UPREGULATION OF MATRIX METALLOPROTEINASES – 2 AND -9 AND TYPE IV COLLAGEN DEGRADATION IN SKELETAL MUSCLE REPERFUSION INJURY

Denise Margaret Roach, MBBS, DDU, FRACS.

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The University of Adelaide, The Queen Elizabeth Hospital, Adelaide, Australia

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Abstract

Aims and objectives: To determine the role of the matrix metalloproteinases, MMP-2 and MMP-9, in reperfusion injury following skeletal muscle ischaemia and to determine whether inhibition of MMPs by doxycycline protects against tissue damage.

Methods: Sprague Dawley rats were anaesthetised and a tourniquet applied above the greater trochanter to occlude blood flow. Sham-operated rats underwent four hours of ischaemia and were sacrificed after 0, 4, 24 or 72 hours. Four hours of unilateral or bilateral lower limb ischaemia was followed by reperfusion for 0, 4, 24 or 72 hours. Two groups of rats received 50 mg/kg or 200 mg/kg twice daily doxycycline for 7 days prior to bilateral ischaemia and 24 hours of reperfusion. Rats were euthanased and skeletal muscle from both limbs, pulmonary and renal tissues were harvested for wet/dry weight lung ratios, histopathological analysis, zymography, western blot analysis and immunohistochemical staining for type IV collagen.

Results: Histopathological analysis confirmed the validity of the animal model with significant tissue damage seen in ischaemic skeletal muscle and kidney. Upregulation of MMP-2 and MMP-9 was seen on zymography in the ischaemic leg and lung but not in the kidney. Western blot analysis with MMP-9 antibody confirmed the zymographic findings. Quantitative immunohistochemical analysis of levels of type IV collagen, showed degradation in reperfused muscle, lung and kidney. There was less upregulation of MMP-2 and MMP-9 in the skeletal muscle seen on zymography following pre-treatment with doxycycline. Doxycycline treated rats showed significant preservation of type IV collagen in skeletal muscle and partial protection from type IV collagen degradation in lung and kidney. The lung wet/dry weight ratios showed no statistical difference between sham-operated and ischaemic animals.

Conclusions: MMP-2 and MMP-9 are strongly upregulated in skeletal muscle ischaemia/reperfusion injury and are also upregulated in remote organs, leading to degradation of membranes. Inhibition of MMP activity may therefore be potentially therapeutically useful in reducing the severity of reperfusion injury.

Statement of Originality

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give my consent to this copy of the thesis, when deposited in the University library, being available for loan and photocopying.

Denise Margaret Roach

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1.1 Introduction

Acute limb ischaemia is a common clinical entity in medical practice, which may be caused by a variety of conditions including thrombosis, embolism and trauma. During the ischaemic period, the limb is depleted of oxygen with consequent utilisation of cellular energy substrates and conversion to anaerobic metabolism. During ischaemia, toxic metabolites accrue, altering membrane integrity leading to extravasation of electrolytes and macromolecules from the cell to the interstitium. Despite the technical advances that have occurred in treatment of ischaemic limbs, including embolectomy, percutaneous transluminal angioplasty, thrombolysis and femoro-distal arterial reconstruction, the morbidity, mortality and failure of limb salvage rates remain significant[1-4].

Reestablishment of blood flow is essential to salvage ischaemic tissues, however reperfusion itself paradoxically causes further damage to the skeletal muscle threatening limb function and viability. There is also a remote effect, most particularly in the lungs, kidney, heart and liver, often resulting in multi-system failure. The clinical symptoms of reperfusion vary according to the length and severity of the primary ischaemia. These symptoms vary from limb oedema, impaired muscle contraction and muscle necrosis to multi-organ failure and death.

Reperfusion is a multifactorial entity that has been extensively investigated with aetiological factors including oxygen free radicals, neutrophil-dependent microvascular barrier disruption, oedema formation and the capillary no-reflow phenomenon. The following review will summarise these pathophysiological factors.

Elucidating other factors involved in skeletal muscle reperfusion injury may have significant benefits when treating patients who have sustained lengthy periods of severe ischaemia. Many therapeutic options have been used to ameliorate skeletal muscle reperfusion injury, but predominantly in experimental models that are not easily applicable to the human situation. The further elucidation of possible therapeutic regimes may allow minimisation of the traumatic effects of reperfusion of an ischaemic limb.

The matrix metalloproteinases (MMPs) are a family of zinc dependent enzymes that have the ability to degrade all components of the extracellular matrix. Together with their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), they are the major physiological

regulators of the extracellular matrix. MMPs are intimately involved in all processes that necessitate degradation or synthesis of the extracellular matrix and important roles for these enzymes have been identified in wound healing, cancer metastasis and vascular disease.

In the brain, MMPs are known to degrade the basal lamina around cerebral capillaries, increasing capillary permeability and contributing to cerebral oedema[5]. In skeletal muscle ischaemia/reperfusion, there are also multiple changes in the microvasculature, vascular permeability and transcapillary filtration, with MMPs as one of the possible mediators of this skeletal muscle ischaemia/reperfusion damage. Type IV collagen is one of the major components of the basement membrane of all tissues and its destruction in ischaemia/reperfusion injury contributes to oedema and tissue damage. MMP-2 and MMP-9 act to degrade type IV collagen. To date, no data has been presented to elucidate the potential role of MMPs in skeletal muscle reperfusion injury, which constitutes the first phase of this study.

This chapter will also review the known pathophysiology of MMPs. Both the natural and exogenous inhibitors of matrix metalloproteinases will be discussed as they have relevance to the research performed in this work.

1.2 Ischaemia

Ischaemia is defined as inadequate blood supply to a tissue such that the oxygen supply is less than the oxygen demand required for function. The majority of studies of ischaemia have been performed on the myocardium. Tolerance to ischaemia varies between tissues, with the lower extremity able to be salvaged up to five to six hours after an acute arterial occlusion[6]. The repartition of blood flow to the ischaemic limb varies, with 71% supplying the skeletal muscle, 15% to the bone, 7% to the skin and the remainder to the tendons, periosteum and nerves[7]. Within the tissue of the limbs it is the skeletal muscle itself, which is the most sensitive to ischaemia[8].

At the cellular level, the mitochondrial synthesis of adenosine triphosphate (ATP) ceases during ischaemia and it is catabolised into adenosine diphosphate (ADP), adenosine monophosphate (AMP) and inosine monophosphate and then further into adenosine, inosine, hypoxanthine and xanthine[9]. There is transient persistence of anaerobic glycolysis to produce ATP and lactate from glycogen. The energy loss and direct membrane damage deranges cell membrane ionic pump function and the transmembrane ionic gradients are lost. Consequently, cytosolic sodium content rises, drawing with it a volume of water to maintain osmotic equilibrium with the surrounding interstitial space and potassium ions escape from the cell into the interstitium. Calcium is released from the mitochondria into the cytoplasm, activating cytosolic proteases, which convert xanthine dehydrogenase to xanthine oxidase by proteolysis. This conversion occurs mainly during the reperfusion phase of tissue damage. Phospholipases are also activated, resulting in deesterification of membrane lipids with increased concentration of free polyunsaturated fatty acids. There is progressive cellular oedema and lysosomal changes.

Irreversible ischaemic changes in skeletal muscle occur after 4 to 6 hours of warm ischaemia[10]. In order for the ischaemic damage process to be reversed, reperfusion must be established.

1.3 Reperfusion injury

Reperfusion injury results from the cascade of events that leads to additional cellular injury when blood flow is restored to a previously ischaemic area. Clinically, this can result in a number of systemic problems characterised by metabolic acidosis, hyperkalaemia, myoglobinaemia, myoglobinuria and renal failure.

After restoration of blood flow to an ischaemic limb, the acidic blood enters the general circulation causing metabolic acidosis. The pH of the venous effluent from a reperfused ischaemic limb is usually less than 7.2 and it may take 15 min or longer to return to normal[11]. The leakage of intracellular potassium leads to hyperkalaemia, which in its severe form, may lead to sudden death of the patient upon reperfusion of a limb[12]. Ischaemic muscle cells undergo rhabdomyolysis releasing creatinine phosphokinase, lactic acid dehydrogenase and glutamic-oxaloacetic transaminase and myoglobin. The myoglobin precipitates in the renal tubules inducing renal failure, thereby further confounding the hyperkalaemia.

Harman, who performed experiments using Bromophenol Blue injection into ischaemic muscles, first described reperfusion in vitro in 1948[13]. Haimovici described the first two clinical cases of skeletal muscle reperfusion injury presenting with severe limb ischaemia, with reperfusion leading to myoglobinuria in 1960[14]. Since that time, multiple studies have been performed aimed at delineating the complex pathophysiological events that occur during reperfusion.

Reperfusion injury is marked by both gross and microscopic changes. Macroscopically, reperfusion injury in skeletal muscle varies from limb oedema to full limb necrosis. The pain may increase in intensity, in spite of revascularisation, due to lack of complete reperfusion of the tissues. In the remote organs, the reperfusion injury can cause symptoms from mild impairment to arrhythmias, cardiac failure, renal failure, non-cardiogenic pulmonary oedema and finally multi-organ failure. There can be marked metabolic changes in the pH, partial oxygen pressure, potassium, liver enzymes and serum creatinine phosphokinase of the patient. These changes are severe in at least 7.5% of cases[15]. Microscopically, there is increased microvascular permeability to macromolecules[16], increased leukocyte adherence to post-capillary venular endothelium resulting in the "no reflow phenomenon". No reflow phenomenon results from occlusion of a large proportion of postcapillary venules by activated white cells, leading to a failure of microvascular perfusion.

There is a time dependent spectrum of severity of reperfusion injury and up to three hours of complete ischaemia in skeletal muscle can be completely reversible, while at four hours there is necrosis of 30% of muscle cells, increasing serially to up to 100% necrosis after five hours of complete warm ischaemia[17]. After up to three hours of ischaemia, ATP can be restored by oxidative rephosphorylation due to a normal functioning of mitochondria and the availability of suitable substrates[18].

No single process can be identified as the critical event leading to tissue injury in ischaemia/reperfusion. It is a multifactorial physiological and pathological process.

1.3.1 Effects of Skeletal Muscle Ischaemia/Reperfusion Injury on Remote Organs

Following ischaemia/reperfusion, systemic release of various mediators may lead to injury at remote sites such as the kidneys, heart, lung and gastrointestinal tract. The myoglobinuria, hyperkalaemia and metabolic acidosis associated with ischaemia/reperfusion were recognized in 1959[14]. A variety of other mediators such as oxygen free radicals, eicosanoids and neutrophil activation have now been recognized as causing remote organ damage. The remote effects of ischaemia/reperfusion are important, as the clinical syndromes that occur such as pulmonary hypertension, myocardial ischaemia and renal failure can be the main events leading to high morbidity and mortality from the ischaemic limb[19].

1.3.1.1 Cardiac effects of Skeletal Muscle Ischaemia/Reperfusion Injury

Skeletal muscle reperfusion can lead to multiple deleterious clinical myocardial events such as arrhythmias, myocardial ischaemia and infarction and cardiac arrest. Aortic cross clamping resulting in lower limb and intestinal ischaemia causes systemic effects such as myocardial ischaemia, low cardiac output states and cardiac death[20], with the likely mediators including complement, thromboxanes, leukotriene D_4 and platelet activating factor[21]. Thromboxane A_2 was originally suggested as the major cause of myocardial depression, although it is now suggested that it induces formation of a specific myocardial depressant factor rather than having a direct effect[22]. Myocardial depressant factor is a short acting peptide synthesized by pancreatic acinar cells in response to splanchnic hypoperfusion following

ischaemia/reperfusion. It exerts its effect by reduction in myocardial calcium and magnesium ATPase activity[23]. Myocardial depressant factor is negatively inotropic, reduces cardiac output and has a profound vasoconstrictive effect, particularly on the splanchnic circulation[21].

1.3.1.2 Lung effects of Skeletal Muscle Ischaemia/Reperfusion Injury

Reperfusion following skeletal muscle ischaemia in humans leads to respiratory failure manifested by pulmonary hypertension, hypoxaemia and noncardiogenic pulmonary oedema[24], known clinically as Adult Respiratory Distress Syndrome (ARDS). The mortality rate in previously healthy individuals developing ARDS remains in excess of 65% and survivors are frequently left with severe pulmonary sequelae[25].

The mechanism of lung injury involves activation of circulating neutrophils, which adhere to pulmonary vascular endothelium and release arachidonic acid products, oxygen free radicals and proteases leading to increased microvascular permeability. The circulating neutrophils are activated directly by metabolites released from the ischaemic reperfused tissue and trapped in the pulmonary circulation[26]. Due to the increased size of activated neutrophils, their delayed passage thought the pulmonary circulation leads to neutrophil-endothelial binding and neutrophil mediated injury. Interleukin–8, thought to be the main mediator of neutrophil activation in ARDS[27] is synthesised by alveolar macrophages and pulmonary endothelial cells and released into the systemic circulation[28]. Post mortem histology shows significant interstitial capillary congestion and polymorphonuclear infiltration suggestive of an early pneumonitis after ischaemia/reperfusion injury[19].

The pulmonary hypertension is related to an increase in thromboxane B_2, leukotriene production, complement activation[29] and the direct toxic effects of oxygen free radicals on the endothelium[30].

The clinical pulmonary oedema that occurs following ischaemia/reperfusion injury is due to increased microvascular permeability and the rapid passage of protein-rich fluids across the endothelial barrier[31]. Certainly after bilateral hind-limb ischaemia, there is evidence histologically of proteinaceous exudates in the lung interstitium and within alveolar spaces[32,33]. Increased lung permeability has also been shown by studying lung lymph flow following ischaemia/reperfusion in sheep, with the lung lymph flow doubling and lymph

protein clearance rising significantly[34]. Complement activation mediates some of the increased permeability and pretreatment with sCR1 complement receptor blocker attenuated the increased lung permeability [35]. Thromboxane elevation in ischaemia/reperfusion also leads to increases in local microvascular permeability in a hindlimb ischaemia/reperfusion model in the dog[36].

1.3.1.3 Gastrointestinal effects of Skeletal Muscle Ischaemia/Reperfusion Injury

Lower limb ischaemia/reperfusion disrupts intestinal mucosal tight junctions leading to endotoxaemia[37]. The mechanism involved in gut injury following lower limb ischaemia/reperfusion principally affects the more active crypt cells, predominantly in the small bowel altering structure and permeability[19,38]. It is possible that the mediators generated in the perfused limb, such as oxygen free radicals and thromboxane A_2, may mediate gut injury by the activation of mucosal mast cells[36,39,40].

1.3.1.4 Renal effects of Skeletal Muscle Ischaemia/Reperfusion Injury

The pathogenesis of renal failure in the setting of skeletal muscle ischaemia/reperfusion injury is not completely understood[41]. Renal failure is more likely in the elderly patients with preexisting atherosclerotic kidney damage, but does not necessarily occur in all patients. Clinically, ischaemia/reperfusion can lead to myoglobinuric renal tubular necrosis and evolve to acute renal insufficiency, manifested from severe oliguria to anuria.

The renal injury that occurs during skeletal muscle ischaemia/reperfusion injury is multifactorial. The myoglobin release resulting from rhabdomyolysis deposits myoglobin in the renal tubules, leading to decreased renal clearance and renal failure[11]. Other products of decomposition such as ferrihemate, uric acid crystals and vasoconstrictive mediators also contribute to renal failure[42,43]. The iron component of myoglobin may play a role by stimulating the formation of hydroxyl radicals, ultimately damaging proximal tubular cell membranes because of lipid peroxidation[41]. Experimentally desferrioxamine, an iron chelator, decreases the renal concentration of malonedialdehyde, a by-product of lipid peroxidation and may improve renal function[8].

Neutrophils and LTB_4 also play a role in ischaemia induced thromboxane synthesis and mediate ischaemic renal injury[44]. Inadequate correction of prerenal dehydration can contribute to renal failure in skeletal muscle ischaemia/reperfusion injury by activating the secretion of constrictor hormones (angiotensin II, catecholamines, vasopressin and intra-renal thromboxane).

1.3.2 Pathophysiology of Reperfusion Injury in Skeletal Muscle

There is a large variety of interlinking pathophysiological processes that contribute to the phenomenon of reperfusion. Initially, these processes were thought to involve release of acid metabolites, potassium and creatinine phosphokinase into the systemic circulation upon establishment of reperfusion[45]. It is now known to be more complex, with oxygen free radical production[46], lipid peroxidation of cell membranes[47,48], raised intracellular calcium levels[49,50], increased microvascular permeability[51], cytokine production[52], complement activation and deposition[35] and the no reflow phenomenon.

These processes will be discussed in further detail in this review.

1.3.2.1 Increased Microvascular Permeability

Reperfusion of ischaemic skeletal muscle leads to an increase in microvascular permeability[53-57]. The increased microvascular permeability that occurs in ischaemia/reperfusion leads to marked oedema and compartment syndromes in the affected limb and in remote organs, leads to pulmonary oedema with respiratory failure and renal oedema manifested by proteinuria.

The magnitude of the changes that occur during reperfusion depend in part, on the length of the ischaemia. The time course for the increases in microvascular protein permeability is rapid with major changes occurring within the first thirty minutes of reperfusion[58,59]. The increased presence of oedema persists for at least 48 hours [60-62]. The initial increases in extravascular albumin and water during reperfusion are due to an increase in microvascular protein permeability. After the return of normal protein permeability, the excess extravascular albumin continues to be oedematogenic[16]. Resolution of the interstitial oedema occurs only after the lymphatics clear the excess extravascular albumin, suggesting that it is the slow lymphatic removal that is responsible for prolonged oedema formation during reperfusion[16]. During ischaemia, the endothelial accumulation of intracellular calcium and release of platelet–activating factor may increase protein permeability. At the start of reperfusion,

xanthine oxidase-derived oxidants[30] from endothelial cells and complement activation[29] may increase protein permeability. Indirectly, the local release of leukotriene B_4 and interleukin-8 attracts and activates neutrophils, which release additional oxidants to increase protein permeability[16]. Thromboxanes cause a marked increase in vascular permeability by disassembling actin microfilaments and widening inter-endothelial tight junctions[36].

1.3.2.2 Reactive Oxygen Species

Reactive oxygen species indicates any compound derived from molecular oxygen that has acquired less than four electrons. These include, the superoxide radical, hydrogen peroxide and the hydroxyl radical. An oxygen free radical (a subset of reactive oxygen species) is defined as a molecule or atom that contains one or more unpaired electrons in its outer orbital. These include superoxide radical but not hydrogen peroxide. However hydrogen peroxide behaves like a free radical. Other biologically important reactive oxygen species in reperfusion injury include lipid hydro peroxide, lipid peroxyl radical, lipid alkoxyl radical, nitric oxide and thiyl radical.

The reactivity of superoxide radicals with other molecules by itself is low (Equation 1: Non-iron catalysed Haber-Weiss reaction) but in the presence of trace amounts of a transition metal, they are converted to the hydroxyl radical rapidly via the Fenton reaction (Equation 2: Iron Catalysed Haber-Weiss (Fenton) Reaction)[63].

Equation 1: Non-iron catalysed Haber-Weiss reaction

$O_2^{\bullet -}$ refers to superoxide radical. OH^- refers to hydroxyl radical Fe^{2+} is ferrous cation, Fe^{3+} refers to ferric cation, H_2O_2 refers to hydrogen peroxide.

$O_2^{\bullet -} + H_2O \Rightarrow OH^{\bullet} + OH^- + O_2$ (non iron-catalysed Haber-Weiss reaction).

Equation 2: Iron Catalysed Haber-Weiss (Fenton) Reaction

$H_2O_2 + Fe^{2+} \Rightarrow OH^{\bullet} + OH^- + Fe^{3+}$ (Iron-catalysed Haber-Weiss (Fenton) reaction).

Equation 3:

$2O_2^{\bullet -} + 2H^+ \Rightarrow H_2O_2 + O_2$ (dismutation catalysed by superoxide dismutase).

Equation 4:

$2H_2O_2 \Rightarrow 2H_2O + O_2$ (breakdown of hydrogen peroxide by catalase).

Physiologically, superoxide dismutase breaks down superoxide radicals into hydrogen peroxide and oxygen (Equation 3). Hydrogen peroxide is then further broken down to water and oxygen by catalase (Equation 4).

Oxygen free radicals are produced from at least five sources; xanthine oxidase system, from activated neutrophils, from the mitochondrial electron transport chain, arachidonic acid metabolism and from auto-oxidation of catecholamines.

During ischaemia, ATP is degraded to ADP, then AMP, adenosine, inosine and finally, hypoxanthine. Xanthine dehydrogenase converts hypoxanthine to xanthine and then to uric acid. The xanthine dehydrogenase enzyme is converted under ischaemic conditions to xanthine oxidase. The xanthine oxidase also converts hypoxanthine and xanthine to uric acid but uses oxygen instead of nicotinamide adenine dinucleotide (NAD) as the electron acceptor, thereby forming superoxide radicals. As illustrated in the equations above (Equation 1-4), the superoxide goes on to form hydroxyl radicals and hydrogen peroxide. Superoxide free radicals are found within ischaemic muscle and in the venous effluent of reperfused limbs. This is supported by the findings of a salvage effect with xanthine oxidase inhibition by allopurinol and superoxide radical removal by superoxide dismutase and catalase[64-66]. The rate of superoxide production varies between tissues, with shorter periods of ischaemia in intestine and liver producing the conversion of xanthine dehydrogenase to xanthine oxidase; however much longer periods are required in skeletal muscle[67-69]. However, two hours of ischaemia is associated with marked increases in muscle xanthine oxidase activity[68,69]. The xanthine oxidase is located within the sarcolemma and mitochondria of aerobic muscle fibres[70] and in capillary endothelial cells [71,72]. The localization of xanthine oxidase in capillary endothelial cells implies that these cells are susceptible to oxidant-mediated reperfusion injury[71]. Damage to the capillary endothelial cells may act as a stimulus to attract and activate inflammatory cells leading to further tissue damage[73].

Activated neutrophils are another source of oxygen free radicals via the enzyme membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Whilst oxidizing

NADPH to NADP+, NADPH oxidase reduces molecular oxygen to form the superoxide anion. In addition, myeloperoxidase, stored in the azurophilic granules of neutrophils can convert hydrogen peroxide to the toxic hypochlorous acid[74].

Other potential sources of superoxide radicals include production of superoxide radicals due to leakage of electrons from the electrons transport system within mitochondria[75,76] and from the cyclo-oxygenase pathway of arachidonic acid metabolism[76-79].

Membrane lipids are, in general, the first target for attacks by reactive oxygen species (lipid peroxidation) as reactive oxygen species are generated in association with membranes[63]. One of the most important actions of oxygen free radicals is to activate the endothelial cell through the transcription factor NFκB[80]. Once activated, the endothelial cell produces E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), endothelial-leukocyte adhesion molecule (ELAM-1) plasminogen activator inhibitor-1 (PAI-1), tissue factor, interleukin-8 (IL-8) and I kappa B-alpha[81]. The ICAM-1 and ELAM-1 molecules interact with complement receptors on the neutrophil surface and allow infiltration of neutrophils through the endothelium, releasing further reactive oxygen species.

1.3.2.3 Lipid Peroxidation

Oxygen free radicals can initiate reactions of lipid peroxidation by subtracting hydrogen ions from unsaturated fatty acids side chains, leading to formation of carbon-centred lipid radicals. The double bond arrangement within these unstable intermediates leads to conjugated dienes, which may react with molecular oxygen leading to peroxyl radicals. These then remove hydrogen atoms from nearby fatty acids, therefore propagating the chain reaction of oxidative membrane injury. Unless attenuated by antioxidants, lipid peroxidation leads to decreased membrane fluidity, disruption of membrane-bound enzyme and transport systems and alteration in cell permeability and cell death[82].

1.3.2.4 Nitric Oxide (Endothelium Derived Relaxing Factor)

Nitric Oxide is a signalling molecule that is synthesised from L-Arginine By Nitric Oxide Synthase. Nitric Oxide is capable of dual functions: as an anti-oxidant in some cases, but in others it may have deleterious effects particularly if it combines with superoxide to form the highly active peroxynitrite radical, which is a potent promoter of lipid peroxidation. Many studies have examined the role of nitric oxide in ischaemia/reperfusion injury of cerebral,

myocardial, mesenteric, gastric and lung tissues[83-85]. The results are paradoxical and arriving at a consensus is difficult. In skeletal muscle, nitric oxide production during reperfusion injury may be deleterious to survival of muscle tissue[86]. A study of rat hindlimb ischaemia suggested that the endothelium derived nitric oxide plays an important role in the maintenance of vascular tone and a reduction in nitric oxide release during reperfusion may predispose vessels to vasoconstriction[87]. Constitutive nitric oxide release is impaired in rat ischaemia/reperfused heart muscle because of endothelial dysfunction and can be ameliorated with L-arginine treatment[88,89]. Nitric oxide can reverse the vessel spasm known to occur during reperfusion[90]. Nitric oxide reduces clotting by inhibiting platelet aggregation and adhesion[91] as well as by inhibiting the adhesion of neutrophils and monocytes[92]. Nitric oxide may increase the resistance of skeletal muscle to fatigue after ischaemia/reperfusion[88]. The post ischaemic inhibition of endothelial mediated vasodilatation may be due to inactivation of nitric oxide by superoxide radical[93]. In summary, the role of nitric oxide in skeletal muscle ischaemia/reperfusion injury remains controversial with evidence for both beneficial and deleterious effects.

1.3.2.5 No-reflow Phenomenon

Reperfusion of ischaemic skeletal muscle is associated with the development of no-reflow phenomenon, where, upon reinstitution of blood flow, a large proportion of the capillaries fail to reperfuse. The cause of no-reflow phenomenon has not been identified but several mechanisms have been proposed, including plugging of the post-capillary venules by leukocytes[94-96] and swelling of the endothelial cells[97-99]. Activated neutrophils are significant contributors to the development of postischaemic capillary no-reflow[100-102] and neutrophil depletion virtually abolishes no reflow in reperfused myocardium, brain and skeletal muscle[101,102]. Partial occlusion of the venular lumen by adherent leukocyte may also alter the haemodynamics within the capillary contributing to no-reflow[103]. Neutrophil/endothelial cell adhesive interactions are required for the development of capillary no-reflow in postischaemic muscle[104]. It has been suggested that microvascular thrombus formation may contribute to the capillary perfusion deficit[105], however intravital studies suggest that this is not the case since microvessel thrombosis is rarely observed[106].

An overall mechanism for no-reflow phenomenon has been proposed by Gute et al[104] .The leukocytes induces microvascular barrier disruption, by adhering to the post capillary venular endothelium in ischaemia/reperfusion injury. The neutrophil emigrates into the tissue spaces and microvascular permeability is increased, leading to enhancement of the transcapillary

fluid filtration rate and consequentially an accumulation of excessive interstitial volume. The rate of oedema formation may be further enhanced by an increase in capillary pressure that occurs as a consequence of the physical reduction in the diameter of post capillary venules induced by leukocytes adhering to the endothelium in these vessels[107]. Many muscles cannot expand due to their surrounding fascial sheaths, hence when ischaemia/reperfusion is induced, neutrophil mediated oedema formation can be accompanied by a significant rise in interstitial pressure, sufficient to produce significant extravascular compression. Since the diameter and intravascular pressure in capillaries is less than in other vessel segments, the capillaries (and venules) are the first to exhibit no-reflow[104].

1.3.2.6 Neutrophils

Neutrophils have a predominant role in ischaemia/reperfusion injury, both in local effects and the effects in remote organs. Both local and remote damage are associated with neutrophil accumulation in the microvasculature[33,108], Depletion of neutrophils effectively prevents increased permeability and oedema formation[109,110]. The interactions between neutrophils and endothelium are a prerequisite for the microvascular injury induced by ischaemia/reperfusion[111].

The initial phase of neutrophil–endothelial interaction is rolling of neutrophils along the endothelial cells and this is mediated by primarily by selectins. Both the integrin family and the immunoglobulin supergene family of adhesion molecules mediate the next phase of adherence and emigration. Following reperfusion, there is enhanced neutrophil adhesion to the endothelium, when compared to the level that occurs during ischaemia [112]. The neutrophil adhesion molecules are members of the CD11/CD18 membrane glycoprotein complex and on the endothelium; ELAM-1 and ICAM-1 play a role in the adhesion. Monoclonal antibodies, which bind specifically to the CD11/CD18 complex on circulating neutrophils, have been shown to prevent the increased microvascular permeability and vascular resistance that follow reperfusion[101]. The adherence of neutrophils to the microvascular endothelium in postischaemic skeletal muscle and other tissues has been demonstrated utilizing the technique of intravital microscopy[113,114].

The production of superoxide by the endothelial cells mediates leukocyte adhesion but the mechanism is not clear[115]. Endothelial cells exposed to anoxia-reoxygenation release a soluble factor that results in the expression and/or activation of CD11b/CD18 on neutrophils[115]. The

adhesiveness of CD11a/CD18 on neutrophils is activated by the tripeptide f-Met-Leu-Phe (fMLP) and interleukin–8[116].

Activated neutrophils, those expressing CD11/CD18 integrins, adhere to and migrate across the endothelium and cause local destruction by releasing free radicals, proteolytic enzymes (collagenase, elastase, cathepsin G) and peroxidase[117,118]. Sequestration of activated neutrophils in the lungs and other organs is a vital step in the development of multi-system organ failure in ischaemia/reperfusion injury[119].

Selectins

Selectins are a family of adhesion molecules with a distinct lectin domain binding to acidic carbohydrate structures in a Ca^{2+}-dependent fashion. The three main selectins play an integral role in initiating leukocyte rolling along the endothelium and hence play a role in reperfusion injury.

L-selectin is expressed constitutively on the surface of neutrophils when in their quiescent state. It is also known as CD62L, LAM-1, LECAM-1, Leu-8, MEL-14 and TQ-1. L-selectin is present on the outer plasma membrane of microvillar projections of leukocytes[120] and initiates the reversible attachment of flowing leukocytes to endothelial cells[121], endothelium-bound leukocytes[122] and immobilised platelets[123], thereby directing neutrophils into areas of acute inflammation[124] and lymphocytes into sites of chronic inflammation and secondary lymphoid organs. Most peripheral blood B cells, T cells, monocytes, granulocytes and some NK cells express L-selectin, as do some spleen lymphocytes, bone marrow lymphocytes, bone marrow myeloid cells and thymocytes. L-selectin can be shed from the cell surfaces upon their activation[125]. Blocking L-selectin with polyclonal[126] and monoclonal antibodies[127] impairs the ability of neutrophils to roll along cultured endothelial cell monolayers and along the endothelial surface of mesenteric venules, indicating that L-selectin-mediated interactions are essential to leukocyte rolling. L-selectin is rapidly shed from the cell surface following leukocyte activation[128], down regulating the cell surface molecule, allowing attached neutrophils to detach from the endothelial surface and extravasate into the extravascular space.

