated in the cell pumped out. With the return of intracellular pH towards normal DNA structure returns to normal and new RNA and protein synthesis resumes.

If on the other hand oxygen supply is not restored to the swollen anoxic cell, which has consumed the last of its glycogen supply anaerobically, the cell will die. This cell death is associated morphologically with severe vacuolization of the mitochondria and the accumulation of calcium (Ca^{2+}) rich densities in the mitochondrial matrix, damage to the plasma membrane and swelling of the lysosomes. Massive Ca^{2+} influx also occurs particularly if the ischaemic zone is reperfused[12].

1.3 The effects of reperfusion on tissues

As mentioned earlier the re-establishment of blood flow to ischaemic tissues is associated with potentially significant pathology. Histological evidence from reperfused tissues shows extensive disruption of the microvasculature[13], in addition there is capillary obstruction by endothelial cell swelling, loss of pinocytotic vesicles, protrusions or blebs, breaks in endothelial cells, disruption of basement membrane and nuclear chromatin clumping[13]. Although leucocytes begin to accumulate and adhere to ischaemic endothelium, neutrophil adherence is significantly increased at the time of reperfusion[14,15], the

neutrophils obstructing capillary lumens usually in association with fibrin and red cells[16,17].

Reperfusion injury has been recognised as a subset of the inflammatory process[18]. The inflammatory processes in response to reperfusion include vascular changes in the form of dilation associated with decreased blood flow in the microvasculature due to plugging and leukocyte migration and adhesion [12]. This is associated with increased exudation of protein rich fluid into the extra-vascular tissues due to increased vascular permeability as a result of leaking from the normally non-penetrable endothelial layer[12]. Leukocytes cause damage during the reperfusion in several ways, apart from causing obstruction to the vascular, by playing part in the formation of plugs and also by the release oxygen derived free radicals (ODFR) and proteolytic enzymes[12]. It has been suggested that damage to endothelial cells at reperfusion is mediated by ODFR or neutrophil-endothelial interaction, or both[19-23]

Another change that occurs during the ischaemic and reperfusion periods is a substantial fall in tissue pH, initially the drop in pH is protective against loss of cell viability during anoxia[24], however later on when as the pH levels returns to normal upon restoration of the blood supply accelerated cell death occurs by activation of latent pH-dependent protease, and phospholipases[25,26].

1.4 Preconditioning

In order to overcome or reduce the effects of IRI various experimental and clinical approaches have been applied, including ischaemic and pharmacological preconditioning. Since first described in 1986 by Murry et al [27] there has been a great expansion in exploring the beneficial effects of

ischaemic preconditioning (IPC) on the various body organs, it has now been established that it confers a beneficial effects on the liver[28]; lungs[29,30]; spinal cord[31]; retina[32]; brain[33]; small intestine[34]; kidneys[35] and skeletal muscles[36,37]. However IPC in skeletal muscle (SM) is still in its early stages and there remains much to be studied. Pharmacological preconditioning is also in its infancy and, even though there has been some progress made in identifying pharmacological agents that offer protection against IRI such as adenosine[38-43] and Ca^{2+} [44-47], these drugs have been used mainly in the cardiac muscle rather than in skeletal muscle, and particularly in the case of Ca^{2+} in ex-vivo models rather than as in vivo work.

Chapter Two

An overview, the story so far

2.1 Introduction

IRI in skeletal muscle occurs during vascular occlusion and revascularisation, in elective vascular surgery, orthopaedic surgery by means of a tourniquet and in transplantation of myocutaneous flaps. Although Skeletal muscle can tolerate warm ischaemia for up to two and a half hours[5], this time limit is sometimes exceeded in clinical situations. Hyperkalemia, acidosis, and myoglobinuria can occur if muscle necrosis is extensive. IRI in the limbs is a major cause of morbidity and mortality in patients with acute limb ischaemia with a mean mortality rate of 25%[1] and an amputation rate of 20% for patients after revascularisation[2]. The pathogenesis underlying muscle necrosis is unclear but experimental evidence suggests that IRI is the cause[5].

In recent years, intensive experimental efforts have been directed to the study of ischaemic preconditioning (IPC) and pharmacological intervention. IPC is achieved by brief episodes of ischaemia followed by periods of reperfusion prior to a prolonged period of ischaemia[27]. IPC has been widely investigated and it has been found to have a protective effect in: cardiac muscle[27]; liver[28]; lungs[29]; spinal cord[31]; retina[32]; brain[33]; small intestine[34]; kidneys[35] and skeletal muscles[36,37]. The first clinical study of IPC in SM was carried out by Laghi Pasini and co-workers[48] in order to reduce claudication in patients with existing peripheral vascular disease (PVD).

An effective understanding of the mechanisms underlying IPC will allow pharmacologic intervention for the prevention of IRI.

2.2 Pathophysiology of IRI

2.2.1 Energy depletion and metabolite accumulation

Skeletal muscles store energy in the form of adenosine tri- phosphate (ATP) and energy depletion during ischaemia is directly proportionate to the extent of muscle damage. ATP is normally produced from the oxidation of free fatty acids when oxygen (O_2) is available. However during ischaemia ATP becomes unavailable. Skeletal muscle energy is therefore provided by the anaerobic pathway firstly by the breakdown of creatine phosphate which provides energy for approximately three hours followed then by the breakdown of muscle glycogen which leads to the accumulation of lactic acid and hydrogen ions from the conversion of pyruvate to lactate. In spite of these compensatory mechanisms for energy production the resultant accumulation of lactate eventually halts further glycolysis. ATP degradation results in the production of inosine-5-monophosphate, adenosine, xanthine and hypoxanthine. These degradation products are vital for restoring ATP stores in SM however they are lipid soluble and enter the cells rapidly, therefore they are lost from further use upon restoration of blood flow [49].

2.2.2 Oxygen-derived radicals (ODFR)

ODFR are O_2 based molecules that react rapidly with other molecules. ODFR are generated from several sources, including xanthine oxidase[50], catecholamines[51], mitochondria[52] and most importantly from neutrophils and monocytes[53]. ODFR are normally neutralised in the body by both enzymatic and non enzymatic mechanisms including superoxide dismutases[54], catalases[55], peroxidases[55,56], glutathione[57] and vitamin E[58]. Xanthine oxidase which is

released during reperfusion of ischaemic muscle is normally eliminated by spontaneous dismutation or through the actions of the enzyme superoxide dismutase[59]. The effect of ODFR is direct oxidation of proteins and DNA, another effect of free radicals is lipid peroxidation of polysaturated fatty acids within the cells leading to loss of cellular integrity[60]. The balance between the production and elimination of ODFR in the body is well maintained and if this balance is disturbed either by an increase of the former or a decrease of the latter it will lead to accumulation of ODFR causing damage to the cells in a process that has been termed oxidative stress[61]. This damage ranges from cell dysfunction to cell death, and can occur either by necrosis or apoptosis[62]. In addition SM contains large amounts of xanthine oxidase and therefore the release of xanthine oxidase during reperfusion causes the generation of free radicals further adding to the damage inflicted on the muscle[63].

2.2.3 Myeloperoxidase and NADPH systems

Two further systems contribute to the generation of ODFR and further cellular damage. Firstly, there is nicotinamide-adenine-dinucleotide phosphate (NADPH) which is a reducing source. NADPH is generated by the action of glucose-6-phosphate dehydrogenase[64]. The NADPH system plays an important role in several sites in the body. NADPH is an essential part of the production of NO by oxidation of L- arginine[65]. It is also involved in the respiratory burst of neutrophils which is an essential part of the body defence against micro-organisms. It can also be activated by cytokines as it is a rich source of ODFR[66]. This ability of the NADPH system to generate ODFR from neutrophils together with xanthine oxidase is an important part of the early phases of reperfusion injury [67-69]. The second involves myeloperoxidase which is secreted

by neutrophils and is another possible source of injury to cells during the reperfusion period, as its presence in both the extra and intra cellular compartments leads to further formation of ODFR and further cellular injury[53,70].

2.3 Protective mechanisms against ischaemic reperfusion injury

Skeletal muscle protection from ischaemic injury by preconditioning can be achieved either by IPC or pharmacological preconditioning. IPC has the advantage of being an endogenously protective mechanism that has few potential side effects. Pharmacological preconditioning is attractive because of the speed of its induction and its theoretical reproducibility. However the results have not been consistently reproducible and no controlled trial has been carried out in order to assess any side effects from its use. Ideally any pharmacological agent used to induce preconditioning should be cheap, easily available, have little or no side effects, be eliminated easily from the body, produce long lasting protection and easily administered with predictable pharmacokinetics.

2.4 Ischaemic preconditioning

IPC was first described in 1986 by Murry et al [27]. Mechanisms by which this protective effect is mediated have to date been quite widely studied, but as yet are still poorly understood.

In SM the protective effects that IPC produces have been related specifically to the effects that pro-inflammatory processes have on the tissue. These inflammatory mediators are modulated by complement and neutrophil activation, reactive oxygen species (ROS) and the formation and subsequent excess production of Ca^{2+} from extra-cellular sources[60,71,72]. Recent work has shown that the ATP-sensitive sodium/potassium channel (discussed later) is

also directly involved, and that IPC results in increased Ca^{2+}-ATP-ase activity of the sarcoplasmic reticulum and of the state 3 respiration level of mitochondria[73,74].

IPC has been shown to minimise infarct size[37], attenuate blood no-reflow within capillaries [75] and to increase the contractile force[76] of skeletal muscle in animal models of IRI. In vitro studies have shown that IPC also offers protection to human skeletal muscle against IRI as demonstrated by preservation of skeletal muscle ultra-structural morphology[77]

Whilst circulating exogenous factors have been shown to produce cellular injury in intact muscle models, intracellular processes have also been shown to be associated with injury. In isolated muscle fibre models elevations in [Ca^{2+} concentrations] and/or generation of ROS from sub-cellular sources have been found to occur[71] particularly during hypoxic conditions (pO_2 = 22 mmHg)[78]. This leads to the activation of proteases and phospholipases which in turn cause non-reversible cellular damage[79].

IPC is thought to share common elements with other signalling pathways. These include the Ras/Erk (extra cellular signal related kinase) cascade and it is thought that there may be other Ca^{2+} dependent pathways that contribute to the development of this tolerance to ischaemic injury in vivo.

One study showed that IPC resulted in significantly higher muscle blood flow which lasts for approximately 3 hours. There was a concomitant increase of phosphocreatine (PCr) by 31%, of ATP by 71% and a significantly higher muscle energy charge potential (ECP) after one and a half hours of reperfusion. In addition muscle inosine 5'-monophosphate (IMP) content is 46% higher in

non-preconditioned muscle after four hours of ischaemia and one and a half hours of reperfusion[37]. (Table 2.1)

However other studies comparing SM morphology and neuromuscular function after IPC (induced by three cycles of 10 minutes of ischaemia followed by 10 minutes of reperfusion) did not show any limitation of ischaemic muscle injury or acceleration in recovery in the seven days after two hours of tourniquet ischaemia. There was also an associated loss of neuromuscular transmission for up to five days after the initial insult. Furthermore the water content of muscle was 5% and 10% higher at three & seven days respectively. This may be due to a difference in the release of muscle proteins from the damaged fibres and increases in vascular wall permeability to plasma proteins or to differences in the total amount of blood flow in the preconditioned muscle following ischaemia[80].

It has also been shown that preconditioning of the cremaster muscle in the rat for 45 minutes of ischaemia followed by 15 minutes of reperfusion significantly attenuated vasospasm and capillary no-reflow after 4-hours of warm global ischaemia and 60 minutes of reperfusion. The mechanism of action for this attenuation was thought to be due to relaxation of vascular smooth muscle[81].

Applying IPC to SM prior to a period of ischaemia generally improves tissue pO_2 during reperfusion and qualitatively restores, to some extent, the hyperoxic phase seen following a short ischaemic period (up to 2hours). Such experiments have demonstrated that tissue oximetry is a sensitive method of assessing micro-vascular flow in reperfused SM [82]. Other studies have shown that IPC significantly attenuates vasoconstriction in the micro-vasculature and

no-reflow in capillaries which occur during the first hour of reperfusion after 4 hours of warm global ischaemia. Furthermore, intermittent reperfusion is effective in attenuating reperfusion-induced vasospasm, particularly in terminal arterioles, although it was not able to attenuate capillary no-reflow by itself [81,83]. Combining IPC and intermittent reperfusion has been found to achieve the best results[83].Mattei et al demonstrated for the first time that IPC improved post-ischaemic function of slow-twitch soleus muscle, as opposed to fast-twitch muscle [84].

Other studies applied IPC with one cycle of 45 minutes of ischaemia 24 hours before four hours of ischaemia and this significantly attenuated ischaemia/reperfusion-induced vasospasm and capillary no-reflow [85], suggesting the effect of late phase of IPC.

The effects of a reperfusion interval and its success in attenuating muscle injury are still not entirely clear. Even though reperfused muscles show a lesser degree of injury and better preservation of function than continuous ischaemia, both groups have the same level of morphological recovery at 14 days[86]. Further study of the molecular mechanisms that are involved in IPC and finding pharmaceutical agents that mirror the protective response of IPC will have the potential to improve the quality of life of patients at risk of ischaemic injury.

Table 2.1: Summary of preconditioning skeletal muscle

DATE	Species	IPC Protocol	Measurement	IT (hrs)	RT (hrs)	Finding
Pang et al, 1995 [37]	Pig	a) No IPC b) 10 min I, 10 min R x 1 c) 10 min I, 10 min R x 2 d) 10 min I, 10 min R x 3 e) 5 min I, 5 min R x 3 f) 30 min I, 10 min R	MI muscle blood flow Levels of PCr	4	48	a) 39% Total infarction. (TI) b) c) & d) 44, 31, & 21% TI respectively. e) 38% Total infarction f) 34% Total infarction Significantly higher Muscle blood flow in IPC Muscles at 1.5 hrs of R Levels of PCr at end of preconditioning 13.5% ↓ than non-preconditioned muscles, levels at 2 & 4 hrs of I & 1.5 hrs of R were 96%, 150% & 31% higher respectively

DATE	Species	IPC Protocol	Measurement	IT (hrs)	RT (hrs)	Finding
Jerome et al, 1995 [75]	Mice	a) 5 min I 10 min R b) a) + Adenosine infusion 0.25 IU/ml. throughout experiment starting 5 min before IPC c) a) + Adenosine infusion 0.25 IU/ml for 10 min, 5 min before IPC and 5 min after start. d) a) + Adenosine during reperfusion only e) Adenosine topically with all the above protocols	Leukocyte migration and adhesion	1	1	a) ↓ in leukocyte emigration and adhesion b) Completely abolished IPC ability to ↓ leukocyte migration and adhesion c) Partial block of IPC ability to ↓ leukocyte adhesion and migration d) Partial block of IPC ability to ↓ leukocyte adhesion and migration e) ↓ in leukocyte migration and adhesion. With protocols a, b, c, &d
Gurke et al, 1995 [87]	Rat	a) 10 min I, 10 min R x 3	Force Performance Contractility	3	2	No loss of Force, Performance or Contractility, in IPC group

DATE	Species	IPC Protocol	Measurement	IT (hrs)	RT (hrs)	Finding
Pang et al, 1995 [88]	Pig	a) 10 min I 10 min R x 3 b) Pre-ischaemic lemakalim 0.18 mg/ms flap c) Post-ischaemic lemakalim 0.18 mg/ms flap d) 5-HD 27 mg/ ms flap. Before preconditioning e) Glibenclamide 0.3 mg/kg before IPC f) 5-HD before pre-ischaemic lemakalim g) 5-HD 27 mg/ms flap before I h) Glibenclamide 0.3 mg/kg before I	MI PCr content in muscle at 2 & 4 hours of I and 1.5 hours of R	4	48	a) 44% ↓ MI Higher levels of PCr at 2 & 4 hrs I and 1.5 hrs R by 108,158 & 30% respectively b) 51% ↓ in MI c) No ↓in MI d) No ↓in MI e) Loss of protective effect of lemakalim and IPC f) Same as e) g) No ↓ in MI
Gurke et al, 1996 [89]	Rat	a) 10 min I, 10 min. R x 1 b) 10 min I, 10 min. R x 2 c) 10 min I, 10 min. R x 3	Force Endurance Performance Contractility	3	2	a) Significant improvement in Endurance, but not in Force, Performance, or Contractility b) Significant improvement of Force,

DATE	Species	IPC Protocol	Measurement	IT (hrs)	RT (hrs)	Finding
						Contractility, but also improvement of Performance & Endurance c) Significant improvement in Force, Contractility, Performance, & Endurance
Lee et al 1996 [90]	Rat	a) 5 min I, 5 min R x 4 b) Adenosine 350 microgm/kg/min x 10 min	Muscle tension ATP & PCr	1	0.5	a) Preservation of muscle function b) Preservation of ATP and PCr levels
Wang et al 1996 [81]	Rat	45 min I, 15 min. R x 1	Arteriole diameter	4	1	89% ↑ in arteriolar diameter after 30 min 90% ↑ in arteriolar diameter after 60 min
Carroll et al, 1997 [36]	Rat	30 min I 10 min R x 2 a) Elevation of flap immediately b) Elevation of flap 24 hrs later	Flap necrosis	N/A	N/A	a) ↓ in flap necrosis from 24% to 14% b) ↓ in flap necrosis from 24% to 11%

DATE	Species	IPC Protocol	Measurement	IT (hrs)	RT (hrs)	Finding
Laghi et al, 1997 [48]	Human	a) 1 min I, 0.5 min R x 10 b) PVD subjects exercised till claudication started, then sub maximal walking performed with 2 hour rest c) PVD subjects + Buflomedil	Blood flow Adenosine levels	N/A	N/A	a) No difference from basal values b) Prolongation of pain free walking distance by 14.3% and total claudication distance by 26.9% c) 84% ↑ in Adenosine levels
Pang et al, 1997[91]	Pig	a) 10 min I, 10 min R x 3. b) Adenosine 0.5 mg/ms pre-ischaemia c) PIA (A_1 agonist), 0.75 mg/ms d) Adenosine 0.5 mg/ms post-ischaemia e) DPCPX (A_1 antagonist) 3 mg/ms before IPC f) DPCPX before pre-ischaemic adenosine g) 5-HD, K_{ATP} channel blocker 27 mg/ms before IPC	MI	4	48	a) 24% ↓ MI b) 18% ↓ MI c) 24% ↓ MI d) 40 % ↓ MI e) 38% ↓ MI f) 46 % ↓ MI g) 37 % ↓ MI

DATE	Species	IPC Protocol	Measurement	IT (hrs)	RT (hrs)	Finding
		h) IPC5-HD, K_{ATP} channel blocker. 27 mg/ms before i) Glibenclamide 0.3 mg/kg before pre-ischaemic adenosine j) DPCPX before I k) 5-HD before I l) Glibenclamide before I				h) 36 % ↓ MI i) 35 % ↓ MI j) 36 % ↓ MI k) 43 % ↓ MI l) 43 % ↓ MI
Whetzel et al, 1997 [80]	Rat	a) I without IPC. b) 10 min I 10 min R x 3	Muscle morphology isometric contractile function, fatigue	2	3 & 7 days	a) No discernible morphological difference between the two groups b) Slower contraction and relaxation in group a than b * No difference in muscle fatigue between the two groups

DATE	Species	IPC Protocol	Measurement	IT (hrs)	RT (hrs)	Finding
Papana -stasiou et al, 1999[92]	Rat	a) 10 min. I, 10 min R x 3 b) Adenosine 1000 micro grams / ml	MPO levels & reduced glutathione. NO metabolites, TNFα & macrophage inflammatory protein	4	2	a) Lower levels of MPO, TNF-α, and higher levels of GSH & NO metabolites b) Lower levels of MPO, TNF-α, and higher levels of GSH & NO metabolites
Atkiss et al, 1999[82]	Rabbit	10 min I 10 min R x 3	Tissue pO_2 levels	a) 2 b) 3.5 c) 3.5+IPC	6	a) Rapid rise in pO_2 on reperfusion followed by hyperoxia for 60-90 min then return to baseline. b) Slow rise in pO2 on reperfusion, lack of hyperoxic period and slower return to baseline c) More rapid rise in pO2 on reperfusion, relative hyperoxia

DATE	Species	IPC Protocol	Measurement	IT (hrs)	RT (hrs)	Finding
Wagh et al, 2000 [93]	Rat	a) No IPC b) Intermittent R after 4 hrs, 5 min R, 5 min I x 3 c) 45min I, 15 min R d) b + c	Arteriolar diameter at 30 & 60 min Capillary perfusion at 30 & 60 min	4	I	a) Capillary diameter 63.4 & 62.1% of baseline. Capillary perfusion 45.3 & 44.8 % of baseline b) Capillary diameter 73.3 & 75% of baseline, capillary perfusion 33 &31 % of baseline c) Capillary diameter 75.7 & 77.6% of baseline Capillary perfusion 16.1 &14 % of baseline d) Capillary diameter 74.8& 78.8 % of baseline Capillary perfusion 17.2 & 14.1 % of baseline
Hopper et al,	Pig	a) 10 min. I 1o min R x 3 b) Adenosine 0.5 mg/[M]	MI	4	24	a) 40 % ↓in MI b) 46 % ↓in MI

DATE	Species	IPC Protocol	Measurement	IT (hrs)	RT (hrs)	Finding
2000[94]		c) Chel 0.6 mg/ Muscle. Immediately after IPC d) Poly B 1 mg/ [M]. Immediately after IPC e) Chel starting 10 min after adenosine f) Chel alone g) Poly B alone h) PMA 0.05 microgm/Muscle before I i) OAG 0.1 mg/ Muscle. Before I j) OAG at onset of Reperfusion k) 5-HD 27mg/kg before IPC l) 5-HD before PMA m) 5-HD before OAG n) 5-HD alone				c) Loss of protective effect of IPC & adenosine d) Same as c) e) Same as c) f) No effect on MI g) Same as f) h) 55% ↓in MI i) 68% ↓in MI j) No ↓in MI k) Complete loss of protective effect l) Same as k) m) Same as k) n) No effect on MI

DATE	Species	IPC Protocol	Measurement	IT (hrs)	RT (hrs)	Finding
Mattei et al, 2000 [84]	Rat	a) No IPC b) 2.5 min I 2.5 min R x 3 c) 5 min I 5 min R x 3 d) 10 min I 10 min R x 3	Muscle function	2.5	2	a)All functional properties significantly reduced b) Functional properties not affected c) Same as b) d) Functional properties not affected
Wang et al, 2000 [95]	Rat	a) IPC by 45 min I b) Adenosine 0.35 mg/100 g. before IPC c) SPT a non-selective adenosine receptor antagonist 0.625 mg/100g before IPC d) SNP + IPC e) L-NA 0.45 mg/100g + IPC	Diameter of terminal arterioles Capillary perfusion	4	1	a) 21% ↑ in diameter of terminal arteriole , same capillary perfusion b) 41%↑ in diameter of terminal arterioles, same capillary perfusion c) 21% ↓ in diameter of terminal arterioles. 36% ↓ in capillary perfusion d) 34% ↑ in diameter of terminal arterioles, same capillary perfusion e) 18% ↓ in diameter of terminal

DATE	Species	IPC Protocol	Measurement	IT (hrs)	RT (hrs)	Finding
						arterioles. 50 % ↓ in capillary perfusion
		f) Adenosine + L-NA + IPC				f) 20% ↓ in diameter of terminal arterioles. 32 % ↓ in capillary perfusion
		g) SPT + SNP + IPC				g) 21% ↓ in diameter of terminal arterioles. No change in capillary perfusion

Key: ATP= Adenosine triphosphate, I= Ischaemia, IPC= ischaemic preconditioning, K_{ATP}= Potassium dependent ATP channels, L-NA= N^{ω}-nitro-l-arginine, MI= Muscle Infarction, MPO= Myeloperoxidase, NO= nitric oxide, OAG= 1-Oleoyl-2-acetyl glycerol, PCr= Phosphocreatinine, PIA= R-phenylisopropyl adenosine, PVD= Peripheral Vascular Disease, R= Reperfusion, SNP= sodium nitroprusside, SPT= 8-sulfophenyl-theophlline, TI= Total Infarction, TNF-α= Tumour Necrosis Factor-α, 5-HD= 5-hydroxydecanoate,↑= increase, ↓= Decrease.

2.5 Pharmacological preconditioning

A number of drugs have been used in order to mimic the effects of IPC. Particular interest has been focused on drugs acting on the sympathetic nervous system and K_{ATP} channels. Buflomedil is a vaso-active drug which induces vasodilatation through its antagonism of α-adreno-receptors and used in the treatment of patients with peripheral vascular disease. Other non selective α-adrenergic antagonists have been used both in clinical and in animal models, as a possible means of pharmacological preconditioning[48]. The results of clinical trials show that there was an 84% increase in the level of adenosine in the blood after the oral or intravenous administration of buflomedil. Another trial[96] showed that an intravenous infusion of between 50-200mg buflomedil and oral administration of 300-900 mg, resulted in an increase in adenosine levels comparable to IPC treatment.

Propionylcarnitine has also been shown to be an effective pharmacological agent for preconditioning, especially in patients with claudication[96] Furthermore pre-treatment with guanethidine, a sympathetic blocking agent, three hours before ischaemia resulted in an initial reduction in blood flow to muscle tissue followed by a higher rate of reperfusion[97].However, the increase in blood flow and walking distance cannot be taken by itself as evidence of IPC[98], as it does not necessarily indicate protection of SM against ischaemic damage.

Gürke et al[99] found that cromakalim, a K_{ATP} channel opener administered ten minutes prior to two and a half hours of ischaemia in a rat model, improved post-ischaemic maximal force, performance and contraction in ischaemic SM but this protective effect was lost when glibenclamide was added (table 1.1).

Furthermore, function of the non-ischaemic contra-lateral SM was not affected[99]. Further studies into the protective effects of cromakalim on SM have shown that pre-treatment with cromakalim repeatedly resulted in higher muscle blood flow during ischaemia and reperfusion with no significant alteration to the systemic haemodynamic circulation[100]. In addition, treatment with lemakalim, another K_{ATP} channel opener, reduced infarct size by 51%[91].

Another method of pharmacological preconditioning is to use a modified University of Wisconsin (UW) solution which does not contain the colloidal hydroxyethyl starch. With infusion of modified UW into the SM prior to a period of cold ischaemia, the tolerance of muscle to ischaemia was significantly improved [93].

A comparable study by Li[101] found that reperfusion with heparinised citrate blood after a combination of four hours warm ischaemia and forty-four hours of cold ischaemia, led to significantly better muscle survival assessed histologically.

Reperfusion in rat hindquarters subjected to 2 hours ischaemia followed by reperfusion with Krebs bicarbonate solution for 15 minutes was found to attenuate dilator responses to the stimulation of cholinergic nerves and also after the injection of acetylcholine and carbachol. Whereas responses to other endothelium-dependent vasodilators, bradykinin and histamine, were not impaired indicating that ischaemia does not cause generalised endothelial dysfunction but a selective impairment of muscarinic receptor-mediated dilatation[102].

A growing number of diabetic patients who have a higher incidence of cardiovascular and PVD are on oral sulphonylureas[103]. These patients are resistant to the effects of IPC as oral sulphonylureas have been documented to inhibit K_{ATP} channels. In light of this Ca^{2+} and alternatives such as glimepiride[104] have emerged as possible substitutes that do not utilise the K_{ATP} channels (discussed later). It has been shown that preconditioning with Ca^{2+} protects against IRI in the rat heart. This work suggests that Ca^{2+} preconditioning acts via a different intracellular signalling mechanism[46,105]. However work done on SM preconditioning with Ca^{2+} to date is very limited and early results suggest that Ca^{2+} preconditioning is a viable alternative[46,103,104].

As muscles contract Ca^{2+} enters the cells by voltage gated Na+ channels [106] or via the Na^{+}/H^{+} and Na^{+}/Ca^{2+} exchangers (figure 2.1) [107]. Ca^{2+} release from the sarcoplasmic reticulum (SR) is also important especially during the early phases of ischaemia. This has been shown in studies whereby modulating SR Ca^{2+} ATP-ase and the SR Ca^{2+} release channel (ryanodine receptor) causes a reduction in SR Ca^{2+} release and this acts protectively against ischaemia (figure 2.2) [108].

The molecular mechanisms involved in the development of Ca^{2+} overload in SM are a result of direct coupling between dihydropyridine and ryanodine receptors. This causes sarcolemmal depolarisation leading to conformational changes in the dihydropyridine receptor, which causes Ca^{2+} release independently of sarcolemmal Ca^{2+} influx [108,109].

Table 2.2: Summary of agents used in pharmacological preconditioning

Family	Drug	Mode of action
A-β-D ribofuranovyladenine	Adenosine	Neuromodulator
K_{ATP} Channel openers	Cromakalim Pinacidil Levcromakalim	K_{ATP} channel opener
Calcium	Sulphonyurea Glimepidine	Cell membrane stabilisation
Non selective alpha-adrenergic antagonist	Buflomedil hydrochloride Pentoxifylline Torbufylline Phentolamine Ifenprofil Isoxvoprine	Vasodilator of microcirculation
Essential factors	Propionylcarnitine	Increased NO production

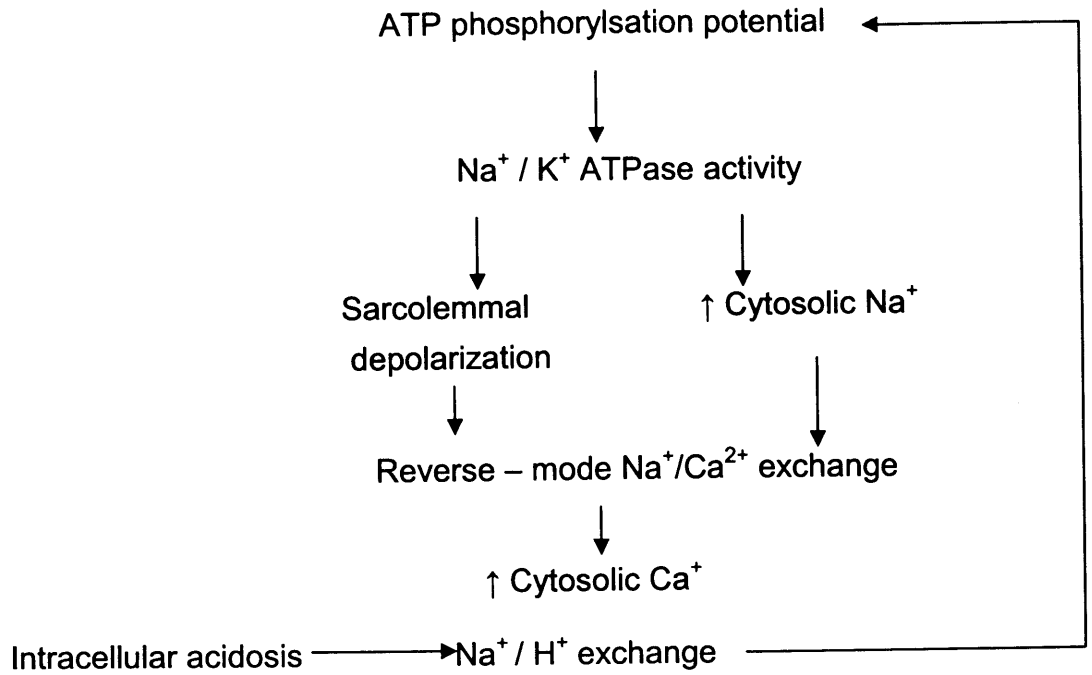


Figure 2.1: A schematic representation of the way in which reverse-mode Na^+/Ca^{2+} exchange leads to an elevation in the Ca^{2+} levels during ischaemia- reperfusion. As adopted from reference[42].

Key: ATP= Adenosine triphosphate, ATPase= Adenosine triphosphatase, Ca^{2+} = Calcium, Na^+ = Sodium, K^+ = Potassium, ↑= increase.

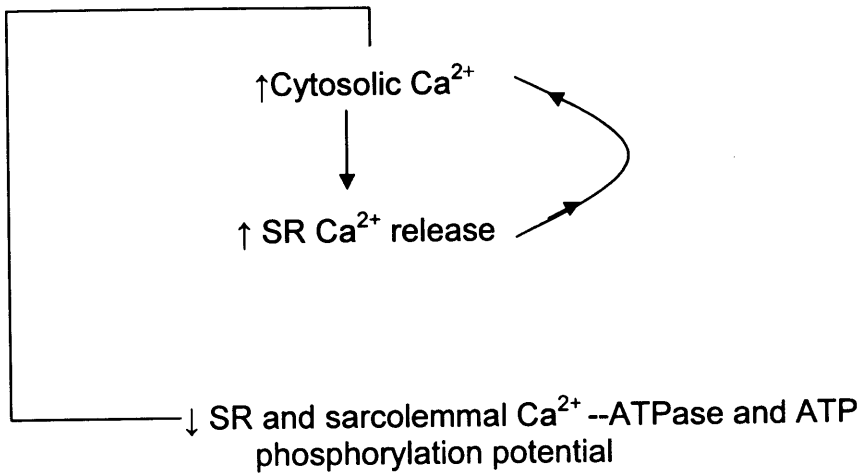


Figure 2.2: A schematic representation of the positive - feedback loop that regulates the release of Ca^{2+} from the sarcoplasmic reticulum as adopted from reference[42].

Key: ATP = Adenosine triphosphate, ATPase = Adenosine triphosphatase, Ca^{2+} = Calcium. SR = Sarcoplasmic reticulum, ↑ = increase, ↓ = decrease.

2.6 Mediators of IPC

2.6.1 Adenosine

Adenosine is a nucleotide precursor, which is a breakdown product of the catabolism of ATP during ischaemia[110,111]. Adenosine can be produced by endothelial cells, platelets, erythrocytes and leukocytes[112,113]. It can also be produced and metabolised extra-cellularly in the interstitial and intravascular compartments both in SM and other body tissues and performs several of its actions by acting on receptors on the surface of skeletal and smooth muscles[96]. The half life of adenosine is short as it undergoes a reuptake by erythrocytes and endothelial cells[114-116]. Adenosine concentration is increased in contracting skeletal muscle. The rate of increase is directly proportional to the blood flow and intensity of muscle contraction[117]. This increase may be directly due to its release from skeletal muscle during contraction[118,119]. Another study which indicates that adenosine is released from skeletal muscle has also shown its release from human SM during systemic hypoxia [120]

It has been proposed that adenosine acts both as "trigger" and "mediator" of IPC[41,76,110]. The reason for considering adenosine as a mediator of IPC is its release in large quantities after onset of myocardial and SM ischaemia[121,122].It has a protective effect that is multifactorial as it inhibits leukocyte adhesion and the expression of adhesion molecules, interferes with neutrophil and platelet function[109,123], reduces free radical production[124,125] and induces vasodilatation[121].

Newby et al proposed that adenosine is a retaliatory metabolite that signals an imbalance between oxygen supply and demand and starts a response to rectify

the imbalance[126]. Adenosine also plays a role in angiogenesis through its action on vascular endothelial growth factor[127] and regulates blood flow in hypoxic or ischaemic tissue[128].

Three adenosine receptors exist in SM: A_1, A_2, and A_{2B}[129]. Preconditioning results in the release of adenosine which in turn leads to the activation of adenosine A_1 receptors and increased protection against ischaemic damage[130-132]. Pre-ischaemic intra-arterial infusion of adenosine in a porcine model reduced the rate of high-energy phosphate and energy charge potential depletion. It was found that IPC acted through A_1 receptors together with post-receptor involvement of the K_{ATP} channels. However, it was suggested that adenosine was unlikely to play a key role in the effector mechanism itself[110].

A further study by Pang et al [88] showed that adenosine mimicked IPC in skeletal muscle, as a local intra-arterial infusion of adenosine (0..5 mg/muscle flap 8-10 min) significantly reduced the infarct size of the Latissmus dorsi (50%) and gracilis muscles (63%) when these were subjected to four hours of warm global ischaemia and forty eight hours of reperfusion. On the other hand, it did not affect the systemic haemodynamics or cause any changes in local muscle blood flow. Furthermore antagonising adenosine receptors with 8-(p-Sulphonyl)-theopylline (8-SPT) (a non-selective adenosine receptor A1 antagonist) and DPCPYX (selective A1 receptor antagonist) prevented the effects of IPC and adenosine [88]. However, another contradictory study showed that the administration of an adenosine receptor antagonist 8-SPT did not reduce the improvement in muscle function offered by IPC [99].

The effects of adenosine have been confirmed in other animal models including rats[76], rabbits[133] and dogs [90]. Clinically, a similar study by Laghi Pasini[48] has demonstrated that adenosine has shown beneficial effect in patients with peripheral obliterative arterial disease (POAD); it prolongs walking distance and reduce haemorheological derangement (table2.1). Further human studies have shown that the administration of caffeine or dipyridamole both an adenosine antagonist abolishes the protective effect of IPC on skeletal muscle[134,135].

It has also been found that metabolite accumulation was reduced in skeletal muscles pre-treated with adenosine, with the level of phosphocreatine, being 37% higher in those skeletal muscles which were treated with adenosine prior to IPC than those that were not. Although adenosine is unlikely to play a key role in the effector mechanism of preconditioning, its anti-infarction effect is associated with a slower rate of energy metabolism and metabolite accumulation during sustained ischaemia and reduced neutrophilic myeloperoxidase activity in post-ischaemic muscle[92,110].

Other work in which the skeletal muscle was subjected to four hours of ischaemia and one and a half hours of reperfusion found no difference in the accumulated muscle content of adenosine during the four hours of ischaemia between the IPC muscle group and controls. However, there was a reduction in the total level of accumulated metabolites and lactate content in the IPC group [37]. These findings might be due to insufficient IPC time or number of cycles.

Stimulation of adenosine A_1 receptors with either adenosine or the adenosine analogue R-phenylisopropyl adenosine (R-PIA), (a specific agonist for A_1 receptors) depressed the systemic blood pressure, but no corresponding

improvement in post-ischaemic SM function was achieved. This variation in the results achieved with adenosine preconditioning might be due to variations in doses, or methods of delivery. Experiments carried out to date however have unequivocally demonstrated that adenosine together with K_{ATP} (discussed later) channels are involved during IPC within SM, further study on the exact mechanism of adenosine preconditioning is required.

2.6.2 Nitric Oxide

It has been proposed that NO plays a key role in triggering and mediating IPC. Work by Parratt [136] has implied that NO mediates its cardio-protective response in IPC by increasing the levels of cyclic GMP (cGMP) thus reducing energy demands on the muscle tissue by limiting cyclic AMP (cAMP) levels by increasing the synthesis of the enzyme cGMP-sensitive cAMP phosphodiesterase.

In skeletal muscle, it has been suggested that depending on its rate of production NO may also play a mediating role in preconditioning [137]. It has been suggested that NO is involved in the release of noradrenaline in SM especially during IPC [138]. Looking at the role of NO and its regulation of noradrenaline release. It has been found that exercise increases the release of interstitial noradrenaline, the source of which seems to be the terminal vesicles of the sympathetic nerves. By administering *N*G-monomethylyl-L-arginine (L-NMMA) an NO synthase (NOS) inhibitor there is a notable increase in the level of interstitial noradrenaline. It may be that the intense exercise increases interstitial noradrenaline release and NO modulates the release of interstitial noradrenaline in skeletal muscles in humans. On the other hand, ischaemia and

intense exercise have a neural preconditioning effect on subsequent exercise in humans. This suggests that NO contributes to protective mechanism during ischaemia [138].

Measurement of the activity of constitutive NO synthase (cNOS) and inducible NOS (iNOS) in a rat model of IPC showed the presence of both NOS isoforms and that the ratio of cNOS to iNOS was reversed after preconditioning. Giving N^{ω}-nitro-L-arginine methyl ester (L-NAME) a NOS inhibitor treatment after ischaemia (without IPC) showed better micro vascular perfusion, whilst administering aminoguanidine (AMG) (without IPC) gave an analogous result[137]. This suggests that at least in the acute phase of IPC, NO may be injurious, but that the source is from iNOS[139]. More importantly observations have shown that NO is essential for the late benefits associated with preconditioning, but that the source of NO was not via iNOS activity, despite elevated iNOS levels in SM in this particular animal model [95].

The role of NO in early and late preconditioning has also been studied. It was found that in the absence of IPC, local intra-arterial infusion of adenosine or sodium nitroprusside (SNP) a NO donor resulted in a similar micro-vascular protective effect as that induced by IPC alone. Furthermore the infusion of 8-sulfophenyl-theophylline (SPT), an adenosine receptor antagonist or N^{ω}-nitro-l-arginine (L-NA), an NO synthetase antagonist eliminated the micro-vascular protection afforded by IPC as did infusion of adenosine together with L-NA. These observations suggested that in late preconditioning of SM adenosine and NO interact with each other to initiate the delayed protective effect[140].

In an effort to determine the exact role of NO in the induction of late preconditioning, a comparison between the outcome of IPC alone, IPC and L-NAME, and IPC and AMG found that even with a lack of parenchymal protection within skeletal muscle from IPC, the level of iNOS activity was 81% higher in IPC treated muscle. However, it was found that the level of cNOS in these muscles was 53% lower and that the ratio of cNOS to iNOS was reversed after IPC. In this study L-NAME treatment without IPC resulted in improved micro-vascular perfusion with a comparable level of improvement in micro-vascular perfusion being observed post AMG treatment. These results suggest that NO is injurious during the reperfusion period and that the source of NO might be iNOS. Further treatment of IPC muscles with L-NAME reduced the beneficial effects of IPC, but treatment with AMG did not reverse the beneficial effect of IPC. These observations led the investigators to conclude that NO is essential for the late effects of IPC, and that the source of this elevated NO was not from iNOS[137]. However one must view the lack of parenchymal protection offered by this study to imply that elevation in levels of NO are not directly involved in late IPC. Functional evidence in the heart indicates that NO plays a prominent role in modulating both early and late preconditioning[141]. In skeletal muscle studies have also shown a protective effect from NO however this was more from the action of endothelial NOS rather than iNOS or nNOS, but again the mechanisms behind the effect of NO are still unclear[142].

2.6.3 ATP-sensitive potassium (K_{ATP}) channels

K_{ATP} channels, which are widespread cellular electrogenic channels have been investigated extensively for their role in IPC in the heart and have been shown to play a definitive role[143,144]. However in skeletal muscles their role has not

been thoroughly investigated. Treatment with lemakalim a K_{ATP} channel opener has been shown to reduce the size of SM infarction by 51%, while the K_{ATP} channel blockers 5-hydroxydecanoate (5-HD) or glibenclamide resulted in the loss of this protective effect[91,145].

It was found from experiments done on latissmus dorsi muscle flaps in porcine animal models that the anti-infarction effect of the PKC activators 1-Oleoyl-2-acetyl glycerol (OAG) and phorbol 12-myristate 13-acetate PMA were blocked by the administration of 5-HD. These observations show that K_{ATP} channels may be involved downstream from PKC in the signal transduction pathway of IPC particularly in skeletal muscle [94].

A role for K_{ATP} channels has also been demonstrated by the observation that administration of K_{ATP} channel antagonist, glibenclamide again 10 min before induction of IPC, abolished the protective effect of preconditioning[75]. This latter work on a rat model was supported by studies performed on a dog model[73,74].

2.6.4 Heat shock protein

Heat shock proteins (HSP) such as HSP_{70} and HSP_{72} are intracellular stress proteins that accumulate after priming protocols such as ischaemic preconditioning or mild hyperthermia[146]. The role of sub-lethal whole body hyperthermia is to produce tolerance to priming stresses and lethal endotoxin exposure, that is hyperthermic preconditioning and associated with the induction of HSP based proteins[83,147].

In skeletal muscle, tolerance to ischaemic injury has also been associated with the production of HSP but in particular HSP_{70}[148]. Lepore et al[149] have shown

that in the rat, ischaemic preconditioning regimens (1 x 5 min ischaemia, 4 x 5 min ischaemia interrupted by 10 min reperfusion, and 1 x 10 min ischaemia or 2 x 10 min ischaemia interrupted by 15 min of reperfusion) induced a significant increase in HSP_{70} at 24 h post preconditioning using the latter two protocols of 1 x 10 or 2 x 10 min as determined by Western blot analysis.

Two previous studies[150,151] showed that thermal stress preconditioning in rat models protected SM against morphological and biochemical changes in the mitochondria of muscle cells by causing the release of creatinine phosphokinase and a significant reduction in the levels of ATP after ischaemic injury. In addition, in these two models electrophysiological function of the tissue was found to be improved[151].

To evaluate the role of HSP_{72} in protecting vascular endothelium and muscle Lille et al[152] looked at neutrophil rolling, adherence and arteriolar diameter after a severe IRI. They found no significance in any of these parameters, as well as muscle oedema. They concluded that if the ischaemic reperfusion injury exceeded the protective capacity of HSP_{72} then the preheated tissues suffered the same amount of damage as the untreated ones.

However a more recent study has shown that increased expression of the HSP_{72} protein is pivotal in mediating the delay phenomenon in preconditioning [153]. Whilst these studies imply that HSP are evidenced post preconditioning, the molecular mechanism(s) behind its accumulation have still not been fully elucidated and as such do not substantiate the theory that HSP are directly involved in preconditioning itself. Other studies looking at the role of heme oxygenase (a heat shock protein) have also shown that there is a protective

effect on skeletal muscle that is not mediated via an altered leukocyte endothelial interaction[154]. Further studies evaluating the role of HSP_{72} in the protection of skeletal muscle during composite tissue transplantation have shown that pre-treatment 24 hours prior to transplant with HSP_{72} resulted in less ischaemic damage[155]

2.6.5 Protein kinase C (PKC)

It was proposed that the PKC mediated pathway was involved in myocardial preconditioning[156] whereby a G-protein which was coupled to the adenosine receptors was activated, leading to PKC activation and its subsequent translocation from the cytosol to the cell membrane where it phosphorylates substrate proteins to induce tolerance to ischaemia [156].

In skeletal muscle, a recent study investigating whether preconditioning with noradrenaline, maintaining sarcoplasmic reticulum (SR) function during metabolic inhibition, it was found that this process was mediated by the PKC pathway[157] in fact it appears that PKC activation played a key role, with an inverse relationship between Ca^{2+} increase during muscle infarction and SR functioning.

Other recent work[94] has shown that eight PKC isoforms (α, βI, βΠ, δ, ε, θ, ζ, ι) exist in the cytosol fraction of muscle, but that only six of them (α, βΠ, δ, ε, θ, ζ,) were detected in the sarcoplasmic reticulum of preconditioned latissmus dorsi muscles before IPC. These observations suggested that PKC plays a central role in the post-receptor signal transduction pathway of IPC against infarction in non-contracted animal SM. Other more recent work by Wang et al has shown that in SM the late phase of micro-vascular protection offered by either IPC or

adenosine is achieved through a PKC dependent pathway[158]. The isoform nPKC alone translocates from the cytosol (within 10 min) after the final ischaemic cycle into a membrane component of the skeletal muscle cell but it does not translocate to the sarcolemmal membrane or sarcoplasmic reticulum itself. Furthermore the anti-infarction effect of IPC and the PKC activator OAG is associated with lower neutrophilic MPO activity in post-ischaemic muscle during 16 hours of reperfusion. Further studies have also suggested PKC plays a protective role that is distinct from NOS as the use of an selective NOS inhibitor did not stop the protective role of IPC[142]

2.7 Future work

Since the first description of the existence of IPC in SM, a plethora of new data has been added, and IPC has been shown to be a powerful, intrinsic mechanisms of protecting skeletal muscle from ischaemic injury both during surgical intervention and treatment of peripheral vascular disease. Current research has demonstrated that IPC is an adaptive modality which can readily be reproduced in a variety of different models of warm and cold ischaemia. The concept of IPC induced by pharmacological intervention opens the door for both safer surgery and a means of treating claudication however this is still in the very early stages and further research into this is required. The causal relationship between initiating events, biochemical signalling pathways and end effector species still remains undefined mechanistically. As this new field looks into more mechanistic based studies of IPC the interrelationships of the preconditioning cascade are likely to be resolved and so defined. The idea of developing a pharmacological agent that will be safe and confer all the protective effects of IPC is an attractive one, as it remains impractical and time

consuming to apply IPC in the operating theatre. Even though a large amount of work has been done on IPC there still remains some difference of opinion on what is the ideal preconditioning time and the number of cycles required. This might be due to the different species used in the work done till now, however there is agreement that IPC does offer protection against IRI.

The aim of this work is to clearly establish the ideal preconditioning time per cycle and the number of cycles required. The identification of a pharmacological agent capable of consistently reproducing the same effects of IPC which must be safe and simple to use clinically.

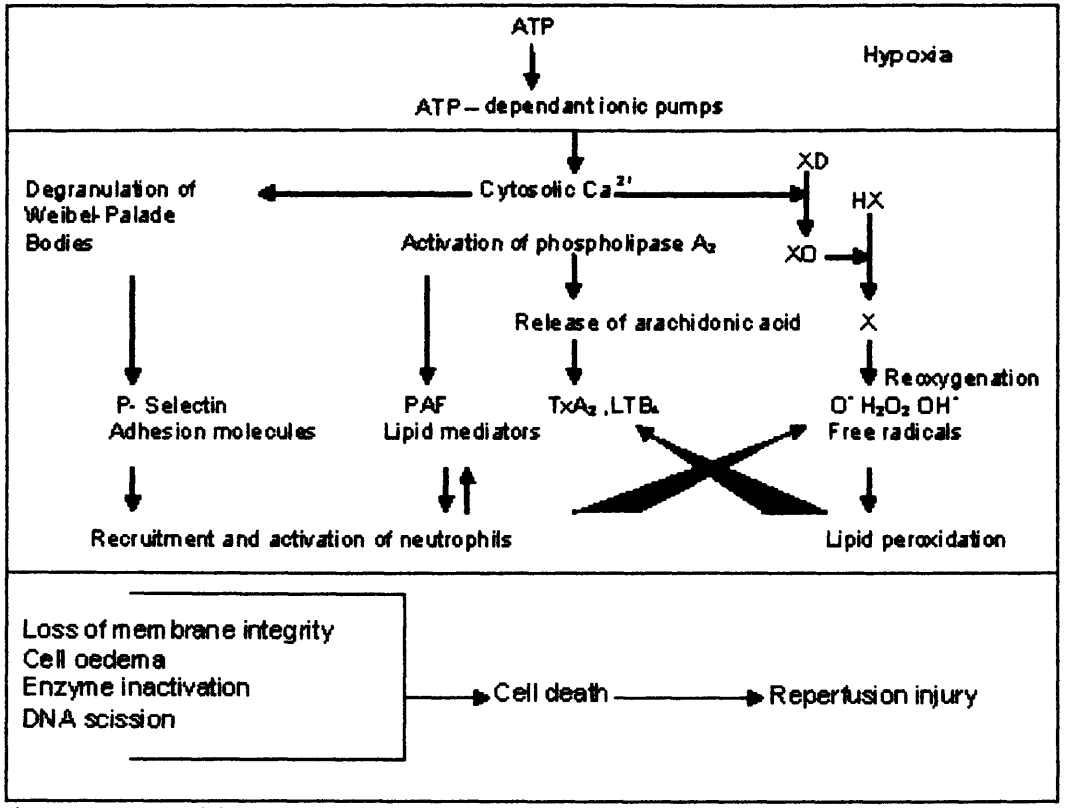


Figure 2.3: The key events which are responsible for the pathogenesis of skeletal muscle reperfusion injury (reproduced with permission from ischaemic reperfusion injury page 35, ch 2, Pierce A Grant, Robert T Mathie, Blackwell science Ltd)

Key: ATP = Adenosine triphosphate, Ca^{2+} = Calcium, LTB_4= leukotriene, PAF= Platelet Activating Factor, TxA2 = Thromboxane A_2, X = Xanthine, HX = Hyroxyl Xanthine, XO = Xanthine Oxidase, XD = Xanthine Derivatives.

Chapter Three

Methods used in the experimental

work for this thesis

3.1 Laser Doppler Flowmetry

Laser Doppler Flowmetry (LDF) is an established method of easily and continuously measuring tissue microcirculation without interfering with blood flow to the tissues[159-161]. The basic principles of its function have been well described[159-161].

3.1.1 Principles of laser Doppler Flowmetry

Light is generated from a 2mV- helium neon laser at 632nm and delivered to the tissues via optical fibres; the light reflected back from the tissues is carried back through the optical fibres and detected by a photo detector. Photons that are reflected back from moving red blood cells (RBCs) will have a Doppler frequency shift unlike those reflected back from static tissues. Mixing of the two by the photo detector generates an electrical signal that contains the Doppler frequency shift information. This electrical signal is further processed to produce an output voltage that is related in a linear fashion to the total number of moving RBCs in the area of a sphere multiplied by the mean velocity of the RBCs. LDF has been shown to be both reliable and sensitive at measuring blood flow to tissues[162].

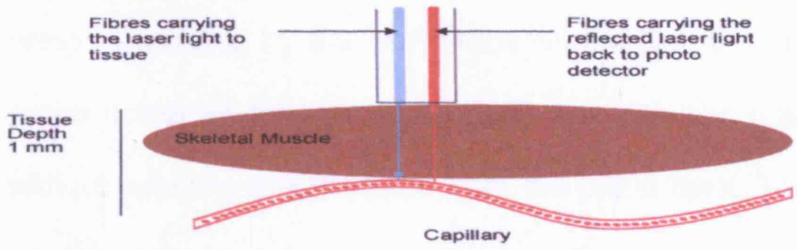


Figure 3.1: Laser Doppler probe applied to skeletal muscle.

3.1.2 Units of measurement

The LDF measures flow in arbitrary perfusion unit (flux), a quantity proportional to the product of the average speed of the blood cells and their number concentration (often referred to as blood volume). This is expressed in arbitrary 'perfusion units' and is calculated using

$Flux = C_{mRBCs} \times V_{mRBCs}$.