P-selectin is stored in the α-granules of platelets and the Wiebel-Palade bodies of endothelial cells. After platelet or endothelial cell activation, P-selectin is rapidly translocated to and expressed on the cell surfaces, under the influence of thrombin, histamine, hydrogen peroxide

and inhibitors of nitric oxide synthase[129]. The translocation process peaks in 10-20 minutes. P-selectin expression may also be induced by exposure of the vascular endothelium to oxygen-derived free radicals[130], the complement complex C5b-9[131] and tumour necrosis factor-α[132]. Histamine, which is normally stored in mast cell granules, can mobilize P-selectin to the endothelial cell surface[133]. Mast cells are also a major source of platelet activating factor. Based on these observations, it appears likely that mast cells also contribute to reperfusion-induced leukocyte endothelial cell adhesive interactions[115,134].

E-selectin is expressed on acutely activated endothelium. It was described initially as "endothelial-leukocyte adhesion molecule-1 (ELAM-1)"[135] but has also been described as CD62E, or LECAM-1. E-selectin must first be synthesised in the endothelial cell before it can be expressed. E-selectin transcription is partly stimulated by proinflammatory mediators such as interleukin-1 and tumour necrosis factor α; therefore expression of E-selectin occurs typically later than that of P-selectin. Focal expression of E-selectin at sites of endothelial activation may promote neutrophil adhesion and emigration[136]. In vitro studies using IL-1 as inflammatory stimulus, show peak expression of E-selectin at four hours[135]. The rapid induction and high turnover rates of E-selectin mRNA and protein are consistent with the transient nature of the neutrophil influx during acute inflammation. In an in vivo ischaemia-reperfusion model in rat kidney, E-selectin mRNA was detected after four hours of reperfusion but to a much lesser extent than after 16 hours of reperfusion[137].

β_2 integrins

The integrins comprise a vast number of cell surface adhesion molecules that mediate intercellular recognition and cellular binding to the extracellular matrix. The neutrophil β_2-integrin adhesion glycoprotein complex consists of a common polypeptide chain, CD18, which is noncovalently linked to three different α-polypeptide chains (CD11a, CD11b, CD11c). CD11a/CD18 is expressed on all leukocytes and mediates the attachment of unstimulated neutrophils to the vascular endothelium through a specific interaction with intercellular adhesion molecule-1 (ICAM-1) and ICAM-2[138]. The CD11b/CD18 and CD11c/CD18 adherence glycoproteins are stored in granules and can be rapidly mobilized to the surface of neutrophils by fusion of granule membranes with the cell membrane on stimulation[139]. The chemotactic agents (CD5a, LTb4 and PAF), cytokines (IL-1, TNF-α) and oxidants superoxide, hydrogen peroxide that have been implicated in the pathogenesis of ischaemia/reperfusion injury, are known to induce neutrophil adherence by CD11/CD18-

dependent mechanisms[140-144]. CD11b/18 interacts with ICAM-1 on the surface of the endothelial cell and both of these molecules are required for the development of lung injury following lower limb ischaemia/reperfusion injury[145]. Inhibition of CD18-mediated leukocyte adhesion using monoclonal antibodies has a protective effect in reperfusion injury[146].

Immunoglobulin Superfamily

The immunoglobin superfamily contains a large number of molecules with multiple IgG-like domains. Five members are involved in leukocyte-endothelial cell interactions: ICAM-1, ICAM-2, ICAM-3, vascular cell adhesion molecule (VCAM-1) and platelet-endothelial cell adhesion molecule (PECAM-1). ICAM-1 is basally expressed on endothelial cells, but its expression is markedly enhanced by endothelial exposure to cytokines or lipopolysaccharide[147]. ICAM-2 is also basally expressed on the surface of endothelial cells, but expression is not increased by cytokine activation. PECAM-1 is expressed constitutively on platelets, most leukocytes and endothelial cells. It has the ability to activate β_1 and β_2 integrins[148]. Immunoneutralization of ICAM-1 attenuates ischaemia/reperfusion induced neutrophil adherence in the mesentery[149,150] and liver[151], reduces post ischaemic pulmonary neutrophil sequestration and oedema[152] and protects kidneys against ischaemia/reperfusion damage[153].

1.3.2.7 Eicosanoids

In reperfusion, oxygen free radicals initiate lipid peroxidation of cell membranes and release arachidonic acid. Eicosanoids are derivatives of arachidonic acid, consisting of three groups; prostaglandins, thromboxanes and leukotrienes. They are metabolised by spontaneous hydrolysis or enzymatic conversion to inactive metabolites in liver and lungs. As eicosanoids are oxygenation products and require molecular oxygen for their synthesis, they are only produced in the reperfusion phase of ischaemia/reperfusion.

Prostaglandins

Prostaglandins are synthesised from arachidonic acid via the cyclo-oxygenase pathway, with the most important being prostacyclin. It is mainly of endothelial origin and has a protective vasodilatory effect in ischaemia/reperfusion[154]. However, it is rapidly depleted in ischaemia/reperfusion injury leading to vasoconstriction with a reduction in local blood flow causing exacerbation of the ischaemia[155]. Prostacyclin analogues have been investigated in the

treatment of ischaemia/reperfusion injury, producing amelioration of the level of metabolic and tissue damage[156-159].

Thromboxanes

Thromboxanes are also derived from arachidonic acid via the cyclo-oxygenase pathway. Thromboxane A_2 is synthesised by neutrophils and promotes vasoconstriction and platelet aggregation. Following release of limb tourniquet, the plasma level of thromboxane A_2 and thromboxane B_2 increase within 10 minutes[160]. This elevation coincides with a rapid rise in pulmonary artery pressure[161] and after 3-6 hours, an increase in microvascular permeability[34,160]. Thromboxane causes disassembly of actin microfilaments, which regulate endothelial cell motility[162] enhancing neutrophil diapedesis through the endothelial cell layer[163]. Anner also showed that thromboxane B_2 induced by ischaemia leads to leukosequestration of polymorphonuclear cells in the lungs[33]. The sequestered, activated neutrophils release oxygen free radicals and eicosanoids leading to further increases in permeability, neutrophil attraction and activation with resultant further tissue damage. The use of thromboxane A_2 receptor antagonists increase muscle blood flow and preserve organ and muscle viability[164-167].

Leukotrienes

The cellular membrane arachidonic acid is converted in myeloid cells via 5-Lipoxygenase to 5-hydroperoxyeicosatetranoic acid (5-HPETE) and then to leukotrienes. Four of the leukotrienes are aminolipids that contain amino acids; leukotriene C_4 (LTC_4), LTD_4, LTE_4 and LTF_4. Activated neutrophils are the major source of leukotrienes, which lead to local injury, both directly by their action on smooth muscle and endothelium and by their effects on neutrophils. Leukotrienes C_4, D_4 and E_4 act on smooth muscle, causing vasoconstriction and act on the endothelial cell resulting in a change in cytoskeletal structure, leading to an increase in size of intracellular junctions and to an increase in vascular permeability[168-170]. Leukotriene B_4 released by activated luminal and extravascular neutrophils leads to further neutrophil accumulation[171]. The lung produces leukotrienes following remote ischaemia/reperfusion injury[172] with direct effects on pulmonary microvessels, increasing permeability, leading to transient pulmonary hypertension[173] and also inducing the pulmonary endothelium to produce thromboxane, resulting in additional vasoconstriction [174]. Inhibitors of leukotrienes reduce neutrophil infiltration[175] and mucosal permeability[176]. 12-HPETE released from both platelets and leukocytes and leukocyte derived-15-HPETE penetrate the

endothelial cell membrane, upon which they are further metabolized producing superoxide radicals in the endothelial cells[115]. The superoxide then induces lipid peroxidation and subsequently causes lysis of endothelial cells.

1.3.2.8 Intracellular Calcium Ion

Intracellular free calcium levels have been shown to increase significantly during ischaemia/reperfusion in a variety of organs[177-180]. The increase in intracellular calcium may activate a number of metabolic pathways, all potentially resulting in cell damage[181-184]. It has been observed that lowering the extracellular calcium produces dramatic protection against lethal cell injury and decreased superoxide radical production during reoxygenation[185,186]. A number of calcium antagonists have shown to protect the tissue against ischaemic injury[181,187-190]. However, the role of calcium ions in ischaemia/reperfusion injury is still debated[191], with some investigators finding a significant increase in intracellular calcium ion during the ischaemic period in myocardium[192,193] and others showing that in skeletal muscle, concentration of intracellular calcium ion either does not change, or does so only moderately during the ischaemic period and then steeply increases during reperfusion[179,194]. Ivanics et al showed that the calcium changes in skeletal muscle during ischaemia depend on the type of muscle, with intracellular calcium increases occurring mainly in slow-twitch muscle fibres[191]. In addition, a more pronounced elevation of calcium ion was observed in experiments with partial recovery of flow as compared to the ones showing no-reflow[191]. The source of the calcium increase in the cytoplasm during both ischaemia and reperfusion is proposed to be either sarcoplasmic reticulum[188] or extracellular calcium[49].

1.3.2.9 Complement

The complement system was originally described after the discovery of antibodies in the late 19th century and was believed to supplement the specific antibody effects that lead to cell lysis. Both in vivo and in vitro studies have shown that the complement system plays an important role in the pathogenesis of tissue damage in ischaemia/reperfusion injury[195-197]. Accumulation of neutrophils and complement activation occur in skeletal muscle ischaemia/reperfusion injury[198,199]. Rubin et al demonstrated that reperfusion was associated with systemic factor B depletion, indicative of alternative complement pathway activation[199]. Hepatic injury following bilateral hind limb ischaemia occurs due to Kupffer cell stimulation via complement dependent mechanisms[200]. Inhibition of the complement cascade improves the initial blood flow and decreases muscle necrosis and injury after prolonged reperfusion in

dogs[198]. Complement blockade also prevents leukocyte adhesion[201], leading to better capillary perfusion and muscle cell viability[201] and attenuates the increase in permeability index[35,202,203]. In humans, there is a relationship between degree of complement activation and the development of organ dysfunction post-operatively after aortic cross clamping[29,204]. The complement membrane attack complex C5b-9 is deposited into the endothelial cell membrane after ischaemia/reperfusion, acting as a pore, leading to unchecked ion flux, resulting in secondary messenger signalling, enzyme activation and potential osmotic lysis[100,202,205]. There is an additive role of complement and neutrophils in mediating skeletal muscle ischaemia/reperfusion injury as observed with 71% reduction in permeability in neutropenic C5-deficient animals, which was greater than neutropenia or C5 deficiency alone[202].

1.3.2.10 Cytokines

Cytokines are a group of polypeptide or glycoprotein mediators of low molecular weight secreted by specific effector cells. They include the interleukins, tumour necrosis factor α, transforming growth factor β, chemokines and platelet activating factor. The first report in which cytokines were implicated in skeletal muscle ischaemia-reperfusion injury involved interleukin-1 (IL-1)[206].

Interleukin-1

Interleukin-1 (IL-1) is produced by tissue macrophages also by neutrophils and the vascular endothelium by the action of tumour necrosis factor α (TNF-α)[207,208]. IL-1 is a potent chemotactic agent and has been shown to be a neutrophil infiltration stimulus in hepatic ischaemia/reperfusion injury[209]. Both IL-1 and tumour necrosis factor-α increase the expression of ICAM-1 on the vascular endothelium[210]. Exposure of the endothelium to IL-1 and TNF-α induces synthesis of E-selectin, which then interacts with L-selectin on the neutrophil surface leading to rolling and initial neutrophil binding to the endothelial surface[211]. Further adhesion of the neutrophil to the endothelium requires further activation by interleukin–8 and platelet activating factor.

Tumour Necrosis Factor-α

Tumour Necrosis Factor-α (TNF-α) is a 17-kilodalton proinflammatory cytokine that produces significant cardiopulmonary dysfunction[212]. It is produced by activated macrophages and monocytes in pathologic conditions and produced by activated T cells, activated killer

cells and fibroblasts. TNF-α is a potent chemoattractant, drawing neutrophils to the site of injury. During the inflammatory response to injury, TNF-α is an early response cytokine[213] and causes expression of interleukins–1, -6 and –8. Upregulation of TNF-α has been seen in transient and prolonged cerebral ischaemia[214] and in skeletal muscle ischaemia/reperfusion[212,215]. TNF-α increases the neutrophil population in the lung by inducing endothelial derived neutrophil chemotactic and adherent factors[135,216] and potentiates the permeability changes in lungs in ischaemia/reperfusion injury[217]. Serum TNF-α increases rapidly during lower extremity ischaemia and causes increased production of nitric oxide from rat lungs by upregulating inducible nitric oxide synthase[218]. TNF-α induces generation of hydrogen peroxide, superoxide anion and other toxic metabolites as well as enhancing the susceptibility of the vascular endothelium to neutrophil mediated injury and inducing expression of ICAM-1[219]. Endothelial cells exposed to TNF-α produce other inflammatory mediators; including IL-1 and platelet activating factor[220]. Attenuation of increased vascular permeability after anti-TNF-α antibody treatment in animal models of sepsis suggests that TNF-α plays a causative role in muscle and lung injury[213,221-224]. Pretreatment with anti-TNF-α antibody significantly attenuated the extent of no-reflow after 2 hours of ischaemia[212], suggesting that TNF-α may play a causative role in the no-reflow phenomenon.

Platelet activating factor

The term platelet-activating factor (PAF) was originally used to describe a substance produced and released by IgE-stimulated basophils that is capable of causing platelet aggregation[225]. It is synthesised by monocytes/macrophages, polymorphonuclear neutrophils, eosinophils, basophils, platelets and endothelial cells in response to cell-specific stimuli. Thus, it is synthesised by most tissues including, lung, kidney, myocardium, brain, liver skin, retina, uterus and embryonic tissue[225,226]. It is a phospholipid that is synthesised from either cellular phospholipid via the remodeling pathway, which involves the inflammatory responses induced by PAF, or via the denovo pathway from alkyl acetyl glycerol, which maintains the resting state levels of PAF[227]. Most of the cells that produce PAF also possess PAF receptors[228]. PAF is a mediator of cell to cell communication, which functions as an intercellular or an intracellular messenger[229]. Platelet-activating factor synthesis is also stimulated by H_2O_2, thrombin, leukotriene C_4, leukotriene D_4, interleukin-1, histamine, bradykinin and adenosine 5'-triphosphate.

PAF has three major effects on the circulation; vasoconstriction[230], chemoattraction for leukocytes[140,231] and an increase in microvascular permeability[232]. PAF is produced by ischaemic skeletal muscles during reperfusion, with peak elevation 15 minutes after commencement of reperfusion[233]. PAF enhances the binding of neutrophils to endothelial cells[129]. A PAF-receptor antagonist (WEB-2086), blocks adhesion to endothelial cells during ischaemia/reperfusion[233,234]. PAF increases microvascular permeability in a dose dependent manner.[232,235,236]. PAF also leads to neutrophil chemotaxis, aggregation and degranulation[237]. Through its production of arachidonic acid, PAF induces elevated activity of prostaglandins, thromboxane A_2, superoxide and leukotrienes. PAF accelerate the inflammatory response by a more delayed production of peptide mediators, such as TNF-α and IL-1.

Interleukin-6

Interleukin-6 (IL-6) is a 19-26 kDa protein produced by monocytes, fibroblasts, keratinocytes and endothelial cells in response to IL-1 and TNF-α. IL-6 is produced during ischaemia[238] and during reperfusion, following aortic cross-clamping for aneurysm surgery[239]. IL-6 may prime and stimulate the oxidative burst (production of reactive oxygen species via NADPH mechanisms) in neutrophils, stimulate endothelial cell expression of ICAM-1 and increase endothelial permeability[240,241].

Interleukin-8

Interleukin 8 (IL-8) is a potent neutrophil chemotactic and activating factor, which is produced by mononuclear phagocytes, T cells, NK cells, fibroblasts, endothelial cells, eosinophils and neutrophils. The production is of IL-8 is stimulated by IL-1, TNF-α, endotoxin, histamine and viruses. IL-8 induces transendothelial neutrophil migration and up-regulation of integrin expression by generating a chemoattractant gradient of immobilized matrix bound Il-8[242,243]. Monoclonal antibodies directed against IL-8 reduce leukocyte accumulation and vascular injury in immune complex injury and in lung reperfusion injury[244,245].

1.3.2.11 Endothelin

Endothelin-1 is a 21 amino-acid vasoconstrictive peptide that is produced by endothelial cells. It constricts vascular smooth muscle cells by increasing intracellular calcium by an inositol triphosphate mechanism. Factors such as hypoxia, growth factors, angiotensin II and

noradrenaline stimulate production of endothelin-1. It has been found to be elevated in skeletal muscle following ischaemia and further increased by a factor of 4 following reperfusion[246]. On the capillary level, endothelin-1 mediates an intense and long lasting vasoconstriction contributing to the phenomenon of 'no-reflow'. In cardiac muscle, endothelin-1 has been shown to cause aggregation of leukocytes and to facilitate neutrophil-endothelial adhesion by stimulating the expression of adhesive molecules on the surface of the neutrophil, leading to endothelial swelling and thereby to increased capillary resistance[247]. Similarly, in renal ischaemia/reperfusion injury, endothelin-1 is a major promoter of early neutrophil accumulation Espinosa, 1996 #1578}. Endothelin-1 activates monocytes by an intracellular calcium ion mechanism and enhances the production of monokines, causing leukocytosis (IL-6), neutrophil activation, superoxide generation (IL-8) and local capillary leakage (PGE_2) with fluid sequestration[248]. In rats treated with an endothelin-1 inhibitor, there was increased muscle viability and functional capillary density, confirming the role of endothelin-1 in ischaemia/reperfusion injury by changes in post ischaemic microvascular perfusion[249,250].

1.3.3 Therapeutic regimes to ameliorate Ischaemia/Reperfusion Injury

A large number of distinct pathophysiological cascades are activated by ischaemia/reperfusion, including oxygen free radical production, complement activation, eicosanoid, cytokine and nitric oxide biosynthesis, altered calcium and phospholipid metabolism, leukocyte activation and endothelial dysfunction. By delineating the mechanisms that contribute to postischaemic microvascular dysfunction and muscle necrosis, therapeutic strategies can be developed. The importance of the development of therapeutic regimes for ischaemia/reperfusion cannot be over-emphasised due to the desire to decrease the morbidity and mortality of ischaemia/reperfusion injury. Multiple therapeutic agents have been used in vitro to attenuate the effects of reperfusion injury. Current therapies are generally directed against ischaemia/reperfusion once it has occurred.

1.3.3.1 Supportive Clinical Measures to decrease the effects of Ischaemia/Reperfusion

To prevent secondary muscle necrosis and nerve compression, early fasciotomy involving all the limb compartments is vital to restore limb functions after severe skeletal muscle ischaemia/reperfusion injury.

Fluid administration restores hypovolaemia that occurs due to the tissue oedema in the skeletal muscle and in remote organs. Mannitol, a hyperosmolar sugar alcohol, causes increased diuresis, which may be helpful in preventing renal tubule obstruction and the development of renal failure. Reperfusion oedema and injury as well as postischaemic compartment pressure in skeletal muscle have been reduced by mannitol[251-253]. Mannitol has been proposed to act as a free radical scavenger, specifically eliminating the highly toxic hydroxyl radicals[251,254,255]. The hyperosmolar property of mannitol appears to be the main mechanism by which postischaemic oedema is reduced[256]. The inability of mannitol to cross cell membranes is thought to increase serum osmolality, which leads to haemodilution and tissue dehydration[257]. When compared to dimethyl thiourea (a potent scavenger of hydrogen peroxide and superoxide radical), mannitol was inferior in reducing microvascular perfusion deficits and leukocyte-endothelial cell interactions and the incidence of cellular injury[258]. Hence, the protective effects of mannitol are largely due to its diuretic action, any nephrotoxic agents (such as ferrihemate and urate) are diluted out of partially obstructed tubules[8].

The hyperkalaemia that is produced after ischaemia/reperfusion injury can be treated with rectal ion-exchange resins, but when severe, requires haemodialysis to keep the potassium down to acceptable levels.

Inhibition of sodium-supported calcium entry would be expected to decrease the excess in intracellular calcium and to improve functional and metabolic recoveries. Amiloride, a potassium-sparing drug, decreases the intracellular sodium concentration and inhibits sodium-hydrogen and sodium-calcium exchange in many tissues[259]. It markedly improves contractile and metabolic recovery during postischaemic reperfusion and so protects against the calcium paradox[259].

1.3.3.2 Controlled Reperfusion

Some data indicate that an overall control of the conditions of the reperfusion injury could prevent the syndrome. Mixing of blood with substances such as citrate-phosphate-dextrose (to lower calcium content), THAM (buffer), glutamate and aspartate (substrate enrichment) and glucose during the actual restoration of perfusion in the operating theatre, has been tried under experimental conditions[8,260-262]. It is more difficult to apply in patients with crush syndrome compared to the embolectomy/thrombectomy situation due to technical operative reasons. It can also incorporate leukocyte depletion filters or other modifications of the initial reperfusate, however this remains an experimental technique. Others have shown that delayed restoration of in venous drainage, leads to a significantly greater local skeletal muscle injury and remote neutrophil-mediated lung injury[263].

1.3.3.3 Free Radical Scavengers and Inhibitors

Free radical scavengers are agents that interact with reactive oxygen species to render them harmless. Oxygen free radical scavengers include mannitol, dimethylurea, dimethylsulphoxide and superoxide dismutase. Circulating proteins that are capable of metabolizing superoxide include caeruloplasmin and extracellular superoxide dismutase[264,265]. However, the role of these circulating agents is unclear because their activity in plasma is low. Superoxide dismutase, a superoxide radical scavenging enzyme, has been shown to attenuate postischaemic microvascular and parenchymal cell dysfunction in a wide variety of tissues[179,266-269]. Catalase is a naturally occurring metalloprotein that catalyses the formation of water and oxygen from hydrogen peroxide and acts in combination with superoxide dismutase in vivo. Dimethylthiourea, dimethylsulphoxide and mercaptopropionyl glycine are all putative scavengers of the hydroxyl radical [270]. All of these agents have been shown to prevent microvascular barrier dysfunction during reperfusion[271].

Free radical production inhibitors include allopurinol and desferrioxamine. Allopurinol is a structural analogue of hypoxanthine and inhibits xanthine oxidase competitively reducing the production of the superoxide anion[272]. Desferrioxamine is a powerful iron-chelating agent. Iron is essential for the Haber-Weiss reaction (Equation 2 on page 10) and production of the hydroxyl radical. Chelation of iron interrupts this process and several studies have shown beneficial effects from the use of desferrioxamine during ischaemia/reperfusion[273-275].

1.3.3.4 Neutrophil Inhibition

Neutrophils play a vast role in the development of ischaemia/reperfusion injury and inhibition of free radical production by neutrophils, or prevention of chemoattraction and neutrophil adherence may modify reperfusion injury. Specific PAF antagonists and 5-lipo-oxygenase inhibitors[112] have inhibited chemoattraction of neutrophils. Monoclonal antibodies against the CD11-CD18 complex also inhibit neutrophil chemotaxis and adherence[110,276]. Transforming growth factor-β inhibits neutrophil adhesion to the endothelium[277]. Adenosine inhibits free radical production by activated neutrophils via a receptor-mediated mechanism[278].

1.3.3.5 Complement Activation

Inhibition of complement by administration of soluble complement receptor (sCR1) in a rat model of ischaemia/reperfusion blocked myocardial infarct size after 7 days, due to a decrease in generation of C5a[279]. In skeletal muscle ischaemia/reperfusion, complement inhibition has been shown to decrease the local and pulmonary albumin leak[35], again by using sCR1.

1.3.3.6 Antioxidants

Antioxidants are agents that interrupt peroxidation; they prevent tissue damage and the production of peroxides and further free radicals. Several antioxidants have been used experimentally, with cytoprotective effects, these include vitamin E, propranolol, calcium channel blockers, captopriul and the lipo-oxygenase inhibitor nafazatrom[272]. During cardiac surgery, the antioxidant, trimetazidine seems to reduce ischaemia-reperfusion damage[280].

1.3.3.7 Ischaemic Preconditioning

Ischaemic preconditioning (IPC) refers to a phenomenon in which a tissue is rendered resistant to the deleterious effects of prolonged ischaemia and reperfusion by prior exposure to brief, repeated periods of vascular occlusion[281]. Ischaemic preconditioning appears to be a biphasic phenomenon that lasts for about 2 hours after the preconditioning stimulus and a second window of protection that occurs 24 hours later[282]. Adenosine and Heat Shock Proteins are implicated in this process. The largest numbers of investigations of ischaemic preconditioning are in the myocardium.

Adenosine

Adenosine, through A_1 receptors is the initiator of the protective mechanism of preconditioning against skeletal muscle infarction and ATP channels are probably involved in the post A_1 receptor signal transduction pathway of this mechanism[283]. Adenosine is unlikely to play a key role in the effector mechanism of preconditioning[283]. The anti-infarct effect of adenosine was associated with a slower rate of energy metabolism and metabolite accumulation during sustained ischaemia[283].

Heat Shock Protein

The heat-shock proteins (HSP) are a family of proteins whose expression constitutes a ubiquitous, intracellular response to stress. Liauw et al investigates preconditioning in skeletal muscle[284]. Using a paired canine gracilis model, one muscle was rendered ischaemic for 5 hours and then allowed to reperfuse, followed by ischaemia of the contralateral limb. The contralateral limb had a mean 60% reduction in muscle necrosis, with significant sparing of ATP utilized in the second muscle group. HSP was detected in both muscles, with a different pattern of expression in the second muscle. This data suggests that reduced ATP utilization and altered HSP expression in the second muscle may play a role in the tissue salvage observed in this sequential muscle ischaemia model[284]. HSP appears to show a protective effect in other studies[285,286]. The mechanism by which HSPs exert protective effects is unclear but experimental work suggests that they are associated with increased production of free radical scavengers, especially catalase and superoxide dismutase[287,288].

1.3.3.8 Other Therapeutic Regimes to ameliorate Ischaemia/Reperfusion Injury:

Pentoxifylline

Pentoxifylline is a xanthine-derived phosphodiesterase inhibitor, which has been shown to have numerous haemorrheologic effects, including inhibition of platelet aggregation and increased synthesis/release of prostacyclin. In addition, pentoxifylline may alter granulocyte function through the inhibition of platelet activating factor[289]. In animals subject to 5 hours of reperfusion and 20 hours of reperfusion, pentoxifylline significantly decreased the levels of platelet activating factor and decreased the extent of muscle necrosis[233,290].

Hypothermia

Hypothermia has been used since the 1960s as adjunct in cardiac surgery and neurosurgery. The major value in the central nervous system has been prevention of haemorrhage and protection from ischaemic by reducing cerebral metabolism[291]. This effect may be due to a reduction in metabolism and preservation of ATP[291] or a fall in the ischaemic release of dopamine and glutamate, both of which may be cytotoxic[292]. Hypothermia has been shown to moderate damage following tourniquet application to a limb[293]. Tourniquet time can be prolonged for 3-4 hours with hypothermia[294] and limb oedema is markedly decreased[295].

Dantrolene

In skeletal muscle, dantrolene reduces free cytosolic calcium by inhibiting calcium release from the sarcoplasmic reticulum. In an isolated buffer perfused rat heart model of global cardiac ischaemia/reperfusion, dantrolene infusion reduced creatine phosphokinase, indicating an attenuation of lethal cellular injury in vivo[296].

Hyperbaric Oxygen

Hyperbaric oxygen treatment raises the levels of high-energy phosphate compounds, indicating stimulation of aerobic oxidation in the mitochondria. In rats with 4 hours of skeletal muscle ischaemia, repeated hyperbaric oxygen treatment significantly increased intracellular adenosine triphosphate and phosphocreatinine and reduced lactate levels compared with untreated rats[297]. In the rat gracilis muscle microcirculation model, a decrease in pedicle arterial leukocyte and neutrophil concentrations was seen following ischaemia-reperfusion injury with hyperbaric oxygen treatment[298].

1.4 Matrix Metalloproteinases

The field of matrix metalloproteinases and the research regarding this group of enzymes is exponentially increasing at present. Indeed, in the year 2001 over 1000 papers were published regarding MMPs. This review introduces the types of MMPs and discusses the activation mechanism of the gelatinases, MMP-2 and MMP-9. The involvement of MMPs in vascular tissue will be discussed. The natural and synthetic inhibitors of MMPs including tissue inhibitors of metalloproteinases (TIMPs) will be reviewed. Finally, a discussion of the brief amount of literature available on the role of MMPs in ischaemia/reperfusion will be reviewed.

1.4.1 Introduction

The proteases consist of exopeptidases and endopeptidases (proteinases). Depending on the catalytic group at their active center, the hydrolytic enzymes are divided into four classes: serine/threonine, cysteine, aspartic and metallo-enzymes. The metalloproteinases consist of over 200 in number with only a small percentage being matrix metalloproteinases. Metalloproteinases are divided into 8 clans and 40 families, with matrixins (MMPs) forming a subfamily of family M10. In order to be assigned to the matrix metalloproteinases subfamily, the enzyme requires a cDNA sequence sufficiently close to that of collagenase.

Matrix metalloproteinases are zinc dependent enzymes that play a fundamental role in the degradation and remodelling of the extracellular matrix components. They have essential roles in normal processes such as reproduction, bone growth and wound healing. They are also extensively studied in pathological conditions such as malignant disease and its metastatic spread, tissue destructive processes such as rheumatoid disease and periodontal disease as well as many other conditions. They have been shown to have a role in vascular disease in aneurysm formation, carotid plaque pathogenesis and ischaemia/reperfusion of the brain and kidneys. They have not been evaluated in skeletal muscle ischaemia/reperfusion injury.

1.4.2 Classification

Currently, MMPs are divided into four main subfamilies, collagenases, gelatinases, stromelysins and membrane-types, as well as some others and non-mammalian groups. These are summarised in Table I. Three MMPs; MMP 4, 5 and 6 reported earlier were later found to correspond to known enzymes and hence no longer exist as separate entities.

MMPs have been defined functionally as having the following characteristics: (1) they are proteinases that degrade at least one component of the extracellular matrix; (2) they contain a zinc ion and are inhibited by chelating agents; (3) they are secreted in a latent form requiring activation for proteolytic activity; (4) they are inhibited by tissue inhibitors of metalloproteinases (TIMPs); and (5) they share common amino acid sequences[299].