Where C_m = concentration, V_m = velocity, RBCs = red blood cells

Due to the variation of the signal across the surface of tissues it is not possible to apply a conversion factor that so that the LDF signal can be expressed in absolute flow units [160,161]

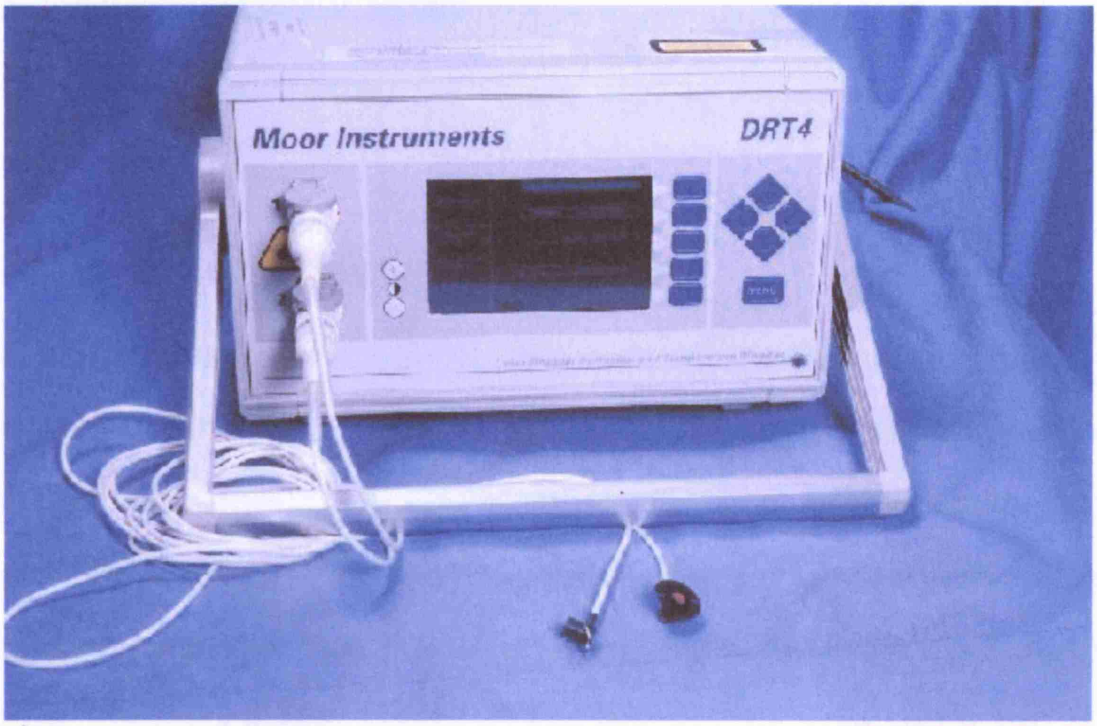
3.1.3 Laser Doppler Flowmeter

Skeletal muscle microcirculation was assessed using a commercially available dual channel surface laser Doppler flowmeter (DRT4, Moore instruments Ltd., Devon, UK) (Figure 3.1)

At the start of each study the LDF was calibrated against a standard reference (Brownian motion of polystyrene micro-spheres in water) provided by the manufacturer. In order to curtail any disturbance to blood flow due to the pressure exerted by the LDF probe on the SM, the probe was attached to a probe holder so that the probe itself was just in contact with the SM surface without applying any pressure from the probe itself. The probe was applied to predetermined point on the vastus lateralis muscle in all experiments in order to minimise any error due to anatomical variations in the SM microcirculation.

3.1.4 Laser Doppler Flowmeter data collection and analysis

Data from the LDF was fed into an analogue digital converter and were recorded at a frequency of 2 Hz. Readings were collected before the application of IPC as baseline and at specific time points during the preconditioning, ischaemic and reperfusion periods.



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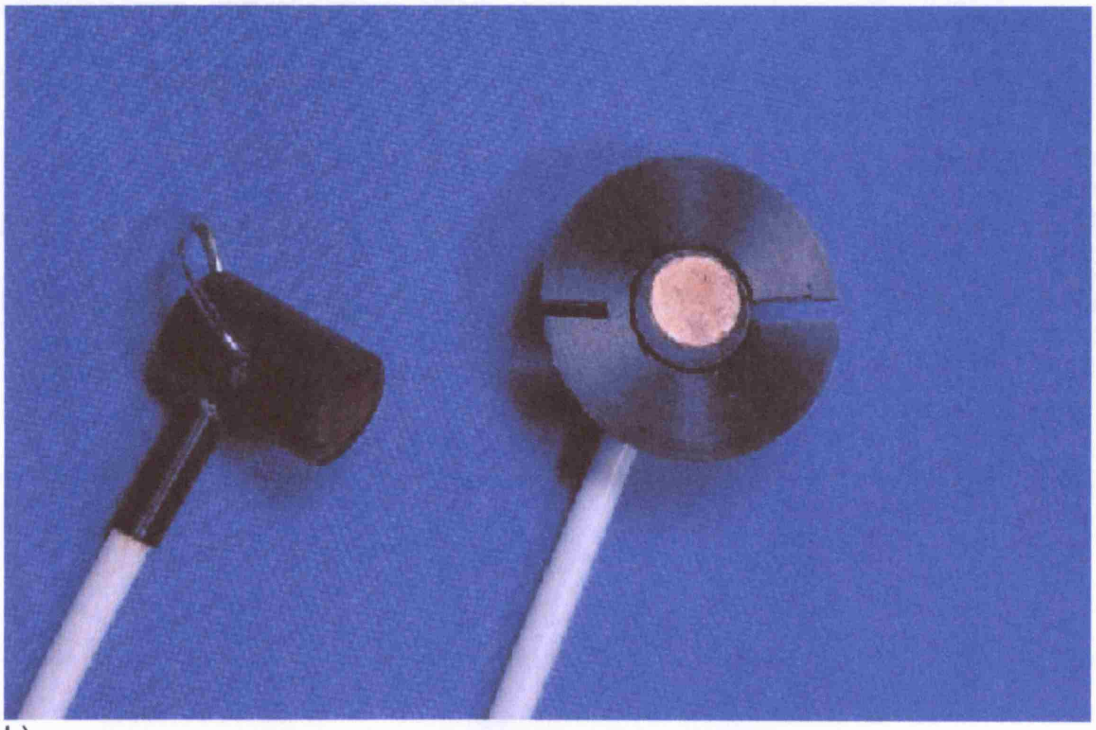


b)

Figure 3.2: (a) Dual channel surface laser Doppler flowmeter and (b) Probes (DRT4, Moor Instruments Ltd., Devon, UK).

3.2 Near Infrared Spectroscopy

Near infrared spectroscopy (NIRS) was used to assess the level of tissue oxygenation.

3.2.1 Principles of near infrared spectroscopy

Biological tissues interact with light in a multitude of ways including scattering, reflection and absorption which is dependent on the type of tissue illuminated and the wavelength of light used[163]. Light in the visible range of the spectrum (450-650nm) is strongly attenuated due to its absorption by haemoglobin and its scattering in tissues. This attenuation is inversely related to the wavelength of light. This leads to the failure of light to penetrate for more than a 1cm into tissues[163-165]. On the other hand light in the near infrared region of the spectrum (700-1000nm) can penetrate for some distance (up to 8cm in the brain) without being significantly absorbed[163,165,166]. Even with the great decrease in absorption of light in the near infrared region there remains a difference in the light absorption spectra of oxygenated and deoxygenated haemoglobin for them to be differentiated spectroscopically using only a few wavelengths[167]. NIRS depends on two basic principles;

1- The relative transparency of biological tissues to light in the near infrared region of the spectrum.

2- The existence of different tissue chromophores with different absorption spectra to light in the near infra red region of light[166,168,169].

Tissue chromophores are colour bearing compounds that exists in all biological tissues in varying amounts. These are oxyhaemoglobin (HbO_2),

deoxyhaemoglobin (Hb) and cytochrome oxidase (CytOx). These compounds have different absorption spectra to light in the near infra red region (Figure 3.2). This difference in light absorption is dependent on the compounds O_2 content[163].

3.2.2 Tissue chromophores

Chromophores are colour-bearing compounds that are present in all biological tissues; these include HbO_2, Hb, and CytOx. Their differing chemical composition gives them different absorption spectra in the near infrared region of light.

Haemoglobin is the red O_2 carrying pigment in red blood cells. It is a protein with a molecular weight of 64,450 it is made up of 4 subunits each of which is made up of a haem moiety attached to a polypeptide chain. Haem is an iron containing prophyrine derivative. The polypeptide chains are otherwise known as globulin. The iron in haemoglobin is in the active ferrous form which is capable of binding an O_2 molecule in a reversible fashion (i.e. the reaction is an oxygenation rather than an oxidation).

Each gram of haemoglobin is capable of binding 1.34ml of O_2. O_2 is released from HbO_2 into the tissues to form Hb. The reaction between O_2 and haemoglobin occurs very rapidly (milliseconds) and is reversible ($Hb + O_2 \leftrightarrow HbO_2$)[170]. CytOx is the terminal enzyme in the cellular respiratory chain inside the mitochondria. It is responsible for the reduction of O_2 to HbO_2, in a four-electron reaction, with the concomitant synthesis of ATP through the oxidative phosphorylation process[171-173]. It contains 4 redox active groups, 2 haem iron (haem and haem a_3) and 2 copper (Cu_A and Cu_B) centres. These four metal

centres accept or donate electrons during the electron transfer through the respiratory chain, changing their redox state. The oxygen-binding site of the enzyme is the binuclear unit formed of the Cu_B and haem a_3. The donation of electrons from this unit to oxygen accounts for the majority of oxygen consumption in the tissues. The Cu_A and haem centres donate electrons to the binuclear unit and therefore are not directly involved in oxygen reduction[171-173]. In the absence of oxygen, electron transfer to oxygen cannot take place. Electrons accumulate on the haem and copper atoms and Cyt Ox becomes reduced. With oxygen availability the electrons are transferred rapidly from the metal centres to oxygen and Cyt Ox becomes oxidised. Many factors can affect the Cyt Ox redox state in vivo, but the most significant factor is likely to be the oxygen concentration[171,174]. All four centres of Cyt Ox exhibit different absorption characteristics depending on their redox state. The copper centres are optically active in the NIR light in contrast with the haem centres that absorb visible light[163,174]. However, absorption of the NIR light by Cyt Ox occurs primarily at the Cu_A centre within Cyt Ox. The oxidised Cu_A centre has a characteristic shape spectrum with a broad peak centred around 845 nm which is absent with the reduced enzyme[163,174]. The contribution of haem iron centres to absorption of NIR is less than 10% of the total signal in the reduced-oxidised spectrum[174,175].

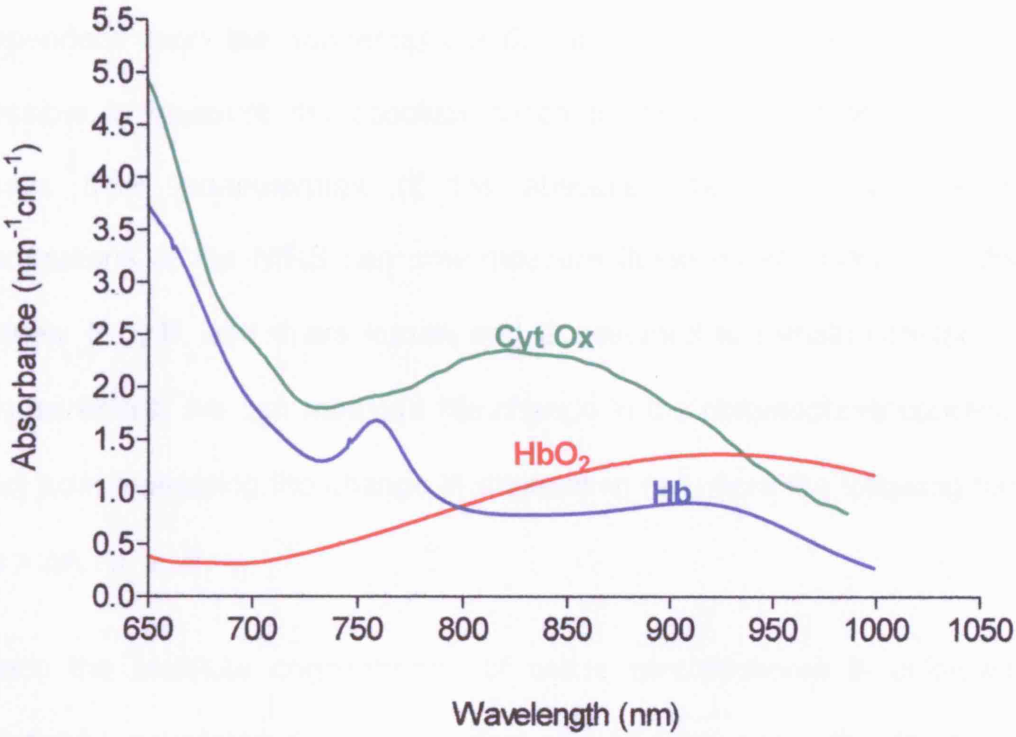


Figure 3.3: Absorption spectra of HbO_2,Hb, & Cyt Ox in the NIR light region[163]

The concentration of Hb, HbO_2, and CytOx in tissues measured in μmol/L, can be calculated using a modified Beer-Lambert law. This law can be applied to tissues with homogenous scattering to calculate the concentration of chromophores from the amount of optical absorption[166].

$$A = \alpha.c.d.\ B + G$$

Where A is the attenuation of light, α is the absorption coefficient of the chromophores ($\mu mol^{-1}.cm^{-1}$), c is the concentration of the absorption compound (μmol/L) and d is the distance between the entry and exit points of light into the tissues (cm). B is the differential path length factor (DPL) which accounts for the increase in optical path length due to light scattering (which causes the optical

path length to be greater than d) and G is a constant factor which accounts for loss of photons by scattering. As G cannot be quantified in vivo and is dependent upon the scattering coefficient of the particular tissue, it is not possible to measure the absolute concentration of the chromophore in the tissue from measurement of the absolute attenuation however newer generations of the NIRS can now measure these concentrations in absolute values. If α, B, and d are known and G assumed to remain constant during measurement, we can measure the change in the chromophore concentration (Δc) from measuring the change in attenuation (ΔA) from the following formula: $\Delta c = \Delta A / \alpha . d . B$.

Since the absolute concentration of tissue chromophores is unknown and cannot be calculated due to the effect of light scattering within the tissue, all NIRS measurements are expressed as absolute concentration changes (μmole/L) from a reference point at the start of the measurement. B depends on the amount of scattering in the medium which can be measured by "the time of flight" method[176].

For simultaneous calculation of the changes in concentration of a number of chromophores from changes in attenuation at a number of wavelength, an algorithm can be used which incorporates the relevant absorption coefficient for each chromophore at each wavelength[166,167,177]. As there are three chromophores of interest in the tissue (HbO_2, Hb, and Cyt Ox) it is necessary to make measurements at a minimum of three wavelengths, if more than three wavelengths are used, standard curve-fitting analysis may be used to increase the accuracy of the calculated concentration changes[166,167,177].

3.2.3 Applications of near infrared spectroscopy

NIRS was first used in a clinical setting to measure myocardial and cerebral tissue oxygenation[178]. Since then it has been used in a variety of clinical situations. It has been most widely used in the assessment of cerebral haemodynamics and oxygenation. Examples of this include the diagnosis of hypoxic brain injury, studying cerebral haemodynamics both intra-partum and in neonates[179-181], the monitoring of cerebral tissue oxygenation during carotid endarterectomy[182], in paediatric cardiac surgery where it has been found to accurately assess the state of cerebral tissue oxygenation[183], and to detect cerebral changes in patients with closed head injuries[184]. In addition it has also been used to detect changes in head injuries associated with delayed traumatic intra-cerebral haematoma before an increase in intracranial pressure, altering neurological signs or CT scan changes[185]. NIRS has also been used in the assessment of intermittent claudication in the lower limbs[186] and in assessing both the intra and extra-cellular effects of hypoxia on hepatic tissue[187,188]

In order to ensure reliability and precision of NIRS validation work has been carried out both in adults[189] and neonates[190]. Readings of cerebral oxygenation obtained by NIRS have been compared to jugular venous oximetry during labour[181], acute brain disease[191] and head injury[184]. In all these studies results from NIRS have correlated well with jugular venous oximetry indicating the reliability and sensitivity of NIRS as a non invasive method of measuring cerebral oxygenation. The measurement of cerebral blood flow by NIRS can be done in adults by measuring the total haemoglobin volume (HbT) from the changes in HbO_2 and Hb in response to changes in arterial oxygen

saturation[190], whereas in infants this can be done by comparing this method with ^{133}xenon clearance[192].

Furthermore NIRS has been shown to reproducibly record intra and extra cellular oxygen levels and blood volume in the heart[193-195] these measurements have been shown to correlate significantly with the duration[195], and degree[194] of occlusion to blood flow as well as with collateral blood flow[193]. Another use of NIRS has been in assessing the mitochondrial respiratory function after IRI in renal transplant in rabbits, which showed a significant correlation between the CytOx redox changes measured by NIRS, NADH fluorescence and histological changes[196].

3.2.4 Application of near infrared spectroscopy in skeletal muscle

In order to apply NIRS to SM it is essential to verify the accuracy and reproducibility of the results of previous studies. Since first used in 1989 to evaluate the state of SM perfusion in patients with heart failure, Wilson et al[197] showed that NIRS was an effective non invasive method in estimating the levels of SM O_2. Since then NIRS has been used in a variety of studies in animal models and humans to estimates the levels of SM O_2 and HbO_2, both in health and disease. Studies to validate NIRS as an effective tool in evaluating SM oxygenation have shown that in human SM, readings obtained from NIRS correlate well with the state of SM oxygenation[198,199]. NIRS has also been used successfully to assess severity of intermittent claudication in patients with PVD[200]. Other studies used NIRS to measure tissue oxygenation and oxygen consumption (VO_2) [201-203] in SM, this was done by calculating the rate of change of HbO_2 and Hb allowing the rate of oxygen consumption to be calculated. This

method of calculation has been evaluated in forearm ischaemia, venous outflow restriction and exercise[201,203] and this technique can be used to determine the severity of PVD[202]. These studies have proved that NIRS is a reliable and reproducible method of estimating VO_2 in human muscles. The use of NIRS to monitor oxygenation and perfusion within the microcirculation of SM and to differentiate between microcirculatory changes due to arterial, venous or total vascular occlusion has been examined in rabbit limbs[204] and SM flaps[205], these studies indicated that SM oxygenation measured by NIRS correlates well with flap vascularity.

The above studies clearly indicate that NIRS is an important tool for measuring SM extra-cellular oxygenation both in healthy and disease states in humans. However the role of CytOx as an indicator of intracellular oxygenation and its importance for measuring critical tissue hypoxia and IRI injury has not been fully evaluated.

3.2.5 Near infrared spectrometer

Intra and extra cellular tissue oxygenation was measured in this study using the NIRO 500[206](Figure 3.3). This machine is the commercial version of an instrument developed by the department of medical physics and bioengineering, University College London[167]. The light source in the NIRO 500 is a monochromatic light generated from semiconductor laser diodes (LD). Light is produced at four wavelengths (774, 826, 849, 906nm). The wavelengths are chosen based on

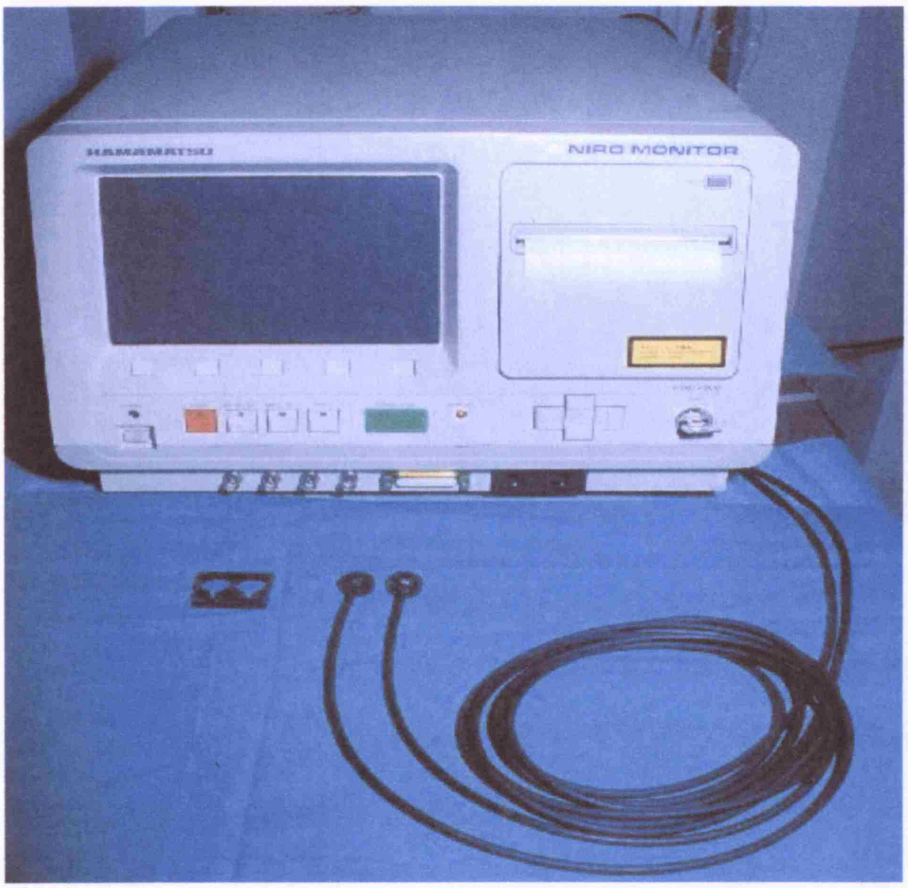
1- 765nm, the absorption maximum for Hb.

2- 810nm, the isobestic wavelength at which the extinction coefficient of HbO2 and Hb are equal, this can be used to calculate haemoglobin concentration independent of haemoglobin saturation.

3- 845nm, the absorption maximum for oxidised CytOx.

4- 900nm, a reference wavelength[166,178].

Light is produced by the laser diodes and transmitted by a bundle of lightproof shielded optical fibre to the surface of the SM. The distal end of the fibres terminate in a small glass prism reflecting the light through 90° and directing it towards the tissue[206]. Photons emerging from the surface of the muscle are collected by a second identical optical fibre, transmitted back to the NIRO 500 and detected by a photomultiplier tube (PMT) light detector[206]. The incident and transmitted light intensities are recorded and based on these changes. The changes in concentrations of tissue chromophores are calculated in (μmol/L) using an algorithm containing the known chromophore absorption coefficient and an experimentally measured optical path length (6.09).



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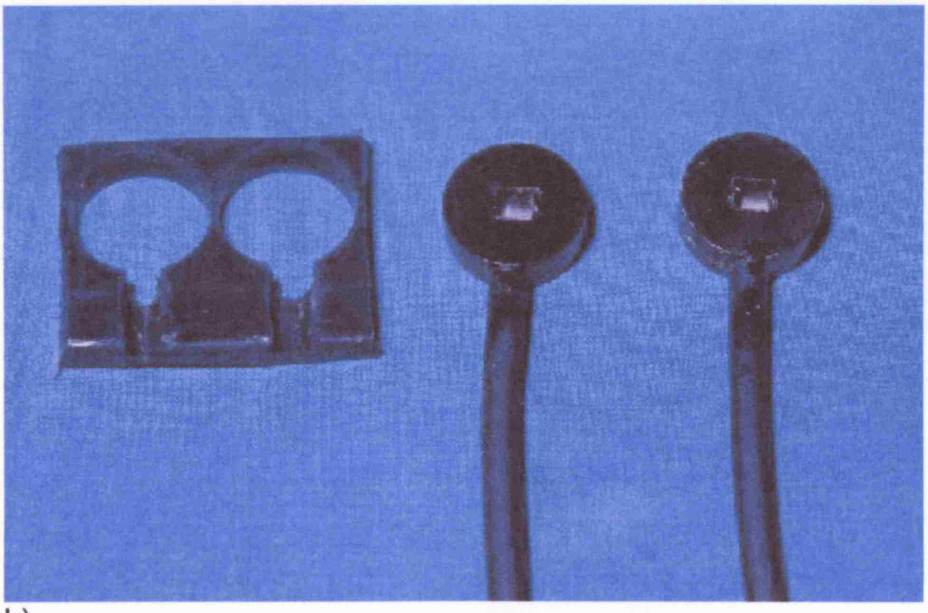


b)

Figure 3.4: a) Near infrared spectrometer probes

b) Rubber optode holder

(NIRO 500, Hamamatsu Photonics KK, Hamamatsu, Japan)

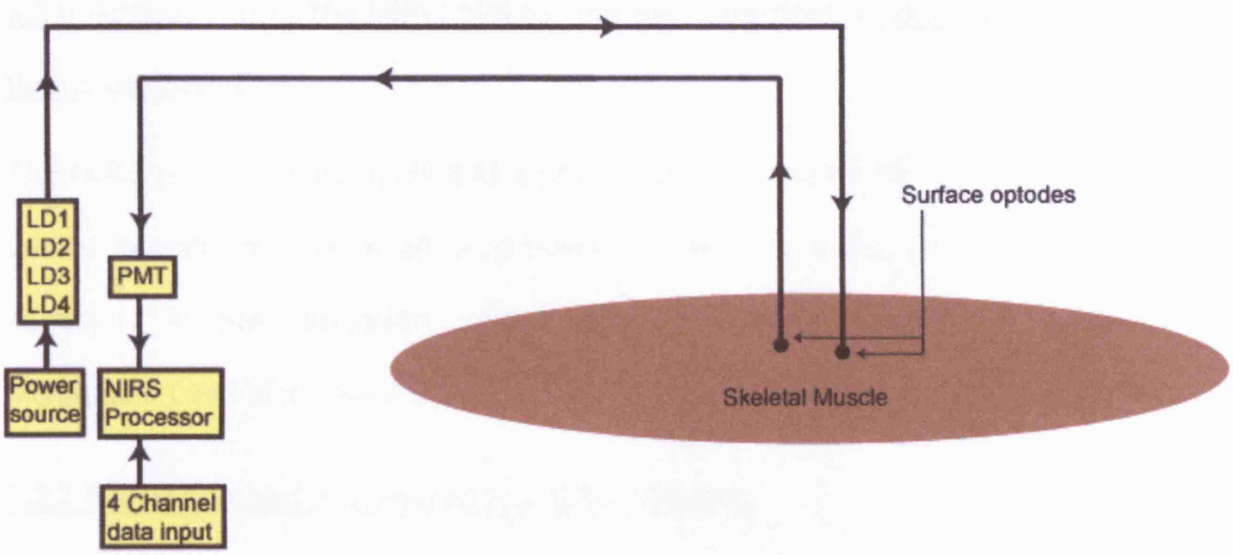


Figure 3.5: Schematic diagram of the NIRO 500 spectrometer. Four laser diodes are used as the monochromatic light source and a photomultiplier tube is used to detect transmitted light from the tissue.

3.2.6 Application of the NIRO 500 for the measurement of skeletal muscle tissue oxygenation

The NIRS probes where applied to a predetermined point on the surface of the vastus lateralis muscle in all experiments in order to avoid any anatomical variations in SM circulation which could have some bearing on tissue oxygenation and blood volume.

3.2.7 Collection and analysis of the NIRO 500 data

The NIRO 500 includes the facility to set the attenuation and therefore chromophore concentration changes to zero with the NIRO's initial setting. Therefore all the measurements are changes from a reference point of zero. For collection of NIRS data, a sampling rate of 2Hz was used. The NIRS data were continuously collected on a laptop computer connected to the NIRS. These data are the changes in light attenuation (optical densities: OD) at four wavelengths due to absorption by the tissue chromophores. A software program called ONMAIN® (Hamamatsu Photonics K.K., Hamamatsu, Japan) was used to convert these data into changes in concentration changes of HbO_2, Hb, and Cyt Ox (μmole/L) using the previously defined algorithm in the NIRO 500. This was then transferred to excel® data sheets (Microsoft Company, Seattle, USA) for analysis. The data at the relevant time points were collected as the mean of 1-minute data and calculated in regard to the baseline value at the start of the experiment.