Table I: Members of the MMP Family

Group Name	MMP number	Other Names/Notes
COLLAGENASES		
Collagenase 1	MMP-1	Interstitial Collagenase
Collagenase 2	MMP-8	Neutrophil Collagenase
Collagenase 3	MMP-13	Rat interstitial collagenase
Collagenase 4	MMP-18	
GELATINASES		
Gelatinase A	MMP-2	Type IV Collagenase
Gelatinase B	MMP-9	Type IV Collagenase
STROMELYSINS		
Stromelysin 1	MMP-3	Transin
Stromelysin 2	MMP-10	Transin-2
Stromelysin 3	MMP-11	RXKR furin cleavage
MEMBRANE-TYPE		
MT1-MMP	MMP-14	Transmembrane domain and RRKR furin cleavage site
MT2-MMP	MMP-15	
MT3-MMP	MMP-16	
MT4-MMP	MMP-17	
MT5-MMP	MMP-24	Isolated from brain and cerebral tumours
MT6-MMP	MMP-25	
OTHERS		
Matrilysin	MMP-7	Pump–1, Lacks hemopexin
Metalloelastase	MMP-12	Macrophage elastase
	MMP-19	
Enamelolysin	MMP-20	
	MMP-23	Isolated from ovarian cDNA
	MMP-26	
NON-MAMMALIAN		
Xenopus XMMP	MMP-21	Frog
CMMP	MMP-22	Chick embryo
MMP-C31		Caenorhabditis elegans (nematode)
MMP-H19		*C. elegans*, furin motif
MMP-Y19		*C. elegans*, furin motif
Envelysin		Sea Urchin
Soybean MMP		*Glycine max,* Protein sequencing
Fragilysin		Bacteroides fragilis

1.4.2.1 MMP Structure

All matrix metalloproteinases (MMPs) are synthesized as pre-pro-enzymes and secreted as inactive proMMPs in most cases. MMPs contain different domain entities, which confer their individual properties. The signal peptide of 17-20 amino acid residues serves as a signal for secretion into the endoplasmic reticulum for eventual export from the cell. It does not exist on MMP-17. The propeptide domain that constitutes the amino-terminus of the enzyme follows the signal peptide and contains around 80 amino acids. It contains a unique conserved sequence with a cysteine residue that ligates the catalytic zinc to maintain the latency of the proenzymes. MMP-23 lacks this sequence but contains a conserved cysteine. The catalytic domain of about 170 amino acids contains the catalytic machinery including the zinc-binding site and a conserved methionine. This domain binds additional zinc and calcium ions which maintain the three dimensional structure of MMPs, required for their stability and enzymatic activities. There are three additional repeats of fibronectin like domains in MMP-2 and MMP-9. These are inserted within the catalytic domain and aid the binding of enzyme to gelatin substrates. The carboxy-terminal hemopexin-like domain has an ellipsoidal disc shape and plays a key role in substrate specificity with collagens and gelatins[300]. MMP-11, MMP-14, MMP-15, MMP-16 and MMP-17 have a furin recognition site, which leads to intracellular cleavage by furin. In the remaining enzymes, a cleavage by external proteases occurs in the middle of the propeptide, partially exposing the zinc and leading to autolytic cleavage by the remainder of the propeptide.

1.4.2.2 Collagenases

This group of collagenases degrade the fibrillar collagens types I, II and III[301]. Collagenase-1 (MMP-1) was the first of the animal metalloproteinases to be identified, as an enzyme released from involuting tadpole tails, described in 1962[302]. Apart from the above substrates, MMP-1 also degrades type X collagen[301], type I gelatin and α1-antitrypsin[303].

Collagenase-2 (MMP-8) was the second MMP to be identified[304]. The enzyme is localized to specific granules and released following phagocytic events. Although originally thought to be confined to neutrophils, it may be expressed in other cells as osteoarthritic chondrocytes[305], synovial fibroblasts and endothelial cells[306]. MMP-8 has the distinction of being the only interstitial collagenase to be stored in cells rather than being synthesized and released on command.

Collagenase-3 (MMP-13) is an interstitial collagenase purified from rat myometrial smooth muscle cells[307]. It displays high levels of gelatinolytic activity in contrast to MMP-1[308]. It is also produced by a variety of other tissues in the rat; osteoblasts, fibroblast and smooth muscle cells[309]. MMP-13 has also been cloned from human breast carcinoma[310]. MMP-13 is localized in chondrocytes and has been found to cleave type II collagen, the major collagen type in cartilage[311].

MMP-18 was reported in 1996 in human mammary gland DNA[312]. MMP-18 mRNA is found in human placenta, lung, pancreas, ovary, small intestine, spleen, thymus, prostate, testis, colon and heart but not detected in brain, skeletal muscle, kidney, liver or peripheral blood leukocytes[312]. It is known to cleave type I collagen[313].

1.4.2.3 Gelatinases

Types

This group consists of the gelatinous matrix metalloproteinases; 72 kDa MMP-2 and 92 kDa MMP-9. They contain additional repeats of fibronectin-like domains, which interact with collagens and gelatins. Liotta first described MMP-2 in 1979 and found that an enzyme secreted by a metastatic murine tumour degraded soluble type IV collagen[314]. This led to the early designation of this enzyme as type IV collagenase. MMP-2 is constitutively expressed in many cells.

In 1974 Sopata et al, purified a neutral protease from human neutrophils that could degrade denatured collagens (gelatins)[315]. Proteolytic activities against type IV and V collagen were subsequently identified in human neutrophils and characterized as metalloproteinases of 90-110 kDa[316]. Following the convention proposed by the Destin Beach matrix metalloproteinases meeting in 1989[317], it was designated as MMP-9. MMP-9 is expressed in neutrophils, macrophages and monocytes. In most cells, MMP-9 is secreted as a complex with TIMP-1. In neutrophils, MMP-9 is secreted complexed with a 25 kDa protein of the lipocalin family[318].

Substrates

Matrix metalloproteinase-2 cleaves gelatin[319], types IV and V collagen, type VII collagen found in anchoring fibrils[320], cartilage type X collagen[321,322], elastin[323], type I collagen[324], fibronectin[325], laminin-1, laminin-5[326] galectin-3[327], aggrecan[328], decorin[329], hyaluronidase-

treated versican, proteoglycan link protein[330] and osteonectin[331]. Matrix metalloproteinases-9 also cleaves gelatin, type IV collagen, type V collagen[332], elastin, aggrecan[328], entactin, galectin-3[327], proteoglycan link protein[330], fibronectin and osteonectin[331]. MMP-9 has a much higher affinity for types IV and V collagen than MMP-2. Only MMP-2 can degrade laminin. Although both MMP-2 and MMP-9 bind to Type I collagen, only MMP-2 has been shown to cleave soluble, triple helical type I collagen generating three quarter amino terminal and one-quarter carbon terminal characteristic of vertebrate interstitial collagenases[324]. A detailed evaluation of the differences in the substrates of MMP-2 and MMP-9 is given in Chapter 4.

1.4.2.4 Stromelysins

Stromelysins consist of three members, MMP-3, MMP-10 and MMP-11. They are derived from stromal cells and degrade various components of the extracellular matrix but not the triple helical regions of interstitial collagens, distinguishing them from collagenases. A non-collagenolytic metalloproteinase activity was first recognized in an extract of human articular cartilage[333]. MMP-3 degrades type IV collagen, aggrecan core protein, fibronectin and α_2-macroglobulin and activates the proMMP forms of MMP-1, MMP-3, MMP-8, MMP-9 and MMP-13. MMP-10 cleaves cartilage link protein[330]. MMP-11 is constitutively expressed on synovial membranes[334].

1.4.2.5 Membrane–type MMPs

These matrix metalloproteinases are grouped together based on the presence of a transmembrane domain. The membrane–type MMPs can also be subdivided into two groups; the type-I transmembrane and the glycosylphosphatidylinositol (GPI)-anchored proteases.
MT1-MMP, MT2-MMP, MT3-MMP and MT5-MMP (MMP-14, 15, 16 and 24) are all type-I transmembrane proteins with a short cytoplasmic tail. They have the ability to degrade many matrix components including type I-IV collagen, gelatin, laminin, fibronectin and fibrin.

MT1-MMP can activate proMMP-2 bound to the cell surface[335]. The soluble forms of MT1-MMP and MT2-MMP are relatively efficient proteinases and degrade denatured interstitial collagens, cartilage aggrecan, perlecan, fibulins-1 and –2, fibronectin, vitronectin, nidogen, large tenascin-C and laminin[336].

MT2-MMP is found in human endometrium[337] and breast cancer[338]. MT3-MMP is also found in breast tumours[338], brain and placenta[339].

MT4-MMP has a unique feature of anchoring the cell membrane by a glycosylphosphatidylinositol (GPI) anchor[340]. MT4-MMP cleaves gelatin and is able to activate MMP-2[341]. MT4-MMP has been isolated from leukocytes, breast carcinoma, brain, ovary, colon and testis tissues[341]. The known substrates of MT4-MMP include fibrinogen, fibrin and proTNF-α[342].

MT5-MMP is predominantly expressed in brain, kidney, pancreas and lung[343]. It is detected at high levels compared to normal brain tissue in a series of brain tumors, including astrocytomas and glioblastomas[344]. The catalytic domain of MT5-MMP, produced in Escherichia coli as a fusion protein with glutathione S-transferase, exhibits a potent proteolytic activity against progelatinase A, leading to the generation of the Mr 62,000 active form of this enzyme. These data suggest that MT5-MMP may contribute to the activation of progelatinase A in tumor tissues in which it is over-expressed, thereby facilitating tumor progression[344].

MT6-MMP is similar in function to stromelysin-1 (MMP-3), being able to cleave type-IV collagen, gelatin, fibronectin and fibrin. It is expressed exclusively in the normal adult in peripheral blood leukocytes and also in many brain tumours[345]. It differs from MMP-3 and MT1-MMP (MMP-14) in its inability to cleave laminin-1 and unlike MMP-3 cannot activate progelatinase B[346]. MT6-MMP could play a role in cellular migration and invasion of the extracellular matrix and its activity may be tightly regulated by all members of the TIMP family[346]. MT6-MMP, like MT4-MMP is very poor at, or unable to activate proMMP-2[342,347]. The other similarity with MT4-MMP is that MT6-MMP is anchored by a GPI protein[347].

1.4.2.6 Others:

MMP-7

Also known as matrilysin and PUMP-1, MMP-7 is a 28 kDa zymogen with the ability to cleave proteoglycan, gelatin, fibronectin, laminin and elastin[348]. Matrilysin represents the 'minimal' enzyme; it consists of a signal peptide, a propeptide and the catalytic domain, lacking the C-terminal domain. MMP-7 has been shown to degrade the extracellular matrix proteins fibronectin[349], gelatins (denatured forms) of types I, III, IV and V[349], collagen type IV[350], laminin[350], and entactin/nidogen. MMP-7 is characteristically of epithelial rather than stromal origin. It is primarily localized to the apical face or lumen of glandular epithelium,

implicating this MMP in extracellular activities unrelated to re-organisation of tissues. It appears to be a sentinel molecule maintaining the gland or duct in a state poised for rapid response when the critical signals are received[351].

MMP-12

Also known as metalloelastase or human macrophage elastase, expression of MMP-12 is largely restricted to tissue macrophages[352]. MMP-12 degrades fibronectin, laminin, entactin, type IV collagen, chondroitan sulfate and heparan sulfate[353].

MMP-19

MMP-19 was recently cloned by Stracke et al[354] and has been identified on activated lymphocytes and in rheumatoid plasma[355]. It will cleave type IV collagen, laminin, nidogen and possibly may have a role in activating MMP-9[354].

MMP-20

A cDNA was cloned from RNA prepared from human odontoblastic cells and named human enamelysin or MMP-20[356]. It is expressed in dental tissues only and is suggested to play a central role in tooth enamel formation. The cleavage of its putative natural substrate, amelogenin was completely inhibited by TIMP-2, providing evidence of the specificity of this proteolytic reaction mediated by human enamelysin[356].

MMP-23

MMP-23 is predominantly expressed in ovary, testis and prostate suggesting that this new MMP may play a specialised role in reproductive processes[357].

MMP-26

MMP-26 was cloned from fetal cDNA and has a highly homologous sequence to macrophage metalloelastase (MMP-12). It includes only the minimal characteristic features of the MMP family: a signal peptide, a prodomain and a catalytic domain. It is specifically expressed in placenta but is also detected in several human malignant cell lines such as HEK 293 kidney cells and HFB1 lymphoma cells[358].

1.4.3 Endogenous MMP Inhibition

1.4.3.1 α_2-Macroglobulin

Human α_2-macroglobulin is a 725 kDa plasma glycoprotein that binds to and inhibits most endopeptidases regardless of their substrate specificity. It was the first natural MMP inhibitor to be identified. The rapid binding properties with MMP-1 suggest that α_2-macroglobulin is the major regulator of collagenolysis. α_2-macroglobulin regulates MMPs mainly in the serum[359]. α_2-macroglobulin acts by offering the MMP a bait region; when this is cut, the molecule changes shape and traps the MMP in a cage like structure[360].

1.4.3.2 Tissue Inhibitors of Metalloproteinases (TIMPS)

One of the cardinal characteristics of MMPs is their inhibition of action by proteins called the tissue inhibitors of metalloproteinases or TIMPS. There are four family members TIMP-1, TIMP-2, TIMP-3 and TIMP-4, with molecular weights of 28, 21, 21 and 22 kDa respectively. The TIMPS are produced by localised cells, often the same cells that release MMPs. The TIMPs bind with high affinity in a 1:1 molar ratio to active matrix metalloproteinases resulting in a loss of activity[361]. The complex of TIMP to MMP can be dissociated by acid pH or EDTA; active TIMP is recovered but the MMP is usually inactive[362].The cell can therefore regulate the activity of the MMPs so that the surrounding matrix is not exposed to uncontrolled degradation. In addition to maintaining control over the activity of MMPs, TIMP-1 and TIMP-2 are able to bind directly to the hemopexin domain of MMP-9 and MMP-2 respectively, exerting further control over the activation process. There are also other roles of TIMPs, which do not seem to be directly attributable to proteinase inhibition, such as growth factor activity, steroidogenesis and cell morphology modulation[363].

TIMP-1

The production of a collagenase-inhibitory protein in the medium of cultured human fibroblasts was first reported in 1975[364]. Human TIMP-1 production is induced by transforming growth factor-β and interleukin-11 in chondrocytes[365]. TIMP-1 has the ability to bind to the hemopexin domain of proMMP-9[366], preventing binding to its active centre. The N-terminal portion of the TIMP-1 possesses the MMP inhibitory activity; it binds and inhibits MMP-1, MMP-2, MMP-3 MMP-7 and MMP-9[367]. TIMP-1 performs various functions in addition to inhibition of MMP activity. TIMP-1 was originally described as erythroid potentiating factor[368] and subsequently, proteins displaying growth factor activity[369] and stimulation of steroidogenesis[370] were found to be identical to TIMP-1.

TIMP-2

TIMP-2 inhibits MMP-1, -2, -3, -7, -8, -9, -10, -13, -14, -15, -16 and -19[362]. TIMP-2 is intimately involved in both the activation and the inhibition of MMP-2. Active MMP-2 can simultaneously bind two molecules of TIMP-2: one at its active site, to inhibit the enzyme, another on its C-domain[371]. The N-terminal domain of TIMP-2 possesses its inhibitory activity[372]. The C-terminal domain also has a role in MMP inhibition also; with the C-terminal of TIMP-2 being held down on the hemopexin domain of MMP-2 in such a way that the N-end is properly aligned with the active site of the enzyme.

TIMP-3

TIMP-3 inhibits MMP-1, -2, -3 and -9[373]. It is distinguished from other TIMPs by being firmly anchored to the extracellular matrix. It is also the only TIMP to be directly associated with a disease, namely, Sorsby's Fundus dystrophy, where inactivating point mutations in the TIMP-3 gene leading to this type of age-related macular degeneration[374].

TIMP-4

TIMP-4 inhibits MMP-1, MMP-3, MMP-7 and MMP-9 and show a particular interaction with MMP-2; binding specifically to its C-terminal domain[375,376]. It is abundant in the human heart but occurs at low levels in most other organs and is upregulated by vascular injury[375].

1.4.4 Control mechanisms and Activation of MMP-2 and MMP-9

All MMPs are synthesised as pre-proenzymes and most of them are secreted from cells as proenzymes consisting of a propeptide, a catalytic domain and a C-terminal domain. The zymogens (proenzymes) of most MMPs can be activated by proteinases and by non-proteolytic compounds such as SH reactive agents (iodoacetate, 4-aminophenylmercuric acid (APMA), HOCL, oxidized glutathione) and denaturants (urea, SDS) and by heat treatment[377].

1.4.4.1 MMP-2

The constitutive, high level of MMP-2 mRNA expression seen in many tissues suggest that the regulation of activation of the proenzyme is more relevant for the control and induction of MMP-2 action than other members of the MMP family in which transcription control is more important[378].

Transcriptional Regulation

Unlike other MMPs, many cell types constitutively express MMP-2 and its expression is not altered by the tumor promoter phorbol myristate acetate (PMA)[379]. MMP-2 is upregulated by transforming growth factor-β, epidermal growth factor, interleukin 1β and interleukin-1α[380]. Romanic and Madri showed that, as part of the process of extravasation, when T cells leave the bloodstream and access the sites of inflammation, MMP-2 synthesis is induced in these T cells and is mediated by their binding to vascular cell adhesion molecule-1 (VCAM-1)[381]. Constitutive MMP-2 production can be down regulated by interferon-β and interferon-γ, although not by interferon-α[382].

Regulation of Activity by TIMPs

There is an interaction between the C-domain of TIMP-2 with the C-domain of MMP-2 as a means to orient the inhibitory N-terminal end of TIMP-2 towards the active centre, thereby increasing the rate of enzyme inhibitor association. In addition, cross-linked proMMP-2/TIMP-2 complex can still be activated and show gelatinase activity, which suggests that the TIMP-2 is not entirely bound to the active site[383]. However, this MMP-2/TIMP-2 complex retains 10% of the proteolytic activity of the enzyme that is free of TIMP-2. Upon cleavage of the pro-fragment, the active site of the MMP-2/TIMP-2 complex becomes available for binding the second domain of TIMP-2. This site is not specific for TIMP-2 and the active site of MMP-2 can be inhibited by all of the TIMPs[384].

Regulation of Proenzyme Activation

In order to develop catalytic activity, the coordination between the unpaired cysteine residue in the pro-domain and the zinc atom of the active site must be broken. Upon the dissociation of this zinc-Cys interaction, MMP-2 undergoes an autolytic cleavage, which removes an 8 kDa peptide from proMMP-2, to form active MMP-2. This processing appears to occur in 2 steps with an initial cleavage giving a 64 kDa intermediate, followed by a second cleavage to give the final 62 kDa active MMP-2. The activation requires formation of a trimolecular complex between MT1-MMP, TIMP-2 and proMMP-2. MT1-MMP on the cell surface serves as a receptor of TIMP-2, which in turn binds proMMP-2[385]. This binding of TIMP-2 to proMMP-2 occurs via the C-terminal ends of the molecules. The formation of this ternary complex is considered critical for the binding of proMMP-2 to the cell surface, where subsequent activation of proMMP-2 by the closely located free MT1-MMP is thought to take place[385]. The requirement of both free MT1-MMP and the TIMP-2-MT1-MMP complex for

proMMP-2 activation was demonstrated[386] by TIMP-2 titration of the MT1-MMP expressed on the plasma membrane. Membrane anchoring of MT1-MMP through the transmembrane domain is essential for its ability to activate proMMP-2[387] but soluble forms of MT1-MMP[388] and MT2-MMP[389] were shown to activate proMMP-2 directly.

The MMP-2 proenzyme is readily activated by 4-aminophenylmercuric acetate (APMA)[390] to a 68 kDa active form. Thrombin, plasmin and u-plasminogen activator (u-PA) will also process the 72 kDa proform to 62 kDa[391]. However, whether all these 62 kDa forms have proteolytic activity is unknown[385]. Trypsin-2 cleaves proMMP-2 but only limited enzymic activity is detected[392]. ProMMP-2 can also be activated by MMP-1[393] and MMP-7[394]. However, MMP-1 and MMP-7 are not very efficient activators of proMMP-2. In the presence of heparin, however, the activation of proMMP-2 by MMP-1 is greatly enhanced[393]. Nonetheless, cell surface activation of proMMP-2 by MT-MMPs is considered physiologically more important. Cell-surface bound proMMP-2 and proMMP-9 were activated by cell surface-associated u-PA/Plasmin system[395].

1.4.4.2 MMP-9

Transcriptional Regulation

Expression of MMP-9 mRNA is stimulated by TGF-α, epidermal growth factor, inteleukin-1β and interleukin-1α[380]. Dexamethasone reduces MMP-9 mRNA levels and Vitamin D_3 also acts as a negative regulator of MMP-9 expression[379].

Regulation of Proenzyme Activation

Activation of the enzyme occurs when the interaction between the zinc molecule in the active site and a cysteine in the pro-domain is disrupted, rendering the active site accessible. This can be achieved by proteolytic removal of the propeptide, or by disruption of the cysteine-zinc interaction by organomercurials and chaotropic agents, leading to an active enzyme which then cleaves the propeptide autocatalytically[396].

In most cells, MMP-9 is secreted as a complex with TIMP-1. When proMMP-9 exists as a complex of proMMP-9-TIMP-1, TIMP-1 readily inhibits other MMPs. A ternary complex, proMMP-9-TIMP-1-MMP-3, is found when the tertiary complex is reacted with MMP-3[397]. Although this ternary complex partly dissociates into free proMMP-9 and the TIMP-1-MMP-3 complex, activation of proMMP-9 requires an excess of MMP-3 or other MMP relative to

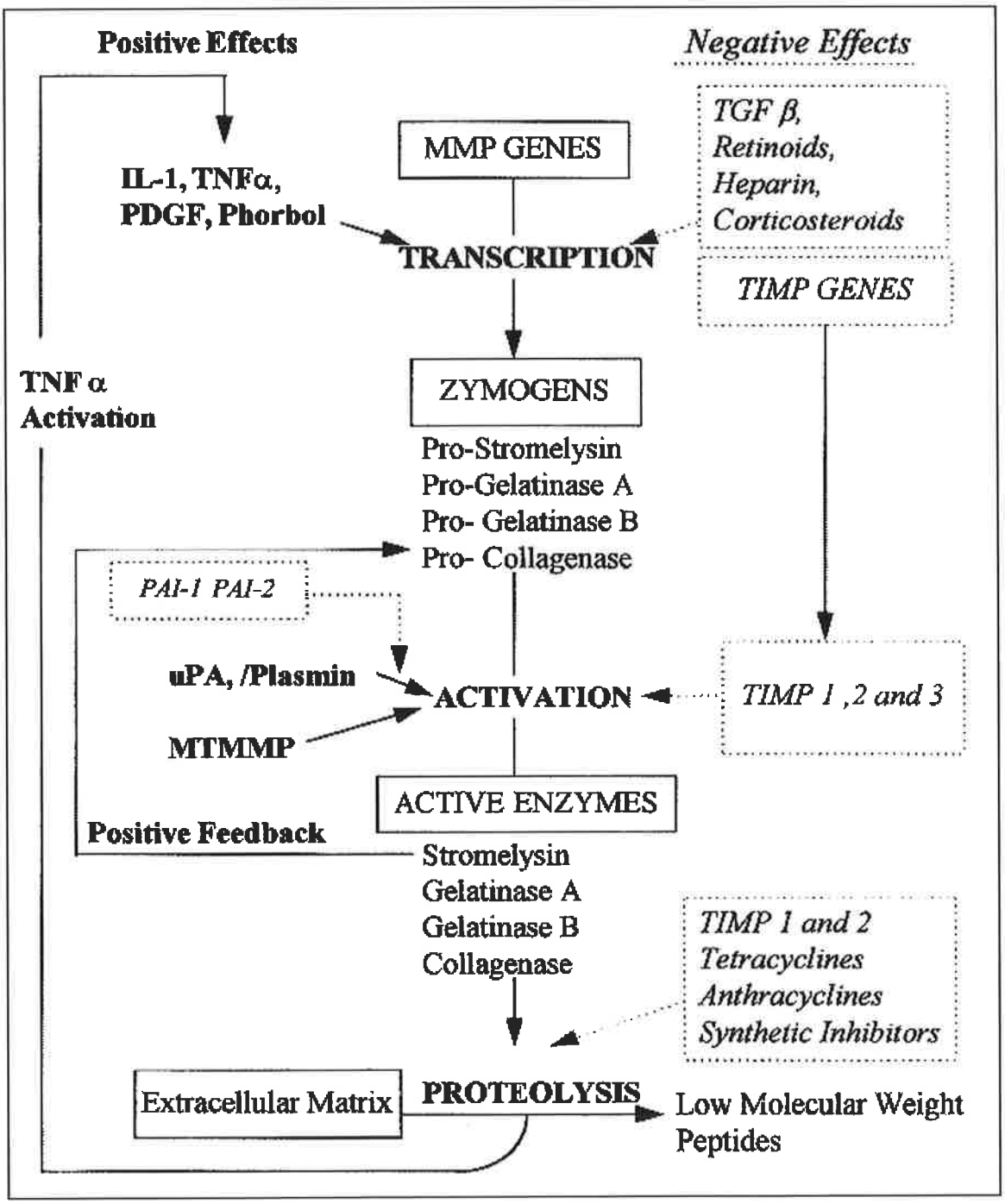
the complex[362]. An alternative pathway for the activation of the proMMP-9-TIMP-1 complex is via specific destruction of TIMP-1. Leukocyte elastase preferentially inactivates TIMP-1[398], leaving the proMMP-9 bound to TIMP-1, which is readily activated by MMP-3[399]. Trypsin also inactivates TIMP-1[400], but this enzyme activates proMMP-9 more readily than inactivating TIMP-1. Thus, in this case, the activated MMP-9 is already inhibited by the cognate TIMP-1[399]. Treatment of the proMMP-9-TIMP-1 complex with APMA activates proMMP-9 and the propeptide is removed by autolysis, but the activated MMP-9 is readily inhibited by TIMP-1[397]. Under these conditions, proteolytic activity of MMP-9 cannot be detected.

ProMMP-9 is also activated by tissue kallikrein[401], cathepsin G, α-chymotrypsin[402], MMP-1[403], MMP-2[404], MMP-3 [405], MMP-7[406], MMP-10[407] and MMP-13[408] by a stepwise mechanism. N-terminal sequence analysis of the initial product generated by MMP-1, MMP-2, MMP-3, MMP-7 or MMP-13 indicated that an intermediate of 86 kDa was produced initially, with a second cleavage to the fully active 82 kDa MMP-9. α2-Macroglobulin binding studies of partially activated MMP-9 demonstrate that the 82 kDa species is proteolytically active, but the initial intermediate of 86 kDa shows no activity[409].

A summary of the activation and control mechanisms of MMPs is shown in Figure 1.

Figure 1: Transcription, activation of latent proenzymes and inhibition of proteolytic activity.

This figure illustrates the control of matrix metalloproteinases at transcription, activation and proteolysis levels, with negative feedback control mechanisms[410].


Positive Effects
Negative Effects
MMP GENES
IL-1, TNFα,
PDGF, Phorbol
TRANSCRIPTION
TGF β,
Retinoids,
Heparin,
Corticosteroids
TIMP GENES
TNF α
Activation
ZYMOGENS
Pro-Stromelysin
Pro-Gelatinase A
Pro- Gelatinase B
Pro- Collagenase
PAI-1 PAI-2
uPA, /Plasmin
MTMMP
ACTIVATION
TIMP 1 ,2 and 3
ACTIVE ENZYMES
Positive Feedback
Stromelysin
Gelatinase A
Gelatinase B
Collagenase
TIMP 1 and 2
Tetracyclines
Anthracyclines
Synthetic Inhibitors
Extracellular Matrix
PROTEOLYSIS
Low Molecular Weight
Peptides

1.4.5 Known roles of Matrix Metalloproteinases in Vascular Tissues

Matrix metalloproteinases have been studied in a large number of physiological and pathological settings in human and animal tissues. These include demonstrating roles for MMPs in parturition and reproduction, wound healing, periodontal diseases, rheumatoid arthritis and malignant diseases. For the purposes of this review, only the topics related to vascular disease will be discussed.

1.4.5.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory process whereby plaques are formed in the intimal layer of the vessel wall as a result of accumulation of lipid-laden macrophages, smooth muscle cells, lipids and extracellular matrix. Plaques may become unstable and rupture, triggering intravascular thrombosis and clinical symptoms of tissue ischaemia. Alternatively, the atherosclerotic vessel wall may dilate due to destruction of the media, leading to aneurysm formation and rupture of the weakened vessel wall. MMPs have been shown to have an inherent role in these processes.

Part of the formation of the atherosclerotic plaque involves smooth muscle cell migration and this action requires MMP activity. Smooth muscle cells in a proliferating state readily migrate across the basement membrane barrier and this ability is inhibited by synthetic peptides that inhibited MMP activity[411,412]. With time, the matrix of the vessel wall becomes modified through the migration and proliferation of cells and the deposition of the extracellular matrix, eventually resulting in the formation of a plaque. MMPs are expressed in human atherosclerotic plaques by both smooth muscle cells and foam cells[413,414]. Stromelysin mRNA transcripts are localized to both smooth muscle cells of both fibrous and lipid-rich atherosclerotic plaques[410]. The accumulation of large numbers of macrophages and foam cells is not a feature of the normal vessel wall and it is likely that the extensive synthesis of stromelysin is pathological event contributing to atherosclerosis.

There is evidence suggesting that MMPs also contribute to the destruction of connective tissue in the atherosclerotic lesion, leading to surface disruption. In non-atherosclerotic arteries, MMP-2 together with TIMP-1 and TIMP-2 were found to be expressed by smooth muscle cells in all layers of the normal artery whereas MMP-9, interstitial collagenase (MMP-1) and stromelysin were not detected[415]. In contrast, atherosclerotic lesions showed

immunoreactivity of all MMPs and TIMPs tested, with MMP-1, MMP-9 and stromelysin being localized to macrophages, lymphocytes, smooth muscle cells and the endothelium in the fibrous cap and shoulders of the lesions[414,415]. The regions expressing MMP-1, MMP-9 and stromelysin, also exhibited gelatinolytic and caseinolytic activity, suggesting that at least some of these enzymes were in active form. MMPs contribute to the vulnerability of atherosclerotic plaques by degrading the component of the fibrous cap: collagens, elastin, fibronectin and proteoglycans[414]. The expression of MMP-1 in atherosclerotic plaques is induced by inflammatory cytokines such as interferon-γ, TNF-α, interleukin-1β[416]. The regulated expression of TIMP-3, in addition to the presence of TIMP-1 and TIMP-2 counteracts MMP activity in atheromas and hence influences plaque stability[417].

These studies suggest that matrix degradation may outstrip synthesis at certain locations in some atheromatous plaques, predisposing them to plaque rupture. In the majority of lesional areas, however, the balance between synthesis and degradation is likely to favour the former since there is a gain in the contents of matrix in all atheromas[415]. There is also evidence to suggest that an imbalance favouring matrix deposition contributes to restenosis after angioplasty and endarterectomy[418].

1.4.5.2 Restenosis at Angioplasty Sites

Percutaneous transluminal angioplasty is a widely used technique for the treatment of vessel stenosis, both in the coronary and peripheral vessels. Initially, this procedure has a high technical success rate, but the usefulness of angioplasty is limited by the fact 25 to 50% of patients have a recurrence of their symptoms within 6 months because of restenosis at the original site. This is due to intimal hyperplasia; migration and rapid growth of medial smooth muscle cells and deposition of extracellular matrix. Rat models of angioplasty with balloon injury to the carotid vessel have been used extensively to study this process.

In the rat carotid artery model of balloon withdrawal injury, medial smooth muscle cell proliferation begins immediately after injury. There is constitutive expression of MMP-2 with some induction 4-5 days after injury, whereas MMP-9 was induced the first day after injury[419]. Active MMP-9 could therefore be controlling the migration of smooth muscle cells from the media to the intima, Studies of the plasminogen system after balloon injury in the rat have shown acute upregulation of u-plasminogen activator activity[420], which could activate the MMP cascade. When a metalloproteinase inhibitor was introduced, there was a 97%

reduction in the early migration of smooth muscle cells into the intima[419]. Arterial injury has also been shown to substantially increase the expression of MT-MMP[421], which precedes the changes in MMP-2 expression, consistent with it potential role as a cell-surface activator of MMP-2[422]. MMP-3 has also been detected within 2 hours of rat carotid arterial injury, with the greatest level 7 days after injury[423]. Expression of TIMP-1 occurs at 24 hours after injury and may play role in protection against further injury[423]. These studies continue to improve the understanding of the pathophysiology of restenosis after angioplasty and may lead to therapeutic measures to decrease this phenomenon.

1.4.5.3 Aortic Aneurysm Formation

Abdominal aortic aneurysm (AAA) is a common and potentially life threatening disease with an estimated incidence of 20 to 40 cases per 100,000 persons per year[424]. The known risk factors for AAA include advancing age, male sex, chronic obstructive airways disease, smoking, hypertension and genetic factors[425]. Most aneurysms are silent until the time of rupture, with the risk of rupture increasing with the size of the aneurysm.

AAAs have been thought to be due to a complication of atherosclerosis for many years[426]. Abdominal aortic aneurysms are characterised by disruption and degradation of the elastin in the media and adventitia, apoptosis and decrease in the number of matrix-synthesizing medial smooth muscle cells and an adventitial and transmural infiltrate consisting of macrophages, lymphocytes, dendritic cells and plasma cells[425,427-431]. Initiation and expansion of AAA is attributed to loss of elastin, normally responsible for the resilience of the aorta, whereas loss of fibrillar collagens (type I and III), the major source of tensile strength is believed to ultimately result in rupture[425,432]. Elastin has a long lifespan of 40 to 70 years, hence, loss of elastin is due to breakdown rather than lack of synthesis. Many animal models used experimentally for AAA utilize elastase to create the aneurysm[433]. Increased local production of several matrix metalloproteinases (MMPs) has been implicated in this process of elastin and matrix destruction leading to AAA formation[434,435].

Aortic aneurysms contain an excess of inflammatory cytokines, such as interleukin-1β, TNF-α and interleukin –6, which increase MMP-9 expression in macrophages. MMPs are also involved in conversion of membrane bound proinflammatory TNF-α to its soluble secreted form[436]. The MMPs overexpressed in AAA tissue are mainly the elastolytic MMPs, MMP-2 and MMP-9[437-439] and MMP-1 and MMP-3[440,441] and MMP-12[442]. In addition to MMPs, the plasmin/plasminogen system has been implicated in the formation of AAAs. Plasmin is

capable of digesting the extracellular matrix directly or indirectly by activating the zymogen (proenzymes) forms of MMPs[434,443]. The predominant source for the MMP-9 in AAAs appears to be the inflammatory cells, primarily monocyte-derived macrophages[429,434,435,444]. The evidence supporting the role of MMPs in AAA includes: (1) evidence for over expression of MMPs in AAA compared with the normal aortic wall[440], (2) evidence for reduced or unchanged expression of TIMPs, (3) in situ and in vitro evidence for an increase in net degrading activity in AAA[445], (4) increased expression of activators of proMMP, such as plasmin and plasmin generating enzymes such as u-plasminogen activator and t-plasminogen activator in AAA[331], (5) experimental studies showing that infusion of elastolytic enzymes initiates the development of AAA[446] and (6) demonstration that inhibition of inflammatory cell recruitment or inhibition of MMP secretion and/or activity of cyclo-oxygenase inhibitors or by tetracycline derivatives inhibits AAA development or expansion[447-450].

1.4.6. Therapeutic Inhibition of MMPs

There are several ways to inhibit the activity of MMPs; reduce the amount of active enzyme, block the active MMPs, or dislodge the MMPs from their receptors, binding to cleavage sites on the substrate. The main interest in inhibitors is two-fold, initially to discover more about the actions of the MMPs themselves and secondly to use the inhibitors in the clinical setting to reduce or negate the effects of MMPs. Given the explosion of knowledge in the role of MMPs in clinical diseases such as atherosclerosis, aneurysms and angioplasty related stenosis, there is a great impetus to discover and put into clinical trials drugs or agent that will decrease the morbidity and mortality of these diseases. The vast majority of research into MMP inhibitors that has reached a clinical level involves inhibitors for treatment of malignant disease, periodontal diseases and arthritis.

The first low molecular weight inhibitors were either chelating agents (e.g. di-sodium ethylenediaminetetraacetate dihydrate (EDTA) or ortho-phenanthroline), which are unusable medically, sulfydryl reagents such as dithiothreitol (DTT) and mercapto-compounds, including thiol peptides[451].

1.4.6.1 Blocking the Production of MMPs

There are some ribozymes that can specifically affect the mRNA of a single MMP; a stromelysin specific ribozyme and an anti-MMP ribozyme have been used under experimental

conditions[452]. The tetracyclines also reduce mRNA levels of MMPs. There are no known agents that block the secretion of MMPs from the cell[452].

1.4.6.2 Inhibition of Active MMPs by Chelators

The majority of synthetic inhibitors are based on the chelation of zinc by the use of thiol, carboxyl, phosphorus and hydroxamate binding groups.

Hydroxamates

These are pseudopeptide derivatives based on the structure of the collagen molecule at the site of initial cleavage by interstitial collagenase. These inhibitors bind reversibly at the active site of the MMP in a stereospecific manner. The zinc-binding group of the inhibitor is positioned to chelate the zinc ion in the active site of the MMP. The hydroxamates have broad specificity for members of the MMP family and display little detectable activity against other classes of metalloproteinases, such as angiotensin converting enzyme and enkephalinase. The initial products included batimastat, however they had poor oral bioavailability. The next phase of development of MMP inhibitors was assisted by X-ray crystallography data on the three-dimensional structure of the collagenase active site. Marimastat was developed and showed improved bioavailability in animals and man. More recently, more specific MMP inhibitors have been developed. These include AG3340 (Agouron); which demonstrates selectivity for gelatinase A over collagenase and Ro32-3555 (Roche); an inhibitor with relatively weak activity against gelatinase A and stromelysin-1 but good activity against interstitial collagenase. Several members of this class of compounds are now in phase III clinical trials[453].

Tetracyclines

The tetracycline group of antibiotics is known to have chelating properties for MMPs. Golub et al in 1983 were the first to note that such compounds, used in treating periodontal disease, could inhibit collagenase from gingival tissues and fluid, both in rats and humans[454]. The effect of tetracyclines on MMPs does not depend on bactericidal action, with the active site of the molecule for each action being separate. Tetracyclines act in a relatively nonspecific fashion with regard to different MMPs, inhibiting disintegrin metalloproteinases and membrane-type MMPs as well as more traditional family members[449]. As it has relevance to the current studies, an in depth discussion on the actions of tetracyclines, in particular,

doxycycline, is included in Chapter 5. Clinical trials have been commenced using tetracycline in the treatment of arthritis[455,456].

HMG CoA Reductase Inhibitors

Hydroxymethylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) have been used extensively as lipid lowering agents. They have also been shown to reduce MMP-9[457,458] secretion by inducing inactivation of NFκB[459], This reduction in MMP-9 associated with HMG CoA reductase inhibitors may contribute to the decrease in incidence of cardiovascular events[460], possibly by stabilizing atheromatous plaque[461]. Reduction in MMP-2 has also been observed in endothelial cells[462]. In human carotid plaques, a reduction in MMP-2 and an increase in TIMP-1, with a stabilizing effect on the carotid plaque, has been observed[463]. Fluvastatin has been shown to decrease MMP-1 in vascular endothelial cells[464]. These studies imply that HMG Co reductase inhibitors may have a role in controlling diseases of vascular tissue, independent of their cholesterol lowering abilities.

1.5 Matrix metalloproteinases and reperfusion injury

Very little has been reported on the role of matrix metalloproteinases in skeletal muscle reperfusion injury. However, there have been some preliminary studies on MMPs in ischaemia/reperfusion in brain, lung, myocardium and kidney.

1.5.1 Brain Ischaemia/Reperfusion injury and MMPs

Reperfusion of the brain damages the blood brain barrier manifested by major alterations in vascular permeability and vessel wall basal lamina structure. Rosenberg et al studied the role of MMPs in reperfused rat brain[465]. MMP-3 was present in microglial cells and ischaemic neurons after 24 hours of reperfusion. Pericytes also contained MMP-3, which is an activator of MMP-9[465]. There was an early rise in MMP-2 seen in the astrocytes around the blood vessels after 3 hours of reperfusion and a second more marked increase after 5 and 21 days of reperfusion. The activation of both MMP-2 and MMP-9 were reduced by a hydroxamate type inhibitor, BB-1101[465]. Earlier studies have shown an increase in MMP-2 within 1 hour of cerebral artery occlusion[466] and MMP-9 only significantly increased in subjects with haemorrhagic transformation following reperfusion[466]. The increase in MMP-2 was correlated with the extent of neuron injury[466]. Rosenberg et al and others have shown that the rise in MMP-2 and MMP-9 correlates with an increase in capillary permeability[467,468], which is a major pathological change in brain ischaemia/reperfusion injury. This effect was reversed at 24 hours with an MMP inhibitor, BB-1101[467]. In a rat model of permanent ischaemia leading to stroke, there was a marked rise in MMP-9, with maximal levels at 24 hours[469]. There was a 30% reduction in cerebral infarct size when an MMP-9-neutralizing monoclonal antibody was administered systemically[469].

1.5.2 Lung Ischaemia/Reperfusion injury and MMPs

Following lung ischaemia/reperfusion in a porcine model of isolated-reperfused lung, both proMMP-9 and MMP-9 increased significantly, correlating with an increase in alveolar-capillary permeability evaluated by the transferring leak index[470]. MMP-2 also increased but not to the same extent[470]. Similarly, in a rat model of lung transplantation, causing severe ischaemia/reperfusion injury, there was a rise in MMP-9 activity and TIMP-1 also increased in the late phase of reperfusion[471].

1.5.3 Myocardial ischaemia/Reperfusion injury and MMPs

In vitro experiments have shown MMP-9 increases during the first few hours of reperfusion[472]. In humans, serum MMP-1 and TIMP-1 showed delayed increases after myocardial infarction, possibly implicated in the healing process[473]. In a porcine model of cardiac ischaemia/reperfusion injury, MMP-1 and MMP-9 activity were increased in the ischaemic/reperfused myocardium[474] compared with non-ischaemic myocardium. After 20 minutes of global no flow ischaemia, there was a marked increase in proMMP-2 in the coronary effluent, which peaked within 1 minute of reperfusion[475]. MMP-2 antibody and doxycycline improved the recovery of mechanical function during reperfusion[475].

1.5.4 Renal Ischaemia/Reperfusion injury and MMPs

In a rat model of renal ischaemia/reperfusion injury, glomerular type IV collagen was decreased from 2 to 16 days following ischaemia, which was accompanied by an increase in MMP-2[476]. In contrast, the level of tubulointerstitial type IV collagen significantly increased by 24 hours after ischaemia, until day 8 when the levels dropped below normal and returned to normal by day 180[476]. Jain et al found an increase in MMP-2 following renal ischaemia/reperfusion injury but it was delayed until 8 weeks after the injury[477].

1.6 Aims of the Current Studies

Skeletal muscle ischaemia has many known contributing factors as discussed in this chapter. There has also been multiple therapeutic regimes examined, predominantly in vitro, aiming to ameliorate ischaemia/reperfusion injury. Matrix Metalloproteinases are a family of zinc-dependent enzymes that are known to degrade all components of the extracellular matrix. MMP-2 and MMP-9, the gelatinase matrix metalloproteinases, degrade type IV collagen, gelatin, fibronectin and laminin and various other components that constitute the basement membrane of tissues. As seen in brain and in the kidney, MMPs have been shown to play a role in reperfusion injury of those organs.

The aims of these studies were to explore the role of MMP-2 and MMP-9 in skeletal muscle ischaemia/reperfusion injury in a rat model. Type IV collagen degradation during skeletal muscle ischaemia/reperfusion injury was used to quantitate the level of damage that occurred. The correlations between changes in MMP levels and type IV collagen levels were studied. The effect of doxycycline, as a MMP inhibitor, was also investigated in skeletal muscle ischaemia/reperfusion injury.

This is the first report that implicates MMP activity in skeletal muscle ischaemia/reperfusion injury and provides evidence that pharmacological modification of MMP activity may be useful for treatment of this condition.

CHAPTER 2:

ESTABLISHMENT AND VALIDATION

OF AN

ANIMAL MODEL OF SKELETAL MUSCLE

ISCHAEMIA/REPERFUSION INJURY

2.1 Introduction

The aims of this chapter are to establish the animal model and validate its use in the study of skeletal muscle ischaemia/reperfusion injury. This is followed in further chapters by investigative studies of matrix metalloproteinases 2 and 9, studies of the effect of these matrix metalloproteinases upon type IV collagen and the role of therapeutic intervention with the matrix metalloproteinase inhibitor, doxycycline.

Three methods of investigation were used to establish and validate the animal model. Firstly, the animal model itself is described in detail, followed by a discussion on the choice and type of model. This particular model utilizes a tourniquet placed around the thigh of the rat, establishing a standardized level of ischaemia as indicated by the lack of pulsatile blood flow on the photoplethysmography trace. Following the experiment, the rat was euthanased and the tissues were processed for further laboratory evaluation.

Secondly, the level of lung oedema was quantified to demonstrate if there was an element of pulmonary oedema caused by the reperfusion in this animal model. Ischaemia/reperfusion injury is characterized by increased capillary permeability, non-cardiogenic pulmonary oedema and a rise in pulmonary vascular resistance[24,478]. The clinical importance of the remote organ effects of skeletal muscle ischaemia/reperfusion was shown in a prospective study of twenty patients that underwent abdominal aortic aneurysm repair[39], where all patients showed increased evidence of increased vascular permeability as demonstrated by an increase in the pulmonary shunt and peak inspired airways pressure. This pulmonary injury is caused by increased microvascular permeability and accumulation of neutrophils[17]. Establishment of the level of oedema was important from the viewpoint of validating the animal model and for the exploration of the potential effect of therapeutic agents.

Thirdly, histopathology studies were performed to confirm that our animal model showed that the tourniquet was producing ischaemic damage with demonstrable histopathological effects to the skeletal muscle and that there was histopathological damage to the remote organs.

2.2 Methods

2.2.1 Animal Model Protocol

The North Western Adelaide Health Service Animal Ethics Committee and The University of Adelaide Animal Ethics Committee approved all animals and procedures. Animals were obtained from Central Animal House, The University of Adelaide and housed at The Queen Elizabeth Hospital Animal House. All animals were fed on standard rat chow and water ad libitum.

A total of 76 male Sprague-Dawley rats weighing 250-300 grams were anaesthetised, with halothane, nitrous oxide and oxygen using a Midget 3 inhalational anaesthetic machine (Figure 2). Anaesthesia was then maintained over a four-hour period. The respiratory rate of the animal was monitored clinically. Core body temperature was measured using a rectal probe (Kane-May Ltd, Welwyn Garden City, Herts) and maintained at thirty-seven degrees Celsius via a heating lamp.

For a unilateral ischaemic animal, the left inner thigh was shaved, a white cotton tape was placed loosely above the level of the greater trochanter. A photoplethysmography probe was attached to the skin over the femoral artery using double-sided sticky tape. The photoplethysmography probe was then connected to the Doppler machine (Parks Medical Electronics Inc. Oregon, USA) (Figure 3) and femoral arterial flow was demonstrated prior to tourniquet placement. The cotton tape was then tightened, forming a tourniquet and secured with an artery forceps to produce ischaemia. The photoplethysmography probe was used to demonstrate absence of arterial flow on the photoplethysmography trace after tourniquet application and documented every fifteen minutes (Figure 4 and Figure 5). In bilaterally ischaemic animals, both lower limbs were shaved and two photoplethysmography probes attached.

After four hours, the rat was either euthanased immediately without releasing the tourniquet or reperfusion was allowed to occur. In the reperfused rats, the tourniquet was removed whilst the rat remained anaesthetised and reperfusion commenced. The returning femoral pulse was demonstrated on the photoplethysmography trace (Figure 6). In bilaterally ischaemic rats, the tourniquets were released approximately 5 minutes apart in order to prevent overwhelming reperfusion and death. Approximately ten minutes after tourniquet release, the anaesthetic

agents were ceased and inhaled oxygen was administered until the rat was completely awake. Reperfusion was then allowed to proceed for either four, twenty four or seventy two hours. During this period, the rat was again fed on standard rat chow and water ad libitum. The rat was also observed for signs of distress or pain during this period.

Following the reperfusion period, rats were euthanased with Pentobarbitone sodium (60 mg/kg) via intraperitoneal injection. The rats were then dissected and tissue harvested from the skeletal muscle of both hind legs, liver, kidneys and lungs. Three horizontal sections of hind limb skeletal muscle were dissected, each section consisting of a cross section of thigh muscles measuring approximately 0.5cm thick (Figure 7). A cross section of skeletal muscles was chosen in order to achieve a combination of slow and fast twitch skeletal muscle fibres. The most proximal cross section of tissue was taken at a sufficient distance from the tourniquet site to avoid the zone of crush injury from the tourniquet itself. These skeletal muscle tissues were processed in three separate ways: either for tissue zymography, frozen in liquid nitrogen or prepared in paraffin blocks for histopathology. The left hepatic lobe, left kidney and left upper lobe of lung were similarly prepared.

A total of 77 rats were used throughout the entire experiments. These groups are summarised in Table II.

2.2.2 Quantitation of Lung Oedema

Following euthanasia, the left upper lobe of the rat lungs was trisected for paraffin blocks, frozen sections or zymography and processed as described in subsequent chapters. The remaining left lower lobe and entire right lung were weighed on a Petri dish immediately after resection. The Petri dish was placed in an oven at 90 degrees Celsius, the lungs allowed to dry out and weighed daily until the weight of the Petri dish and lungs reached a steady state dry weight. This procedure took an average of seven to ten days. Hence, a ratio was obtained of the wet to dry weight of the lungs, as an indicator of the level of lung oedema that occurred as a result of the anaesthesia, the ischaemia and the reperfusion.

Statistical analysis of Wet/Dry Weight Ratios in Lung Tissue

Data were analysed using an unbalanced 2-way ANOVA 3x4 factorial design with interaction. ANOVA partitions the variation values into variation between and within groups. If the overall F-test is significant, this means that the model as a whole is significant, that is, there is evidence to suggest that the individual cell means are different.

The R-square is interpreted as the percent of variation in unit that can be accounted for by the model.

Type III sums of square are preferred in testing the effects of reperfusion, group and the interaction between them in unbalanced designs. An unbalanced design occurs when the number of observations per treatment combination are not equal or when there are missing cells.

The interaction term in the model tests the hypothesis that the effect of reperfusion does not depend on group and vice versa. If the interaction term is significant this means that the effect of reperfusion depends upon which group the animal is assigned.

2.2.3 Histopathological Assessment of Tissue Damage

Each section of skeletal muscle from left and right legs, lung and kidney tissue was initially placed in 10% neutral buffered formalin. The tissue was then processed into paraffin blocks by the Histology Department, The Queen Elizabeth Hospital, using standard techniques. A selection of the blocks were chosen and prepared into haematoxylin and eosin slides by the Histology Department, The Queen Elizabeth Hospital.

The tissues were then assessed in two ways. Firstly, a qualitative review of the level of damage that occurred in the all tissues was performed. This was done in the all the different sham-operated and ischaemia/reperfusion groups.

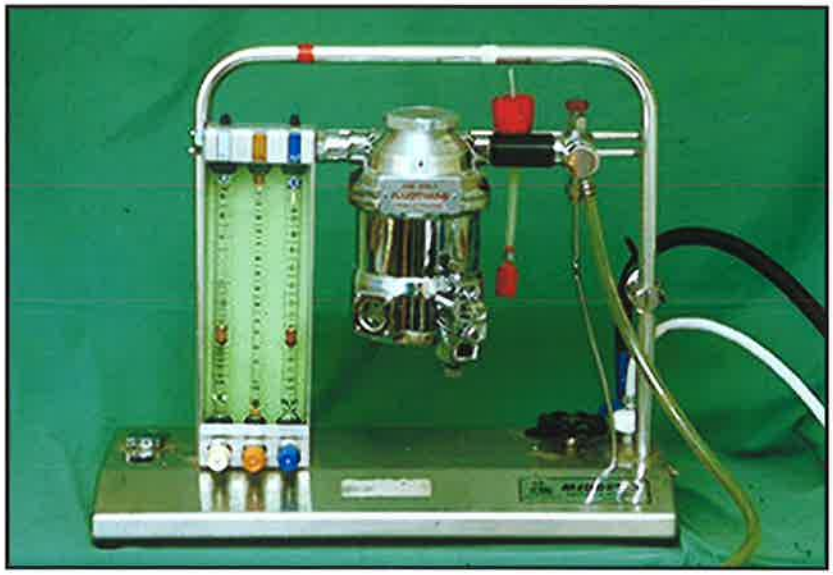
Secondly, an independent observer (Ms S. Millard, Department of Surgery, The Queen Elizabeth Hospital) performed a quantitative analysis of level of damage. This second analysis was only performed on animals that were sacrificed at the 24 hour time point, omitting the

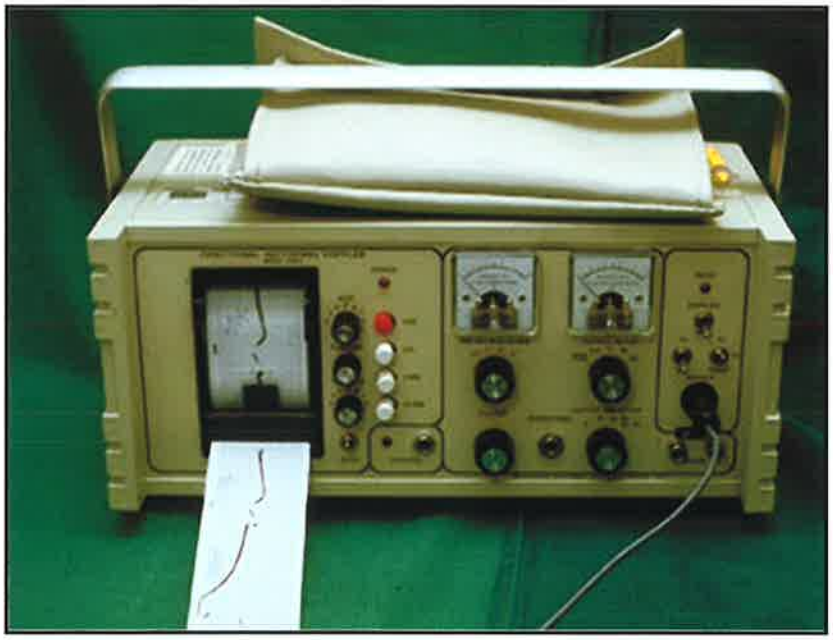
analysis of the right limb. In this study, Carter's scoring system for histopathological changes to skeletal muscle during ischaemia/reperfusion was used[479]. This scoring system was adapted for damage in lung and kidney, in conjunction with Dr Michael Texler, Histopathology Department, The Queen Elizabeth Hospital. The details of these scoring systems are outlined in Table III, Table IV and Table V. The schemata of these tables were given to an independent observer, who then analysed the slides. The observer was blinded to the study group from which each individual slide was produced.

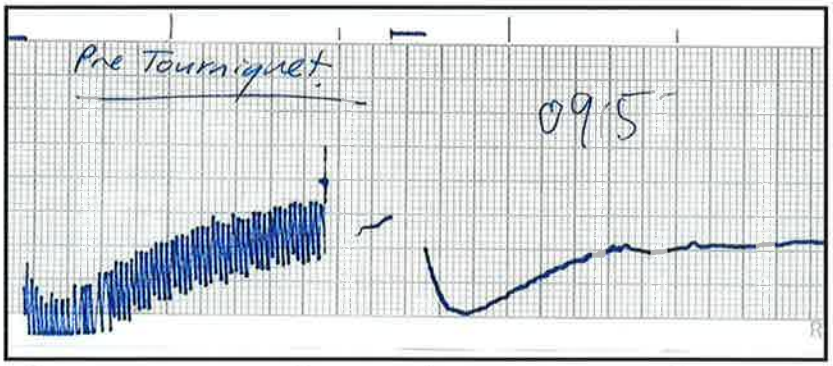
Figure 2: Anaesthetic Machine used for all rats.

Figure 3: Doppler machine used for monitoring femoral arterial blood flow.

Figure 4: Photoplethysmography traces before tourniquet placement and immediately after application of tourniquet.







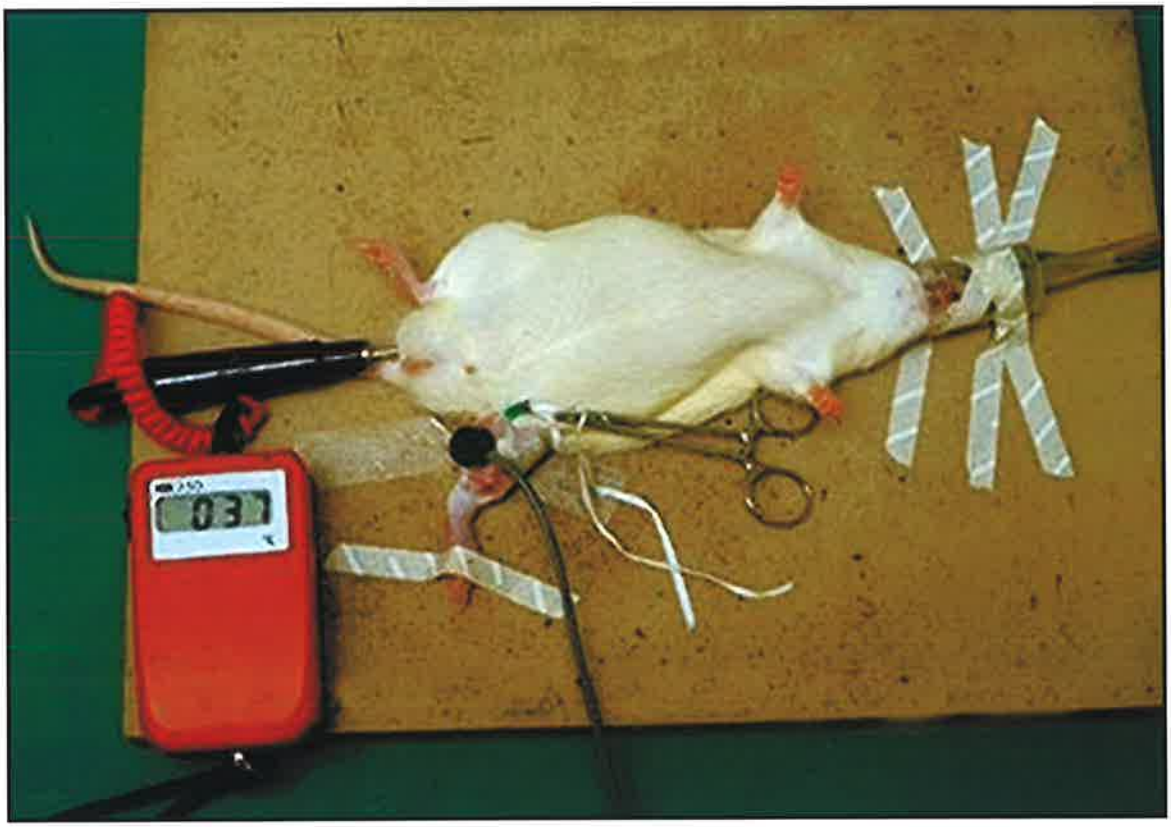
No-tourniquet

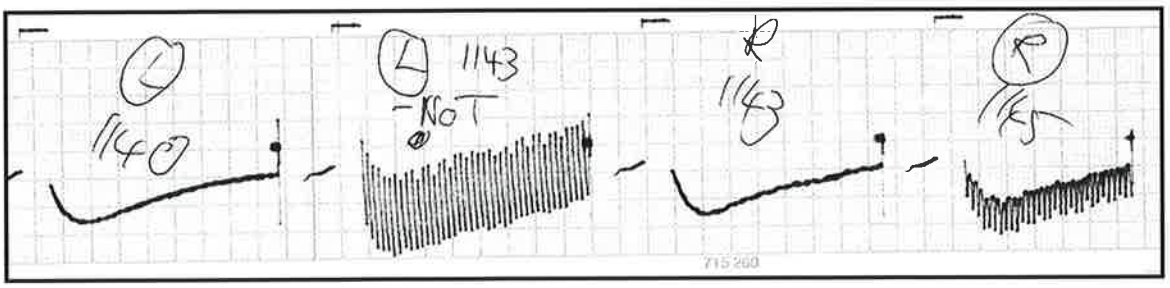
Tourniquet applied

Figure 5: Rat showing anaesthetic, tourniquet and rectal temperature probe.

Figure 6: Photoplethysmography traces immediately before the removal of the tourniquet and after tourniquet release for left and right legs on a rat that underwent bilateral limb ischaemia.

Figure 7: Muscles of Lower Limb of rat, medial aspect, showing Tissue slices used for analysis[480].