3.2.8 Interpretation of NIRO 500 measurements

NIRS allows the continuous monitoring of the changes of various parameters including HbO_2 which is the amount of oxygenated haemoglobin concentration

within the blood vessels and capillaries. It increases in response to an increase in blood flow or oxygen saturation and vice versa[204,207,208], Hb which is the amount of deoxygenated haemoglobin within the blood vessels and capillaries. It changes in the opposite direction to HbO_2. It also represents the state of the venous outflow and increases with its impairment[204,207 208]. It also measures HbT: which is the total haemoglobin (the sum HbO_2 and Hb) which reflects changes in the blood volume and hence provides an indirect indication of the blood flow and tissue perfusion. It increases with increasing blood flow and with impedance of the venous outflow[204,207,208]. Another parameter measured is HbD: this reflects the difference between HbO_2 and Hb this gives an indication of the net changes in haemoglobin oxygenation independent of any blood volume changes[204,207,208].

Another parameter measured by NIRS is Cyt Ox: which reflects the measurement of the Cyt Ox redox state providing a good index of intracellular oxygen. Cyt Ox becomes more reduced with decreasing blood flow or oxygen desaturation and more oxidised with increasing the blood flow or oxygen saturation [174,209]. Changes in the glucose concentration, as a substrate for the electrons generated in the respiratory chain, also affects the redox state of Cyt Ox. With hypoglycaemia Cyt Ox becomes more oxidised and with glucose administration it becomes more reduced[174,209]. The cell metabolic activity can affect the redox state of Cyt Ox. With increasing metabolic activity, it becomes more oxidised provided that adequate oxygen and substrates are available[174,209].

3.3 Transcutaneous pO_2/pCO_2 monitoring system

Skin pO_2 and pCO_2 (SpO_2, $SpCO_2$) was measured using a transcutaneous pO_2/ pCO_2 monitoring system

3.3.1 Principles of transcutaneous pO_2/pCO_2 monitoring

Measurement of tissue oxygenation by using a transcutaneous pO_2/pCO_2 probe ($TcpO_2/pCO_2$) probe is an established method; it offers easy and continuous measurement that does not interfere with the circulation in the underlying muscle mass[210,211]. The basic principles of its function have been well described[212].

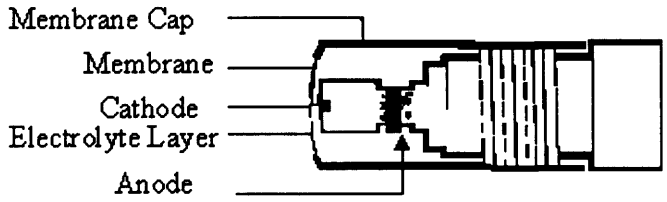


Figure 3.6: schematic representation of Clark probe

A Clark-type surface $TcpO_2/pCO_2$ probe is placed on the skin overlying the muscle. The measurement of pO_2 and pCO_2 was performed as a direct polarographic measurement based on an electro-chemical electrode chain, consisting of the platinum cathode (the sensor electrode) and the silver anode (the reference electrode) and a pH measurement which was converted to a CO_2 reading on the basis of the linear relationship between pH and log CO_2. The calibration of the $TcpO_2/pCO_2$ system was performed with 5% CO_2 and 20.9%

O_2 standard calibration gas supplied from the TCC3 TC Calibration Unit. The electrode temperature was set ranging from 43°C to 45°C. Following calibration, the probe was placed on the skin overlying the adductor muscle mass through a fixation ring with S44416 contact liquid (1, 2-propanediol, de-ionized water) supplied by the manufacturer between the probe and the skin.

When the electrode is attached to the skin the generated heat is transferred from the probe to the skin surface. The heating produces local vasodilatation and increases the permeability of the skin to O_2 and CO_2.

The Transcutaneous pO_2/pCO_2 monitoring system measures oxygen and carbon dioxide tension in mmHg

3.3.2 Transcutaneous pO_2/pCO_2 monitor

Skeletal muscle pO_2/pCO_2 was assessed using a commercially available transcutaneous pO_2/pCO_2 monitoring system (Radiometer Medical A/S, Copenhagen, Denmark).

At the start of the study the transcutaneous pO_2/pCO_2 monitoring system was calibrated using 5% CO_2 and 20.9% O_2 standard calibration gas supplied by the manufacturer. In order to curtail any disturbance to blood flow due to the pressure exerted by the $TcpO_2/pCO_2$ probe on the SM, the probe was attached to a probe holder so that the probe itself was just in contact with the skin surface without applying any pressure by the probe weight. The probe was applied to the same point in all experiments (overlying the adductor muscle mass) in order to minimise any error due to anatomical variations in the SM microcirculation.

Data from the transcutaneous pO_2/pCO_2 monitoring system was recorded before the application of hypoxaemia as baseline and at specific time points during the hypoxaemic periods.

3.4 Intravital Fluorescence Microscopy (IVFM)

3.4.1 Introduction.

The first intravital fluorescent microscope (IVFM) was made by Ellinger and Hirt in Germany, it was a modified version of the fluorescence microscope (developed in the early 20th century) so that it could be used to examine opaque tissues (Kasten, 1993).

The IVFM can be divided into two separate measuring systems. The first represents the microscope alone, with the light source, lenses, filters and fluorescence acting as the functional elements (Fig 3.7)

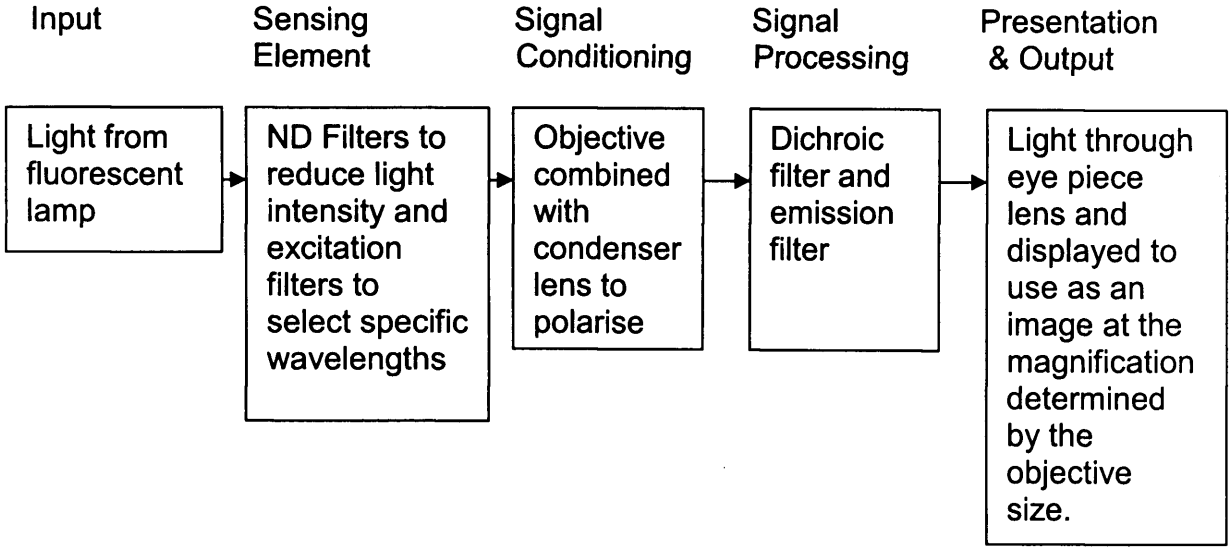


Figure 3.7: Intravital fluorescence microscope – instrumental components.

The second system begins with the image seen from the microscope being converted via the CCD camera, passing through the frame grabber and onto the computer monitor display

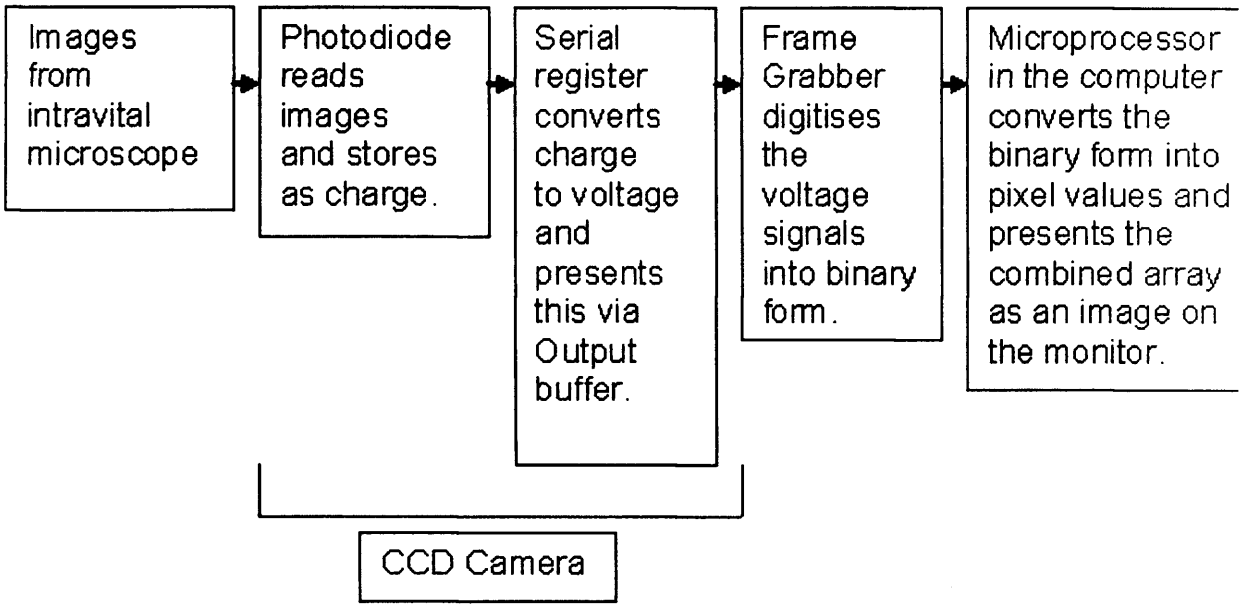


Figure 3.8: Image recording system in the intravital microscope

The IVFM used for this study was a custom built Nikon Epi-illumination system with filter block set suitable for Texas Red, FITC and DAPI dyes as detailed in table 3.1. (Fig.3.9)

Fluorochrome	Excitation (nm)	Emission (nm)	Colour	Type of label
DAPI	360	450	Blue	Nuclear Stain
FITC	495	525	Green	Protein Conjugation
Texas Red	596	620	Red	Protein Conjugation

Table 3.1: The details of filter set for the Nikon Epi-illumination system

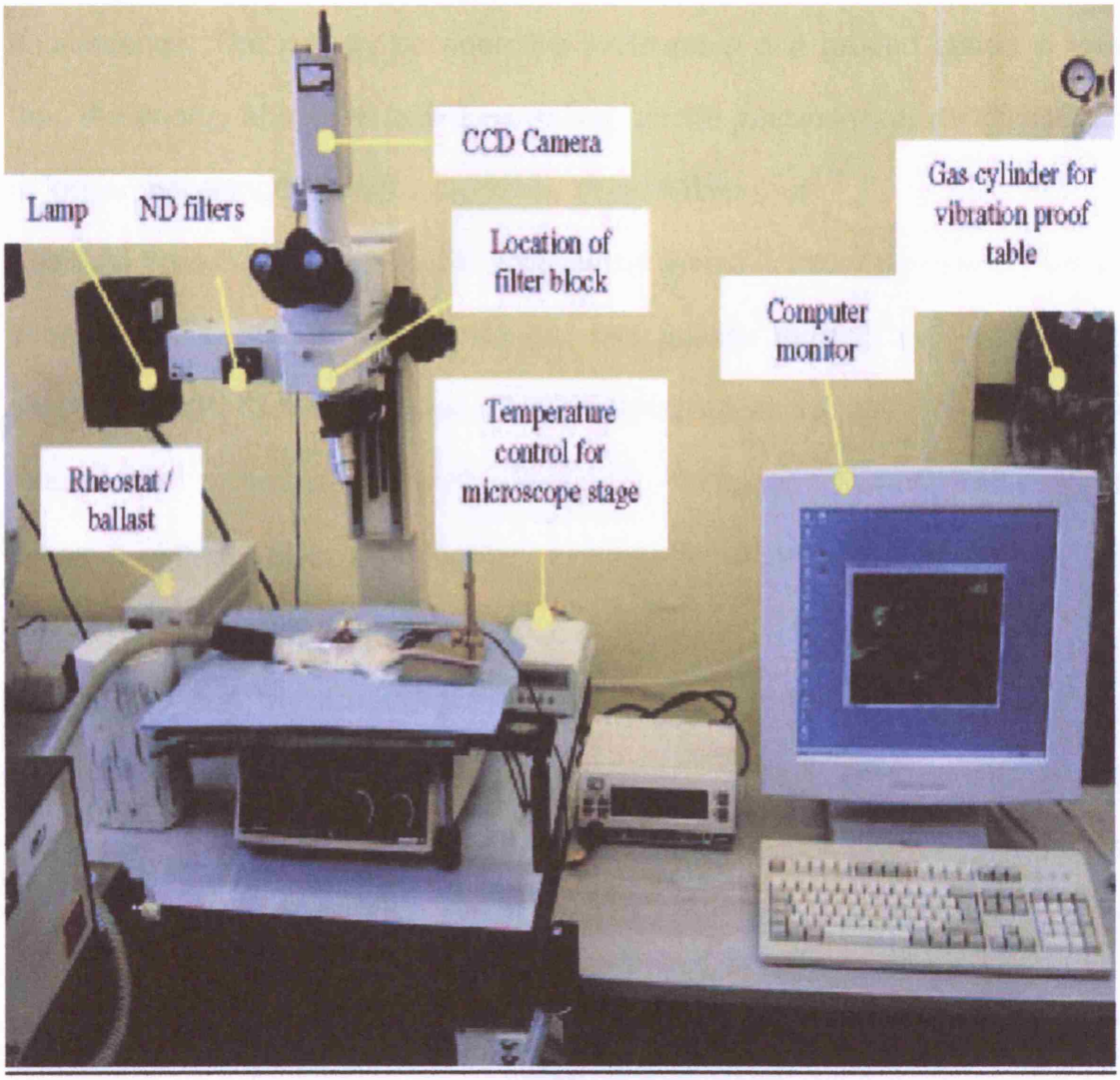


Figure 3.9: The complete setup of intravital microscopy.

3.4.2 Principles of action of Intravital Microscopy[213]

One of the main characteristics of the intra-vital fluorescence microscope is that it uses fluorescence to aid visualisation. The chromophores are excited by an external light source; they absorb the energy and pass into a higher energy state. After entering the higher energy state, the molecules undergo internal changes. The electrons in certain molecules instead of returning to the ground state enter a metastable state. When the molecules pass down to the ground

state they emit the excess energy as electromagnetic radiation[214] – seen as fluorescence. The energy between the metastable and ground states is less than the energy absorbed during excitation, so the emission wavelength will be of longer wavelength than the absorbed or excitation light[214].

The light from the epi-illumination light source (which is usually a mercury arc or xenon lamp in standard IVFM setups) first passes through an excitation or short-pass (SP) filter allowing only the excitation waves through. These are then passed onto a chromatic beam splitter (dichromatic mirror) where wavelengths below a certain value are reflected onto the specimen while any above the wavelength value are passed through and dissipated.

The light hitting the specimen then activates the fluorescence probe which produces emission light of a particular wavelength. A dichromatic mirror allows passage of excitation wavelengths of certain values and emission wavelengths of certain values. According to Stokes' law the wavelength of emission is of lower energy and therefore longer wavelength than that of the excitation.

The dichromatic mirror is designed to allow the transmission of longer emission wavelengths and reflect the shorter *excitation* wavelengths. The three filters are usually contained within a filter box as illustrated in figure 3.10

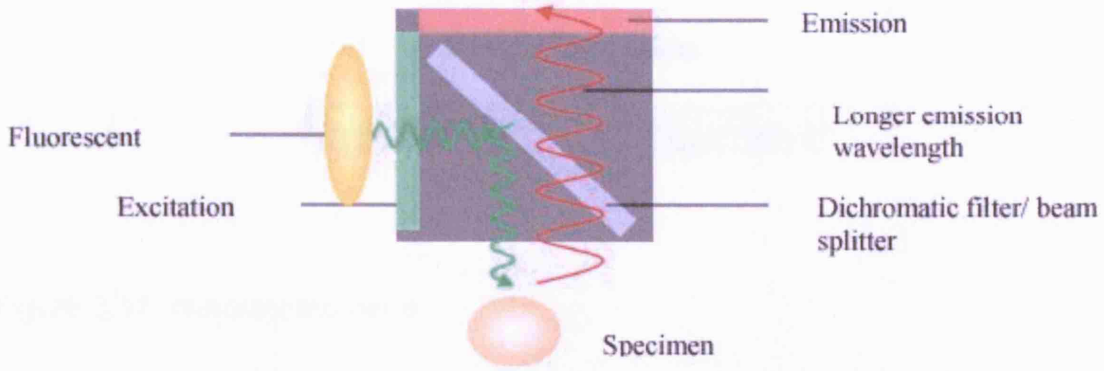


Figure 3.10: Filter block

After passing through the dichromatic beam splitter the excitation wavelength then passes through a barrier filter that removes any light of shorter wavelength that may have mistakenly passed through the dichromatic beam splitter. This emission wavelength passes into a detector that sends the signals to the eye pieces for operator observation or into a video camera for image recording.

3.4.3 Components

3.4.3.1 Light source

The light of the IVFM is a fluorescent mercury arc lamp (Fig 3.11), which is protected in a lamp house. Since the development of epi-illumination, microscope optics has developed with the incorporation of laser light sources increasing the resolution of the image and improving the signal-to-noise ratio. A rheostat or ballast alters the current applied to the lamp to control the intensity of the light produced. In a gas discharge, such as a fluorescent lamp, current causes resistance to decrease. This is because as more electrons and ions flow

through a particular area, they bump into more atoms, which frees up electrons, creating more charged particles

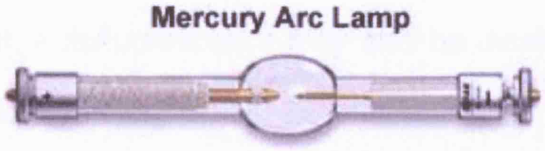


Figure 3.11: Mercury arc lamp

IVFMs usually use water immersion objectives as these reduce the refractive index in living in vivo imaging. The water immersion objective delivers contrast and resolution values nearly equivalent to the theoretical limits, and maintains its performance when water layers of 80 and 153 micrometers are added between the target specimen and cover slip, a simulation of the situation encountered in imaging deep within aqueous material such as living cells or tissue.

There are four types of filters in the intra-vital fluorescence microscope system, neutral density (ND) filters, an excitation filter, an emission filter and a multi-band pass filter.

Special neutral density filters are built in the light path to block 90-95% of the incidental light passing into the microscope to prevent damage to the eye as well as reduce overexposure of fluorescence in specimens[213]. There are three neutral density filters which are placed in front of the optical light path to reduce illumination without altering the colour balance.

The excitation filter passes only a selected range of wavelengths of light to cause the fluorescently labelled specimen to fluoresce and filters the rest. The

bandwidth of a filter determines the brightness of the fluorescent image. If the bandwidth is narrow then the image appears dark but minimal auto-fluorescence and photo-bleaching occur. With a wide bandwidth, although the image appears bright, autofluorescence may also be detected with the added disadvantage of photo-bleaching[213].

The emission or barrier filter allows only light wavelengths that have been emitted from the specimen. These are usually longer according to Stoke's law. In older epi-illumination microscopes only a dichromatic beam splitter is present allowing use of one fluorescent dye at a time. However newer developed models have a multi-band pass filter or a polychromatic beam splitter that can allow visualisation of three different coloured fluorescent dyes.

3.4.3.2 Charged Coupled Device Camera

The image obtained from the IVFM is then recorded by a charged coupled device (CCD) camera. For this study a JVC TK-C1360B colour video camera was used. CCD sensors are light integrating devices that accumulate photo charges until image readout. The CCD chip in the camera contains an array of pixels that transform light (wavelength 400nm to 1000nm) into a charge, which during readout is transformed into a voltage.

Once photo charges are shifted to the storage area, images are erased from the CCD light sensing area[215]. New photo charges can not pile up on top of the previous images. In video rate CCD cameras, this process occurs at regular video-rate of 60 Hz in RS 170 format. However the JVC camera used in this study produces an image acquisition rate of 50 Hz.

The photo charges are shifted in block from the sensing area to the storage area, then each line is individually read to the serial registers and finally photo charges are transferred to the output buffers. Each readout cycle is initiated by a vertical sync Pulse (vertical blanking sync) which activates the parallel driver and triggers the shift block of photo charges accumulated in the sensing area to the CCD storage area. Exposure time is the main factor that determines the sensitivity of CCD cameras. Long exposure times improve camera sensitivity and reduce the noise levels of the images, since the accumulation of the photo charge in the CCD sensor is proportional to the duration of the exposure period. The analogue video images are digitised at varying resolutions typically around 8 bits resolution by a frame grabber board.

JVC TK-C1360B Colour Video Camera	
Image Pickup device	½ Inch Interline- transfer CCD
Effective pixels	440,000 pixels [752(H) x 582(V)]
Sync Systems	Internal, external power sync (50 Hz areas only)
Scanning frequency	15.625 kHz (H), 50.0 Hz (V)
Horizontal resolution	470 TV lines
Minimum illumination	0.95 lux (25%, F1.2, AGC 18 dB) 0.03 lux (25%, F1.2, AGC 18 dB, SLOW 32/50)
Ambient Temperatures	-10 to 50°C (operation), 0- 40°C (recommended)
Video signal to noise ratio	48 dB
	Y/C output connector

Table 3.2: JVC TK-C1360B Colour video camera

3.4.3.3 Frame Grabber

The Matrix Meteor II/Standard frame grabber used in this study allows image acquisition at 25 frames per second. The main parts of the frame grabber are

the low-pass filter, the decoder, the trigger and the image coding components. The low pass filter reduces the high frequency noise and aliasing effects from the analogue CCD signals and passes the refined signals to the video decoder. This is the component of the frame grabber that performs the actual analogue to digital conversion of the component (Y/C) analogue video signals.

3.4.3.4 Image analysis software

Adjusting the output image can enhance the resolution and contrast of the image. Light intensity and colour are represented in numbers between 0 (black) and 255 (white). Converting the image into binary form enables it to be converted into a black and white image. This is done by assigning threshold values which determine the distribution of pixels into two populations either of value 0 or 255.

An image can be transformed into binary form using many different types of software packages. The one used in here is Laboratory Universal Computer Image Analysis (LUCIA).

7.2.5.3 Lucia G System

LUCIA is a multispectral image analysis software developed specifically for image processing independently, on red, green and blue components and then combining them together into the RGB image at the same time. Most Nikon microscopes are supplied with Lucia software and there are many versions available denoted by the letter following Lucia. Lucia G is the top of the range package that allows 24-bit colour image analysis with the function to allow user to create specialized macros.

Most of the frame grabbers require the PC resolution of 800x600 pixels to work with an analogue camera and show live images. If the VGA resolution is higher than 800x600, it needs to be switched to a lower resolution when running LUCIA. Systems with digital cameras accept resolutions up to 1280x1024 pixels.

Work using the Intravital microscope and the Lucia G software has been used in assessing the assessment of microcirculation with success[216-218].

3.5 Skeletal muscle preparation

The tibialis anterior muscle was harvested after the end of each experiment and cut into four blocks of approximately 0.5cm each mounted on a mounting disc using gum tragacanth as a mounting agent. Blocks were frozen in isopentane cooled in liquid nitrogen.

Each muscle block was subsequently cut into sections 7 microns in thickness and stained with haematoxylin and eosin.

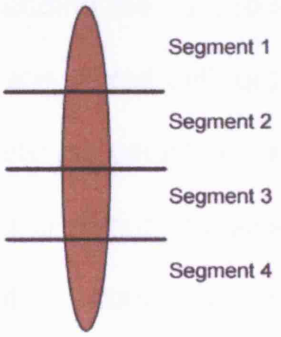


Figure 3.8: Tibialis anterior muscle harvested & sectioned.

3.5.1 Gum tragacanth for use as a freezing mountant

3.5.1.1 Reagents used in the preparation of gum tragacanth as a mounting agent

1- Gum Tragacanth 5mg.

2- Distilled water 80ml.

3- Thymol crystals.

3.5.1.2 Reagents used in the staining and fixation of SM slides

1- Xylol.

2- Haematoxylin Harris.

3- Eosin Y.

4- 0.5% HCL in 70% ethanol.

5- 70% ethanol.

6- Absolute ethanol.

Method:

Gum is heated in distilled water until dissolved. This is done gradually by adding the gum to the water which is placed on a hot plate set to medium heat and stirred until quite thick. The stirrer is removed as the mixture thickens and stirring continued manually adding the gum until dissolved, heating constantly and stirring vigorously with a spatula to avoid clumping. The mixture is left until it has cooled down after which thymol crystals are added. This is stored in the fridge until used.

3.5.2 Haematoxylin and Eosin staining

SM sections were de-waxed with xylol. This was followed by staining with haematoxylin for 2 minutes. Excess haematoxylin was washed away with

distilled water for 1 minute and staining confirmed microscopically. This was followed by staining in 1% eosin for 1 minute, the excess eosin was washed off with distilled water for 1 minute and staining was checked microscopically.
Following this, sections were dehydrated in alcohol and clear xylol and mounted. Muscle fibres were seen as deep pink thick fibres with dark staining nuclei.

3.5.3 Assessment of the extent of muscle infarction

The extent of muscle infarction was assessed on frozen section slides stained with haematoxylin and eosin. The area of muscle infarction was assessed under a light microscope.

The extent of muscle infarction was calculated using a Zeiss KS 400 image analysis system (Carl Zeiss Ltd, Hertfordshire, UK). A matrix of image fields was captured, shading correction applied and a montage image created for each whole section. The montage was digitally enhanced and segmented; a series of binary imaging procedures was used to define total section area and the area of muscle damage. A number of sections were analysed for each slide; mean area values were used to define the percentage of damage at each muscle level.

3.6 Assessment of Nitric Oxide

Plasma nitrite + nitrate (NO_3^-) was measured using a 280 nitric oxide analyser (Sievers Instruments) by a chemiluminescence method[219].
NO was determined as its decay products, nitrite (NO_2^-) and nitrate (NO_3^-):

$$3\ NO + 2O_2 \rightarrow NO_3^- + 2\ NO_2^-$$

The method is based on the gas phase chemiluminescence reaction between NO and ozone (O_3), viz:

$NO + O_3 \rightarrow NO_2^* + O_2$ and $NO_2^* \rightarrow NO_2 + O_2$

Briefly, the plasma sample (0.5 ml) was diluted one in ten and centrifuged at 1,000 g for 60 minutes at room temperature to remove the protein. NO_3^- was then reduced by nitrate reductase prior to chemiluminescence which involved incubating the sample with 40 μM NADPH, 1 μM FAD and 10 mU nitrate reductase in 20 mM Tris buffer pH 7.4 at 37 °C for 60 minutes. Standard solutions of NO_2^- or NO_3^- reduced sample were then added to the purging (reaction) vessel which contained a reducing solution of 1.5 ml KI (50 mg/ml) and 200 μl anti-foaming agent in 6 ml concentrated acetic acid to generate NO. NO then reacted with O_3 produced from O_2. The Nitric Oxide Analyser was attached to a PC computer and with a NOA-Excel linked program, which allowed real time NO_2^- and NO_3^- measurements to be recorded. Results were compared with a standard NO_2^- calibration graph.