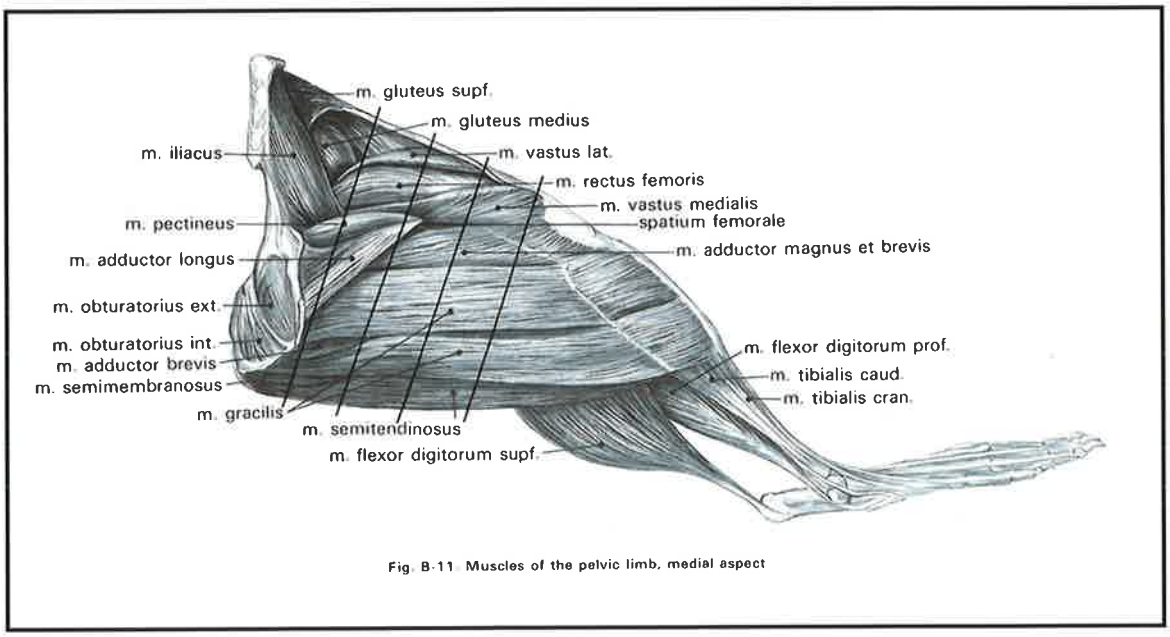


Fig. B-11. Muscles of the pelvic limb, medial aspect

Table II: Distribution of All Rats Used for experiments

Group	Duration (Hours)			Rat numbers/group
	Anaesthesia	Ischaemia	Reperfusion	
Sham-operated	4	nil	nil	5
Sham-operated	4	nil	4	5
Sham-operated	4	nil	24	5
Sham-operated	4	nil	72	5
Unilateral Ischaemia	4	4	nil	5
Unilateral Ischaemia	4	4	24	5
Unilateral Ischaemia	4	4	72	5
Bilateral Ischaemia	4	4	nil	5
Bilateral Ischaemia	4	4	4	5
Bilateral Ischaemia	4	4	24	5
Bilateral Ischaemia	4	4	72	5
Bilateral Ischaemia + Low Dose[1] Doxycycline	4	4	24	5
Bilateral Ischaemia + High Dose[2] Doxycycline	4	4	24	5
Other[3]				4
Died During Anaesthesia – 4 sham-operated, 1 unilateral and 3 bilateral ischaemia				8
Total number of rats				77

[1] Low Dose Doxycycline was defined as 50 mg/kg twice a day.

[2] High Dose Doxycycline was defined as 200 mg/kg twice a day.

[3] Included two rats used for mastering the animal model technique, one extra 4 hour unilateral ischaemia/4 hour reperfusion rat and one rat sacrificed outright without preceding four-hour anaesthetic.

Table III: Scoring system to quantitate the degree of histopathological damage in skeletal muscle following skeletal muscle ischaemia/reperfusion injury, based on Carter et al[479].

PMN = polymorphonuclear cells.

Numerical score	Skeletal Muscle Histopathology
0	No abnormal findings - cigar shaped nuclei, muscle cross striations complete, no cellular infiltrate
1	Mild focal swollen muscle nuclei, mild localized mononuclear cell infiltration, muscle striations complete
2	Mild multifocal swollen muscle nuclei, mild multifocal mononuclear cell infiltration, muscle striations complete
3	Moderate generalized swollen muscle nuclei, moderate mononuclear cell infiltration, rare loss of banding/cross striations
4	Moderate cell infiltration including polymorphonuclear cells (PMN), mild loss of banding/cross striations and mild multifocal fibre necrosis
5	Marked cell infiltration including PMN, moderate loss of banding/cross striations and moderate multifocal fibre necrosis
6	Marked cell infiltration including PMN, marked loss of banding/cross striations and moderate generalized fibre necrosis
7	Severe cell infiltration including PMN, marked multifocal fibre necrosis
8	Severe cell infiltration, haemorrhage possible with severe fibre necrosis
9	Massive cell infiltration, haemorrhage possible with severe generalized fibre necrosis
10	Massive cell infiltration and complete loss of tissue architecture

Table IV: Scoring system to quantitate the degree of histopathological damage for lung tissue, following skeletal muscle ischaemia/reperfusion injury, adapted from Carter et al[479].

PMN = polymorphonuclear cells.

Numerical score	Lung Histopathology
0	No abnormal findings - no cellular infiltrate, no collapse, no consolidation
1	Mild localized mononuclear cell infiltration, no collapse, no consolidation
2	Mild multifocal mononuclear cell infiltration, occasional areas of collapse
3	Moderate mononuclear cell infiltration, focal areas of collapse
4	Moderate cell infiltration including PMN, multifocal areas of collapse and mild vessel congestion
5	Marked cell infiltration including PMN, generalized areas of collapse and moderate vessel congestion
6	Marked cell infiltration including PMN, marked collapse and consolidation and marked vessel congestion
7	Severe cell infiltration including PMN, severe collapse and consolidation and severe vessel congestion
8	Severe cell infiltration, haemorrhage possible with severe alveoli necrosis
9	Massive cell infiltration, haemorrhage possible with severe generalized alveoli necrosis
10	Massive cell infiltration and complete loss of tissue architecture

Table V: Scoring system to quantitate the degree of histopathological damage for renal tissue following skeletal muscle ischaemia/reperfusion injury, adapted from Carter et al[479].

PMN = polymorphonuclear cells.

Numerical score	Kidney Histopathology
0	No abnormal findings - no cellular infiltrate, normal glomeruli and tubules
1	Mild localized mononuclear cell infiltration, normal glomeruli and tubules
2	Mild multifocal mononuclear cell infiltration, occasional congested/swollen tubule
3	Moderate mononuclear cell infiltration, focal areas of congestion and swollen tubules
4	Moderate cell infiltration including PMN, multifocal areas of tubule congestion and mild glomeruli congestion
5	Marked cell infiltration including PMN, generalized areas of tubule congestion and moderate glomeruli congestion
6	Marked cell infiltration including PMN, marked tubule congestion and marked glomeruli congestion
7	Severe cell infiltration including PMN, severe tubule congestion and marked glomeruli congestion, focal nuclear dropout
8	Severe cell infiltration, haemorrhage possible with severe tubule and glomeruli necrosis, moderate nuclear dropout
9	Massive cell infiltration, haemorrhage possible with severe generalized tubule and glomeruli necrosis, severe nuclear dropout
10	Massive cell infiltration and complete loss of tissue architecture

2.3 Results

2.3.1 Animal Model and Utilization

Out of a total of 76 rats given anaesthesia, 8 (10.5%) died during the anaesthetic. All rats that died during the experiments died during the 4 hour anaesthetic. Of these rats, 4 (50%) were sham-operated animals, 1 (12.5%) underwent unilateral lower limb ischaemia and 3 (37.5%) underwent bilateral lower limb ischaemia. No rat inadvertently died during the reperfusion period. There was no evidence that the rat was under any stress or duress following reperfusion, eating and drinking as normal.

2.3.2 Quantitation of Lung Oedema

The results of the rat wet/dry weight ratios are expressed in tabulated form Table VI. There was no significant difference in wet/dry weight seen throughout the groups.

Statistical analysis of Wet/Dry Weight Ratios in Lung Tissue

The overall F test was not significant (F=0.88, P=0.5570) indicating that there is no evidence that the means for the 11 cells are different. Each cell consists of the 5 individual animals in each treatment group.

The type III Sums of squares are preferred in testing effects in unbalanced designs because they test a function of the underlying parameters that is independent of the number of observations per treatment combinations.

Since the interaction term is not significant, it is valid to look at the main effects of group (sham-operated, unilateral and bilateral groups) and reperfusion duration (F=1.35, P=0.2604). This means that the effect of group does not depend on the level of reperfusion and vice versa.

The main effect of the group is not significant (F=0.05, P=0.9478). This is the comparison between sham-operated, unilateral and bilateral ischaemia groups. The main effect of reperfusion duration is also not significant (F=0.65, P=0.5889).

Table VI: Wet/Dry Weight Ratios in Lung Tissue

Ratios of wet weight to dry weight of lung tissue in individual rats and the average ratios for the five rats in each group are shown. The average was calculated as the arithmetic mean.

	Sham-operated – no ischaemia	4 hours of Unilateral Ischaemia	4 hours of Bilateral Ischaemia
Sacrificed at end of four hour anaesthetic	4.45	5.34	4.32
	4.51	4.92	4.43
	4.6	4.42	4.27
	3.91	4.51	4.32
	4.64	4.41	4.91
Mean	**4.422**	**4.720**	**4.450**
Sacrificed 4 hours after end of anaesthetic	3.55	Not Done	4.29
	4.32		4.49
	4.66		4.61
	4.34		4.67
	4.7		4.31
Mean	**4.314**		**4.474**
Sacrificed 24 hours after end of anaesthetic	4.28	4	4.76
	4.52	4.5	4.51
	3.42	4.35	4.7
	4.98	3.6	4.63
	5.06	4.44	4.69
Mean	**4.452**	**4.178**	**4.658**
Sacrificed 72 hours after end of anaesthetic	3.94	4.1	4.42
	4.22	4.79	4.51
	4.54	4.82	4.46
	4.52	3.65	3.51
	4.89	4.58	4.04
Mean	**4.422**	**4.388**	**4.191**

2.3.3 Histopathological Assessment of Tissue Damage

2.3.3.1 Qualitative Analysis

The following four figures (Figure 8, Figure 9, Figure 10 and Figure 11) demonstrate representative examples of the histological changes that occur during reperfusion injury. Each figure shows sham-operated, unilateral and bilateral ischaemic tissue at 0 hours (sham-operated is 4 hours of anaesthesia only; unilateral and bilateral 4 hours of ischaemia and no reperfusion), 4 hours, 24 hours and 72 hours.

Left leg Skeletal Muscle

(Figure 8)

The sham-operated animals had minimal pathological changes seen across all time points. The striated muscle fibres are well preserved, with no oedema or cellular infiltration. In both the unilaterally and bilaterally ischaemic animals sacrificed immediately after the anaesthetic, there was loss of cross striations and banding within the striated muscle, with these effects being due to ischaemia alone. However, as the time period of reperfusion increased, there was increasing striated muscle destruction, oedema and cellular infiltration. These effects were more marked in the bilaterally ischaemic group compared to the unilaterally ischaemic groups.

Right leg Skeletal Muscle

(Figure 9)

The sham-operated animals had minimal pathological changes seen across all time points. In the unilaterally ischaemic animals, the left leg was rendered ischaemic and the right leg was not ischaemic. Again, there were minimal pathological changes seen across all time points in this contralateral limb. In the bilaterally ischaemic animals, the images show infiltration with polymorphonuclear cells, loss of muscle fibre banding and striations and moderate fibre necrosis. There was minor damage seen in the bilaterally ischaemic animal sacrificed immediately after anaesthetic with the level cellular infiltration and tissue destruction increasing after 4 hours reperfusion, 24 hours of reperfusion and becoming marked after 72 hours of reperfusion.

Lung

(Figure 10)

Throughout the images at all time points, there was some cellular infiltration and focal areas of collapse. There were minimal differences seen in these representative images between sham-operated, unilaterally ischaemic and bilaterally ischaemic animals.

Kidney

(Figure 11)

The sham-operated animals showed preservation of renal tissue with normal renal glomeruli and minimal cellular invasion. Similar preservation was seen in both the unilateral and bilateral ischaemic animals sacrificed immediately after the four-hour anaesthetic, indicating minimal renal damage due to the effects of ischaemia alone. However, upon reperfusion at 4, 24 and 72 hours with the unilateral and bilateral ischaemic animals, there was evidence of oedema, haemorrhage and congestion of glomeruli.

2.3.3.2 Quantitative Analysis

The results are shown in Table VII. The histopathological assessment of tissue damage data was ranked and analysed using analysis of variance, as shown in Table VIII.

Left Leg Skeletal Muscle

For the statistical analysis, the left leg score for rat 56 in bilateral group was excluded as an outlier, as statistically it was so far out of the range within that group that it was considered that there was a technical error in processing. There was a significant difference between the histopathological scores for the sham-operated and the bilaterally ischaemic animals ($P=0.0420$). The median score for sham-operated animals was 0; indicating no abnormal findings, with cigar shaped nuclei, muscle cross striations complete and no cellular infiltrate. In the animals subjected to bilateral limb ischaemia, the median score for the left leg skeletal muscle was 6.5. As there was an even number of rats in this group, the median was calculated as the average of the two numbers in the middle. This score of 6.5 indicates marked to severe cellular infiltration including polymorphonuclear cells, marked loss of banding/cross striations and moderate to marked multifocal fibre necrosis.

Lung

There was no significant difference seen in the histopathological analysis between sham-operated and animals subjected to bilateral limb ischaemia. The median score for sham-operated animals was 2 and for bilateral ischaemic animals was 3. This correlates to mild to moderate mononuclear cell infiltration with occasional to focal areas of collapse.

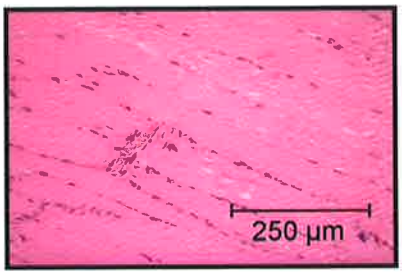
Kidney

There was a significant difference between the histopathological scores for sham-operated and bilaterally ischaemic animals (P=0.0276). The median score for sham-operated animals was 1; indicating mild localized mononuclear cell infiltration with normal glomeruli and tubules. For the bilaterally ischaemic animals, the median score was 3; indicating moderate mononuclear cell infiltration, focal areas of congestion and swollen tubules.

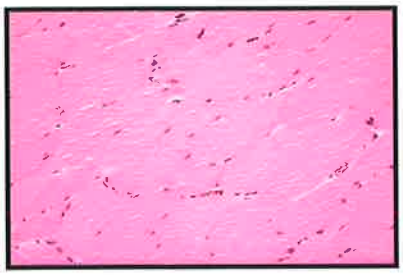
Figure 8: Representative Images of Left leg Skeletal muscle Histopathology.

Sham refers to an animal that underwent a 4 hour anaesthetic and no ischaemia. Unilateral refers to unilateral left leg skeletal muscle for 4 hours. Bilateral refers to bilateral lower limb ischaemia. 0 hours, indicates that the rat was sacrificed immediately at the end of the four hour anaesthetic/ischaemia period. Four, 24 or 72 hours implies the animal was sacrificed at that duration after the anaesthetic/ischaemia was completed.

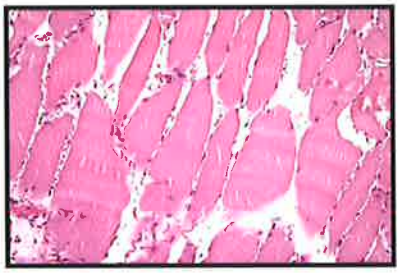
Paraffin blocks, Haematoxylin and Eosin Stain, Magnification 20X.



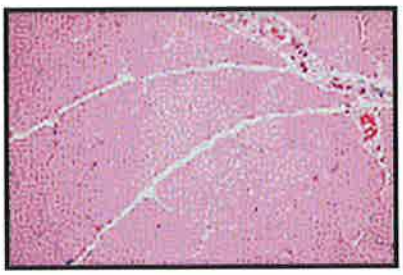
Sham – 0 hours



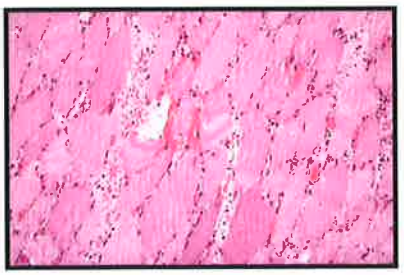
Unilateral – 0 hours



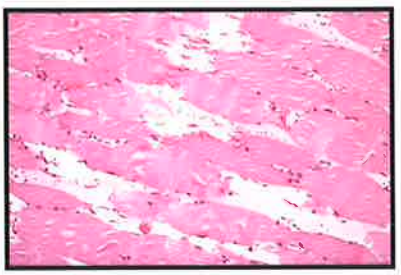
Bilateral – 0 hours



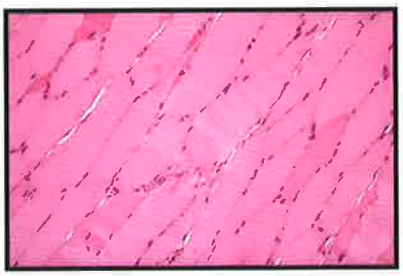
Sham – 4 hours



Unilateral – 4 hours

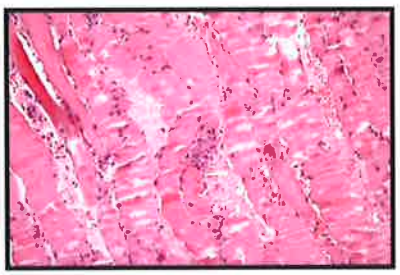


Bilateral – 4 hours

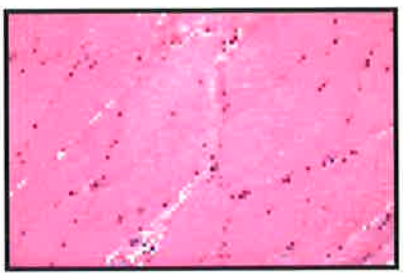


Sham – 24 hours

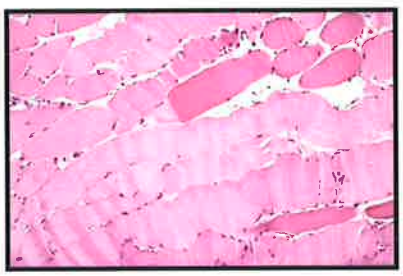
Unilateral – 24 hours



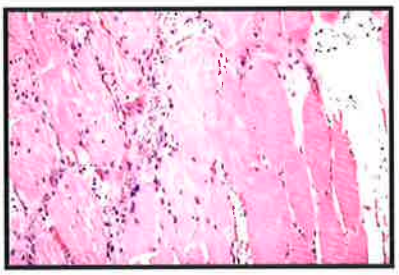
Bilateral – 24 hours



Sham – 72 hours



Unilateral – 72 hours

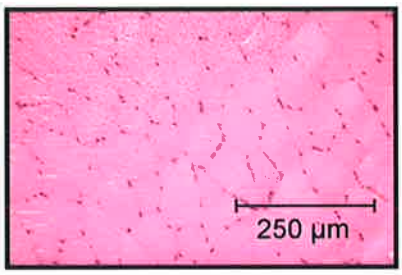


Bilateral – 72 hours

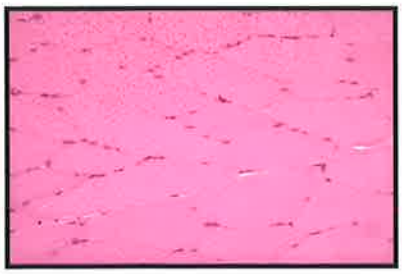
Figure 9: Representative Images of Right leg Skeletal muscle Histopathology.

Sham refers to an animal that underwent a 4 hour anaesthetic and no ischaemia. Unilateral refers to unilateral left leg skeletal muscle for 4 hours, so in this case the images shown are of the contralateral limb. Bilateral refers to bilateral lower limb ischaemia. 0 hours, indicates that the rat was sacrificed immediately at the end of the four hour anaesthetic/ischaemia period. Four, 24 or 72 hours implies the animal was sacrificed at that duration after the anaesthetic/ischaemia was completed.

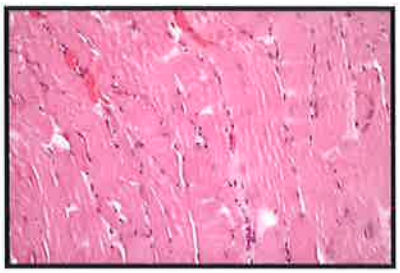
Paraffin blocks, Haematoxylin and Eosin Stain, Magnification 20X.



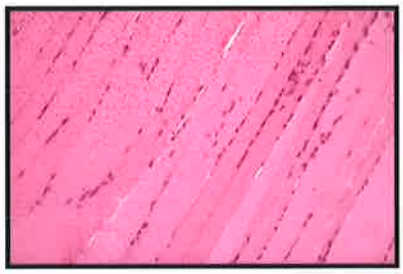
Sham – 0 hours



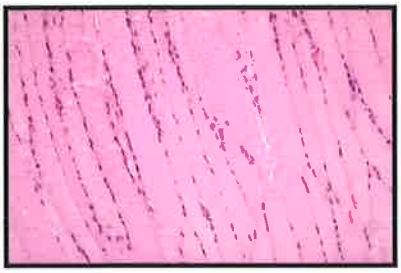
Unilateral – 0 hours



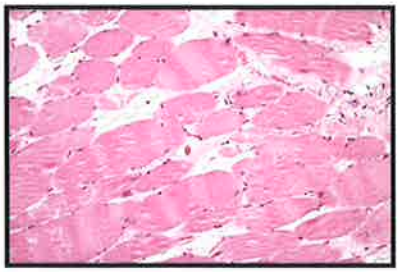
Bilateral – 0 hours



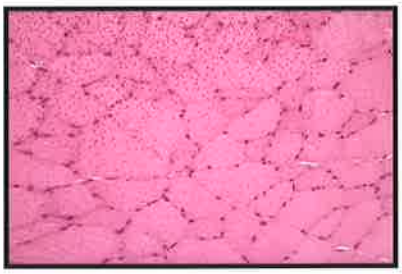
Sham – 4 hours



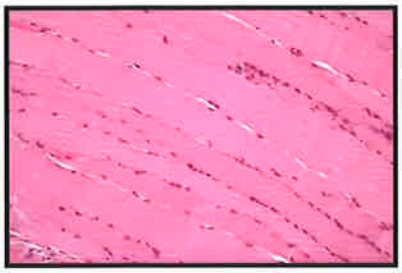
Unilateral – 4 hours



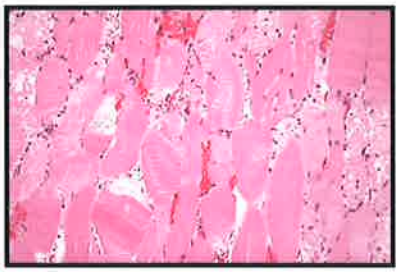
Bilateral – 4 hours



Sham – 24 hours

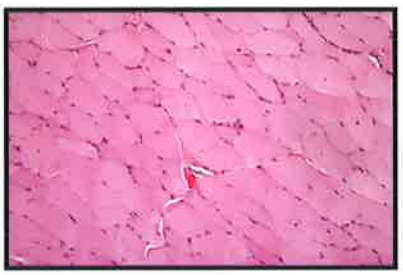


Unilateral – 24 hours

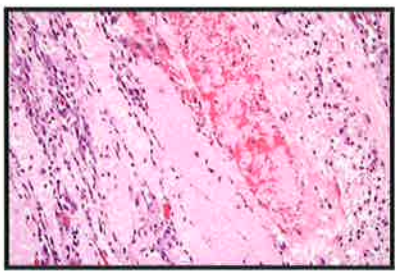


Bilateral – 24 hours

Sham – 72 hours



Unilateral – 72 hours

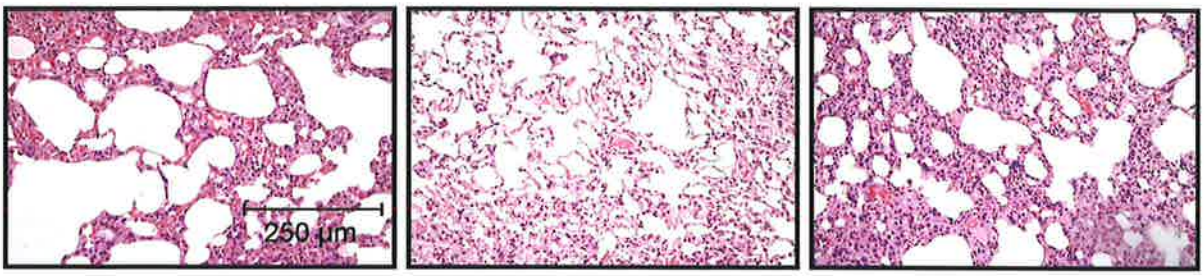


Bilateral – 72 hours

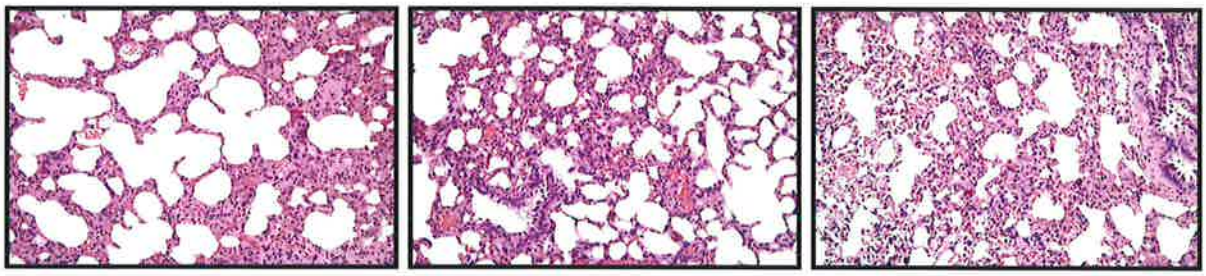
Figure 10: Representative Images of Lung Tissue Histopathology.

Sham refers to an animal that underwent a 4 hour anaesthetic and no ischaemia. Unilateral refers to unilateral left leg skeletal muscle for 4 hours. Bilateral refers to bilateral lower limb ischaemia. 0 hours, indicates that the rat was sacrificed immediately at the end of the four hour anaesthetic/ischaemia period. Four, 24 or 72 hours implies the animal was sacrificed at that duration after the anaesthetic/ischaemia was completed.

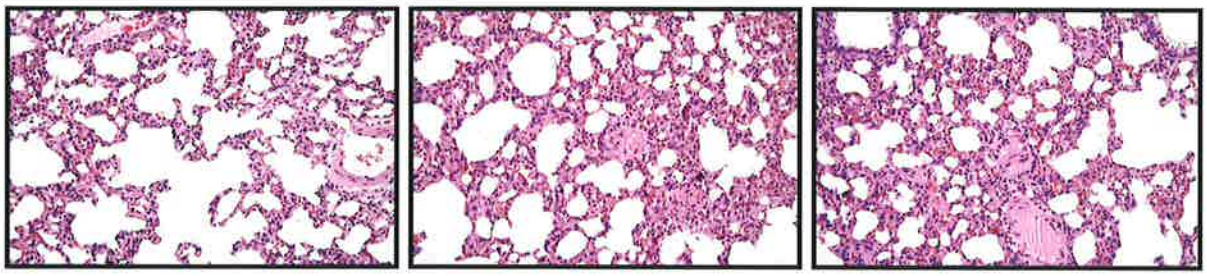
Paraffin blocks, Haematoxylin and Eosin Stain, Magnification 20X.



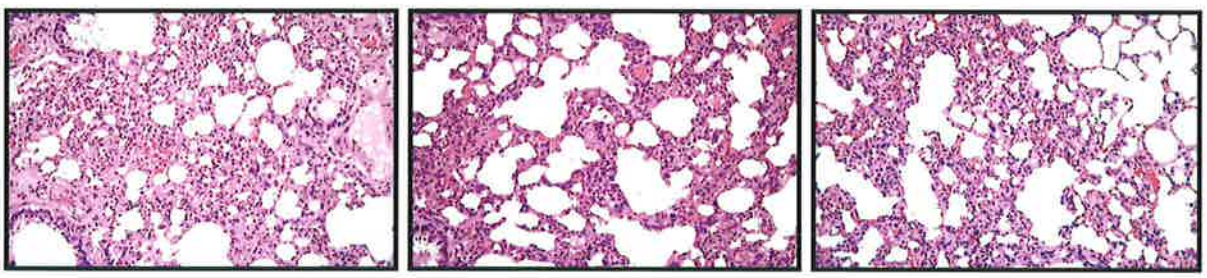
Sham – 0 hours | Unilateral – 0 hours | Bilateral – 0 hours



Sham – 4 hours | Unilateral – 4 hours | Bilateral – 4 hours



Sham – 24 hours | Unilateral – 24 hours | Bilateral – 24 hours

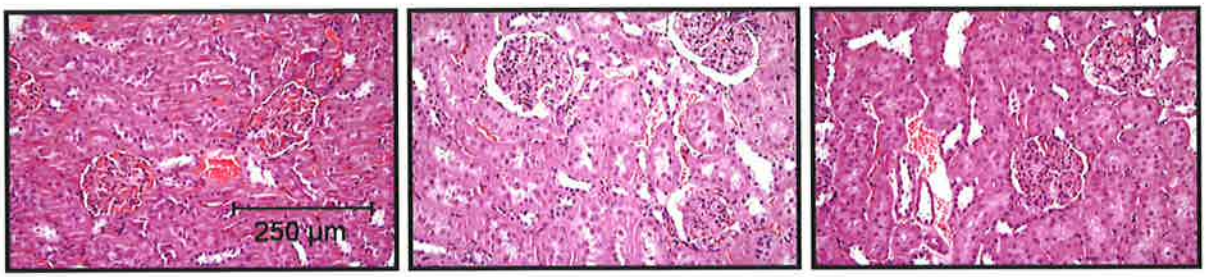


Sham – 72 hours | Unilateral – 72 hours | Bilateral – 72 hours

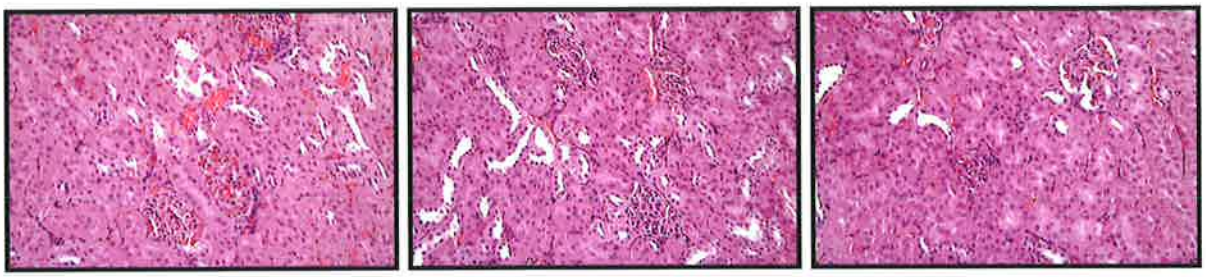
Figure 11: Representative Images of Renal Tissue Histopathology.

Sham refers to an animal that underwent a 4 hour anaesthetic and no ischaemia. Unilateral refers to unilateral left leg skeletal muscle for 4 hours. Bilateral refers to bilateral lower limb ischaemia. 0 hours, indicates that the rat was sacrificed immediately at the end of the four hour anaesthetic/ischaemia period. Four, 24 or 72 hours implies the animal was sacrificed at that duration after the anaesthetic/ischaemia was completed.