3.7 Assessment of local nitric oxide receptors

Nitric oxide receptors were assessed by immuno-staining. This was done on Cryostat-cut sections fixed in 3% para-formaldehyde (Sigma, Sigma-Aldrich, Dorset, England) at 4°C for 30 minutes this was followed by rinsing them twice (for 5 min) in 0.01M PBS (Sigma, Sigma-Aldrich, Dorset, England) and incubating in 1mg/ml β-nicotinamide adenine dinucleotide phosphate (reduced form) [=β-NADPH], 0.1 mg/ml nitroblue tetrazolium and 0.3% triton made up in 0.01M PBS (Sigma, Sigma-Aldrich, Dorset, England) for 1 hour at 38°C, this was followed by rinsing for 5 min in water. Samples were then counterstained with 1% eosin. Dehydrated (50-100% ethanol), de-waxed (3xhistoclear) (Sigma,

Sigma-Aldrich, Dorset, England) and mounted on cover slips with XAM. Slides were then visualised on an Olympus BX50 microscope and photographed where appropriate. NOS was identified by blue staining[220,221].

Chapter Four

Measurement of critical lower limb tissue hypoxia, validating chemical and optical techniques with arterial blood gases

4.1 Introduction

In order to assess the accuracy of NIRS at measuring the levels of oxygenation in SM it is necessary to validate the results obtained against other parameters. This was done in this chapter by comparing the results obtained from the NIRS to those of arterial blood gases and a $TcpO_2/pCO_2$ monitor. There has been extensive research done on $TcpO_2/pCO_2$ monitors in human experiments[222-225]. These experiments have shown that $TcpO_2$ monitors are a reliable predictor of the state of oxygenation of tissues. The use of the NIRS500 in our experiment has been validated in accurately predicting the level of tissue oxygenation in the liver[226] when compared to a $TcpO_2$ monitor and arterial blood gases. The use of a $TcpO_2$ in estimating the level of amputation in ischaemic limbs and as a predictor of the state of oxygenation of muscles has been studied in humans. Results obtained have been varied regarding the speed of responsiveness of the $TcpO_2$ monitors to the drop in the levels of oxygenation, however they have all shown that using a $TcpO_2$ monitor as a predictor of the state of oxygenation is reliable[227-230]. Based on these previous findings, it would therefore be reasonable to compare the NIRS500 to these established methods.

4.2 Materials and Methods

This study was conducted in accordance with the Animals Scientific Procedures Act (1986) and with licence from the Home Office. New Zealand white rabbits (3.2 ± 0.2 kg, n=6) were used.

Anaesthesia was induced with hypnorm (fentanyl and fluanisone) (Janssen, Saunderton, UK) 0.5 mg/kg im, and maintained by 0.5-2% isoflurane (Baxter healthcare, Norfolk, UK) mixed with nitrous oxide and O_2 via a standard anaesthetic circuit. Animals were tracheostomized and a tracheostomy tube

with an internal diameter of 3mm was inserted in the trachea. Body temperature was maintained at 36-38°C by means of an electronic heating blanket and monitored continuously using an endorectal probe (Harvard apparatus LTD, UK). A pulse oximeter (Ohmeda Biox 3740-pulse oximeter, Ohmeda, Louisville, USA) was used for monitoring of systemic arterial oxygen saturation (SaO_2) and heart rate (HR).

The right ear artery was cannulated using a standard IV cannula (22G) for the continuous monitoring of arterial blood pressure and the collection of blood samples (0.5 ml) for blood gas analysis at the end of each hypoxic period using a commercial blood pH/gas analyzer (Mode l860, Bayer PLC, Newbury, Bucks, UK). The marginal ear vein was also cannulated using a standard IV cannula (20G) for the administration of fluids at a rate of 10ml/kg/hour. The rabbits were placed in supine position, and the muscle mass in the hind limb was exposed by incising the covering skin.

A pair of optical probes for NIRS was placed directly on the gracilis, and adductor muscle mass, while the $TcpO_2/pCO_2$ probe was placed on the hind limb skin overlying the muscles.

4.3 Experimental protocol:

After a 30-minutes stabilization period with the fraction of inspired oxygen (FiO_2 = 30%), baseline measurements for the above parameters and blood samples were obtained. Graded hypoxaemia was induced by the stepwise reduction of the FiO_2, (20%, 15%, 10%, 8% and 6%) by increasing the ratio of NO_2/O_2 in the inspired gas mixture. Five minutes were allowed at each hypoxaemic level before blood samples were taken. Recovery between the hypoxaemic periods

was achieved by increasing the FiO_2 to 30% for 10 min and confirming this by normalized arterial blood gas values. After completion of the experiment, the animals were destroyed by an overdose of anaesthesia. Data from the NIRS, and $TcpO_2$ /pCO_2 monitors were recorded continuously during the experiment.

4.4 Data collection and statistical analysis

The data from the pulse oximeter, blood pressure monitor and NIRS were collected continuously on a laptop computer. The changes in muscle tissue oxygenation were averaged before induction of hypoxemia and at the one minute end of each hypoxaemic period were calculated relative to the baseline. Values are expressed as mean ± SD. Student t test was used with Tukey's adjustment for multiple comparisons. The correlation between muscle tissue Hb, HbO_2, Cyt Ox and skin pO_2/pCO_2 was calculated using linear regression. $P<0.05$ was considered statistically significant.

4.5 Results

4.5.1 Systemic haemodynamic and arterial blood gas changes with hypoxaemia

The systemic haemodynamic changes during graded hypoxaemia are outlined in table 4.1. The animals tolerated all grades of three minutes hypoxaemia induced when FiO_2 was not less than 6%. The heart rate slowed during the hypoxaemic period but this drop became significant at a FiO_2 level below 10%. MABP and pH remained stable during the experiment. Systemic arterial pO_2 (Sa-pO_2) declined significantly corresponding to the drop in FiO_2 as expected. Systemic arterial pCO_2 (Sa-pCO_2) gradually dropped during the experiment and this drop became significant at a FiO_2 level below 10%.

FiO_2 (%)	SaO_2 (Kpa)	HR (beat/min)	MABP (mmHg)	pH	Sa-pO_2 (kPa)	Sa-pCO_2 (kPa)
30	98 ± 8	232 ± 23	72 ± 2	7.36 ± 0.08	21.0 ± 1.3	7.9 ± 1.9
20	85 ± 5	230 ± 13$^{\#}$	67 ± 9	7.38 ± 0.07$^{\#}$	10.7 ± 1.7*	7.8 ± 2.2$^{\#}$
15	80 ± 5	214 ± 26$^{\#}$	66 ± 10	7.4 ± 0.06$^{\#}$	7.6 ± 1.4*	7.5 ± 2.5$^{\#}$
10	66 ± 8	204 ± 34*	62 ± 4	7.42 ± 0.06$^{\#}$	5.4 ± 1.3*	6.7 ± 1.8*
8	58 ± 4	197 ± 28*	69 ± 6	7.44 ± 0.05$^{\#}$	5.3 ± 2.2*	5.7 ± 1.7*
6	39 ± 9	194 ± 45*	70± 11	7.45 ± 0.06$^{\#}$	4.0 ± 1.5*	6.2 ± 1.4*

Table 4.1: Changes in systemic haemodynamics and arterial blood gas pressure during systemic graded hypoxia

Values are presented as mean ± SD of 6 animals ($^{\#}$p=not significant; *p < 0.05 Vs baseline values).

Keys: FiO_2, fraction of inspired oxygen; SaO_2, oxygen saturation; MABP, mean arterial blood pressure; HR, heart rate; Sa-pO_2, systemic arterial oxygen partial pressure, Sa-pCO_2 systemic arterial carbon dioxide partial pressure.

4.5.2 Skeletal muscle oxygenation changes with hypoxaemia

Muscle tissue oxygenation changes measured by NIRS with hypoxaemia, are presented in table 4.2. More specifically HbO_2 decreased significantly at FiO_2 below 15%. On the other hand Hb levels increased during hypoxaemia, and this increase became significant at FiO_2 below 20%. Cyt Ox became reduced during hypoxaemia; however this reduction was not statistically significant during the experiment.

4.5.3 Skin pO_2 and pCO_2 changes with hypoxaemia

The SpO_2 and $SpCO_2$ changes measured by $TCpO_2/pCO_2$ probe during each hypoxaemia period are presented in table 4.2. SpO_2 and $SpCO_2$ showed a progressive decrease with increasing levels of hypoxaemia which was

significant in all levels for SpO_2; however $SpCO_2$ did not drop significantly until the later stages of hypoxaemia (when $FiO_2 \leq 8\%$) (Figure 4.1).

FiO_2 %	HbO_2	Hb	Cyt Ox	spO_2	$spCO_2$
30	-0.8 ± 2.0	1.5 ± 2.2	0.01 ± 0.65	8.5 ± 2.5	8.4 ± 2.1
20	-2.9 ± 5.1$^{\#}$	5.8 ± 6.3*	-0.25 ± 0.60$^{\#}$	7.1 ± 2.2*	8.2 ± 2.7
15	-5.6 ± 4.1*	9.1 ± 7.8*	-0.44 ± 0.43$^{\#}$	6.2 ± 2.5*	8.1 ± 2.3
10	-6.5 ± 5.3*	14.1±15.2*	-0.49 ± 0.27$^{\#}$	5.4 ± 2.7*	7.9 ± 1.8
8	-10.5 ± 6.7*	16.4±16.1*	-0.35 ± 0.33$^{\#}$	4.2 ± 2.3*	7.4 ± 2.1*
6	-11.4 ± 6.6*	18.1±16.5*	-0.28 ± 0.45$^{\#}$	3.9 ± 2.6*	6.9 ± 1.6*

Table 4.2: Muscle tissue oxygenation recorded by NIRS and the $TcpO_2/pCO_2$ monitor system. Values are mean ± SD of 6 animals,

($^{\#}$p=not significant; *p < 0.05 Vs baseline values).

Key: HbO2 = Oxyhaemoglobin, Hb = Deoxyhaemoglobin, CytOx = Cytochrome oxidase, spO_2 = Skin pO_2, spO_2 = Skin $spCO_2$.

4.5.4 Correlation between tissue oxygenation measured by the PO_2/PCO_2 meter and NIRS with systemic arterial gas pressure

There was a significant correlation between the changes in spO_2, FiO_2 and Sa-pO_2 (Table 4.3, Fig 4.1). There was also a significant correlation between the changes in HbO_2 as recorded by NIRS, FiO_2 and Sa-pO_2 (Table 4.3, Fig. 4.2). The changes also in SpO_2 as recorded by the $TCpO_2/pCO_2$ probe and muscle HbO_2 as recorded by NIRS correlated well (Table 4.3).

	Correlation coefficient	P value	Equation
FiO_2 vs. spO_2	r = 0.92	p < 0.001	Y= 0.2x + 4.5
pO_2 vs. spO_2	r = 0.89	p < 0.001	Y =0.3x + 3.9
FiO_2 vs. HBO_2	r = 0.90	p < 0.001	y = 0.5x -12.8
pO_2 vs. HBO_2	r = 0.88	p < 0.001	y = 0.7x -14.0
HbO_2 vs. spO_2	r = 0.92	p < 0.001	Y = 0.4x + 9.4

Table 4.3: Correlations between SpO_2, HBO_2, and pO_2 after induction of hypoxaemia.

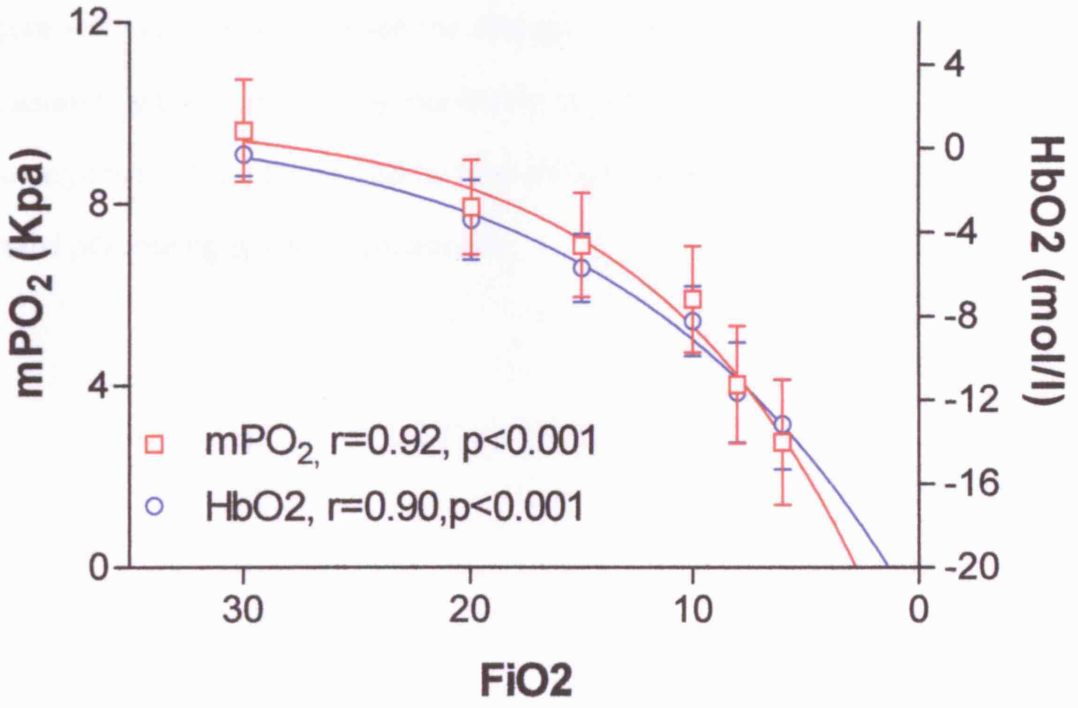


Figure 4.1: Correlation between the changes in skin oxygenation (SpO_2) measured by the $TcpO_2/pCO_2$ monitoring system and muscle tissue. oxyhaemoglobin (HbO_2) measured by near infrared spectroscopy (NIRS) and FiO_2 values during graded hypoxaemia. mpO_2 = muscle pO_2.

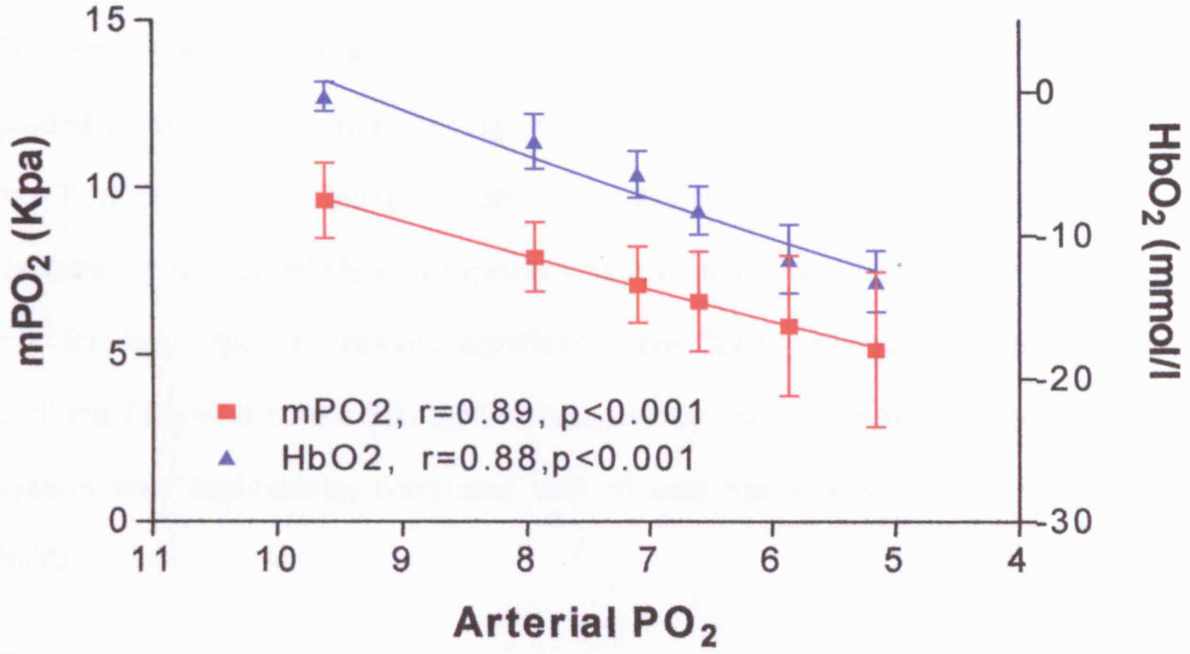


Figure 4.2: Correlation between the changes in skin oxygenation (spO_2) measured by the $TcpO_2/TCO_2$ monitoring system and muscle tissue haemoglobin (HbO_2) measured by near infrared spectroscopy (NIRS) and arterial pO_2 during graded hypoxaemia.

4.6 Discussion

This study has measured the changes in muscle tissue oxygenation during graded systemic hypoxaemia using the non-invasive techniques of NIRS and the $TCpO_2/pCO_2$ monitoring system. The results showed that there was a decrease in muscle HbO_2 with hypoxia and a simultaneous increase in muscle Hb. Similarly, spO_2 decreased significantly but $SpCO_2$ remained unchanged until the FiO_2 was below 8%. SpO_2 measured by the $TCpO_2/pCO_2$ monitoring system was significantly correlated with muscle tissue HbO_2 monitored by NIRS.

The animal model adopted for this experiment was designed so that there would be strict control of the FiO_2 inspired through a tracheostomy in order to be able to manipulate the Sa-pO_2 and assess the response in the local pO_2. We used a rabbit model, rather than a rat model due to larger size of muscle compared with the rat and better control of FiO_2 inspired via tracheostomy. Also the amount of blood taken from the animal during the experiment would not be possible in a rat model. Arterial cannulation for the monitoring of systemic blood pressure and the repeated measurements of arterial blood gases was achieved through the ear artery. This was chosen because of the easy access, and minimal trauma.

The detection of tissue oxygenation in terms of measuring concentrations of Hb, HbO_2 and Cyt Ox by NIRS is based on an optical technique. NIRS relies upon the relative transparency of tissue to near infrared light and the presence in this wavelength region of two natural chromophores exhibiting oxygen-dependent absorption spectra, namely haemoglobin and Cyt Ox. Readings are

instantaneous and reflect the changes in tissue; however the system measures changes relative to an initial baseline value which is usually zero, whereas the $TCpO_2$ / pCO_2 system measures absolute value in kPa.

There was a significant drop in heart rate at FiO_2 levels lower than 10%. Similar findings have been observed in other experimental studies of systemic hypoxaemia[231] and this might be a direct effect of the sustained hypoxia on the cardiac muscle[232].

Hypoxaemia was maintained at each level to obtain accurate measurements from the $TCpO_2$/pCO_2 probe. At the end of each level of hypoxaemia the level of FiO_2 was returned to the baseline in order to allow the animal to recover.

The systemic pH remained stable during the experiment. This could be explained by the compensatory increase in respiratory rate that resulted in the drop in the Sa-PCO_2.

A significant decrease was seen in muscle HbO_2 (after $FiO_2 \leq 15\%$) and a significant increase in muscle Hb, as recorded by NIRS, in all grades of hypoxaemia. The sensitivity of NIRS at detecting changes in muscle HbO_2 has been demonstrated in other studies[186,233] and our results confirmed these findings.

CytOx is the terminal electron carrier of the mitochondrial respiratory chain that catalyses the reduction of oxygen to H_2O in a four electron reaction with the concomitant synthesis of ATP through the oxidative phosphorylation process. In the presence of oxygen electron transfer occurs and the enzyme becomes oxidised, and in the absence of oxygen electron flow decreases and CytOx

becomes reduced[174]. Thus, assessment of the redox state of Cyt Ox could be used as an indicator of intracellular oxygenation and mitochondrial redox state[234,235]. In the present study Cyt Ox was reduced during hypoxaemia but this reduction was not significant in any level of hypoxaemia in comparison to baseline levels. There are several factors that could explain why muscle tissue CytOx levels show only a small reduction during systemic hypoxaemia. CytOx has a low Km for O_2 and the myoglobin acts as a large oxygen reserve[236]. Also during general anaesthesia the muscles are in resting state and the oxygen consumption is minimal[237]. Another factor is that the contribution of cytochrome oxidase to the NIRS light attenuation is very small (about 2-5 %)[238] and this can lead to difficulties in separating its signal from the generally much larger signals due to Hb and HbO_2[238,239]. For this reason significant efforts have been made recently to develop special algorithms to magnify the signal to provide sufficient resolution[240] and to differentiate changes in CytOx independent of haemoglobin[241] with various degrees of success.

In the present study, the use of the $TCpO_2/pCO_2$ monitoring system recorded significant changes in SpO_2 with all grades of hypoxaemia and in $SpCO_2$ only with severe hypoxaemia (FiO_2<8%). The fact that the local $SpCO_2$ as recorded by the $TCpO_2/pCO_2$ probe decreased during severe hypoxaemia is not what we would expect. It is well known that at severe levels of hypoxaemia the fall in oxygen delivery inhibits aerobic metabolism and leads the metabolic process to anaerobic pathways with the production of lactic acid. The hydrogen cations (H^+) that are produced during severe hypoxaemia are buffered by the bicarbonate (HCO_3^-) buffering system as described by the formula $H^+ + HCO_3^- \leftrightarrow H_2CO_3 \leftrightarrow H_2O + CO_2$. For this reason we would expect a rise to regional

pCO_2 levels with severe hypoxaemia. There are several possible explanations for the finding that the PCO_2 levels were not elevated during hypoxaemia. Firstly, the amount of O_2 present in muscle myoglobin is sufficient for the metabolic needs of muscles in a resting state under general anaesthesia. Secondly would be that the $TCpO_2/pCO_2$ probe was not sensitive enough to record the changes in $SpCO_2$.

The $TCpO_2$ / pCO_2 monitoring system used in this experiment monitors skin oxygenation based on chemical detection of O_2 and CO_2 that diffuse to the surface of the skin. However in this study we would like to confirm whether measurement of skin pO_2 reflects accurately the underlying muscle oxygenation state. A significant correlation ($r=0.92$, $p<0.001$,) was found between the changes in regional pO_2 (measured by the TCpO2/pCO_2 probe) and regional HbO_2 (measured by NIRS). This suggests that pO_2 of the skin tissue measured by the $TCpO_2/pCO_2$ system correlates with oxygen diffusion from blood to muscle tissue.

The changes of SpO_2 as recorded by the $TCpO_2/pCO_2$ system as a response to systemic hypoxaemia took place much more slowly when compared to the changes in HbO_2 as recorded by NIRS. This is probably due to the delay for the chemical reactions that occur on the surface of the tissue to be detected by the sensor of the $TCpO_2/pCO_2$ system.

Both skin pO_2 values as recorded by $TCpO_2/pCO_2$ system and HbO_2 values as recorded by NIRS correlated very well with the changes in Sa-pO_2. This is an indication about the reliability and validity of these monitoring systems.

In summary, the data from this study suggest that skin oxygenation measured by the $TCpO_2/pCO_2$ monitoring system as indicated by tissue pO_2 and pCO_2 correlates with skeletal muscle tissue Hb and HbO_2 measured by NIRS and with systemic arterial pO_2 and pCO_2. Therefore it is reasonable to conclude that NIRS gives reliable information about muscle oxygenation by probe placement over the muscle to be studied.

Chapter Five

Preconditioning of skeletal muscles; finding the right formula

5.1 Aim

Having described the various preconditioning protocols available in chapter two, the aim of this chapter was to find if there was any difference between the most commonly used preconditioning protocols and then compare these with the pharmacological preconditioning protocols.

5.2 Materials & Methods

5.2.1 Animal model and surgical preparation. (Figure 5.1)

This study was conducted in accordance with the Animals Scientific Procedures Act (1986) and under licence from the Home Office. Rats (Sprague-Dawley) weighing (300-350gm) were placed in supine position. Anaesthesia was induced with 1.5-2% isoflurane (Baxter healthcare, Norfolk, UK) delivered via a face mask, and maintained with thiobutabarbital sodium (Inactin; Sigma, Sigma-Aldrich, Dorset, England) 80-100mg/Kg intra-arterially. A polythene catheter (internal diameter 0.8mm) (Portex limited, Hythe, Kent, England) to monitor mean arterial blood pressure (MABP) was inserted into the internal carotid artery. A tourniquet was placed proximally around the right thigh and a small longitudinal incision was made through the skin of the lower leg and the muscle was exposed. Near infrared spectroscopy (NIRS) probes were positioned onto the muscle mass. Following each given preconditioning protocol a pressure of 200mmHg was applied via the tourniquet in order to occlude the blood supply for 2.5 hours. This was followed by 3.5 hours of reperfusion. Heart rate (HR), MABP, temperature and tissue oxygenation were monitored throughout the ischaemia and reperfusion period. Blood samples of 0.5ml were collected from the carotid line at baseline and at the end of the ischaemia and reperfusion periods to measure nitrate and nitrite. At the end of the experiment the whole of

the tibialis anterior muscle was harvested to assess the extent of muscle infarction.

A pulse oximeter (Ohmeda Biox 3740-pulse oximeter, Ohmeda, Louisville, USA) was used for the monitoring of arterial oxygen saturation and HR. Body temperature was maintained at 36-37.5ºC by means of an electronic heating blanket and monitored continuously using a rectal probe (Harvard apparatus LTD, UK). Mean arterial blood pressure (MABP) was monitored continuously using an invasive blood pressure monitor (F-LM1; Datex-Ohmeda Ltd, Hertfordshire, UK).

Ca^{2+} preconditioning was induced using Calcium chloride 1 mM (Martindale Pharmaceuticals Ltd, Romford, UK). K^{+} channel opener preconditioning was performed using Levcromakalim (Sigma, Sigma-Aldrich, Dorset, England)

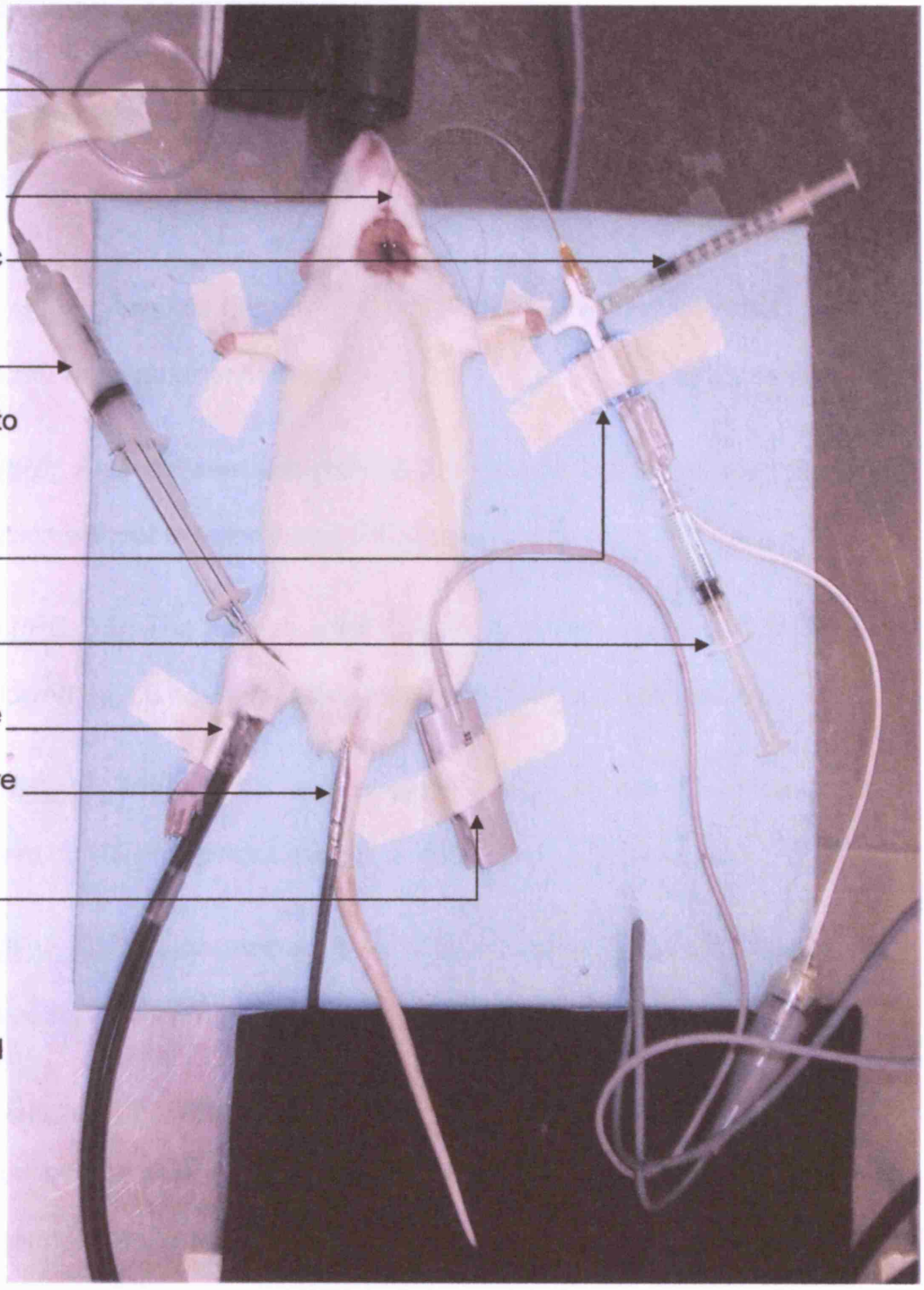

O_2 mask
Carotid line
Anaesthetic agent
Pneumatic tourniquet connected to pressure monitor
Pressure transducer
Saline
NIRS probe
Temperature probe
Pulse oximeter

Figure 5.1

5.3 Experimental protocols

Forty two adult male Sprague-Dawley rats weighing between 300 and 350g were used. Animals were divided into 7 experimental groups (n=6 each group) (Table 5.1).

Group 1 (sham): Animals had an operation under general anaesthesia for the same procedure as other experimental groups without inducing ischaemia.

Group 2 (IRI): Animals were subjected to 2.5 hours of ischaemia and 3.5 hours of reperfusion without any preceding IPC protocol.

Groups 3 (IPC 2.5): The animals were subjected to three cycles of 2.5 minutes of IPC followed by 2.5 hours of ischaemia and 3.5 hours of reperfusion.

Group 4 (IPC 5): The animals were subjected to three cycles of 5 minutes of IPC followed by 2.5 hours of ischaemia and 3.5 hours of reperfusion.

Group 5 (IPC 10): The animals were subjected to three cycles of 10 minutes of IPC followed by 2.5 hours of ischaemia and 3.5 hours of reperfusion.

Group 6: Calcium preconditioning (CPC): Animals were subjected to 2.5 hours of ischaemia followed by 3.5 hours of reperfusion preceded by the administration of calcium chloride ($CaCl_2$). Preconditioning was induced by infusing 1 mM $CaCl_2$ over 2 min, 10 min before the ischaemia period

Group 7: K^+- Channel opener preconditioning (K^+PC): Animals where subjected to 2.5 hours of ischaemia followed by 3.5 hours of reperfusion preceded by the administration of K^+- Channel opener (Levcromakalim). Preconditioning was

induced by infusing 80-100 mg/kg 10 minutes before the induction of 2.5 hours of ischaemia.

Group	IPC Protocol	Ischaemic time (hours)	Reperfusion time (hours)
Control	None	0	0
IRI	None	2.5	3.5
IPC 2.5	3 X ($I_{2.5min}$+$R_{2.5min}$)	2.5	3.5
IPC 5	3 X (I_{5min}+R_{5min})	2.5	3.5
IPC 10	3 X (I_{10min}+R_{10min})	2.5	3.5
CPC	$CaCl_2$ (1 mM)	2.5	3.5
K^+PC	Levcromakalim 80-100 mg/kg	2.5	3.5

Table 5.1: Experimental groups 1-7 & protocol applied to each

Key: I = Ischaemia, R = Reperfusion, IRI = Ischaemia reperfusion, IPC = Ischaemic preconditioning, CPC = Calcium preconditioning, K^+PC = Potassium preconditioning.

5.4 Data collection and statistical analysis

Data from the NIRS, pulse oximeter, and blood pressure monitor were collected continuously on a laptop computer. After allowing ten minutes for stabilisation, the data was averaged for one minute at baseline and during the periods of ischaemia and reperfusion. Muscle oxygenation changes were calculated at various stages of the ischaemia & reperfusion periods and compared to baseline.

The comparison of data between the groups is assessed by one way analysis of variance (ANOVA) with Tukey's adjustment for multiple comparisons.

5.5 Results

5.5.1 Systemic haemodynamics (table 5.2)

There were no significant changes in HR or SaO_2 from baseline and during the periods of ischaemia and reperfusion. However there was a significant change in MABP between the end of ischaemia and reperfusion period between the control group and the CPC group.

Group	$Sa0_2$			HR			MABP		
	BL	EOI	EOR	BL	EOI	EOR	BL	EOI	EOR
Control	99±1	99±1	99±1	255±12	255±11	255±13	113±12	107±11	115± 11
IRI	97±2	98±1	98±1	255±11	255±11	255±11	122±12	120±12	111± 11
IPC2.5	98±1	98±1	98±1	255± 9	255±10	255±10	111±11	109±11	116± 12
IPC 5	99±1	99±1	99±1	255±10	255± 9	255±12	114±11	117±10	113± 10
IPC 10	99±1	98±1	98±1	255±11	255±11	255±11	100±14	115± 9	118± 11
K^+ PC	99±1	97±2	98±1	255±13	255±13	255±10	104±16	113± 9	109±113
CPC	99±1	98±1	98±1	255±10	255±11	255±12	112±11	85*±12	81*±11

Table 5.2: Systemic haemodynamics during ischaemia & reperfusion

Key: SaO_2 = Oxygen saturation, HR = Heart rate, MABP = Mean arterial blood pressure, BL = Baseline, EOI = End of ischaemia, EOR = End of reperfusion.

* $P < 0.05$ compared to controls

5.5.2 Tissue Oxygenation (figure 5.2)

A significant decrease in levels of tissue HbO_2 was found in all groups after one and a half hours of ischaemia (P=0.04) vs. baseline (pre-ischaemia) and at the end of the ischaemia period (p= 0.007) vs. baseline (pre-ischaemia). During

reperfusion no significant difference in tissue HbO_2 levels between the different groups was found in the early phase (first two hours), however there was a significant difference between the 2.5, 5 & 10 min IPC groups compared to the IRI group at 3hours post reperfusion (p= 0.0202, 0.0358, 0.0184 respectively). The level of HbO_2 in the pharmacologically preconditioned groups was consistently lower during the reperfusion period when compared the other groups, however this difference was statistically significant only after 3 hours of reperfusion vs. IRI group.

There was a rise in the level of Hb at the start of ischaemia and a drop with the beginning of reperfusion in all groups however these changes did not reach statistical significance (figure 5.3). No significant change in Hb was found across the cohort of experiments and between individual groups. No significant difference was found in the level of CytOx between the various groups at any stage during the ischaemia and reperfusion periods. There was also no significant difference between the various groups compared individually.

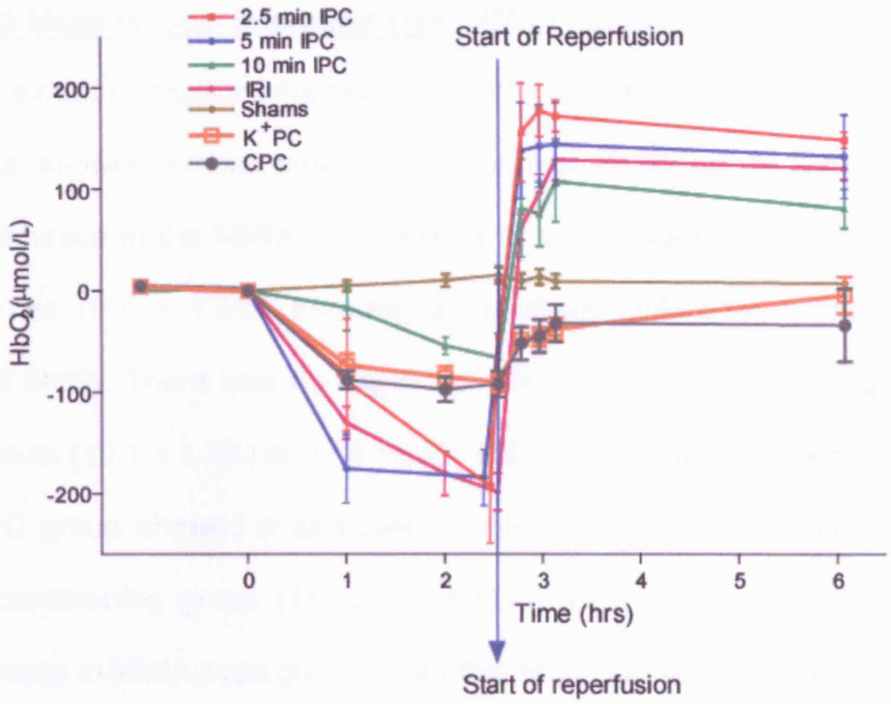


Figure 5.2: Changes in HbO2 during ischaemia and reperfusion in experimental groups 1-7

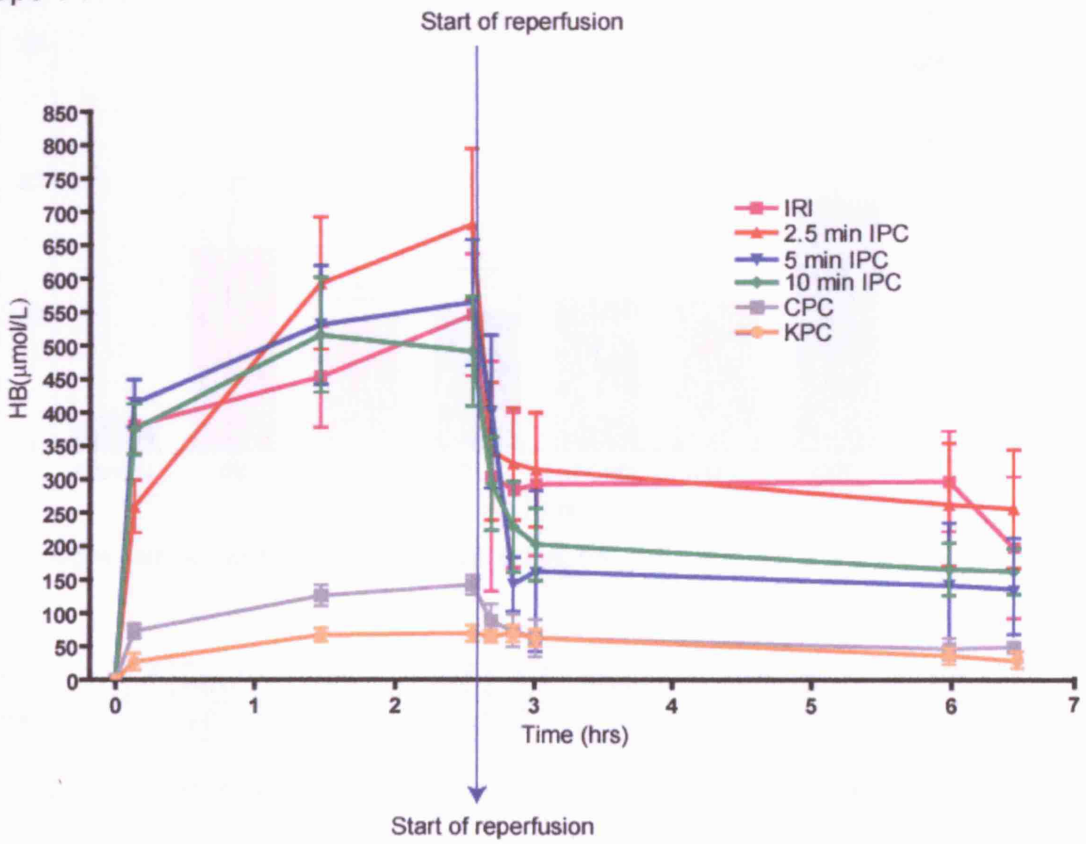
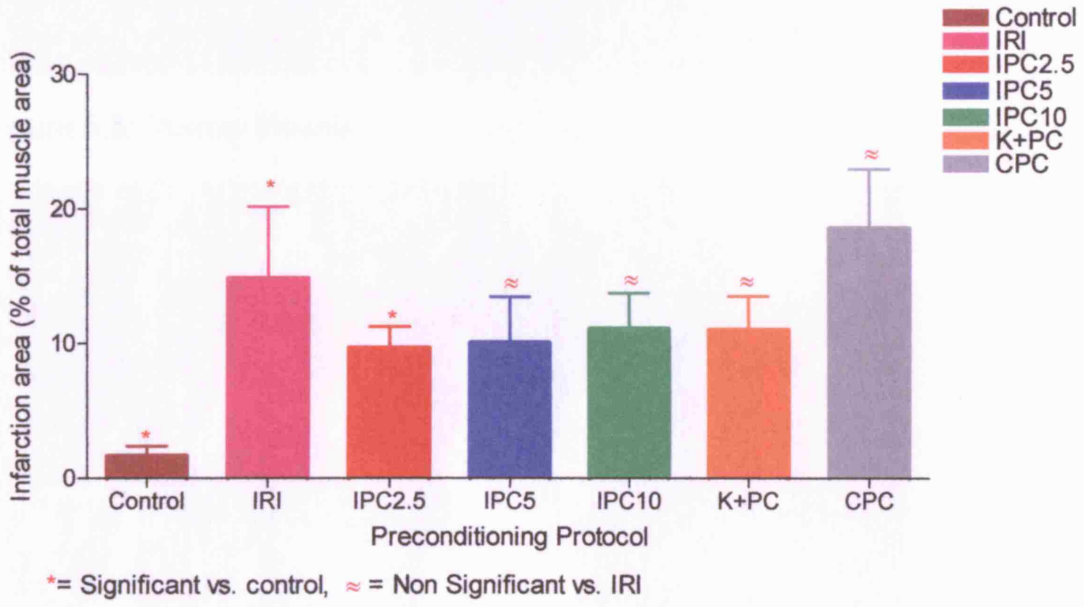


Figure 5.3: Changes in Hb during ischaemia & reperfusion in experimental groups 1-7

5.5.3 Mean muscle infarction size (MMIS) (figure 5.6-5.9)

The extent of muscle infarction for each group is shown in (figure 5.4). The IRI group showed a mean muscle infarction area (MMIA) of 14.2 ± 4.6%.there was a decrease in the MMIA in all groups of preconditioning with the group of 2.5 minutes (9.7 ± 1.5%) showing a significant difference with the IRI group (p=0.0456). There was no significant change between the IRI group and the 5minute (10.1 ± 3.2%) and 10 minute (11.1 ± 2.6%) preconditioning groups. The K^+PC group showed a decrease in MMIA comparable to that of the 10 min preconditioning group (11.03 ± 2.4%), whereas the CPC group showed an increase in MMIA even greater than that of a reperfusion injury (18.59 ± 4.3%).



Group	Control	IRI	IPC2.5	IPC 5	IPC 10	K^+PC	CPC
Infarction(%)	1.6±0.7	14.2±6.4	9.7±1.5	10.1±3.2	11.1±2.6	11.03±2.4	18.5±4.3

Figure 5.4: Extent of skeletal muscle infarction in IRI & preconditioning groups with different cycle's ± SD.

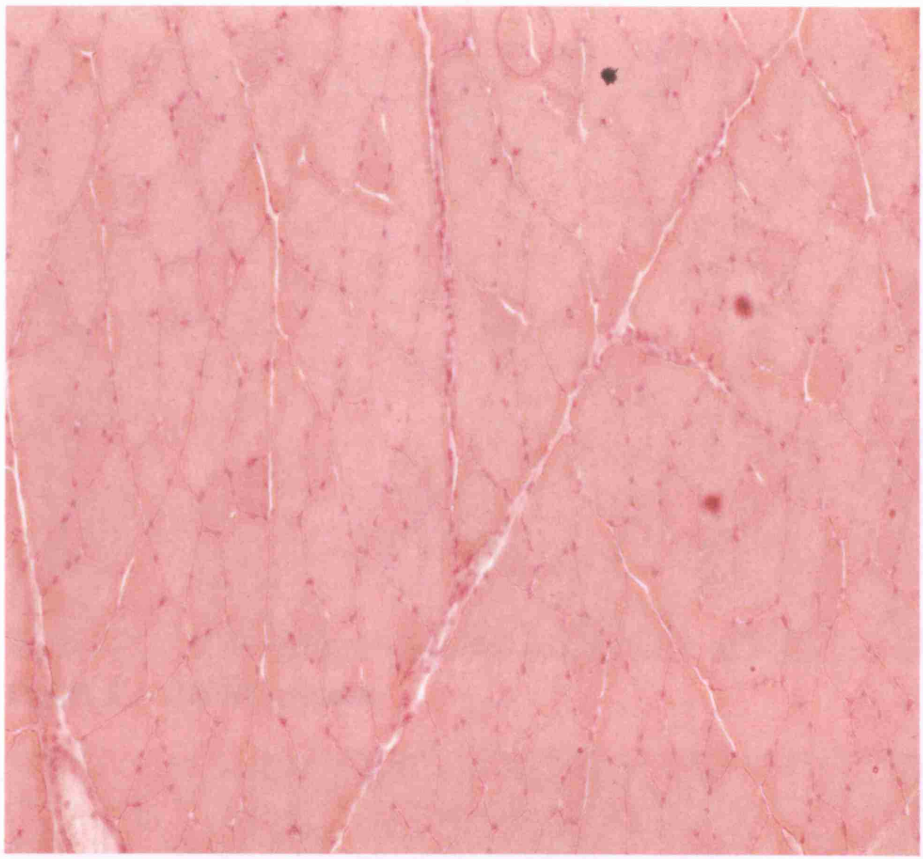


Figure 5.5: Normal Muscle

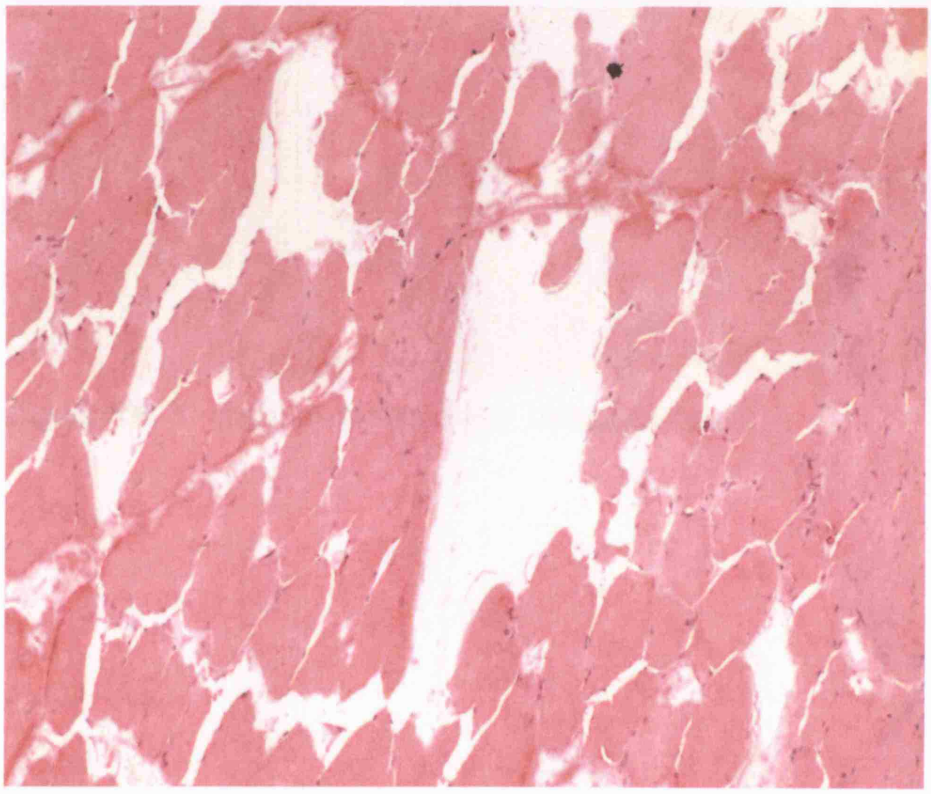


Figure 5.6: Ischaemia reperfusion injury muscle

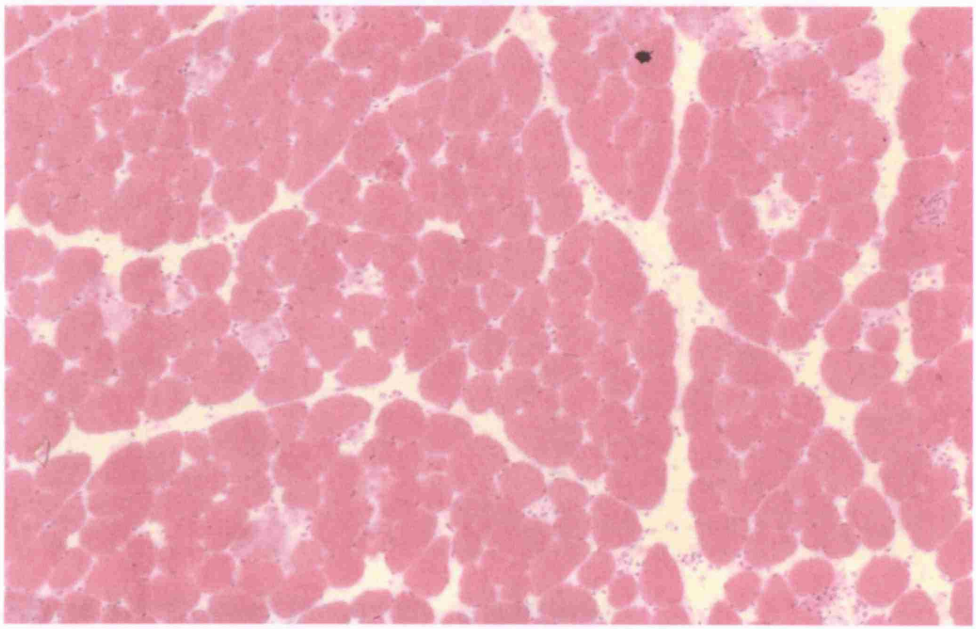


Figure 5.7: 2.5 IPC muscle

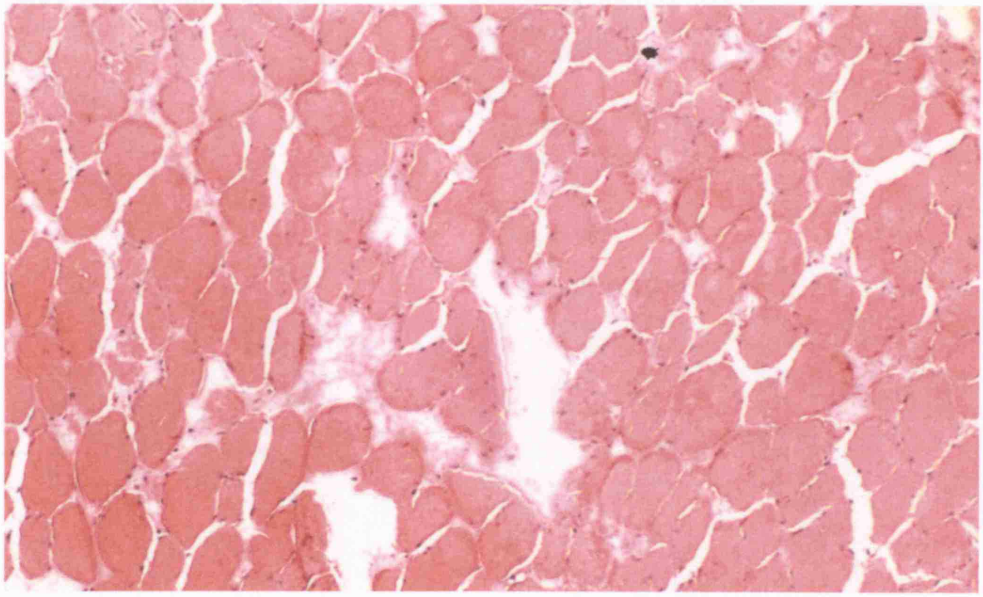


Figure 5.8: K^+ channel opener preconditioning muscle

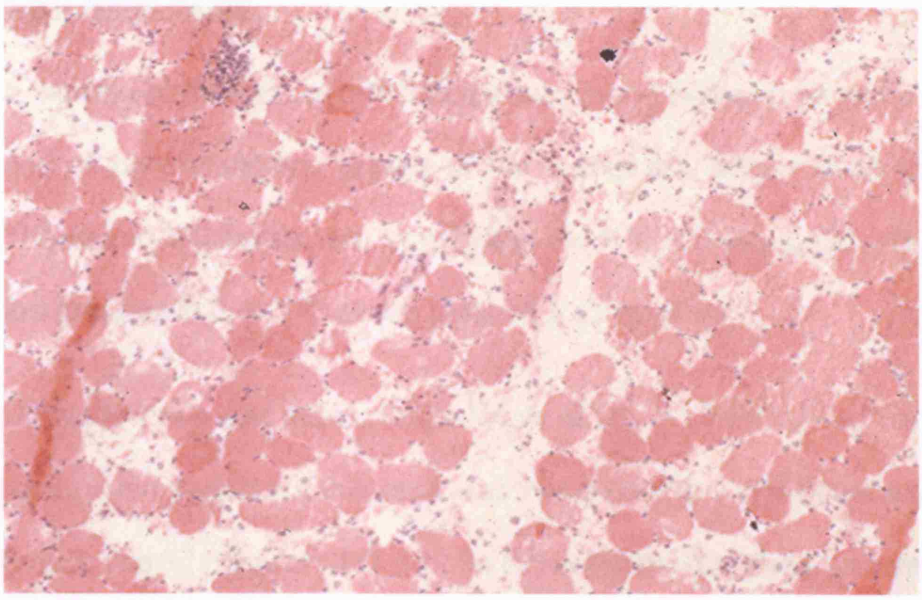


Figure 5.9: Calcium preconditioning muscle

5.5.4 Nitric oxide

A significant difference in NO levels was found between baseline (0.36±0.3 uM) and all preconditioning groups at the end of the ischaemic period (Table 5.3). The rise in NO levels was smallest in the IPC 2.5 group (23.01±7.70 uM), and the greatest in the CPC group (140.41 ± 42.82 uM).

However there was a drop in the levels of NO in all IPC groups at the end of the reperfusion period but this drop was not statistically significant except in IPC 10 group. The K^+ PC and CPC groups did not show the same pattern of drop in NO levels. No significant change in the number of local nitric oxide receptors was found in the skeletal muscle in all groups.