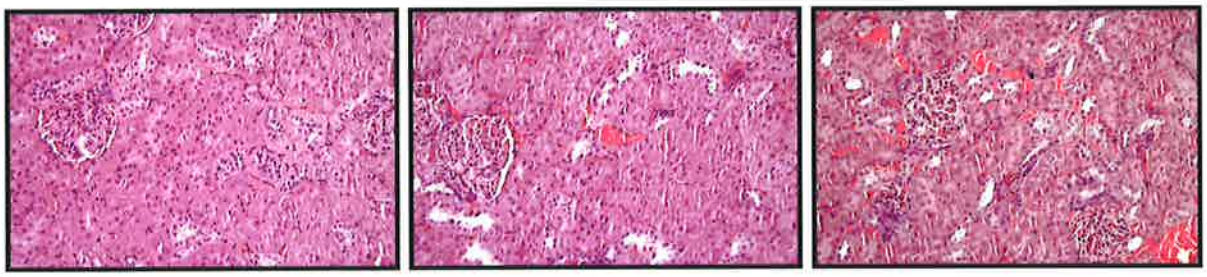
Paraffin blocks, Haematoxylin and Eosin Stain, Magnification 20X.



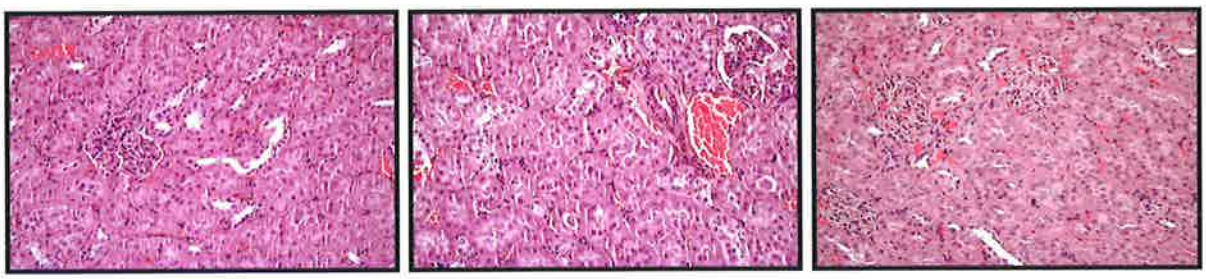
Sham – 0 hours | Unilateral – 0 hours | Bilateral – 0 hours



Sham – 4 hours | Unilateral – 4 hours | Bilateral – 4 hours



Sham – 24 hours | Unilateral – 24 hours | Bilateral – 24 hours



Sham – 72 hours | Unilateral – 72 hours | Bilateral – 72 hours

Table VII: Histopathological Score of Level of Tissue Damage, based on Carter et al.

RAT GROUP	RAT Number	Left leg Skeletal muscle	Lung	Kidney
SHAM-OPERATED – sacrificed at 24 hours	19	0	2	1
	21	0	3	1
	33	0	3	0
	34	0	2	0
	36	0	2	1
BILATERAL ISCHAEMIA – 24 hours reperfusion	52	4	4	3
	53	6	3	3
	54	7	3	3
	56	0	3	3
	60	7	2	3

Table VIII: Statistical Analysis of Histopathological Scoring.

	Sham-operated, sacrificed at 24 hours (median)	Bilaterally ischaemic, reperfusion for 24 hours (median)	P-value	Difference between Means	Simultaneous 95% confidence Limits ***P<0.05
Left leg skeletal muscle	0	6.5	0.0420	9.750	3.769 - 15.731 ***
Lung	2	3	0.1669		
Kidney	1	3	0.0276	16.200	12.267 - 20.133 ***

2.4 Discussion

2.4.1 Animal Model and Utilization

For the investigation of skeletal muscle ischaemia/reperfusion using an in vivo model, at least 4 different methods are reported in the literature. These include; a tourniquet model (thigh or forearm) with interruption of both arterial and venous blood flow; vascular pedicle occlusion in an isolated in vivo or ex vivo single skeletal muscle (canine gracilis, rat cremaster); in situ occlusion of arterial inflow, with maintenance of venous outflow and intravital microscopy of thin striated muscles prior to and following complete interruption of blood flow.

Many authors have used a tourniquet model of ischaemia/reperfusion injury[9,202,481-483]. These methods commonly apply a rubber band or tape tourniquet around the level of greater trochanter of either the rat or mouse. The advantages of this method are that it was easy to learn and establish and was readily reproducible. It also includes muscles of each fibre type in the ischaemic area and thus the overall effect of a mixture of fibre types was included in the element of reperfusion, hence making this model akin to the human situation of ischaemic limbs arising due to trauma, embolism or thrombosis. This model differs from the isolated canine model in that the rat hindquarter contains detectable levels of xanthine oxidase, an important contributor to the events of ischaemia/reperfusion[484]. Tourniquets essentially model complete ischaemia; however, they do not occlude bone blood flow, although this has been shown to be less than 1 percent of normal blood flow in primate limbs[485]. Tourniquets have the significant disadvantage of inflicting a crush artefact on underlying muscle and nerve, with myocytes directly beneath the tourniquet showing greater damage than do distal cells[486,487]. The experiments in this study aimed to avoid the effects of crush artefact by dissecting tissue at a distance from this area. The tourniquet model also renders the entire limb ischaemic, not just the skeletal muscle, hence the ischaemic skin and subcutaneous tissue is also reperfused with removal of the tourniquet. Although these tissues are remarkably resistant to ischaemia in comparison to skeletal muscle, blood flow to skin during the reactive hyperaemic response may actually divert blood flow from skeletal muscle[487].

Vascular pedicle techniques have also been used extensively. These techniques include the use of in vivo canine gracilis muscle[290,488,489], ex vivo canine muscle[484], rectus abdominus muscle[490], spinotrapezius[491,492], rectus femoris[86] and cremaster muscles[493,494].

The ex vivo technique using canine gracilis has many advantages; including proximity of the muscle to the skin for ease of excision and an isolated set of vessels supplying the muscle allows for control of perfusion. Although demanding to prepare and maintain in stable states, this model has advantages for certain kinds of studies[64]. Extracorporeal perfusion allows varying degrees of ischaemia to be established. However, as the muscle is isolated from the systemic circulation it is not under the influence of the overall treatment-induced systemic cardiovascular changes that occur in the tourniquet model. The systemic changes that occur in the tourniquet model can have further effects on the skeletal muscle and on remote organs. However, the ex vivo model can be used to selectively modify the blood constituents of leukocytes, platelets, complement and fibrin to investigate their individual effects on the skeletal muscle ischaemia/reperfusion process. In addition, the blood volume is small within this closed system, which allows for potential therapeutic interventions of agents that may be in scarce supply or expensive. A further problem with this model is that exposure of blood to the extracorporeal circuitry leads to complement and neutrophil activation, which may in turn induce microvascular injury.

The isolated in vivo muscle experimental models involve isolating the selected muscle within the animal and measuring a variety of components of the muscle during both the ischaemia and reperfusion phases. Many of these utilize the canine gracilis muscle, again for ease of surgical preparation and repeated access for sequential tissue biopsies can be performed[488,495]. Unlike the tourniquet model, compartment syndromes due to oedema formation may have less pathogenic influences in this model since during its preparation fascial planes are opened[60].

In situ occlusion of arterial flow with maintenance of venous outflow has also been extensively utilized in the investigation of skeletal muscle ischaemia/reperfusion injury[479,496]. This model involves surgical isolation of arterial vessels to the limbs and is hence more technically demanding than a tourniquet model. However, it does closely resemble that of human clinical ischaemia arising from thrombotic or embolic occlusions to the arterial vessels or that of aortic surgery. This model has the potential advantage of studying the effect of partial ischaemia, which may be more likely to exhibit prolonged abnormalities during recovery than muscle rendered completely ischaemic by total arterial inflow occlusion for an equivalent period[497-499]. These models are also attractive because collateral perfusion and venous drainage are preserved, however quantifying the variability can be difficult because of differences in the level of occlusion. The extent of ischaemia after clamping the aorta is not

uniform with wide variability in the anatomy of pelvic collaterals among species[500]. This makes the use of this model less valid for the experiments used in the current studies. The second major disadvantage of this model is that placement and removal of the aortic clamp may have profound haemodynamic consequences independent of any effects on skeletal muscle[501,502].

Intravital microscopy is used to directly examine the microcirculation, providing the opportunity to quantify both the temporal and spatial changes in microcirculatory flow[503]. Microscopic images are taken of skin or striated muscle under conditions of ischaemia or reperfusion using tissue staining or enhancing techniques to study specific components of the circulation[504]. This technique can be used to evaluate microvascular perfusion, vessel diameter, red blood cell velocity, macromolecular leakage, polymorphonuclear cell–endothelial reactions and functional capillary density[504]. Two examples of muscles used include extensor digitorum longus[505] or cremaster muscles[506]. Intravital microscopy is the only method for analysing skin and striated muscle microcirculation that allows direct visualization of all individual segments in the musculature[507]. An advantage of this method is that the exact site of permeability change in the microcirculation can be identified and the time frame of transient changes can be established[487]. The analysis of the microcirculation using these techniques allows the study of skeletal muscle ischaemia/reperfusion in reconstructive microsurgery.

A four-hour time course of ischaemia was chosen for the current study. The length of ischaemia must be sufficiently long to produce an ischaemic injury followed by reperfusion changes. This is counter balanced by long ischaemic periods causing marked necrosis of muscle and possible death of the animal upon reperfusion. The four-hour time point has been previously established in our laboratory as a sufficient length of time to create histological damage to the skeletal muscle[508]. After two hours of skeletal muscle ischaemia, there is a rapid restoration of ATP following 15 minutes of reperfusion, with creatine phosphate levels reaching normal levels within 30 minutes[509]. The ultrastructure of muscle after 2 hours of ischaemia was normal, apart from dilatation of the sarcoplasmic reticulum and T tubules[509]. Muscles exposed to 3, 4 and 5 hours of ischaemia develop 2 ± 0.9%, 30.3 ± 6% and 90.1 ± 3.5% necrosis, respectively as measured by technetium pyrophosphate uptake and nitroblue tetrazolium staining[17]. It is apparent from this study and others[510,511], that ischaemia longer than 5 hours produces a large percentage of tissue necrosis leading to metabolically non-functioning muscle. After 7 hours of ischaemia, the metabolic enzymes and organelles are not

capable of creatine phosphate or ATP synthesis leading to irreversible metabolic failure[509]. Hence, a four-hour time point was used to achieve the balance of sufficient ischaemic damage to achieve reperfusion effects versus overwhelming muscle necrosis.

The rat experiments were initially performed on the sham-operated and unilateral animals in random order. The experiments using bilaterally treated animals were performed secondarily for reasons outlined below. The interim results of the sham-operated and unilateral ischaemic rats showed no significant damage occurred in the wet/dry weight lungs so the decision was made to proceed with bilaterally ischaemic animals. Ultimately, the aims of these experiments were to show a quantifiable effect with the treatment used for reperfusion, hence, it was important to have a demonstrable tissue damage effect on the initial animals After reviewing the literature regarding rats subjected to bilateral lower limb ischaemia, there was only one article that quoted a mortality rate. Using bilateral limb ischaemia, they observed 30% mortality rate at 12 hours and 50% mortality rate at 24 hours (n=10). They did not divulge more information about at which stage the rats died and the sample size was small[19]. Following further application to The North Western Adelaide Health Service Animal Ethics Committee and The University of Adelaide Animal Ethics Committee, experimentation was allowed to proceed with the initial five animals to a four-hour duration of reperfusion only. An interim report was then generated to the Ethics Committees stating that only one out of the initial six animals died. The initial death with bilateral ischaemia occurred under the anaesthetic and was thought to be anaesthetic related and not due to reperfusion. The Ethics Committees allowed further work to proceed to the 24 hour reperfusion period and then ultimately to the 72 hour reperfusion time point. The overall mortality rate was 10.5% during the current studies, which were all anaesthesia related and this mortality rate is within acceptable limits for similar procedures for ischaemia/reperfusion experiments[19,202].

The time points for sacrifice of the animal were ischaemia only, reperfusion at 4 hours, 24 hours or 72 hours. At the completion of the 4 hour anaesthetic, rats were sacrificed to establish the level of tissue damage due to ischaemia and/or anaesthesia alone. At four hours, many of the early events of reperfusion have occurred, with further effects seen at 24 hours. Seventy two hours was chosen as the time point when most of the initial events of reperfusion had occurred and thus would simulate a clinically relevant endpoint.

The establishment of ischaemia was monitored via use of a photoplethysmography probe over the femoral artery. This probe uses an infrared transducer to beam light through the epidermis

to the subdermal pool of vessels and a proportion of this light is reflected back. Blood is more opaque to red light than the other components of the skin and subcutaneous tissue, so the absorption of light is affected by the amount of blood under the source beam. The output of the probe varies with the blood content of the subdermal capillary bed. When the probe is used in alternating current coupling mode, it displays rapid changes and pulsatile characteristics. The pulsatile trace represents arterial flow and flat trace represents lack of arterial flow, hence an indication of femoral arterial and capillary flow is obtained. This gave an indication of flow to the limb and hence the flow to the actual skeletal muscle was inferred. However, the technique is limited to tissue depths of 1.5 mm and skeletal muscle perfusion per se was not measured[512].

Experimental techniques for measuring skeletal muscle perfusion; include plethysmography[513], electromagnetic probes[514], tracer washout techniques[515,516] and injection of radioactive microspheres[517]. The advantages of these techniques are that substantial methodological validation is available to document the level of perfusion and quantitation of perfusion is feasible. However, the disadvantages include requirement for technical expertise, use of expensive equipment and individual problems such as need for continuous calibration with electromagnetic flow probes and disposal of radioactive carcasses after injection with radioactive microspheres[487].

The choice of the animal model used in these studies was based on the following factors; ability of the process of injury and reperfusion to resemble that of a human model of embolism, thrombosis or trauma. The animals were capable of surviving for a period of extended reperfusion, the model was inexpensive and used on a readily available laboratory animal. The model can be easily taught for ongoing work in this field in our laboratory. The period of four hours of ischaemia has been previously established to cause definite histological injury[508]. The rat model utilized in these studies meets the above criteria and was used throughout these experiments.

2.4.2 Quantitation of Lung Oedema

Reperfusion following lower torso ischaemia in humans, leads to respiratory failure manifested by pulmonary hypertension, hypoxaemia and noncardiogenic pulmonary oedema. Pulmonary hypertension can be measured via pulmonary arterial pressure measurements; the

level of interstitial pulmonary oedema can be documented with roentgenograms and hypoxaemia can be directly measured in arterial blood gas samples.

In animal models, wet/dry weight ratios of lung tissue are used as an indicator of level of pulmonary oedema. This method has been used not only for lung but also for the skeletal muscle itself, liver and kidney in animal models of ischaemia /reperfusion[503,518,519].

The results showed no significance when the means of the wet/dry ratios of lungs in the five rats in each group were compared statistically. In this study, an inhalational anaesthetic technique was used. The effect of 4 hours of halothane, nitrous oxide and oxygen to the lung tissue causes an element of pulmonary damage in itself[520,521]. Lung histology reveals patchy atelectasis, dystelectasis and interstitial oedema after inhalational anaesthesia with halothane[521]. Hence, in this study, there was no difference between the sham-operated and the ischaemic-treated animals as the anaesthetic itself caused a level of lung damage that could not be differentiated from damage caused by reperfusion injury. The results may have reached significance if a large number of animals were used in each group, but as this was essentially a pilot study to validate the animal model and determine if this method was useful, we could not ethically justify using larger numbers of rats. The technique of wet/dry weights in skeletal muscle ischaemia/reperfusion tissues has also been shown by others to be relatively insensitive in small number groups[496]. Anner et al also found that despite the rise in mean pulmonary artery pressure and foci of proteinaceous exudate and polymorphonuclear cells seen in alveoli, they did not demonstrate a rise in wet/dry weight of lung tissue[32].

Other potential avenues could be used to show damage to the lungs caused by skeletal muscle reperfusion injury. The first is the use of a different type of anaesthetic, such as an intraperitoneal or intravenous technique, which would negate direct effects of the anaesthetic on the lungs. To maintain an intraperitoneal anaesthetic for four hours is technically more difficult given the resources for monitoring available in our laboratory, particularly to keep the level of consciousness titrated to an even level when comparing sham-operated and ischaemic animals. Hence, the established method of inhalational anaesthesia in our laboratory was chosen for these experiments.

Elevation of myeloperoxidase concentration in lungs has been interpreted as an indication of leukocyte accumulation and thus showing leukocyte mediated injury[35,52,263,518,522]. Myeloperoxidase is a haem-containing enzyme stored in the azurophilic granules of

neutrophils, constituting 5 percent of their dry weight. By comparing the result of myeloperoxidase levels in frozen lung tissue to known control standards, an estimation of myeloperoxidase and hence neutrophil activity is achieved. This is inherently different from showing the level of oedema as in wet/dry weight ratios of lung tissue but albeit it is another method of showing skeletal muscle ischaemia/reperfusion damage. Lung leukosequestration occurs progressively over several hours, an event associated with permeability oedema[523].

Increased lung permeability can also be shown with either lymph protein flux or tracer uptake techniques. Accurate interpretation of lymph data depends on achievement of steady state conditions because changes in lymphatic protein flux generally lag behind changes in transvascular protein flux[16,524,525]. In the current studies, it was not known if steady state conditions of lymphatic protein flux were achieved, hence this method was not utilized for investigating increased permeability. Tracer uptake techniques aim to demonstrate the clearance of a radiolabelled substance through the lungs. This is either measured in the lung tissue, residual values in the vascular space or demonstrated by bronchoalveolar lavage-blood ratios following an intravenous bolus of isotope[35,522].

Bronchoalveolar lavage techniques can also be used by measuring the protein and neutrophil counts, which are higher in the lungs of skeletal muscle ischaemia/reperfusion animals[518].

2.4.3 Histopathological Assessment of Tissue Damage

The quantitative analysis of histopathological damage was performed at 24 hour time point, as it is known that the morphological manifestations of the reperfusion injury generally peak within 24 hours[511]. The quantitative analysis was based on a method used by Carter et al[479] in which they developed a murine model of skeletal muscle reperfusion injury. They found good correlation between the level of histopathological skeletal muscle damage and clinical signs, creatinine phosphokinase and lactate dehydrogenase. They ranked the level of damage on a 0 to 10 scale encompassing muscle nuclei changes, level of cellular infiltration and level of skeletal muscle fibre necrosis. This method was adapted for use in pulmonary and renal tissue in these studies.

In the skeletal muscle, the damage that occurs with lower limb tourniquet ischaemia and reperfusion confirms that this experimental model is reproducible and valid for use in other tissue analyses. Others have shown that the level of histopathological damage seen in

haematoxylin and eosin staining is comparable to the level of tissue necrosis seen with nitroblue tetrazolium staining[526]. The oedema that is easily seen on haematoxylin and eosin staining has been confirmed to be due to increased permeability by using quantitative Evan's blue staining method[526]. The level of histopathologically discernible damage increased after revascularization, with minimal damage due to ischaemia alone, this effect has been confirmed to occur in animal models and in human tissue upon reperfusion[527-530]. There was an increased level of cellular infiltration that occurred with increasing reperfusion times. Knight et al showed pavementing of neutrophils in venules and small veins after thirty minutes of reperfusion and by eight hours, many neutrophils were present in extravascular tissues[527].

The lung tissue failed to show a significant difference in damage between sham-operated and bilaterally ischaemic animals. The most likely reasons for this lack of difference is that the inhalational anaesthetic over four hour causes significant damage in itself as discussed in detail in the wet/dry lung analysis (Section 2.4.2). No experiments were performed on animals using any other type of anaesthesia. In order to completely demonstrate the theory that lung damage is predominantly anaesthetic related, all experiments would need to be repeated using different anaesthesia, such as an intraperitoneal technique. Others have shown that with an intraperitoneal anaesthetic, there is histopathological damage in the lung with neutrophil sequestration and alveolar haemorrhage after hind-limb ischaemia/reperfusion that is greater than in the sham-operated animal[531]. The use of inhalational anaesthetic is one of the limitations of the animal model used in these experiments. It was not possible to discern damages secondary to the anaesthetic versus damages secondary to skeletal muscle ischaemia/reperfusion itself using techniques of wet/dry lungs or histopathology.

In the reperfused animals, the kidneys were damaged and this effect was seen to increase with increasing duration of reperfusion. Rhabdomyolysis of reperfused muscle releases myoglobin into the circulation that precipitates in the renal tubules and causes renal tubular necrosis[11]. There was significant renal oedema after skeletal muscle ischaemia/reperfusion injury seen in the histopathological slides, confirming the findings of other investigators[476,518]. Although there was haemorrhage within the renal tissue after skeletal muscle ischaemia/reperfusion injury, there was minimal increase in neutrophils seen in the histopathology slides. This finding has been confirmed in a rat model of supradiaphragmatic aortic cross clamping[532] and by failure of protection against renal ischaemia-reperfusion injury by introducing neutropenia[43]. The finding of evidence of endothelial injury in the absence of neutrophil

infiltration may imply that some other agent generated during reperfusion may be capable of producing endothelial injury independent of the neutrophil[518,533,534].

Other methods of quantifying levels of tissue damage after skeletal muscle ischaemia/reperfusion have been used. These include histopathological scales of damage, tracer techniques, different staining techniques such as that with nitroblue tetrazolium staining and measurement of various plasma and haematological factors. A semi-quantitative evaluation of presence of oedema, disruption of normal muscle architecture and individual myocyte detachment over a 0 to 3 scale has been used[535]. Although they showed significant damage between sham-operated and animals subjected to ischaemia/reperfusion, a more extensive scale was chosen for the current experiments, aiming to prove a difference between ischaemia/reperfusion animals and drug treated ischaemia/reperfusion animals. Extravasation of ^{125}I-albumin to assess hind limb vascular permeability index has been used to show the level of oedema in skeletal muscle[535] and in lung tissue[134,522]. Technetium 99m pyrophosphate has been shown to be effective in quantifying the level of histopathological skeletal muscle damage after ischaemia/reperfusion[495,536]. Skeletal muscle viability can be assessed by nitro blue tetrazolium staining[249,537], which can differentiate between viable and necrotic skeletal muscle and provides a reproducible method for estimating the extent of necrosis. Plasma urea nitrogen levels are significantly increased in skeletal muscle ischaemia/reperfusion mice compared to sham-operated mice[479], however, the urea nitrogen levels are influenced by changes in renal infiltration and dietary nitrogen and catabolism of tissues. Plasma creatine phosphokinase is a cytosolic enzyme found primarily in striated muscle and is used as an indicator of muscle damage, with levels correlating linearly with estimation of histopathological tissue damage[479]. Haematologic parameters such as lymphocyte and neutrophil levels can also be measured as an indicator of histopathological damage. The absolute lymphocyte count is significantly reduced in skeletal muscle ischaemia/reperfusion animals compared to sham-operated animals, with the reduction most likely representing a stress-induced lymphopaenia. The level of circulating neutrophils increases in animals subjected to skeletal muscle ischaemia/reperfusion compared to sham-operated animals[479].

Many investigators have reported conflicting results regarding the susceptibility of various muscle groups and fibre types to skeletal muscle ischaemia/reperfusion injury[9,538-545]. Given the variability in the literature, it is probable that many factors such as the animal model utilized, duration of ischaemia, temperature, maintenance of blood flow during reperfusion and animal variations were not controlled between these experiments. No attempt was made

in the studies in the current experiments to differentiate between the different muscle fibre types. By contrast, a cross section of muscles was chosen to give a combination of different fibre types. This was an attempt to emulate the ultimate situation of human skeletal muscle ischaemia/reperfusion, which naturally includes all skeletal muscle fibre types.

In summary, the experiments described in this chapter have shown that our chosen animal model is producing significant skeletal muscle and renal damage following skeletal muscle ischaemia/reperfusion. This justifies the validity of the technique for further analysis using investigation with matrix metalloproteinases specific experiments. It also allowed further investigation in the therapeutic role of doxycycline using this model.

CHAPTER 3:

ELEVATED ACTIVITY

OF

MATRIX METALLOPROTEINASES-2 AND -9

DURING

SKELETAL MUSCLE

ISCHAEMIA/REPERFUSION INJURY

3.1 Introduction

The aims of this chapter are to observe and analyse the changes in matrix metalloproteinase-2 and –9 in skeletal muscle ischaemia/reperfusion injury. Two methods are used to explore the role of MMPs; zymography and western blot analysis.

Zymography is an electrophoretic technique used to identify proteolytic activity in enzymes separated in polyacrylamide gels under nonreducing conditions. It has been used extensively in the qualitative evaluation of proteases present in tumours and cell culture conditioned media. Zymography utilizes a large protein substrate, often gelatin, which is copolymerised with acrylamide during the casting of the gel. As the enzymes MMP-2 and-9 degrade gelatin, a 0.10% gelatin solution was used in the zymograms. Following processing and staining, the resulting MMP-2 and MMP-9 enzyme activity show as white bands on a blue background.

A series of confirmational studies were performed to verify the nature of the MMP-2 and MMP-9. All zymograms were run with a standard molecular weight marker, to show the sizes of the gelatinolytic bands. A zymogram was performed using the normal development buffer and compared to a replicate gel where phenanthroline was added to the development buffer. Phenanthroline is known as a zinc chelator. If the zinc is chelated in the matrix metalloproteinase molecule, then the matrix metalloproteinase will no longer act as a gelatinase and hence will not be seen on a gelatin based gel. Therefore, if the bands that occur in the gelatin gel disappear with phenanthroline, these bands can be confirmed as matrix metalloproteinases.

Zymographic analysis was performed on left leg skeletal muscle, right leg skeletal muscle, lung and kidney. In each tissue, a comparison was made of the changes that occurred over increasing duration of reperfusion as well as between sham-operated animals and animals with either unilateral or bilateral skeletal muscle ischaemia. The figures presented are of representative animals in each experimental group. No attempt to quantify the level of MMP induced was made. The quantitation of the level of tissue damage occurring during reperfusion was performed on type IV collagen, the main substrate of MMP-2 and MMP-9. These studies are discussed in Chapter 4.

Western blot analysis using an anti-MMP-9 antibody was also performed to confirm the findings of zymography.

3.2 Methods

3.2.1 Zymography

Gelatin zymography was carried out using a modification of the method of Porter et al[546].

3.2.1.1.Tissue Preparation

Each section of tissue was cut into match head size pieces and weighed. It was combined with 1 ml of homogenising buffer (Appendix 7.4.1 Homogenising Buffer) per 100mg of tissue and then homogenised thoroughly (B.Braun. Melsungen AG) at 1500 rpm. The tissues samples are then centrifuged at 11000 rpm for 60 minutes. Following centrifugation, the supernatant was aspirated and placed in Visking dialysis tubing (14 kDa cut-off), which had been boiled for two minutes. The pelleted cell debris was discarded. With the dialysis tubing sealed at both ends, the tissue supernatant was dialysed for 18 hours at 4 degrees Celsius in dialysis buffer (Appendix 7.4.2 Dialysis Buffer) in order to remove the urea used in the homogenising buffer. The supernatant was then divided into aliquots, frozen in liquid nitrogen and stored in an -80° Celsius Freezer.

3.2.1.2 Bio-Rad Protein Assay

The Bio-Rad® Protein Assay is a dye-binding assay, in which a differential colour change of Coomassie® Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. The manufacturer's instructions for Microassay procedure were used to determine the level of protein in the tissue dialysate for each rat sample.

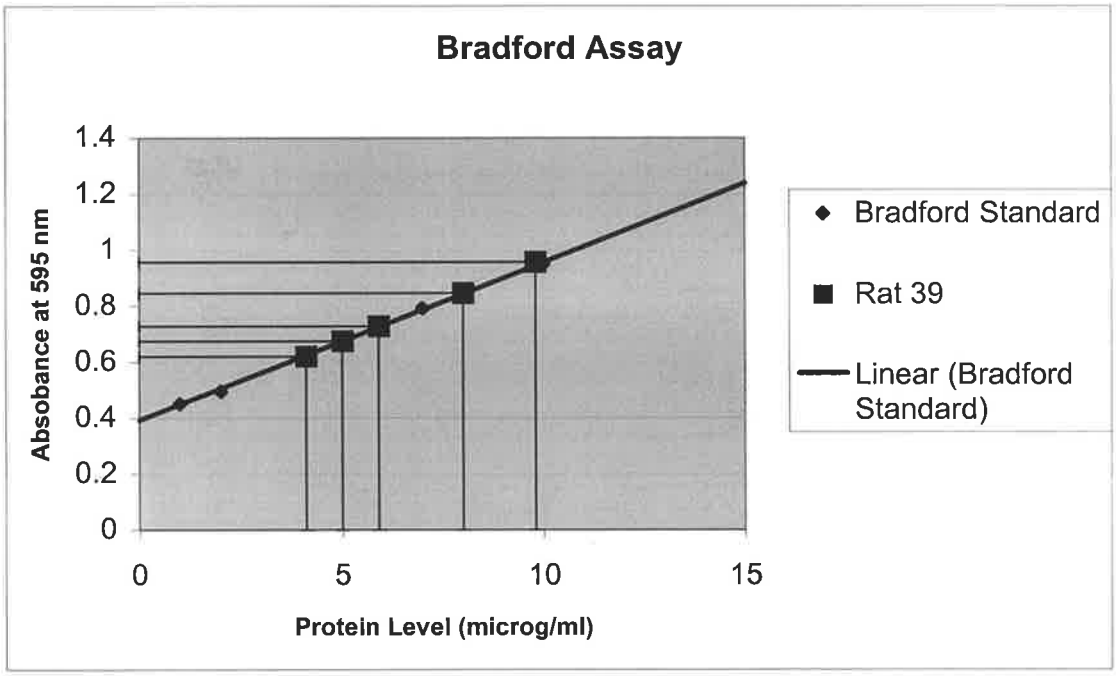
The Bio-Rad® Protein Assay Dye Reagant was mixed with varying concentration of bovine serum albumin (BSA) as a standard. Five dilutions of BSA from 1–10 µg/ml were used. Two hundred µl of Bio-Rad® Protein Assay Dye Reagant was mixed with the BSA at the appropriate dilution and Milli-Q H_2O to make 1 ml. The solution was incubated at room temperature for at least five minutes. Absorbance was measured within the next hour at 595nm on the spectrometer. A graph was drawn from the result of these known standard protein concentrations.

The tissue sample, was diluted with Bio-Rad® Protein Assay Dye Reagant and Milli Q H_2O. The absorbance of the solution was measured twice to allow for technical errors and the final absorbance represents an average of the two results.

A graph was drawn of serum protein level (BSA) versus Absorbance at 595nm. A sample of these graphs for Rat 39 is shown in Figure 12. Each tissue sample was analysed separately and the result of the absorbance used to calculate the total level of protein in each sample.

Figure 12: Example of Bradford Protein Assay

The small stars represent the spectrometer absorbance for the known concentration of protein. A black trendline is shown for the standard protein level. The large squares represent the absorbance of the samples with unknown protein levels, left leg skeletal muscle, right leg skeletal muscle, lung and kidney. By extrapolating a line down to the x-axis the protein concentration for that unknown sample was determined.