Group	End of ischaemia(uM)	End of reperfusion(uM)	% Drop
Control	0.36 ± 0.38	0.36 ± 0.38	0
IRI	42.01 ±12.10	28.21 ±12.31	35.7%
IPC 2.5	23.01 ± 7.70	8.04 ± 2.41	65.2%
IPC 5	55.12 ± 18.30	19.01 ± 3.23	65.4%
IPC 10	56.03 ± 10.13	35.04 ± 2.01	37.5%
K^+PC	94.17 ± 38.21	285.52± 38.70	↑303.19%
CPC	140.41 ± 42.82	501.01 ± 119.10	↑ 356.81%

Table 5.3: Changes in Nitric oxide during ischaemia & reperfusion ± SEM

The levels of NO were inversely proportional to capillary diameter in the IPC 2.5 and K^+PC groups (figure 5.10). This may be due to tachyphylaxis which is defined as rapid decrease or reduction in response to a identical dose of an agonist over a short period of time[242,243] and is thought to apply particularly to agents that act by releasing endogenous transmitters and is probably the result of transmitter exhaustion[242].

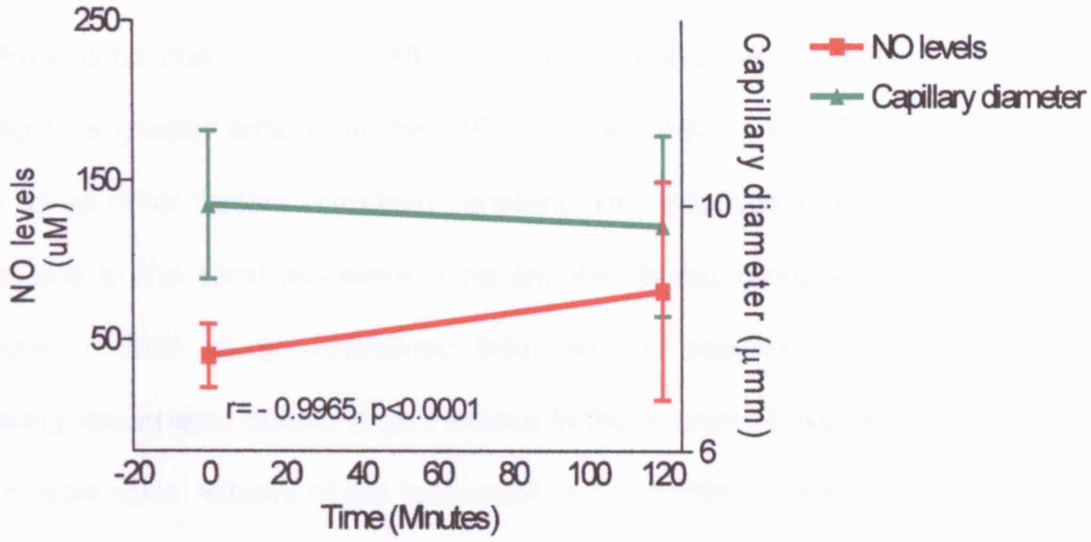


Figure 5.10: relationship between NO levels and capillary diameter over two hours

5.6. Discussion

IPC in skeletal muscle was first described by Pang et al [37] in 1995. The protection it offers to skeletal muscle from infarction has already been demonstrated[37,81], however, the controversy about the ideal cycle time and the best pharmacological agent to use for preconditioning continues. In this study we investigated different cycle times and pharmacological agents. Muscle infarction was taken as the endpoint in this experiment while NIRS was used to assess the state of tissue oxygenation during the experiment. I also looked at the state of local NO receptors and the levels of serum NO.

I have looked at levels of oxygenation in skeletal muscle in different groups of IPC. Significantly increased levels of HbO_2 were observed in skeletal muscle subjected to 2.5 min of IPC. This increase is consistent with the finding of a

decrease in the extent of muscle infarction in this group compared to the five and ten minute preconditioning groups (figure 5.11). This observed increase is most likely to be due to a direct effect of IPC in attenuating vasospasm[81],this occurred to a greater extent in the IPC 2.5 group than in the IPC 5 and 10 groups as all other factors remained constant. This attenuation in vasospasm may be due to the short ischaemic time adopted in this group which did not contribute in itself as an ischaemic insult to the muscles. The effect of decreasing vasospasm results in an increase in the amount of oxygen delivered to the muscle upon release of the tourniquet and in addition the increased flow of blood to the muscles will result in the rapid washout of the accumulated products of anaerobic metabolism.

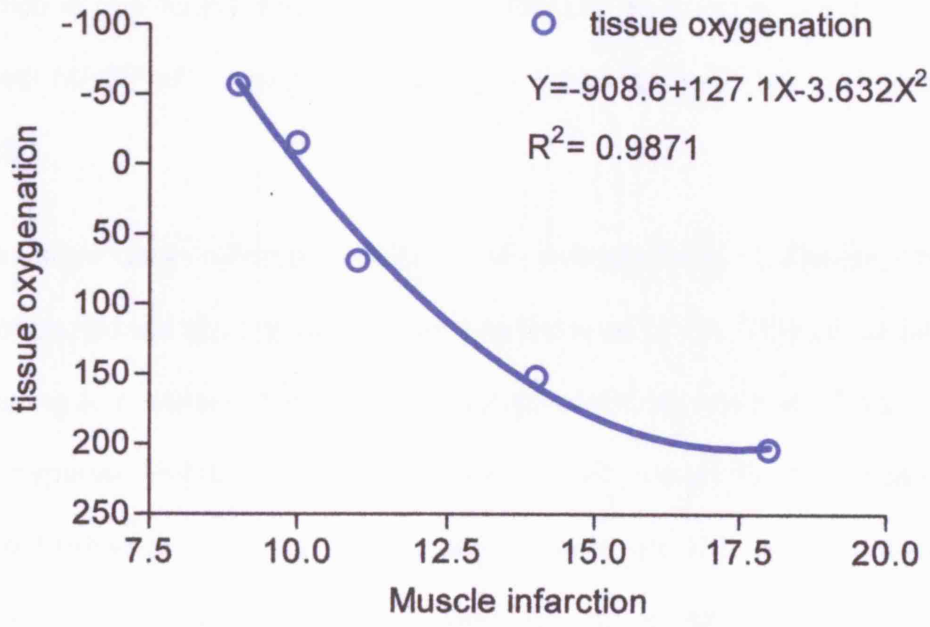


Figure 5.11: correlation between SM oxygenation and infarction extent

The levels of HbO_2 in the K^+PC and CPC groups were consistently significantly lower than in the IPC groups and they both showed a comparable trend and only slowly returned to baseline values at the end of the reperfusion period. In the CPC group this is consistent with the level of skeletal muscle infarction seen. However in the K^+PC group this is not in keeping with the extent of infarction, one would expect the K^+PC group to show HbO_2 levels comparable to those seen in group 5, as both groups showed the same extent of muscle infarction. However, if the systemic haemodynamics of both groups are taken into consideration particularly the MABP, this could account for the low levels of tissue oxygenation in the K^+PC group. There was a statistically significant difference in the MABP between the two groups; furthermore the K^+PC group did show a consistently lower MABP throughout the period of the experiment which is due to the antihypertensive effect of K^+-channel opening agents. This lower MABP will consequently lead to a lower perfusion rate and oxygenation of limbs.

No significant difference could be demonstrated between the different preconditioned groups with regards to the level of Hb. This could be due to IRI leading to systemic disturbances such as metabolic acidosis. This usually leads to hyperventilation and the reduction of CO_2 (respiratory alkalosis) which in effect will lead to an overall lowering of the levels of CO_2, thus maintaining the overall low levels of Hb. As with Hb no significant changes could be demonstrated in the levels of CytOx between the different preconditioning groups. There are several factors that could explain why muscle tissue Cyt Ox levels show only a small reduction during systemic hypoxaemia. Firstly Cyt Ox has a low Km for O_2 and secondly, the myoglobin acts as a large oxygen

reserve[236]. Also during general anaesthesia the muscles are in resting state and the oxygen consumption is minimal[237]. Another factor is that the contribution of cytochrome oxidase to the NIRS light attenuation is very small (about 2-5 %)[238] and this can lead to difficulties in separating its signal from the generally much larger signals due to Hb and HbO_2[238,239]. For this reason significant efforts have been made recently to develop special algorithms to magnify the signal in order to provide sufficient resolution[240] and to differentiate changes in Cyt Ox independent of haemoglobin[241].

Even though ischemia leads to decreased levels of NO due to decreased intracellular levels of NADPH and oxygen, which are both essential for NOS activity[244], the observed rise in NO during the ischaemic period may well be due to remote preconditioning. This may result from IRI activation of the complement system[245,246] which in turn leads to the production of NO by liver endothelial cells and Kuppfer cells[247]. These can be activated through activation of the complement system either via the classical or alternative pathway[248]. The lack of any elevation in the concentration of NO receptors in the skeletal muscle may be due to an absence of any decrease in the levels of circulating NO, thus not triggering any up regulation of local NO receptors.

The protective effects of IPC in skeletal muscle have been well documented; however the optimal number of cycles and the duration of each are less well described. A recent study by Saita et al[249] showed that preconditioning with 3 or more cycles confers protection against ischaemic injury. Studies on the extent of muscle infarction have shown that in a pig, three ten min cycles of preconditioning offer optimal protection. However these studies were done in

comparison to 5 minute cycles or variations on the 10 minute cycles[37,250]. On the other hand, work done comparing skeletal muscle morphology and neuromuscular function after three cycles of 10 minutes of ischaemia followed by 10 minutes of reperfusion did not show any reduction of post-ischaemic muscle injury or acceleration in recovery for up to seven days after two hours of tourniquet ischaemia. There was also an associated loss of neuromuscular transmission for up to five days after the initial insult. Furthermore the muscle water content was 5% and 10% higher at 3 & 7 days respectively[80].

5.7 Conclusion

Three cycles of 2.5 minutes of preconditioning are associated with a significant decrease in the extent of muscle infarction. Pharmacological preconditioning is achievable with the use of K^+ channel openers, however the use of Ca^+ lead to an overall increase in mean muscle infarction area.

Chapter Six

Late effects of preconditioning

6.1 Introduction

Having looked in the previous chapter at the early effects of IPC on skeletal muscle, here we will look at the effects on skeletal muscle later on (after 24 hours). It is known that in cases of IRI the extent of skeletal muscle damage is not fully apparent from the outset and that over a period of time the damage to muscles can extend a great deal more particularly when muscles are allowed to return to normal room temperature[251]. It is also know that IPC offers protection from any further ischaemic insult[252,253] for up to two to three days.

In order to determine the full benefit of any pharmacological preconditioning protocol it is important to fully evaluate the extent of muscle injury both short and long term. This injury to muscle is not influenced by the second window of IPC protection as this offers protection from a second ischaemic insult rather than prevent further damage[254-256].

6.2 Materials and Methods

6.2.1 Animal model and surgical preparation

This study was conducted in accordance with the Animals Scientific Procedures Act (1986) and under licence from the Home Office. Animals were placed in supine position. Anaesthesia was induced and maintained with 1.5-2% isoflurane (Baxter healthcare, Norfolk, UK) delivered via a mask. A tourniquet was placed proximally around the right thigh and a small longitudinal incision was made through the skin of the lower leg and the muscle was exposed. A laser Doppler flowmeter (LDF) probe was positioned onto the muscle mass (vastus lateralis). The reason why LDF was used in this instance rather than NIRS is that NIRS measures changes in the absolute value of tissue oxygenation from a reference point of zero which is set at the start of each

experiment. Since animals in this experiment were recovered and brought back 24 hours later in order to assess the late effects it would not be possible to obtain an accurate reading with the use of a NIRS probe. This is because the original reference point of zero would be lost and a new reference point would need to be established. Following each given preconditioning protocol a pressure of 200mmHg was applied via the tourniquet in order to occlude the blood supply for 2.5 hours. This was followed by a recovery period for the animal of 24 hours of reperfusion. Heart rate (HR), temperature and tissue microcirculation were monitored. Blood samples of 0.5ml were collected at baseline and at the reperfusion periods to measure nitrate and nitrite levels. At the end of the experiment the tibialis anterior muscle was harvested to assess the extent of muscle infarction. (Chapter 3 section 3.4-3.4.3)

A pulse oximeter (Ohmeda Biox 3740-pulse oximeter, Ohmeda, Louisville, USA) was used for the monitoring of arterial oxygen saturation and HR. Body temperature was maintained at 36-37.5°C by means of an electronic heating blanket and monitored continuously using a rectal probe (Harvard apparatus LTD, UK).

K^+ channel opener preconditioning was achieved using Levcromakalim (Sigma, Sigma-Aldrich, Dorset, England)

6.3 Experimental protocols

24 adult male Sprague-Dawley rats weighing between 300 and 350g were used. The animals were divided into 4 experimental groups (n=6 each group) (Table 5.1).

Group 1 (Control): The animals had an operation under general anaesthesia for the same procedure as other experimental groups without inducing ischaemia.

Group 2 (IRI): The animals were subjected to 2.5 hours of ischaemia and 24 hours of reperfusion without any preceding IPC protocol.

Groups 3 (IPC 2.5): The animals were subjected to 2.5 hours of ischaemia and 24 hours of reperfusion preceded by three cycles of 2.5 minutes of IPC.

Group 4: (K^+- Channel opener preconditioning, K^+PC): Animals were subjected to 2.5 hours of ischaemia followed by 24 hours of reperfusion preceded by the administration of K^+- Channel opener (Levcromakalim). Preconditioning was induced by infusing 80-100 mg/kg 10 minutes before the induction of 2.5 hours of ischaemia.

6.4 Data collection and statistical analysis

Data from the pulse oximeter and LDF were collected at specific time points. After allowing ten minutes for stabilisation the data was averaged for one minute at baseline and during the periods of ischaemia and reperfusion.

Muscle microcirculation changes were calculated at various stages of the ischaemia & reperfusion periods and compared to baseline.

The comparison of data between the groups is assessed by one way analysis of variance (ANOVA) with Tukey's adjustment for multiple comparisons.

6.5 Results

6.5.1 Mean muscle infarction size

The extent of muscle infarction for each group is shown in (figure 6.1, Table 6.1). The IRI group showed a mean muscle infarction area (MMIA) of (13 ± 1.7%). There was a decrease in the MMIA in all preconditioning groups with the 2.5 minutes group (11 ± 1.0%) and the K^+PC group (12± 0.60%) showing a comparable decrease in MMIA. However these results did not reach statistical significance.

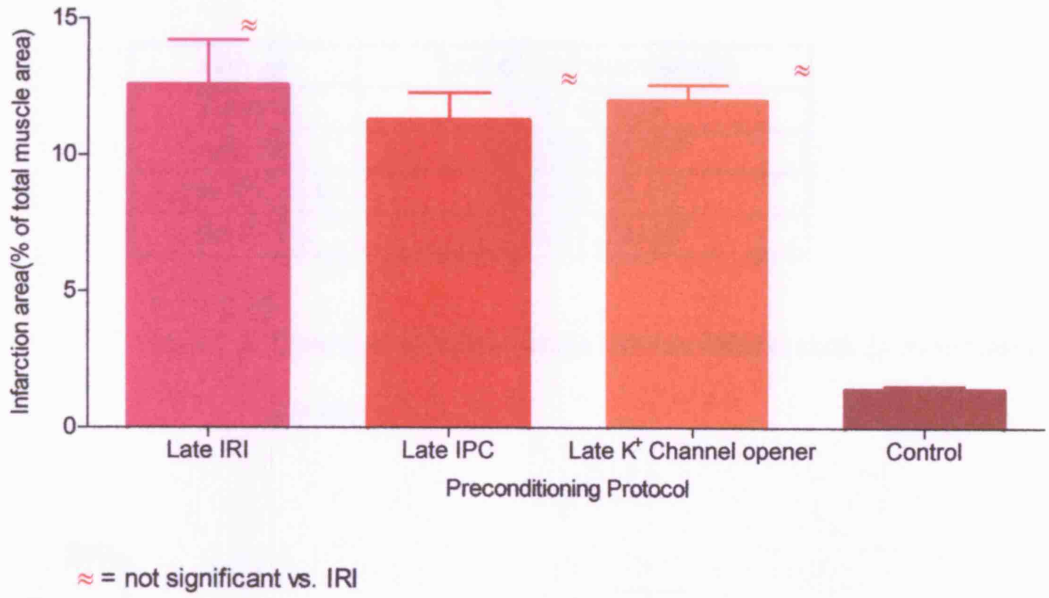


Figure 6.1: Extent of skeletal muscle infarction in IR & preconditioning groups with different cycles. Results are displayed as mean ± SD.

Group	Mean muscle infarction area (%)
Group 1	1.4 ± 0.3
IRI	13 ± 1.7≈
IPC 2.5	11 ± 1.0≈
K^+PC	12 ± 0.6≈

Table 6.1: Mean Muscle infarction area ± SD.,

≈ = Not significant vs. IRI

6.5.2 Nitric oxide (Table 6.2) (Figure 6.2)

A significant difference in NO levels was found between baseline (0.37±0.39) and all groups at the end of the 24 hours reperfusion. This increase was most marked in the IPC group (128± 53.08 uM). The rise in NO levels was smallest in the IRI group (101± 36.67 uM).

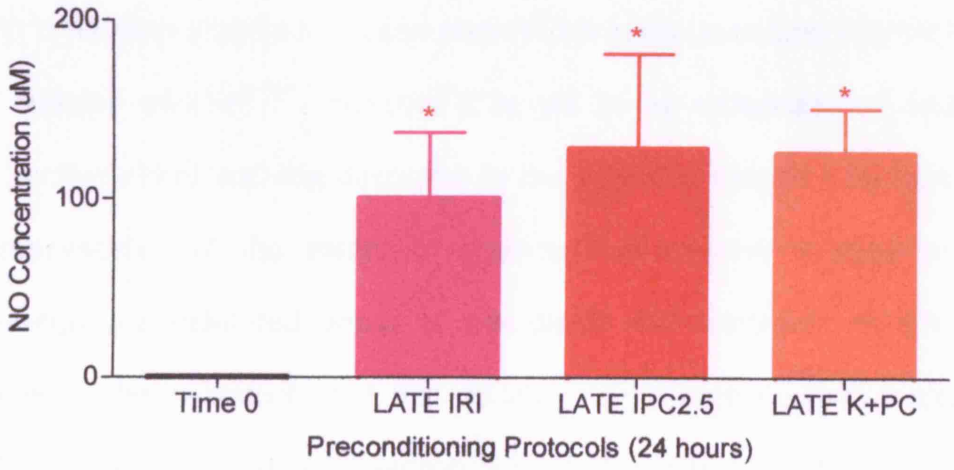
The pharmacological preconditioning group also showed a rise in the levels of NO at the end of the reperfusion period comparable to that of the IPC group (126± 24.68 uM)

Group	End of ischaemia(uM)
Time 0	0.37 ± 0.39
Late IRI	100.60± 36.67*
Late IPC 2.5	128.40± 53.08*
Late K^+PC	125.90± 24.68*

* = P< 0.05

Table 6.2: Changes in Nitric oxide concentration during reperfusion

± SEM



* = Significant vs. baseline

Figure 6.2: NO levels at the end of the reperfusion period.

6.5.3 Muscle Microcirculation

Muscle microcirculation (MMC) was measured at baseline, post preconditioning and at 24 hours after the induction of ischaemia. There was no significant difference between the various groups at baseline. There was a significant increase in the levels of MMC immediately post IPC vs. baseline ($p < 0.001$). There was a significant increase in the levels of MMC in the K^+PC groups at 24 hours from baseline ($p=0.01$). At 24 hours post reperfusion there was a significant increase in the level of MMC between the IPC and K^+PC groups and the IRI group ($p= 0.01, 0.001$ respectively).

	Baseline	Post IPC	24 post Reperfusion
IRI	$155.20 \pm 6.53^{*\approx}$	N/A	$118.70 \pm 8.72^{\ddagger}$
IPC	$162.01 \pm 7.97^{*\approx\dagger}$	$282.70 \pm 23.02^{\approx}$	$204.30 \pm 15.52^{\dagger\ddagger}$
K^+PC	$168.80 \pm 15.63^{*\dagger}$	N/A	$264.30 \pm 21.78^{\ddagger}$

* = $p > 0.05$, $\approx$ = $p < 0.05$, † = $p < 0.05$, ‡ = $p < 0.05$

Table 6.3:MMC at baseline & 24 hours post preconditioning ± SD

6.6 Discussion:

IPC in skeletal muscle has been shown to provide protection against ischaemia in skeletal muscle[37,81], however it is yet to be demonstrated whether this protective effect and the decrease in the extent in muscle infarction is a true representation of the extent of protection or whether skeletal muscle will undergo a accelerated phase of cell death with continued reperfusion. The previous demonstration that IPC as well as pharmacological preconditioning offers protection (Chapter 5 section 5.5.3) which was evident in skeletal muscle harvested after 3.5 hours reperfusion, provides a reference point for comparison compared to after 24 hours of reperfusion. The level of decrease in muscle

infarction in our two preconditioning groups (IPC, K^+PC) (2% & 1% respectively) is consistent with results from the previous chapter, when compared to the IRI group. However there was an increase in the extent of infarction in both groups (1-1.5%) after 24 hours of reperfusion, this increase (1-1.5%) was not mirrored in the IRI group which showed a decrease of 1%. These changes in the extent of muscle infarction can be due in the preconditioning groups to the increase in microcirculation in the skeletal muscle this might lead to the infiltration of the interstitial tissues with lymphocytes and other inflammatory cells which will lead to an increase in the amount of damage[19-23] when compared to the IRI group.

The levels of NO in all 3 groups showed a significant increase from the baseline, this increase was inversely proportional to the extent of muscle infarction in the three groups, this is in keeping with the view that NO plays a key role in preconditioning and protecting skeletal muscle from reperfusion injury[257-262].

The significant elevation in the MMC in both the IPC and K^+PC groups at the end of the preconditioning and reperfusion period demonstrates that the protective effect on MMC is maintained up to 24 hours post ischaemia and not only during the early period[100].

6.7 Conclusion

These experiments have shown that the decrease in the extent of muscle infarction observed in the early phase of reperfusion does continue for up to 24 hours post reperfusion. This is accompanied by an increase in the levels of microcirculation in the preconditioned groups and these effects are probably related to the elevated levels of NO observed.

Chapter Seven

Skeletal muscle microcirculation

7.1 Introduction

From previous chapters we have seen that IPC confers a protective effect on skeletal muscle both in the early and late phases of reperfusion. It is however still not clear whether this protection is due to the systemic effects of increased levels of circulating nitric oxide as was shown in chapter 5 (page 115), or whether there is a local protective effect exerted on the local microcirculation. There have been previous studies that have shown that IPC does offer local benefits either in the way of muscle function, blood flow or capillary diameter[37,48,75,81,87,93,95]. These studies however are variable in the protocol of preconditioning used and the species they were used on.

7.2 Materials & Methods

7.2.1 Animal model and surgical preparation (figure 5.1)

This study was conducted in accordance with the Animals Scientific Procedures Act (1986) and under licence from the Home Office. Rats (Sprague-Dawley) weighing (300- 350gm) were placed in supine position. Anaesthesia was induced with 1.5-2% isoflurane (Baxter healthcare, Norfolk, UK) delivered via a face mask and maintained with thiobutabarbital sodium (Inactin; Sigma, Sigma-Aldrich, Dorset, England) 80-100mg/Kg intra-arterially. A polythene catheter (internal diameter 0.8mm) (Portex limited, Hythe, Kent, England) used to monitor mean arterial blood pressure (MABP) was inserted into the internal carotid artery. A pulse oximeter (Ohmeda Biox 3740-pulse oximeter, Ohmeda, Louisville, USA) was used for the monitoring of arterial oxygen saturation and HR. Body temperature was maintained at 36-37.5°C by means of an electronic heating blanket and monitored continuously using a rectal probe (Harvard apparatus LTD, UK). Mean arterial blood pressure (MABP) was monitored

continuously using a blood pressure monitor (F-LM1; Datex-Ohmeda Ltd, Hertfordshire, UK).

A tourniquet was placed proximally around the right thigh and following each given preconditioning protocol a pressure of 200mmHg was applied via the tourniquet in order to occlude the blood supply for 2.5 hours. This was followed by 2 hours of reperfusion. Heart rate (HR), MABP (Table 7.2) and temperature were monitored throughout the ischaemia and reperfusion period. The reason why the reperfusion period was shorter than the other experimental protocol used in the previous chapters (namely 3.5 hours of reperfusion) is that after 2 hours there is decay in the fluorescent dye labelled red blood cells which makes their detection difficult.

The adductor muscle mass was exposed at the end of the end of the ischaemia period with a longitudinal incision in the skin overlying it in preparation for the application of the Intravital microscope. This part of the surgical procedure was done immediately prior to placing the animal under the microscope in order to minimise any effects the exposed muscle mass might have on the animals haemodynamic state.

K^+ channel opener preconditioning was done using Levcromakalim (Sigma, Sigma-Aldrich, Dorset, England)

7.2.2 Experimental protocols (Table 7.1).

24 adult male Sprague-Dawley rats weighing between 300 and 350g were used. Animals were divided into 4 experimental groups (n=6 each group)

Group 1 (sham): The animals had operation under general anaesthesia for same procedure as other experimental groups without inducing ischaemia.

Group 2 (IRI): The animals were subjected to 2.5 hours of ischaemia and 2 hours of reperfusion without any preceding IPC protocol.

Groups 3 (IPC 2.5): The animals were subjected to three cycles of 2.5 minutes of IPC followed by 2.5 hours of ischaemia and 2 hours of reperfusion.

Group 4: K^+- Channel opener preconditioning (K^+PC): Animals where subjected to 2.5 hours of ischaemia followed by 2 hours of reperfusion preceded by the administration of K^+- Channel opener (Levcromakalim). Preconditioning was induced by infusing 80-100 mg/kg 10 minutes before the induction of 2.5 hours of ischaemia.

Group	IPC Protocol	Ischaemic time (hr)	Reperfusion time (hr)
Group 1(Control)	None	0	0
Group2 (IR)	None	2.5	2
Group3 (IPC 2.5)	3 X ($I_{2.5min}$+$R_{2.5min}$)	2.5	2
Group 4 (K^+PC)	Levcromakalim 80-100 mg/kg	2.5 hours	3.5 hours

Table 7.1: Experimental groups & protocol

7.2.3 Fluorescent dye preparation

The parameters that will be measured can only be clearly analysed if labelled with a fluorescent dye. Red blood cells were labelled with fluorescein isothiocynate (FITC).

Fluorescein isothiocynate (FITC) has been used to label blood taken from donor animals according to the protocol indicated in Zimmerhackl et al[263].

7.2.4 Red blood cell labelling

Eight millilitres of blood or as much as needed in is collected in a heparinised container. This is then centrifuged at 400g or 2000rpm for 10 minutes. The plasma and buffy coat are removed and the cells washed with glucose saline buffer 5 times. For every 1 ml of blood collected 1 ml of glucose buffer and 0.4ml of FITC is added. The mixture is left to incubate for 1.5 to 2hrs at room temperature. This is followed by washing the labelled cells with glucose buffer 3 times or until the supernant is clear and then suspend in glucose buffer in 1:2 dilution.

Material used in Red blood cell labelling with FITC*					
Stock 1		Stock 2 (1M)		Stock 3 (0.03M)	
Barbital sodium*	2.55g	MgSO4*. 7H2O*	24.6g	CaCl2*.2H2O*	4.41g
1M HCl*	10ml	Distilled water	100ml	Distilled water	100ml
NaCl	6.8g				
Glucose Buffer		FITC solution			
Stock 1	50ml	FITC*	40mg		
Stock 2	0.1ml	Glucose Buffer	2ml		
Stock 3	0.1ml	NaOH	10µl		
Glucose	4.2g				

* = (Sigma, Sigma-Aldrich, Dorset, England)

7.2.5 Application of Intravital fluorescent microscopy (IVFM) to the study of skeletal muscle microcirculation

After the completion of all the surgical procedures and the ischaemia and reperfusion periods the intravital microscope was prepared by switching on the lamp and setting the stage heater to 37°C. A plastic covering was placed on the stage for ease of cleaning. The animal was transferred onto the pre-heated stage. The exposed muscle section was covered with a cover-slip and a drop of

saline or distilled water was pipetted onto the centre of the cover-slip. With the x10 objective in line the nose of the microscope was lowered so that the lens was immersed in the aqueous liquid.