3.2.1.3 Zymography Gel Preparation

Zymography gels were prepared according to Appendices 7.4.3 Resolving gel and 7.4.4 Stacking gel. The Bio-Rad Mini-Protean® II Electrophoresis Cell system was used. The resolving gel was poured and allowed to set overnight. The stacking gel was then poured with the comb in place. A standardized amount of protein (40μg) of the tissue samples was mixed with an equal volume of zymogram loading buffer (Appendix 7.4.5 Zymogram Loading Buffer) and loaded into the wells. Amersham rainbow marker® (RPN 800) was used for comparison of molecular weights on the gel. The tank was filled with running buffer

(Appendix 7.4.7 Zymogram and Western Blot Tank/Running Buffer) and the gels were run at 100 volts for approximately 90–120 minutes until the tissue samples reached the bottom of the gel. The gels were then washed three times for fifteen minutes on each occasion in 2.5% Triton-X 100 and then placed in development buffer (Appendix 7.4.8 Zymogram Development Buffer), and incubated at 37°C for 18 hours with gentle agitation. The gels were then stained with Coomassie Blue stain (Appendix 7.4.9 Coomassie Blue Stain) for approximately one hour at room temperature. The gels were then incubated in destain (Appendix 7.4.10 Destain) until clear bands appeared against the blue background, approximately 30-60 minutes. The gels were photographed using a digital camera and images stored as Tagged Image File Format (TIFF files).

3.2.1.4 Zinc Chelation

Phenanthroline was added in a concentration of 10milliMolar to the development buffer (Appendix: 7.4.8 Zymogram Development Buffer). Two zymogram gels were run simultaneously, loaded with tissue samples from Rat 15 (Unilaterally ischaemic for 4 hours, with 72 hours of reperfusion). One gel was developed in development buffer as described in Appendix 7.4.8 Zymogram Development Buffer and the other gel was developed in buffer with the added 10 mM of phenanthroline.

3.2.2 Western Blots[*]

SDS Poly acrylamide gels were prepared using resolving gel (Appendix 7.4.11 Resolving Gel for Western Blot Analysis) and stacking gel (Appendix 7.4.4 Stacking gel For Zymography and Western blots). The protein samples were loaded (see Appendix 7.4.6.Western Loading buffer), the tank filled with running buffer (Appendix 7.4.7 Zymogram and Western Blot Tank/Running Buffer) and the gel electrophoresced at 60 Volts for 30 mins, and then at 100 Volts for 90 minutes. The gel was removed from the apparatus, washed and allowed to equilibrate in transfer buffer (Appendix 7.4.12 Western Transfer Buffer) for 30 minutes at room temperature. The gel was then placed against a nitrocellulose membrane and sandwiched between 2 sheets of Whatman chromatography paper, which had been pre-soaked in transfer buffer. The gel was the placed in tank with transfer buffer and cooling block of ice

[*] The example of the MMP-9 western blot analysis included in these studies was performed by Dr Peter Laws, MBBS, FRCS, Vascular Research Fellow, The Queen Elizabeth Hospital.

and subjected to 400mA for 60 minutes. The nitrocellulose membrane was washed in PBS for 1 minute, followed by 5 minutes in Tris Buffered Saline (Appendix 7.4.14 Tris Buffered saline for Western Blots).

Non-specific binding sites on the nitrocellulose membrane were blocked by incubation with 5% non-fat skim milk powder (Appendix 7.4.13 Non-fat Powdered Milk Solution) for 2 hours by gentle agitation. The membrane was transferred to a bag containing the primary antibody; Rabbit Anti-Rat MMP-9 (AB19016, Chemicon International, Inc), at a dilution of 1 in 2000 in western antibody buffer (Appendix 7.4.15 Western Antibody Buffer) and incubated for 16 hours at room temperature with gentle agitation. Unbound antibody was removed by washing twice in tris buffered saline for 10 minutes. The membrane was then transferred to anew bag containing 1 in 1000 dilution of secondary antibody and western antibody buffer. The secondary antibody used was Swine Anti-Rabbit IgG Horseradish Peroxidase (Dako PO399). The membrane was then incubated for an hour and washed for 2 X 10 minutes in tris buffered saline to remove unbound antibody.

The membrane was placed on clean glass plate and blotted with tissue paper. The ECL method of detection was used according to the manufacturer's instructions. One ml of Reagent 1 (ECL) was mixed with 1ml of Reagent 2 (ECL) and poured onto membrane. A second glass plate was placed over membrane and incubated for 1 minute. The glass plate was removes and unbound reagents blotted with a tissue. In the dark room, the membrane was placed between sheets of plastic wrap and covered with photographic film. The film was then developed in the radiology department at The Queen Elizabeth Hospital.

3.3 Results

3.3.1 Zymography

3.3.1.1 Confirmational Studies

Figure 13A shows the zymographic analysis of tissues from a rat that underwent 4 hours of unilateral left leg ischaemia and 72 hours of reperfusion. Baseline low levels of MMP-2 are seen in the right leg skeletal muscle, liver and kidney, with upregulation of both proMMP-2 and active MMP-2 seen in the ischaemic left leg and lung. These changes are completely abolished when 1,10 phenanthroline was added to the development buffer as seen in Figure 13B. In Figure 13C, the rat without experimentation and was killed outright and shows low levels of MMP-2 and MMP-9 in the kidney but MMP expression was not detected in any other tissues.

3.3.1.2 Comparative studies of Zymographic detection of Gelatinolytic activity at varying Reperfusion times and between Sham-operated, Unilaterally and Bilaterally ischaemic animals.

Left leg Skeletal Muscle

Figure 14 illustrates the zymographic analysis of skeletal muscle from the left leg of rats subjected to varying degrees of ischaemia and reperfusion. Tissues from the sham-operated animals in both Figure 14A and Figure 14B show a constitutive low level of expression of MMP-2 in the left leg skeletal muscle but there was no MMP-9 activity in these tissues. A similar result was seen when the leg was subjected to 4 hours of ischaemia and no reperfusion (Figure 14A, Lane 5 and Figure 14B, Lane 4). However, when the rats were subjected to 4, 24 or 72 hours of reperfusion, there was a marked induction of activity of proMMP-9 (92-97 kDa) as well as elevated levels of proMMP-2 (72 kDa). Skeletal muscle from rats subjected to 72 hours of reperfusion also showed an increase in the levels of the 62 kDa active from of MMP-2 and a reduction in the amount of MMP-9 activity when compared to 24 hours of reperfusion. Similar findings were seen in Figure 14B, with upregulation of MMP-9 and proMMP-2 and MMP-2, starting initially following 4 hours of bilateral reperfusion and increasing with 24 hours of reperfusion. In Figure 14C, unilateral ischaemic animals were compared with bilaterally ischaemic animals; proMMP-2 but not MMP-9 were detected in muscle from animals without reperfusion. The changes of induction of proMMP-2, MMP-2

and MMP-9 were seen at 4, 24 and 72 hours in both the unilaterally and the bilaterally ischaemic animals, with no discernible differences between the 2 levels of ischaemic insult.

Right leg Skeletal Muscle

Zymograms of right leg skeletal muscle from sham-operated, unilaterally and bilaterally ischaemic animals are shown in Figure 15. The right leg underwent no experiment in the sham-operated animal. In the unilaterally ischaemic animal, the right leg was the contralateral limb. In the bilaterally ischaemic animal, the right leg was rendered ischaemic for 4 hours. In Figure 15A and in the lanes1-4 of Figure 15B and lanes 1-4 of Figure 15C, representing the sham-operated and unilaterally ischaemic animals there were baseline low levels of proMMP-2 observed in the right leg skeletal muscle. In the bilaterally ischaemic animals, there were similar changes in the limbs subjected to 4 hours of ischaemia and no reperfusion. However after 4, 24 and 72 hours of reperfusion in the bilaterally ischaemic animals, there was a marked induction of activity of proMMP-2, MMP-2 and proMMP-9. The increase in both active MMP-2 and MMP-9 was maximal at 24 hours of reperfusion (Lane7, Figure 15B and Lane7, Figure 15C).

Lung

The zymograms of lung tissue from sham-operated, unilaterally and bilaterally ischaemic animals are shown in Figure 16. Sham-operated animals showed zymographic activity corresponding to both proMMP-2 and MMP-9 with the highest activity in animals sacrificed immediately after 4 hours of anaesthesia. Levels of gelatinolytic activity decreased as the interval between anaesthesia and euthanasia increased, presumably reflecting recovery from anaesthetic damage. In animals subjected to 4 hours of ischaemia and 4, 24 or 72 hours of reperfusion, levels of MMP-2 and MMP-9 were elevated at 4 hours of reperfusion and decreased to lower levels by 72 hours of reperfusion.

Kidney

The zymograms of kidneys from sham-operated, unilaterally and bilaterally ischaemic animals are shown in Figure 17. All samples of renal tissue expressed baseline levels of proMMP-2 only, which did not alter following ischaemia and reperfusion.

3.3.2 Western Blot Analysis

The western blot analysis confirms that the zymographic bands shown in the above zymographic figures are indeed MMP-9. The result of the MMP-9 Western blot analysis is shown in Figure 18. A comparison is shown between ischaemic skeletal muscle and non-ischaemic skeletal muscle with marked upregulation of MMP-9 in the ischaemic muscle. Similarly, in the lung tissue there is marked upregulation of MMP-9 in lung tissue that came from a rat that underwent 4 hours of skeletal muscle ischaemia and 24 hours of reperfusion, compared to the lung of a sham-operated rat that underwent an anaesthetic but no ischaemia and was sacrificed 24 hours later.

Figure 13: Zymography to confirm nature of Matrix Metalloproteinase bands.

Panel A: Rat underwent 4 hours of unilateral left leg ischaemia followed by 72 hours of reperfusion. The first lane is the molecular weight marker showing sizes in kilodaltons. The zymographic bands of MMP-9, proMMP-2 and active MMP-2 are indicated. A standardized load of 40 μg of protein was loaded in each well.

Lane 1: Left leg skeletal muscle

Lane 2: Right leg skeletal muscle

Lane 3: Liver

Lane 4: Lung

Lane 5: Kidney

Panel B: The same rat samples were used as in Panel A. This gel was incubated with 10 mM of phenanthroline added to the development buffer. The lane structure is the same as Panel A.

Panel C: Rat 28, killed by stunning and cervical dislocation. The lane structure is the same as Panel A.

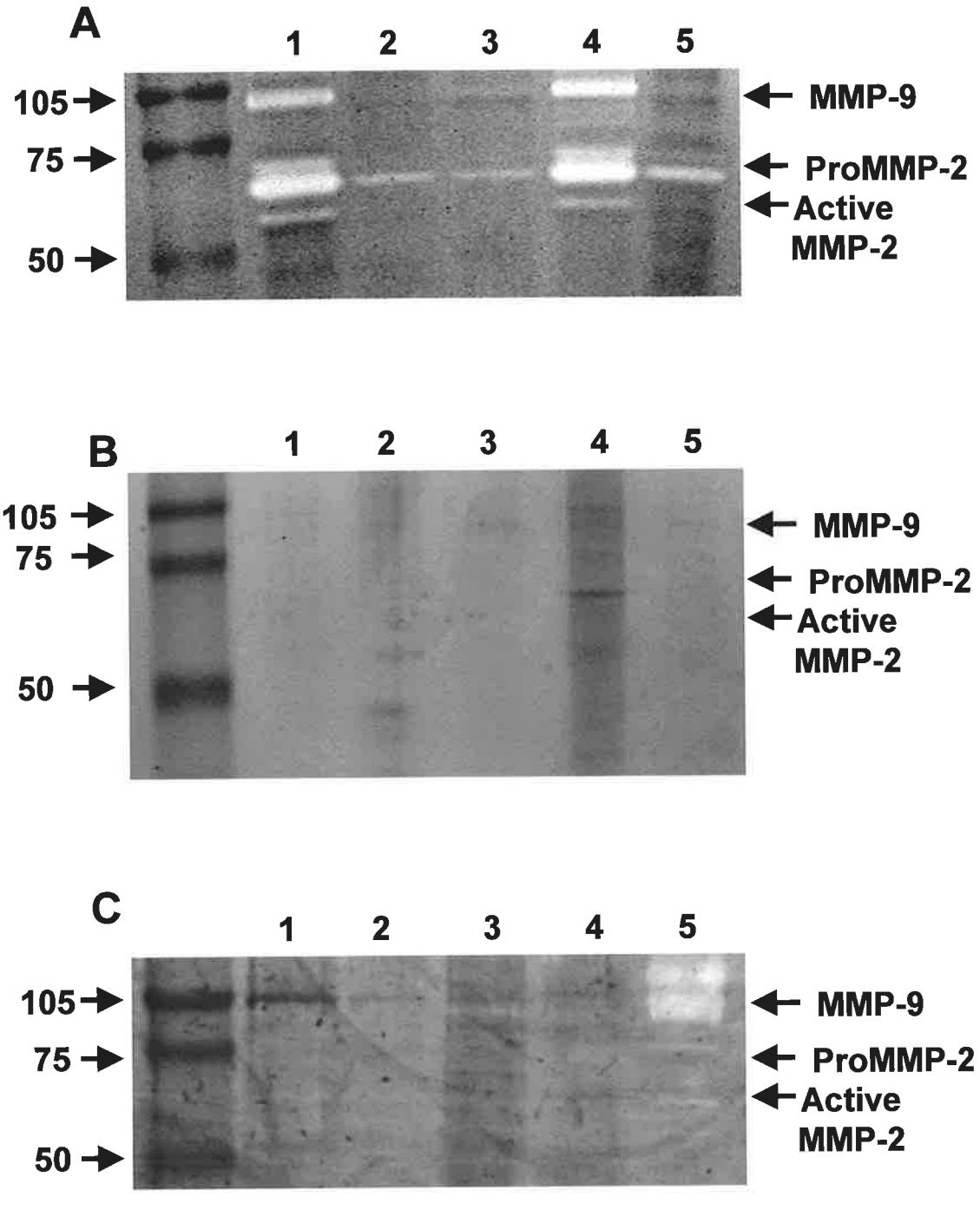

A
1
2
3
4
5
105
75
50
MMP-9
ProMMP-2
Active
MMP-2
B
1
2
3
4
5
105
75
50
MMP-9
ProMMP-2
Active
MMP-2
C
1
2
3
4
5
105
75
50
MMP-9
ProMMP-2
Active
MMP-2

Figure 14: Zymographic detection of Left leg Skeletal Muscle gelatinolytic activity.

For each panel: The lane on the left side is the molecular weight marker showing sizes in kilodaltons. The MMP-9, proMMP-2 and active MMP-2 are shown. A standardized load of 40 μg of protein was loaded in each well.

Panel A: Sham-operations/Unilateral Ischaemia

Lane 1: Sham-operation, no ischaemia, sacrificed at end of anaesthetic.

Lane 2:Sham-operation, no ischaemia, sacrificed at 4 hours after end of anaesthetic

Lane 3:Sham-operation, no ischaemia, sacrificed at 24 hours after end of anaesthetic

Lane 4:Sham-operation, no ischaemia, sacrificed a 72 hours after end of anaesthetic

Lane 5: Unilateral left limb ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 6: Unilateral left limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 7: Unilateral left limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 8: Unilateral left limb ischaemia, sacrificed after 72 hours of reperfusion

Panel B: Sham-operations/Bilateral Ischaemia

Lane 1: Sham-operation, no ischaemia, sacrificed at end of anaesthetic

Lane 2:Sham-operation, no ischaemia, sacrificed at 4 hours after end of anaesthetic

Lane 3:Sham-operation, no ischaemia, sacrificed at 24 hours after end of anaesthetic

Lane 4: Bilateral leg ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 5: Bilateral lower limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 6: Bilateral lower limb ischaemia, sacrificed after 24 hours of reperfusion

Panel C: Unilateral/Bilateral Ischaemia

Lane 1: Unilateral left limb ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 2: Unilateral left limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 3: Unilateral left limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 4: Unilateral left limb ischaemia, sacrificed after 72 hours of reperfusion

Lane 5: Bilateral leg ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 6: Bilateral lower limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 7: Bilateral lower limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 8: Bilateral lower limb ischaemia, sacrificed after 72 hours of reperfusion

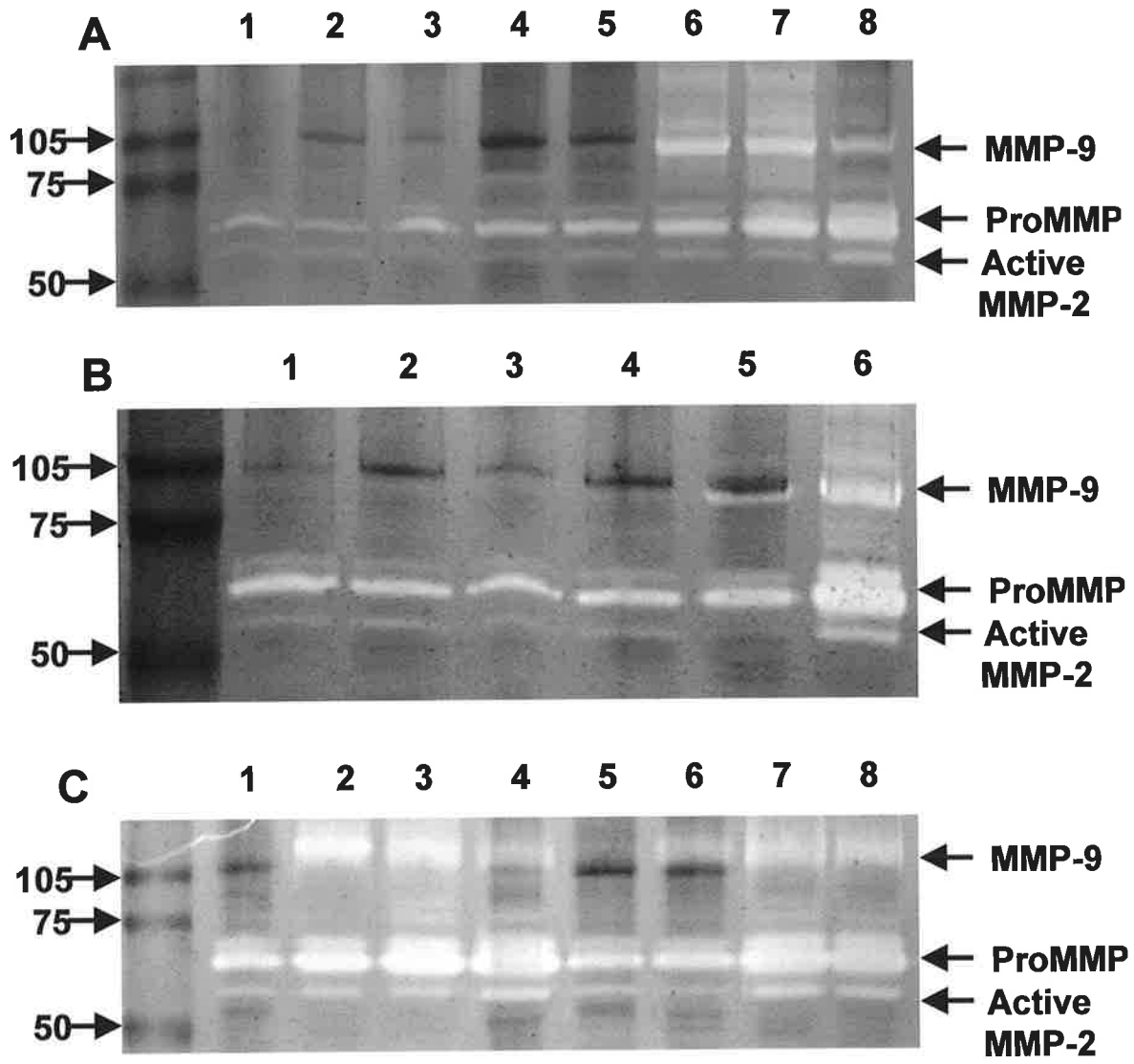

A
1 2 3 4 5 6 7 8
105
75
50
MMP-9
ProMMP
Active
MMP-2
B
1 2 3 4 5 6
105
75
50
MMP-9
ProMMP
Active
MMP-2
C
1 2 3 4 5 6 7 8
105
75
50
MMP-9
ProMMP
Active
MMP-2

Figure 15: Zymographic detection of Right leg Skeletal Muscle gelatinolytic activity.

For each panel: The lane on the left side is the molecular weight marker showing sizes in kilodaltons. The MMP-9, proMMP-2 and active MMP-2 are shown. A standardized load of 40 μg of protein was loaded in each well. In the sham-operated animal, there was no lower limb ischaemia. In the unilaterally ischaemic animal, the right leg was the contralateral limb. In the bilaterally ischaemic animal, the right leg was rendered ischaemic for 4 hours.

Panel A: Sham-operations/Unilateral Ischaemia

Lane 1: Sham-operation, no ischaemia, sacrificed at end of anaesthetic

Lane 2:Sham-operation, no ischaemia, sacrificed at 4 hours after end of anaesthetic

Lane 3:Sham-operation, no ischaemia, sacrificed at 24 hours after end of anaesthetic

Lane 4:Sham-operation, no ischaemia, sacrificed a 72 hours after end of anaesthetic

Lane 5: Unilateral left limb ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 6: Unilateral left limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 7: Unilateral left limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 8: Unilateral left limb ischaemia, sacrificed after 72 hours of reperfusion

Panel B: Sham-operations/Bilateral Ischaemia

Lane 1: Sham-operation, no ischaemia, sacrificed at end of anaesthetic.

Lane 2:Sham-operation, no ischaemia, sacrificed at 4 hours after end of anaesthetic

Lane 3:Sham-operation, no ischaemia, sacrificed at 24 hours after end of anaesthetic

Lane 4: Sham-operation, no ischaemia, sacrificed a 72 hours after end of anaesthetic

Lane 5: Bilateral leg ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 6: Bilateral lower limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 7: Bilateral lower limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 8: Bilateral lower limb ischaemia, sacrificed after 72 hours of reperfusion

Panel C: Unilateral/Bilateral Ischaemia

Lane 1: Unilateral left limb ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 2: Unilateral left limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 3: Unilateral left limb ischaemia, sacrificed after 24 hours of reperfusion

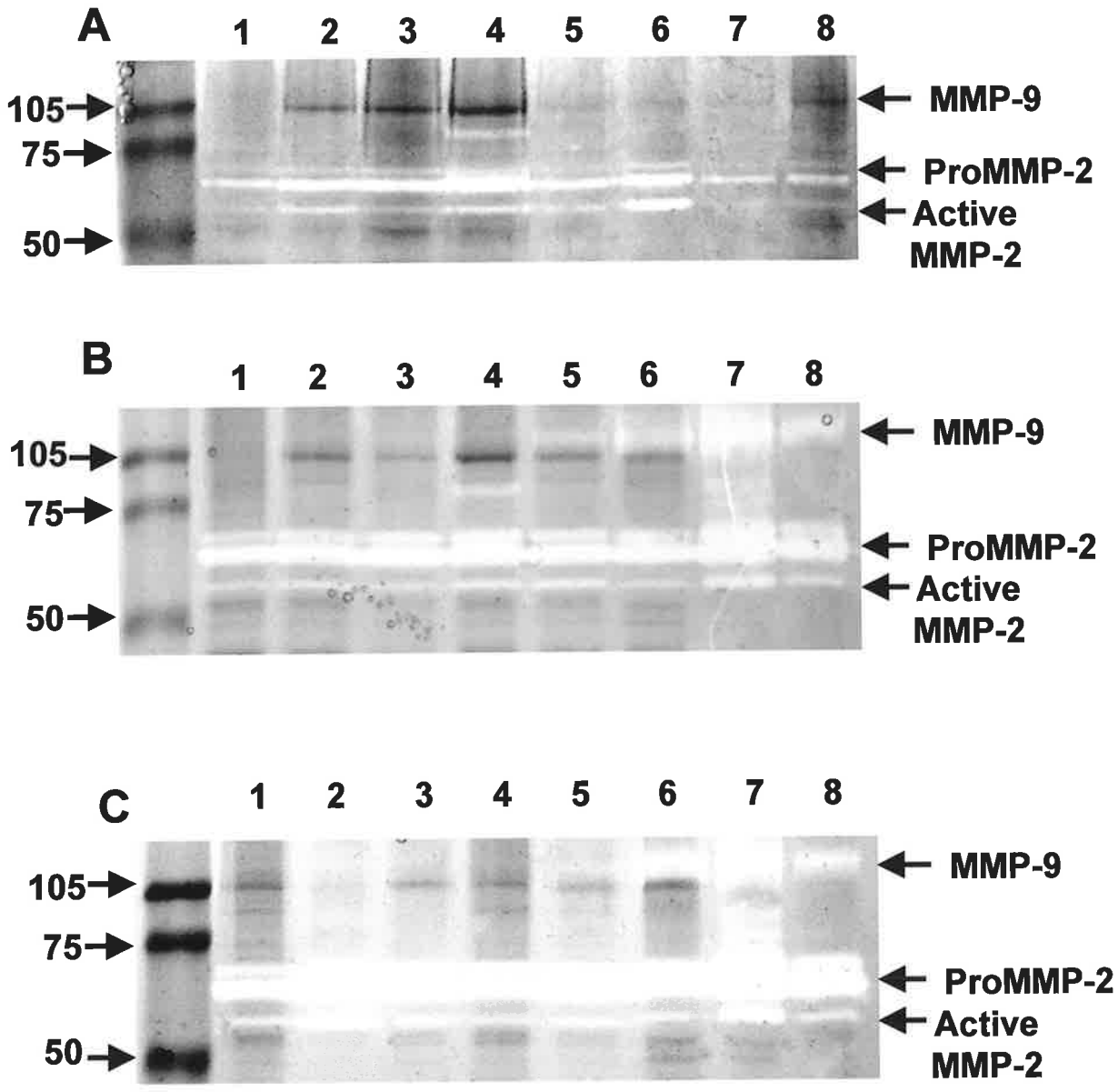
Lane 4: Unilateral left limb ischaemia, sacrificed after 72 hours of reperfusion

Lane 5: Bilateral leg ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 6: Bilateral lower limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 7: Bilateral lower limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 8: Bilateral lower limb ischaemia, sacrificed after 72 hours of reperfusion


A
1 2 3 4 5 6 7 8
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MMP-9
ProMMP-2
Active
MMP-2
B
1 2 3 4 5 6 7 8
105
75
50
MMP-9
ProMMP-2
Active
MMP-2
C
1 2 3 4 5 6 7 8
105
75
50
MMP-9
ProMMP-2
Active
MMP-2

Figure 16: Zymographic detection of Lung gelatinolytic activity.

For each panel: The lane on the left side is the molecular weight marker showing sizes in kilodaltons. The MMP-9, proMMP-2 and active MMP-2 are shown. A standardized load of 40 μg of protein was loaded in each well.

Panel A: Sham-operations/Unilateral Ischaemia

Lane 1: Sham-operation, no ischaemia, sacrificed at end of anaesthetic.

Lane 2:Sham-operation, no ischaemia, sacrificed at 4 hours after end of anaesthetic

Lane 3:Sham-operation, no ischaemia, sacrificed at 24 hours after end of anaesthetic

Lane 4:Sham-operation, no ischaemia, sacrificed a 72 hours after end of anaesthetic

Lane 5: Unilateral left limb ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 6: Unilateral left limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 7: Unilateral left limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 8: Unilateral left limb ischaemia, sacrificed after 72 hours of reperfusion

Panel B: Sham-operations/Bilateral Ischaemia

Lane 1: Sham-operation, no ischaemia, sacrificed at end of anaesthetic.

Lane 2:Sham-operation, no ischaemia, sacrificed at 4 hours after end of anaesthetic

Lane 3:Sham-operation, no ischaemia, sacrificed at 24 hours after end of anaesthetic

Lane 4: Sham-operation, no ischaemia, sacrificed a 72 hours after end of anaesthetic.

Lane 5: Bilateral leg ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 6: Bilateral lower limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 7: Bilateral lower limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 8: Bilateral lower limb ischaemia, sacrificed after 72 hours of reperfusion

Panel C: Unilateral/Bilateral Ischaemia

Lane 1: Unilateral left limb ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 2: Unilateral left limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 3: Unilateral left limb ischaemia, sacrificed after 24 hours of reperfusion

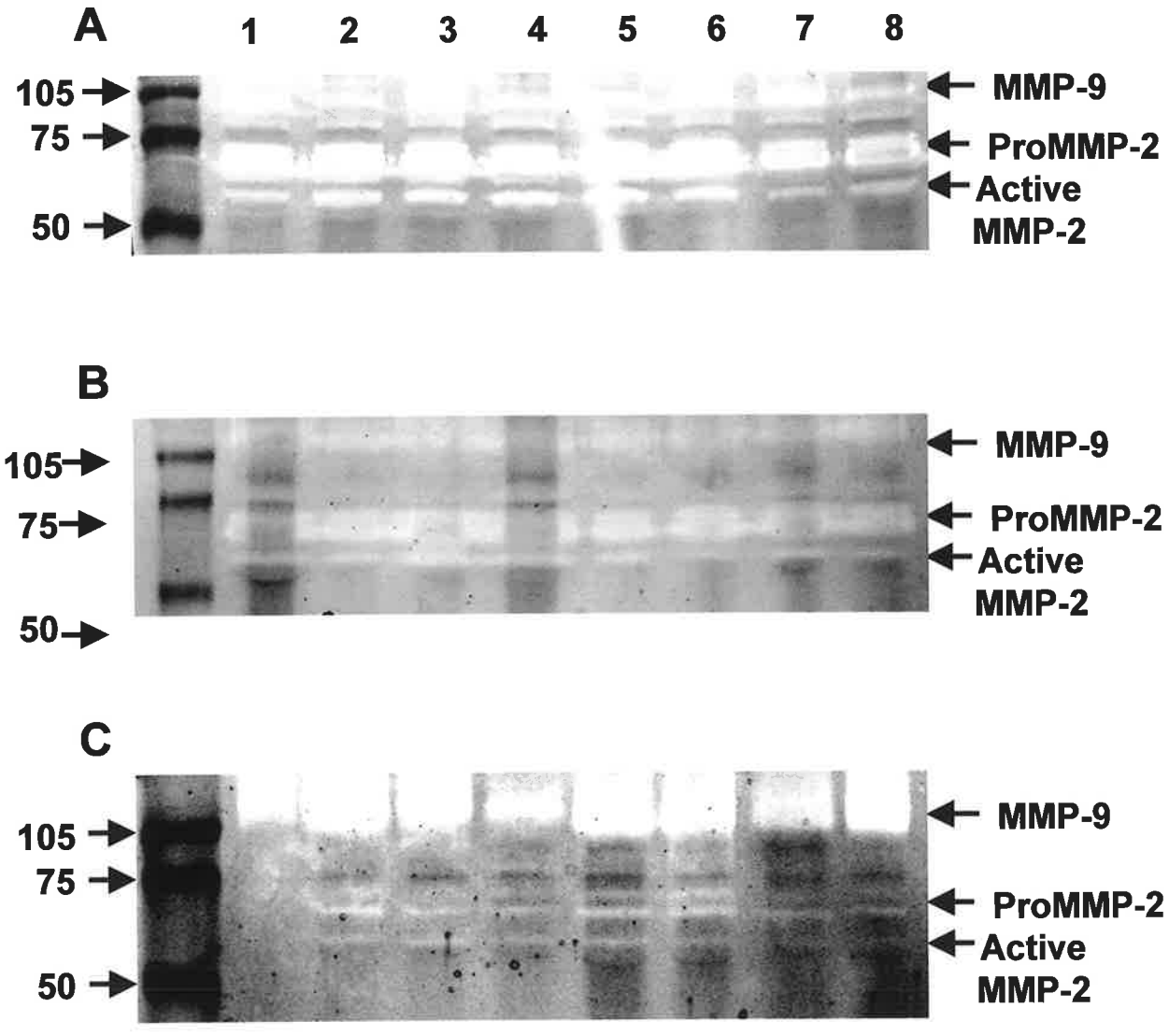
Lane 4: Unilateral left limb ischaemia, sacrificed after 72 hours of reperfusion

Lane 5: Bilateral leg ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 6: Bilateral lower limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 7: Bilateral lower limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 8: Bilateral lower limb ischaemia, sacrificed after 72 hours of reperfusion


A
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105
75
50
MMP-9
ProMMP-2
Active
MMP-2
B
105
75
50
MMP-9
ProMMP-2
Active
MMP-2
C
105
75
50
MMP-9
ProMMP-2
Active
MMP-2

Figure 17: Zymographic detection of Kidney gelatinolytic activity.