7.2.6 Hardware and software

This has been described in detail in Chapter Three pages 62-95

7.3 Data collection and statistical analysis

Data from the pulse oximeter, and blood pressure monitor were collected continuously on a laptop computer. After allowing ten minutes for stabilisation, the data was averaged for one minute at baseline and during the periods of ischaemia and reperfusion. Data obtained from the IVFM was recorded on a desk top computer at specific time points during the reperfusion period namely at (0, 30, 60, 90, and 120) minutes and analysed at a later stage.

The measurement of the capillary blood flow and capillary diameter was achieved by measuring the capillary diameter and the blood flow in at least 6 vessels in the field at the specified time points (0, 30, 60, 90, and 120). These reading were then used to give a mean value that was used in the final analysis.

The comparison of data between the groups is assessed by one way analysis of variance (ANOVA) with Tukey's adjustment for multiple comparisons.

7.3.1 Off line microcirculatory analysis

Numerous methods have been described to measure red cell velocity, and blood vessel diameter using intravital microscopy software. The line shift method or the histogram peak methods could have been used however, this is

time consuming and not more accurate than the method used here described in detail in appendix 2 of this thesis.

7.3.2 Measurement Parameters

No measurements of flow where taken during the ischaemic period as an initial experiment performed verified that no flow was seen during the ischaemic phase of the experiment and hence a value of zero is given to flow during this period. A detailed account of how the various measurements are obtained and calculated from the computer can be found in appendix 2 of this thesis.

7.3.2.1 Capillary blood flow

Capillary blood flow (CBF) can be calculated to determine the blood flux within capillaries where v_{rb} refers to the red blood cell velocity for each vessel[264].

The blood flow can easily be calculated by using data obtained from the methods described in section 7.3.1.1.

$$CBF = v_{rbc} \times \pi \times (d/2)^2$$

7.4 Results

7.4.1 Systemic haemodynamics (Table 7.2)

There were no significant changes in HR or SaO_2 from baseline and during the periods of ischaemia and reperfusion.

Group	$Sa0_2$			HR			MABP		
	BL	EOI	EOR	BL	EOI	EOR	BL	EOI	EOR
Control	99±1	99± 1	98± 1	255±10	255±12	255±15	109±14	116±15	119± 15
IRI	97±2	96± 1	99± 1	255±12	255±13	255±13	123±10	126±16	115± 13
IPC2.5	98±1	98± 1	99± 1	255±10	255±11	255±12	107±13	119±15	117± 14
K^+ PC	99±1	97± 2	98± 1	255±12	255±11	255±13	111±09	123±11	122± 16

Table 7.2: Systemic haemodynamics during ischaemia & reperfusion in groups 1-4

Key: SaO_2 = Oxygen saturation, HR = Heart rate, MABP = Mean arterial blood pressure, BL = Baseline, EOI = End of ischaemia, EOR = End of reperfusion.

7.4.2 Capillary blood flow (Figure 7.1)

A significant difference in flow was found between all groups except for the difference between the IPC vs K+ PC groups where the mean difference was 0.294 with a P value > 0.05. The mean difference between the IRI group vs K+PC group was 2.039 with a P value < 0.001. Similarly the mean difference between IPC group vs IRI group was 2.445 with a P value of < 0.001. The mean difference between sham vs IRI group was 3.016 with a P value of < 0.001. However this difference fell to 0.6822 and 0.9768 between the sham and IPC and IRI groups respectively.

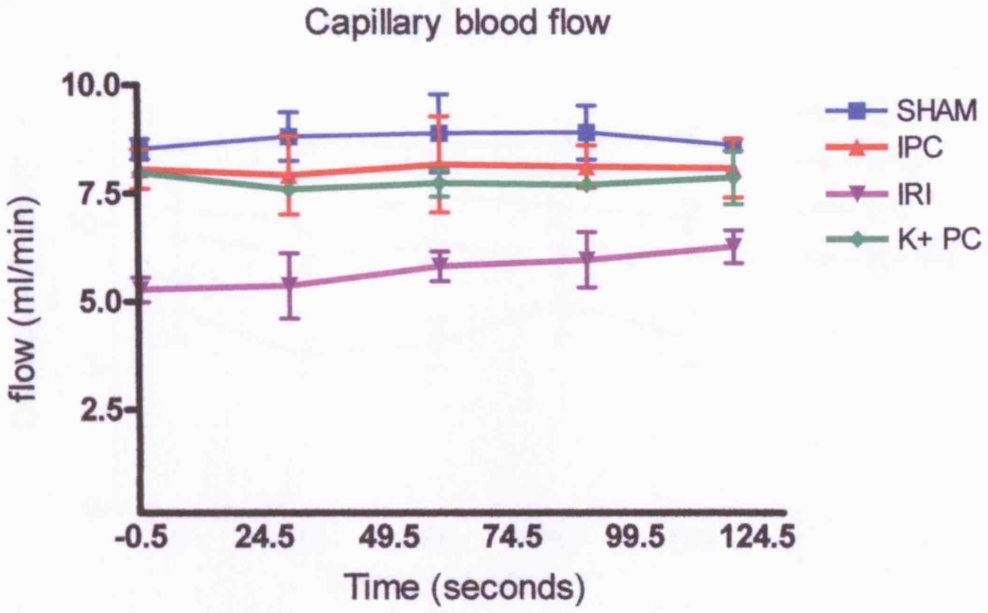


Figure 7.1: Capillary blood flow

7.4.3 Capillary diameter (Table 7.3), (Figure 7.2)

Table 7.3 shows the P value between the various groups

Groups	P value
Sham vs IPC	P < 0.001
Sham vs IRI	P < 0.001
Sham vs K+ PC	P < 0.001
IPC vs IRI	P < 0.001
IPC vs K+ PC	P < 0.01
IRI vs K+ PC	P < 0.001

Table 7.3: P value of the difference in blood vessel diameter between the various groups

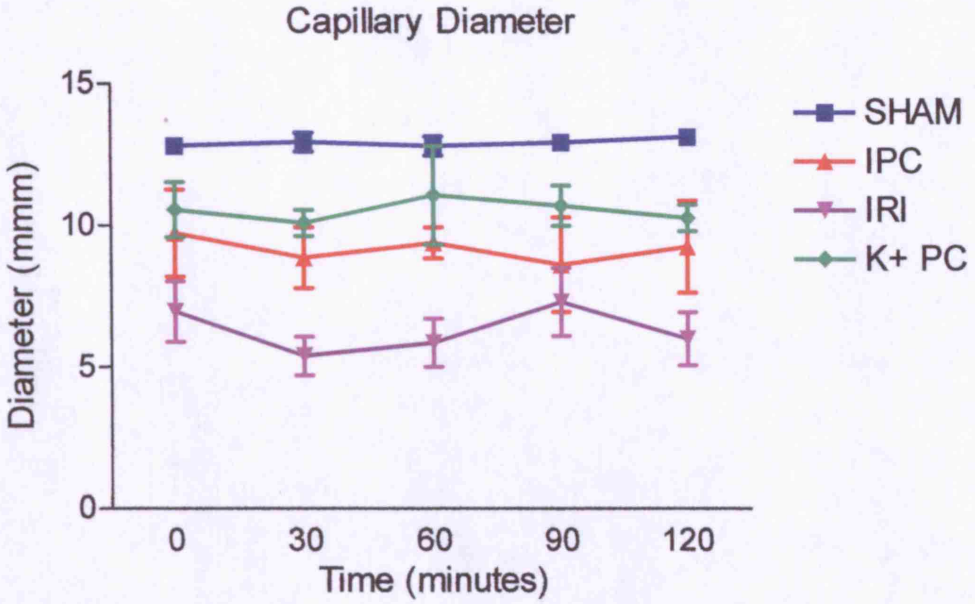


Figure 7.2: Capillary diameter

7.5 Discussion

From the work done in previous chapters I have shown that IPC in skeletal muscle does provide protection to skeletal muscle. This is in keeping with work done by other investigators[37,81]. Looking at skeletal muscle microcirculation will provide further insight into the mechanisms involved in this protection. Previous work done shows a decrease in the extent of rolling, adhering, and transmigrating leukocyte[265,266]. Regarding capillary diameter, the use of a K+ channel opener maintains a capillary diameter at higher levels than IPC however this result is to be expected as a result of its pharmacological properties[267]. This maintenance of capillary diameter was not statistically different from that achieved by IPC alone, which might overall be an indication as to why both modalities offered a similar level of protection against muscle ischaemia in previous chapters. Based on this it is should be possible to look into the pharmacological properties of K+ channel openers and try and eliminate and unwanted effects that they might have such as the development

of transient hypotension when initially administered in inducing K+PC (Table 5.2, Page124). This would bring us a step closer to developing an ideal pharmacological agent for preconditioning. The level of flow seen in the capillaries is a reflection of the maintenance of capillary diameter both in the IPC and K+PC groups. Both show maintenance of flow levels comparable to those seen in the sham group. This is in contrast to the IRI group in which even after two hours of reperfusion failed to return any level of normality both in flow and capillary diameter.

7.6 Conclusion

These experiments have shown that the decrease in the extent of muscle infarction observed in the early phase of reperfusion is associated with a preservation of capillary diameter and flow.

Chapter Eight

General discussion

8.1 General problem

Ischaemia reperfusion injury of skeletal muscle occurs in acute vascular occlusion and revascularisation; it also develops in elective vascular surgery, orthopaedic surgery (tourniquets) and in transplantation of myocutaneous flaps. IRI to skeletal muscle following episodes of acute ischaemia remains a major cause of morbidity and mortality in hospital practice. Attempts to define the basic mechanism of IRI have lead to a great increase in our understanding of this phenomenon and hence our attempts to limit the amount of damage created upon restoring the circulation to ischaemic tissues.

This damage is manifested locally by the increased leakage from capillaries and venules, extensive disruption of the microvasculature[13], and the formation of oxygen derived free radicals, capillary obstruction by endothelial cell swelling, loss of pinocytotic vesicles, protrusions or blebs, breaks in endothelial cells, disruption of basement membrane, and nuclear chromatin clumping[13] and a substantial fall in tissue pH.

Systemic consequences of IRI are complex. These include the development of inflammation, hyperkalaemia, acidosis, myoglobinuria, myonephropathic-metabolic syndromes[7], ARDS and renal dysfunction[7,8]. Systemic effects develop from the generalised release of cytokines and neutrophil infiltration leading to the development of SIRS and MODS[9].

Since described in 1986 by Murry et al [27] there has been a great expansion in exploring the beneficial effects of IPC on the various body organs. It is now established that IPC is protective to various body organs[28,29,31-35] and skeletal muscle[36,37]. However IPC in skeletal muscle is still in its early stages and there remains much to be studied.

8.2 Aim and methodology

8.2.1 Aim

My aim was to study the beneficial effects of IPC on skeletal muscle in the lower limb to identify a preconditioning protocol that achieves the best results from the various protocols described in the literature, and to study pharmacological agents that might mimic the beneficial effects of IPC.

There are different methods of preconditioning skeletal muscle. Ischaemic and pharmacological. Both offer protection against ischaemic damage. Applying ischaemic preconditioning to skeletal muscle prior to a period of ischaemia generally improves tissue pO_2 during reperfusion and qualitatively restores, to some extent the hyperoxic phase seen following a short ischaemic period (up to 2hours). IPC has the advantage of being an endogenously protective mechanism that has few potential side effects. Pharmacological preconditioning is attractive because of the speed of its induction and reproducibility. A number ischaemic preconditioning[36,37,76,80,82,89,91,92,94,268] and pharmacological[75,100,269] protocols have been used in order to mimic the effects of IPC, however the results have not been consistently reproducible and no controlled trial has been carried out in order to assess any side effects from their use. In addition the end points that these experiments have looked at have varied. Ideally any pharmacological agent used to induce preconditioning should be cheap, easily available, have no or minimal side effects, be eliminated easily from the body, produce long lasting protection and easily be administered.

8.2.2 Methodology

Most of the ischaemic preconditioning protocols that have been used to date have employed a number of cycles of preconditioning[36,37,75,80-82,84,87,89,90,92-94,268,270]. I have therefore chosen to take these protocols and directly compare them against each other using skeletal muscle infarction as the end point of my experimental work. In addition I have added a 3 cycle of 2.5 minutes preconditioning to these in order to assess if a shorter cycle time offers as much protection as a longer one.

I have chosen two compounds for the use in my pharmacological preconditioning protocols and directly compared them against the ischaemic preconditioning protocols with muscle infarction as the end point. The reason for choosing these two compounds namely ca^{2+} and a K^+ channel opener is that work done before has shown them both to be promising agents in the fights against ischaemia reperfusion injury[46,100]. Calcium as a preconditioning agent has been used in the cardiac muscle with success particularly in diabetic patients who form a considerable proportion of the patients who present with peripheral vascular disease. Cromakalim has been shown to produce significantly higher skeletal muscle microcirculation[100].

8.3 Results

In my first experimental chapter I have compared the use of near infrared spectroscopy as a tool to measure the levels of Hb and HbO_2 with the levels of skin oxygenation by the $TCpO_2/pO_2$ monitoring system and systemic arterial pO_2 and pCO_2. This was an important step in setting up my experimental model as in addition to using the extent of skeletal muscle infarction as an end point to

my experiment I used NIRS as a measuring tool during the ischaemic and reperfusion periods to monitor the changes in the oxygenation levels and to see if oxygenation levels correlated later with the extent of muscle infarction.

I have shown that IPC is achievable in the experimental in vivo setting and that this is usually associated with early elevation of NO. This is followed by a drop in NO levels that is proportional to the level of muscle infarction; however, the early increase in the levels of circulating NO was not mirrored by an increase in the levels of local nitric oxide receptors. In addition my results have shown that IPC with a shorter cycle of 2.5 minutes is more effective than the longer cycles that have been used to date in offering protection against ischaemia and that this decrease in the extent of muscle infarction is associated with improved oxygenation levels in the muscles.

I have also been able to show that pharmacological preconditioning of skeletal muscles using a K^+ channel opener (Levcromakalim) reduces the extent of muscle infarction, which is in line with previous experiments that have shown an improvement in skeletal muscle microcirculation[100]. However, the use of Ca^{+2} as a preconditioning agent has led to an increase in the extent of skeletal muscle infarction which is in contrast to what has been shown in cardiac muscle. In addition pharmacological preconditioning is also associated with a rise in NO, indicating that nitric oxide plays an integral role in preconditioning.

8.4 Limitations

I have shown that pharmacological preconditioning is possible in skeletal muscle and that there is a decrease in the extent of skeletal muscle infarction. However, it was not possible, due to the financial and time constraints of this

thesis, to further explore if there is any change in the extent of infarction with different doses of a K^+- channel opener particularly with lower doses as this would also lead to fewer side effects namely hypotension, which would be a step towards applying them in a clinical setting. My attempts at calcium preconditioning were based on studies that have shown that its use particularly in diabetic patients has been beneficial in protecting the cardiac muscle, I therefore used the same dose that was used in that study to investigate its effects in skeletal muscle reperfusion injury. It was surprising to see that the use of Ca^{+2} resulted in an increase in the extent of muscle infarction. It is however a possibility that changing the dose of calcium given or the time frame of administration prior to the onset of ischaemia or if administered during the ischaemic period itself then this might show a protective effect as that seen in cardiac muscle. In my study of skeletal muscle microcirculation I was not able to perform this experiment for the same duration as my other experiments; this was primarily due to degradation of the fluorescent dye making it undetectable by the microscope. It is well established that ischaemic preconditioning has both an early and late window of protection and these might be associated with their own microcirculatory changes that could not be studied.

8.5 Conclusion

Since the first description of the existence of IPC in skeletal muscle, a plethora of new data has been added and IPC has been shown to be a powerful, endogenous modality to protect SM from ischaemic injury both during surgical intervention and treatment of peripheral vascular disease. Current research has demonstrated that IPC is an adaptive modality which can readily be reproduced in a variety of different models of warm and cold ischaemia. The concept of IPC

induced by pharmacological intervention opens the door for both safer surgery and a means of treating claudication however, this is still in the very early stages and further research into this is required. The causal relationship between initiating events, biochemical signalling pathways and end effector species still remains undefined mechanistically. As this new field looks into more mechanistic based studies of IPC the interrelationships of the preconditioning cascade are likely to be resolved and so defined. The idea of developing a pharmacological agent that will be safe and confer all the protective effects of IPC is an attractive one as it remains impractical and time consuming to apply IPC in the operating theatre. Even though a large amount of work has been done on IPC there still remains some difference of opinion on what is the ideal preconditioning time and the number of cycles required, this might be due to the different species used in the work done till now, however there is agreement that IPC does offer protection against IRI.

8.6 Future work

Further work in order to clearly establish the mechanism of action of each mediator involved in IPC and their interaction with each other is obviously still required. Further work on animal models in order to clearly establish a pharmacological agent that is capable of offering protection against IRI in SM to an extent that is comparable to that offered by the most appropriate preconditioning cycle is still required. This clearly will need to be followed by attempting to establish the most appropriate dose required and attempting to reduce the incidence of any side effects that may occur from its use. The identification of a pharmacological agent capable of consistently reproducing

the same effects of IPC and being safe and easy to use in the clinical setup is the long term goal of research in this field.

Appendix 1

1 Red blood velocity

1. Open a file and select the frame sequence

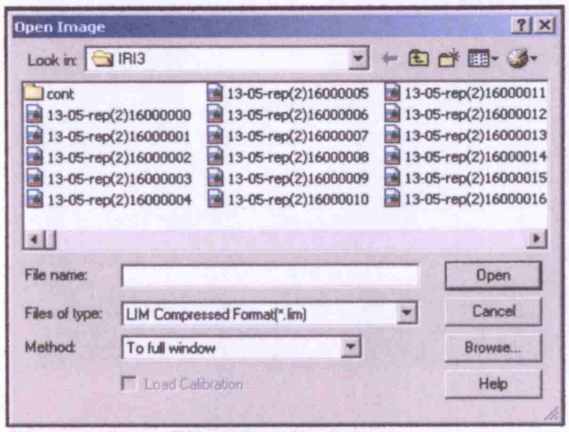


Figure 8.1: File opening

2. Once opened hold Pg Up or Pg Dn to move the frame sequence forward or backward respectively

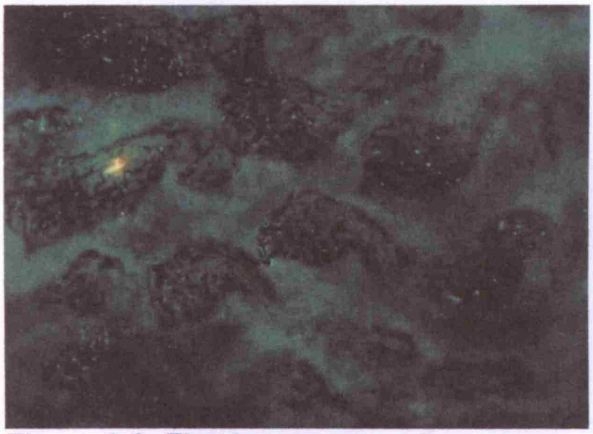


Figure 8.2: First image opened

3. Select the taxonomy measuring tool and a class marker

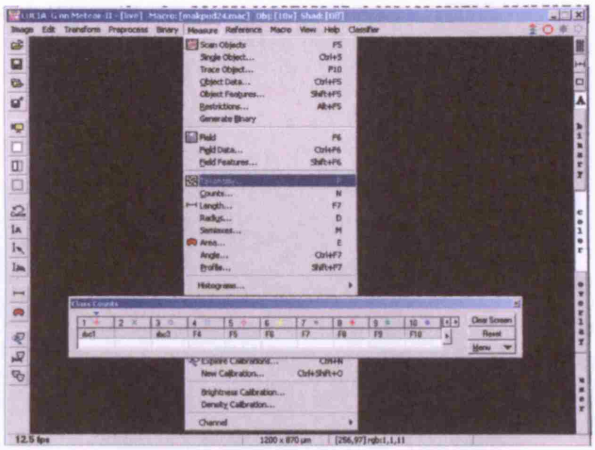


Figure 8.3: Taxonomy tool & class marker selection

4. From the sequence identify an RBC to measure then frame by frame mark the RBC's movement with the class markers.

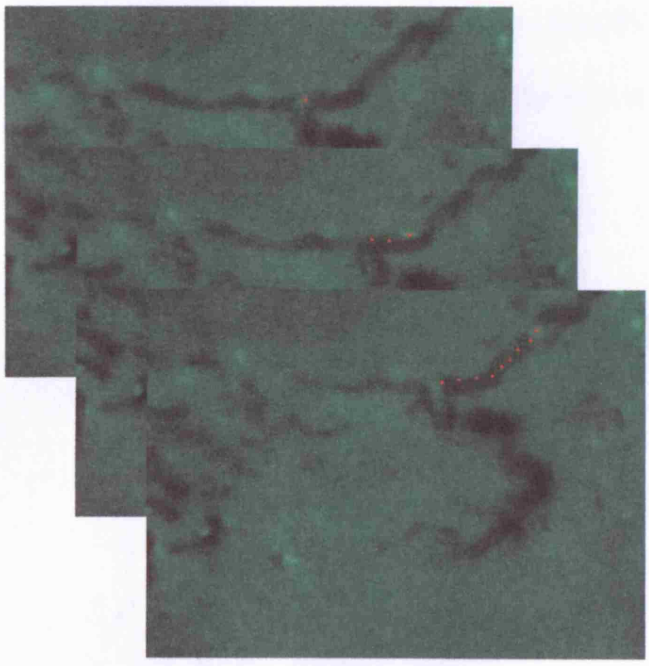


Figure 8.4: Progress of RBC marked

5. Select the appropriate objective calibration setting as highlighted in white (figure 7.5).

6. Select the length tool as highlighted in yellow, then the 'polyline' highlighted in lime green (figure 7.5).

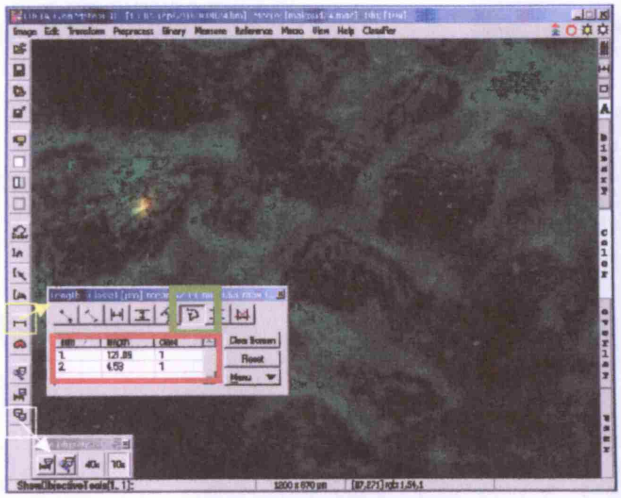


Figure 8.5: Objective tool & length tool selection

7. Using the polyline tool join the markers to calculate the distance moved (figure 9.6). More than one RBC movement is usually measured in order to increase the accuracy of assessment. The measurement will be shown in a table as highlighted in red (figure 9.5).

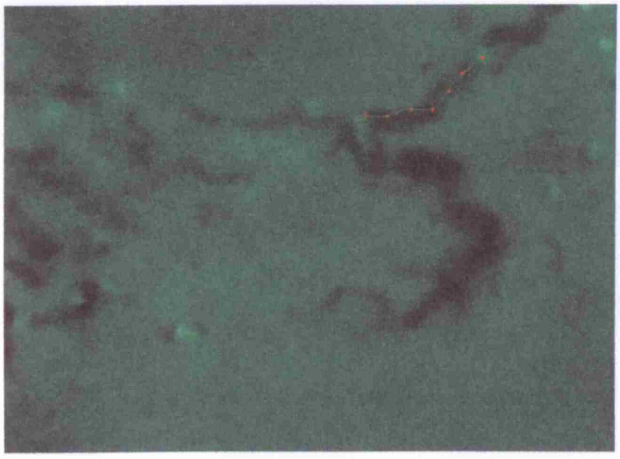


Figure 8.6: Distance calculation using the polyline tool

2 Vessel Diameter

1. Select image
2. Select appropriate objective calibration (shown in white)
3. Select length tool – 2 points (shown in yellow)
4. Then measure the diameter as shown in the red circle
5. The data in the table can then be pasted into excel for further analysis
6. More than one blood vessel diameter is assessed in order to increase the accuracy of measurement.

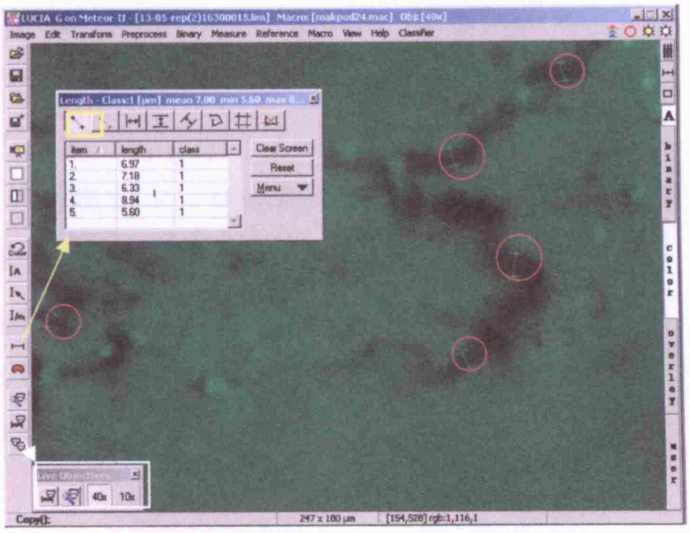


Figure 8.7: measurement of vessel diameter

Appendix 2

Publications and presentations from this thesis:

Publications:

1- M Salman, J Muddle, R King, G Hamilton, A Seifalian, F Myint. Pharmacological preconditioning: Calcium versus Potassium. BJS 91 (9) 1208

2- M Salman, G Glantzounis, W Yang, G Hamilton, F Myint, A Seifalian. Measurement of critical lower limb tissue hypoxia, coupling chemical and optical techniques: comparison with arterial oxygen partial pressure, BJS 91 (9)

3- M Salman, J Muddle, R King, G Hamilton, A Seifalian, F Myint. Ischaemic preconditioning of skeletal muscle. BJS 91 (9) 1235

10- Salman M, G Glantzounis, W Yang, G Hamilton, F Myint, A Seifalian. Measurement of critical lower limb tissue hypoxia, coupling chemical and optical techniques. *Clin Sci (Lond)* 2004

Presentations:

1- M Salman, J Muddle, R King, G Hamilton, A Seifalian, F Myint. Pharmacological preconditioning: Calcium versus Potassium. 14-16 January 2004, Oral presentation, Society of Academic and Research Surgery Annual Forum

2- M Salman, G Glantzounis, W Yang, G Hamilton, F Myint, A Seifalian. Measurement of critical lower limb tissue hypoxia, coupling chemical and optical techniques: comparison with arterial oxygen partial pressure, 14-16 January 2004, Society of Academic and Research Surgery Annual Forum

3- M Salman, J Muddle, R King, G Hamilton, A Seifalian, F Myint. Ischaemic preconditioning of skeletal muscle. 14-16 January 2004, Society of Academic and Research Surgery Annual Forum

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