For each panel: The lane on the left side is the molecular weight marker showing sizes in kilodaltons. The MMP-9, proMMP-2 and active MMP-2 are shown. A standardized load of 40 μg of protein was loaded in each well.

Panel A: Sham-operations/Unilateral Ischaemia

Lane 1: Sham-operation, no ischaemia, sacrificed at end of anaesthetic.

Lane 2:Sham-operation, no ischaemia, sacrificed at 4 hours after end of anaesthetic

Lane 3:Sham-operation, no ischaemia, sacrificed at 24 hours after end of anaesthetic

Lane 4:Sham-operation, no ischaemia, sacrificed a 72 hours after end of anaesthetic

Lane 5: Unilateral left limb ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 6: Unilateral left limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 7: Unilateral left limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 8: Unilateral left limb ischaemia, sacrificed after 72 hours of reperfusion

Panel B: Sham-operations/Bilateral Ischaemia

Lane 1: Sham-operation, no ischaemia, sacrificed at end of anaesthetic.

Lane 2:Sham-operation, no ischaemia, sacrificed at 4 hours after end of anaesthetic

Lane 3:Sham-operation, no ischaemia, sacrificed at 24 hours after end of anaesthetic

Lane 4: Sham-operation, no ischaemia, sacrificed a 72 hours after end of anaesthetic

Lane 5: Bilateral leg ischaemia, sacrificed at end of anaesthetic, no reperfusion.

Lane 6: Bilateral lower limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 7: Bilateral lower limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 8: Bilateral lower limb ischaemia, sacrificed after 72 hours of reperfusion

Panel C: Unilateral/Bilateral Ischaemia

Lane 1: Unilateral left limb ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 2: Unilateral left limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 3: Unilateral left limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 4: Unilateral left limb ischaemia, sacrificed after 72 hours of reperfusion

Lane 5: Bilateral leg ischaemia, sacrificed at end of anaesthetic, no reperfusion

Lane 6: Bilateral lower limb ischaemia, sacrificed after 4 hours of reperfusion

Lane 7: Bilateral lower limb ischaemia, sacrificed after 24 hours of reperfusion

Lane 8: Bilateral lower limb ischaemia, sacrificed after 72 hours of reperfusion

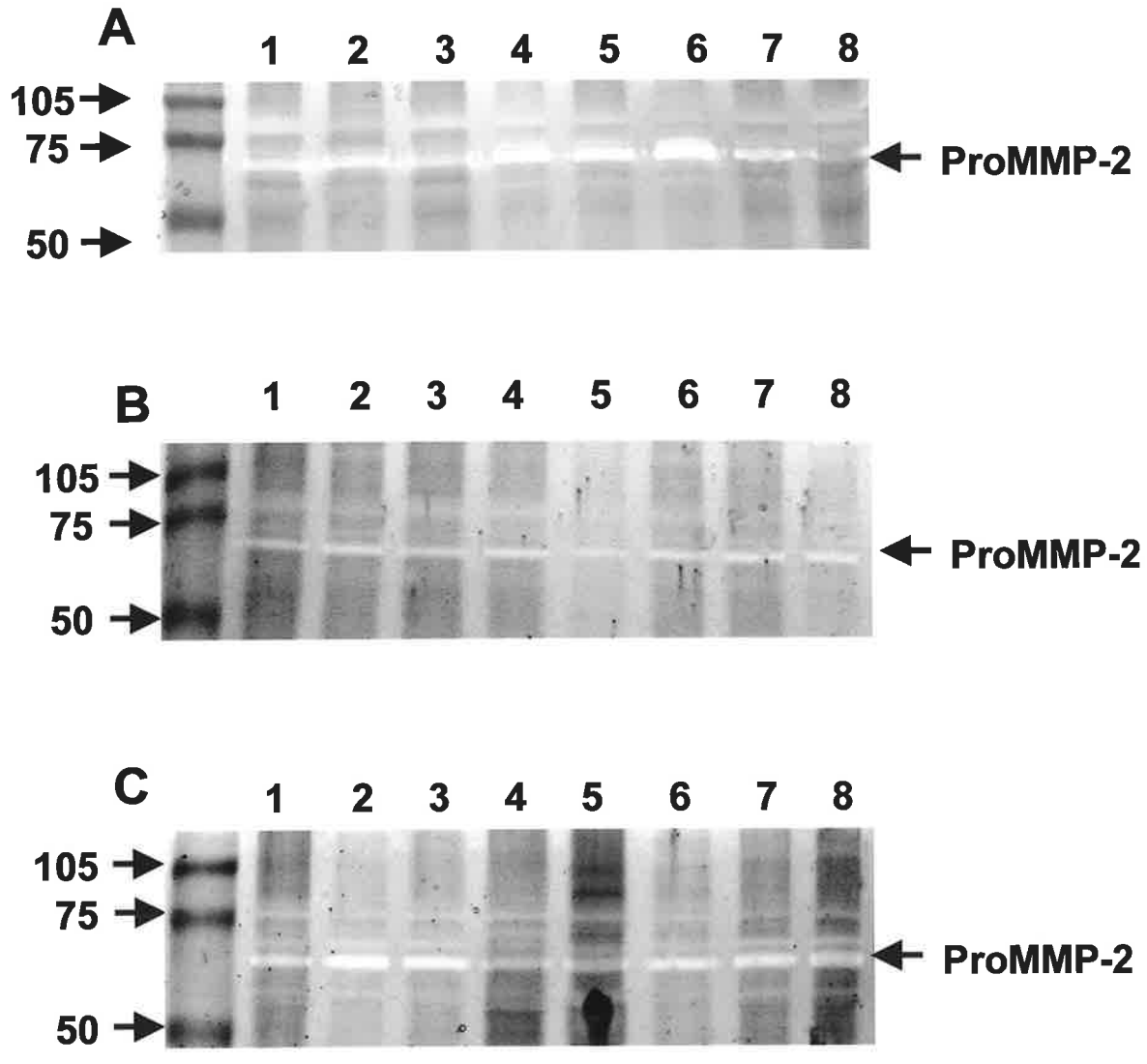

A
1 2 3 4 5 6 7 8
105
75
50
ProMMP-2
B
1 2 3 4 5 6 7 8
105
75
50
ProMMP-2
C
1 2 3 4 5 6 7 8
105
75
50
ProMMP-2

Figure 18: Western Blot Analysis

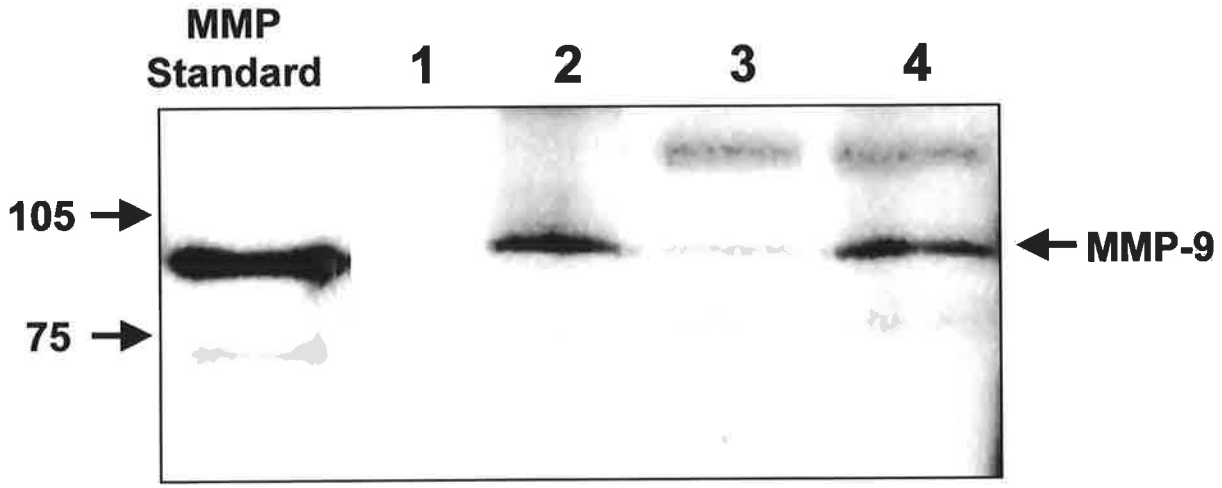
The left lane was MMP Control-1 (Sigma) showing MMP-9 and a small amount of MMP-2.

Lane 1: Skeletal muscle from the left leg of a sham-operated animal, 4 hour anaesthetic, no ischaemia and sacrificed after 24 hours

Lane 2: Skeletal muscle from the left leg of an animal that underwent 4 hours of unilateral left leg ischaemia, sacrificed after 24 hours of reperfusion

Lane 3: Lung from sham-operated animal, 4 hour anaesthetic, no ischaemia and sacrificed after 24 hours

Lane 4: Lung from an animal that underwent 4 hours of unilateral left leg ischaemia, sacrificed after 24 hours of reperfusion


MMP
Standard
1
2
3
4
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MMP-9

3.4 Discussion

The current studies have shown that gelatinolytic activity corresponding to MMP-2 and MMP-9, is markedly increased during ischaemia/reperfusion in a rat model of skeletal muscle lower limb occlusion. Elevated activity of MMPs was observed both in the skeletal muscle that has undergone ischaemia/reperfusion and to a lesser extent, in the lungs of these animals. This was in marked contrast to the gelatinolytic activity of an animal that underwent no experimental procedures, which showed very little expression of either MMP-2 or MMP-9 in either tissue. Zymographic activity in the kidneys appeared unchanged under all conditions, showing only baseline levels of MMP-2. The changes in both MMP-2 and MMP-9 were maximal at 24 hours following reperfusion in the ischaemic limbs. As MMP-9 is known to be produced from polymorphonuclear cells, monocytes and macrophages, the rise in MMP-9 coincides with the time at which the maximal influx of these inflammatory cells would be expected.

The identity of the gelatinolytic activities observed on the zymograms was defined by the molecular weights of the species observed, in accordance with published sizes. The pro-form of MMP-2 is ubiquitously expressed and has a molecular weight of 72 kDA, with an active form of 64 kDa[475,547]. ProMMP-9 has been widely reported as being 92-96 kDa[548] along with an 84 kDa activated from[402,465]. The MMP-9 band observed is of a size consistent with it being proMMP-9, activated MMP-9 was not seen. Incubation of zymograms with the zinc chelator, (1,10)-phenanthroline, abolished all gelatinolytic activity, confirming the identity of these bands as metalloproteinases. The western blot analysis using an antibody against MMP-9 showed that the bands of upregulated MMP-9 in ischaemic skeletal muscle and lung were confirmed to be MMP-9.

These findings are in agreement with Frisdal and co-workers, who used a rat model of permanent ligation of the femoral artery and demonstrated an increase in the gelatinolytic activity of MMP-2 and MMP-9, accompanied by a degradation of basement membrane components, including type IV collagen and laminin in the soleus muscle[549]. Although collateral circulation in the limb would provide some level of reperfusion, ischaemia was permanent and there was no formal reperfusion phase in these studies. This model is less relevant to the clinical situation and they were not able to examine mechanisms of damage to distant organs, which are commonly affected in reperfusion injury.

The patterns of MMP-2 and MMP-9 reported above are also in agreement with the results of studies of both cardiac and cerebral ischaemia/reperfusion. In an in vitro model of ischaemia/reperfusion in isolated rat hearts, reperfusion resulted in increased levels of MMP-2 in the perfusate as well as in the ventricular tissue[475]. This rise in MMP-2 levels was partially inhibited by doxycycline, resulting in improved cardiac function[475]. However, in an in vivo porcine model of cardiac ischaemia (90 minutes) followed by brief reperfusion (90 minutes), marked elevation of MMP activity was observed but without degradation of collagen, presumably a reflection of the brief duration of reperfusion[550]. Increased levels of both collagenase (MMP-1) and gelatinase (MMP-9) activity were also observed following cardiac ischaemia/reperfusion in a pig model[474], with MMP-9 immunolocalised to infiltrating leukocytes in the ischaemic myocardium.

There are no published reports on the influence of reperfusion injury on the levels of MMPs in remote organs, which might be predicted to suffer damage. Soccal et al showed that in an animal model of ex vivo lung transplantation ischaemia/reperfusion, MMP-2 and MMP-9 were increased after 8 hours of ischaemia[470]. It is difficult to compare these findings to the current studies, in which the lung is not directly ischaemic, but undergoes pathological changes secondary to the remote organ effects of skeletal muscle ischaemia/reperfusion injury and due to the action of the inhalational anaesthetic. However, 8 hours of lung ischaemia probably represents significantly more pathological insult than would occur in the skeletal muscle ischaemia/reperfusion model and hence resulted in a more marked elevation of MMP-2 and MMP-9. Pardo induced lung injury in a rat model of hyperoxia induced by 100% oxygen and showed a marked elevation in MMP-2, MMP-9 and MMP-13[551]. Insitu hybridisation and immunohistochemistry revealed that all three MMPs were expressed in alveolar macrophages and in varying degrees by interstitial and alveolar epithelial cells. Again, the lung injury model used by Pardo et al[551] probably represents more significant pathological damage than in the current studies and hence the greater elevation in MMP-2 and MMP-9.

Forbes et al used a rat model of renal ischaemia/reperfusion injury and showed an immunohistochemical increase in MMP-2 at 8 days after 45 minutes of renal ischaemia, with an associated decrease in glomerular type IV collagen[476]. At 2 days, there were only minor immunohistochemical changes in MMP-2. No zymographic studies were performed[476]. Jain et al, showed in a renal model of ischaemia/reperfusion injury that the rise in MMP-2 occurred, albeit at 8 weeks, after 45 minutes of renal ischaemia[477]. Ziswiler et al utilized a rat model of

renal ischaemia/reperfusion and showed no evidence for an alteration in the activity or expression of MMP-2 or MMP-9 after 60 minutes of renal ischaemia[552]. They used zymography, reverse transcriptase polymerase chain reactions and creatinine levels to assess the levels of MMP expression and tissue damage at 12 and 24 hours post injury. Despite the lack of expression of MMP-2 and MMP-9, the creatinine levels significantly increased in the ischaemic group, which was not prevented by MMP inhibitor, BB-94[552]. Hence, it appears that changes in matrix metalloproteinases in the renal tissue following injury, either do not occur or do so after at least 8 days. As the current studies only continued for 3 days, no changes in MMP levels were detected.

The elevation of MMP-9 in damaged tissues, demonstrated above, is in agreement with previous studies in our laboratory[508] and others, showing leukocyte infiltration during skeletal muscle ischaemia/reperfusion injury. Schlag and co-workers demonstrated considerable extravasation of leukocytes into muscle tissue that increased with increasing reperfusion times and correlated closely with the degree of tissue injury[553]. These infiltrating leukocytes are capable of secreting high levels of MMP-9[554], confirming the elevation in gelatinolytic activity observed on zymography.

In summary, marked increases in MMP-2 and MMP-9 occurred in skeletal muscle ischaemia/reperfusion injury, which were maximal at 24 hours of reperfusion. There were minor changes in gelatinolytic activity in the lung, maximal immediately after the anaesthetic and after 4 hours of reperfusion, with declining levels at 24 and 72 hours. There were minimal changes in the gelatinolytic activity of MMP-2 and MMP-9 in the kidney.

CHAPTER 4:

DEGRADATION OF TYPE IV COLLAGEN

DURING

SKELETAL MUSCLE

ISCHAEMIA/REPERFUSION INJURY

4.1 Introduction

The current studies have shown that levels of MMP-2 and 9 are upregulated during skeletal muscle ischaemia/reperfusion injury, as seen on zymography and western blots (Chapter 3). The aim of the studies illustrated in this chapter was to show the association of the upregulation of MMP-2 and –9, with the degradation of the main substrate of these enzymes, type IV collagen.

Matrix metalloproteinase-2 cleaves gelatin[319], types IV and V collagen, type VII collagen found in anchoring fibrils[320], cartilage type X collagen[321,322], elastin[323], type I collagen[324], fibronectin[325], laminin-1, laminin-5[326] galectin-3[327], aggrecan[328], decorin[329], hyaluronidase-treated versican, proteoglycan link protein[330] and osteonectin[331]. Matrix metalloproteinase-9 also cleaves gelatin, type IV collagen, type V collagen[332], elastin, aggrecan[328], entactin, galectin-3[327], proteoglycan link protein[330], fibronectin and osteonectin[331]. MMP-9 has a much higher affinity for types IV and V collagen than MMP-2.

Immunohistochemical techniques were chosen to quantify the effects of ischaemia/reperfusion injury on the substrates of matrix metalloproteinases 2 and 9 in both ischaemic tissues and reperfused tissues. The quantitation of type IV collagen using immunohistochemical techniques has not been extensively utilized in the literature in this context[555-557], especially with the use of fluorescein isothiocyanate. Before performing the definitive quantitation of type IV collagen degradation following ischaemia reperfusion, other studies were performed to establish and validate the technique of type IV collagen quantification using fluorescein isothiocyanate immunohistochemistry.

4.1.1 Type IV Collagen

Collagen is the most abundant protein in the human body, representing 30 percent of its dry weight. The word *collagene* is a French neologism from the nineteenth century meant to designate the constituent of connective tissue that means glue[558]. There are over fourteen different types of collagen described and these are divided into four different classes[558,559]. The fibril-forming collagens including types I, II, III, V and XI which aggregate to form fibrils clearly visible under the electron microscope. The fibril-associated collagens, types IX and XII, are short structures that bind collagen fibrils to one another and to other components of the extracellular matrix. Network forming collagen is type IV collagen, whose molecules assemble in a meshwork that constitutes the structural component of basement membranes. Anchoring collagen is type VII collagen, present in the anchoring fibrils that bind collagen fibres to the basement membrane.

Type IV collagen is derived from three polypeptide chains [two α1(IV) and one α2(IV) chain], measures 400 nm and possesses a distinctive globular domain at its carboxy-end terminus[560]. Other chains are reported to be involved in the triple helix, such as α3, α4, α5 and α6 and the existence of six chains allows for many different kinds of isoforms of triple helix monomer that differ in type and stoichiometry[561]. The α1(IV) and α2(IV) chains appear to be ubiquitous, whereas the other chains have a restricted distribution[562]. In the kidney, the α3(IV) and α4(IV) chains have a similar distribution and are localized to the glomerular basement membrane[563], as is the α5(IV) chain[564]. The α1(IV) and α2(IV) chains are found in the mesangial matrix, glomerular, vascular and tubular basement membranes[563]. The α5(IV) chain is also expressed in brain tissue[565]. In muscle, the α3(IV) and α4(IV) chains occur in synaptic muscle fibres whereas α1(IV) and α2(IV) occur in extrasynaptic muscle fibres[566]. In the aorta, the classical α1(IV) and α(IV) are found, along with small amounts of α3(IV), α4(IV) and α5(IV)[567]. In the lung, α1(IV) and α3(IV) have been identified[568]. The production of type IV collagen for the basement membrane takes place in cells arising from mitoses in young capillaries[569], arising along a pathway extending from the rough endoplasmic reticulum through Golgi saccules to secretory granule that release their content to the outside[569].

Type IV collagen triple helical monomers can self-assemble into a stable three-dimensional basement membrane network involving tetramers and dimers of type IV collagen molecules connected and cross-linked via their like ends[570]. The network includes lateral interactions along the triple helix and additional binding of further terminal domain NC1 segments into

triple helical domains[571,572]. The three-dimensional network of type IV collagen forms the basic superstructure upon which the other components of the basement membranes are attached[570,573].

4.1.2 Basement membrane structure

Basement membranes are found in every organ in the body and their components are synthesized by the cells resting upon them, including epithelial, endothelial muscular and adipose cells[574].

Basement membranes are specialized extracellular matrices, which serve as a support, a sieve and a barrier, keeping cells on one side and proteins on the other side. The nomenclature of "basement membranes" has been variable and confused in the past[575]. The International Anatomical Nomenclature Committee recommended the successive layers of the basement membranes to be called: "lamina lucida", the pale layer in immediate contact with the plasmalemma of the associated epithelial or other cells; "lamina densa", the dark layer below; "lamina fibroreticularis", the incomplete layer in continuity with connective tissue[576,577]. This classification is justified by the fact that the specific components of the basement membrane type IV collagen, laminin and heparan sulphate proteoglycan) are present in the three layers and little or none is found elsewhere. The term basement membrane is used to specify a periodic acid-Schiff-positive layer, visible beneath the light microscope, beneath epithelia and in the kidney glomerulus and lung alveoli[559]. The structure of a typical basement membrane consists of a thin sheet like layer sandwiched between a cell layer and a thick collagenous stroma.

Although basement membranes are widespread tissue components, their fine structure and composition varies from tissue to tissue as well as within the same tissue at different developmental periods and during repair. All basement membranes contain laminins, entactin-1/nidogen-1, type IV collagen and heparan sulfate proteoglycans and of these components, type IV collagen and laminin account for the greatest mass as studied in vitro[578]. Entactin/nidogen acts as a bridge between the two major basement membrane proteins, type IV collagen and laminin. The large heparan sulfate proteoglycan molecules are firmly anchored to the basement membrane via laminin[560]. Osteonectin (also known as BM-40 and SPARC) is also found in basement membranes, with its interaction mediated by calcium but not via laminin or the laminin/entactin complex[579]. Type VII procollagen forms dimers that

are the anchoring fibrils, connecting basement membrane components deep within the stroma to each other[560]. Other basement membrane components include fibulins, which are bound to the network via calcium binding[580], fibronectin and the newly discovered proteoglycans, agrin and type XVIII collagen[581].

The tissue support provided by the basement membrane is a direct consequence of the formation of a cohesive polymer of type IV collagen and laminin. The mesh-like structure of the basement membrane network is part of the formation of the tissue sieve, however, the charged heparan sulfate possess very large spheres of hydration which leave little free water between macromolecules, permitting passage of only small macromolecules across the basement membrane[560]. The basement membrane also provides an interactive surface for the regulation of cell function, with the binding of one component to the next possibly blocking the access to a cell determinant or altering conformation and cell binding of another component[560].

The basement membrane was once thought to be an inert membrane, however the complexity and diversity of the basement membrane continues to unfold. Remodelling of extracellular membrane is seen in physiological processes throughout life from ovulation[582,583] and morphogenesis[584] to involution and cell death[585,586]. In ischaemia/reperfusion injury, there is disruption of the basement membrane as seen in the brain with decrease in fibronectin, type IV collagen and laminin[587,588] and in the lung[471], myocardium[589] and skeletal muscle[590].

4.1.3 Immunohistochemical Techniques

Immunohistochemistry utilizes the antibody specificity of immunoglobulins to detect specific constituents within tissue sections prepared for microscopy. Immunohistochemistry requires specific antibodies, with high affinity antibodies increasing reaction specificity and reliability and decrease background as well as cross reactions with undesired antigens. In order to reveal reaction of antibody with antigens in tissue sections, it is necessary to mark the antibody with labels to allow visualisation by light or electron microscopy. The marker must be of sufficient intensity to be detected and these include highly fluorescent compounds, such as fluorescein and rhodamine; or enzymes such as horseradish peroxidase, glucose oxidase and intestinal alkaline phosphatase that are used for light microscopy[591].

Ideally, only the specific antibody should react with the desired antigen and the remaining constituents of the reagent are removed by washing. However, the polarity of fluorescein can create non-specific attachment to tissue constituents, that is 'method non-specificity'[591]. In addition, reaction of the isothiocyanate with amino groups of proteins increases the negative charge of the conjugate and thus invites non-specific staining. These errors are largely avoided by using indirect immunofluorescence where the primary antibody is unlabelled. The second step used in the studies described in this chapter involved biotinylated anti-rabbit IgG that binds to the primary antibody. Biotin is a small molecular weight vitamin that functions as a prosthetic group to a number of transcarboxylases. Biotin has a very strong affinity for Streptavidin, which is prepared from the culture supernatant of Streptomyces avidinii. Unlike avidin, Streptavidin has an isoelectric point close to neutrality and contains no carbohydrate, making it less prone to non-specific binding[592]. The higher sensitivity of multiple layer techniques allows the use of lower titres of the primary antiserum and produces positive staining results even in tissue fixed under less than optimal conditions[593], so indirect immunofluorescence is preferred. In order for a molecule to emit its absorbed light as fluorescence, that part of the molecule that is responsible for light absorption must possess a structural rigidity sufficient to prevent dissipation of energy by rotation. Fluorescein and rhodamine fulfil these conditions[591], also their green and red emission colours respectively, make them distinguishable from the bluish autofluorescence of nucleic acids and the proteins, tryptophan and tyrosine in tissues.

Once the tissue slides are prepared, an image analysis system must be used to measure the fluorescent signal intensity of the fluorochrome–labelled antibody. The phenomenon of luminescence occurs when a substance absorbs ultraviolet light and converts its energy to light of a longer wavelength. The type of luminescence that stops immediately after excitation ceases is fluorescence. The relationship between the wavelength of the exciting light (λ_E) and that of the emitted light (λ_F) is governed by Stoke's Law, $\lambda_E<\lambda_F$. Since the fluorescent light is extremely weak in comparison to the exciting light, if the latter contained light of the same wavelength as the former, it would be impossible to distinguish the fluorescence. Therefore, it is necessary to filter the exciting light so that it contains light of predominantly shorter wavelength, resulting in maximal fluorescence emission. Other filters are used as a barrier to the exciting light, which is not absorbed by the specimen, so that it does not follow the same optical path to the eye and therefore the fluorescence that is emitted by the specimen can be recognized. This makes the microscopic field dark except for the specifically stained fluorescent component. Even though the fluorescent tissue may emit only 1% as much light as

is seen in light microscopy under full transmission, this 1% is viewed in a surrounding field of practically 0% transmission and can, therefore, be easily discerned. Intensity of the light source does increase the sensitivity because it increases fluorescent emission against a background that remains dark. The fluorescent molecule that is used should not be able to absorb the wavelength at which is emits light; as this would create internally quenching[591]. Quenching is the deterioration of the image produced, because the tissue absorbs the wavelength of light it emits and thus the immunofluorescence seen in the image diminishes. This is explored further in Chapter 4.2.4 on page 127.

Reflected light fluorescence microscopy involves the specimen being illuminated from above with a mercury lamp, allowing most of the light that is not absorbed to pass through. The use of a dichroic mirror reflects almost all of the light of a wavelength shorter than a specific value and allows most of the light with a wavelength longer than that value to pass through. After passing through the barrier filter, the emitted light is captured on the camera, converted to a digital signal and displayed on the computer monitor. The image can be then stored on the computer hardware.

4.2 General Immunohistochemical methods

Frozen sections of tissues were cut using the Microm HM 505N cryostat with the temperature set at minus 28°C. Tissue was removed from the -80°C freezer and kept frozen, with a maximum temperature of minus 28°C. A small sample of tissue was embedded into Tissue Tek® OCT and cut at the chosen thickness. Three to four slices of tissue were placed reasonably close together on a prepared Poly-L-lysine coated slide. (Appendix 7.5.1 Slide preparation for Immunohistochemistry). Two slides of each tissue were prepared, one for positive staining with primary antibody and one for negative staining without primary antibody. The tissue was immediately re-wrapped in foil and frozen in liquid nitrogen before storing back in the minus 80°C freezer. The slide was air-dried and then placed in 20% acetone in the -20°C freezer for twenty minutes. The slide was then allowed to dry. The area of tissue was circled with a PAP pen and each slide was washed for 5 minutes, three times in phosphate buffered saline (PBS) (Appendix 7.4.16 Phosphate Buffered Saline for Immunohistochemistry) with 0.1% Bovine Serum Albumin (BSA).

The primary antibody used to detect type IV collagen was anti-collagen type IV (Affinity purified Anti-Collagen Type IV [Rabbit], Rockland, Gilbertsville, PA, USA), diluted to chosen concentration in PBS with 0.1% BSA. One hundred µl of the anti-collagen Type IV solution was pipetted onto the tissue on the positive slides or 100µl of PBS with 0.1% BSA was pipetted onto the tissue on the negative slides. All slides were placed into a sealed container lined with paper towel soaked in Milli Q Plus H_2O, in order to keep tissue moist and incubated at 4°C for 15 hours.

Each slide was washed for 5 minutes at room temperature, three times in PBS with 0.1% BSA. The secondary antibody used was Biotinylated anti-rabbit IgG antibody (Vector Laboratories), diluted to 1:400 in PBS with 0.1% BSA. On hundred µl of the biotinylated anti-rabbit antibody was pipetted onto the area surrounded by PAP pen on all the slides. The slides were then incubated for 60 minutes in a humidified container at room temperature.

Each slide was washed for 5 minutes, three times in PBS with 0.1% BSA The tissues were then labelled with 100µl Streptavidin-fluorescein (Streptavidin– fluorescein RPN 1232, Amersham Pharmacia, Biotech UK Limited), diluted to 1:200 with PBS with 0.1% BSA. The slides were then incubated for 60 minutes in the humidified container at room temperature.

Each slide was washed for 5 minutes with three changes of PBS with 0.1% BSA. One drop of Dako® Fluorescent Mounting Medium was placed on each slide and a coverslip (Menzel GLÄSER) placed over the top. The slides were covered with foil and placed in a 4°C refrigerator until images were captured.

Images were collected on an Olympus BH2 microscope with Reflected Light Fluorescent Attachment (Olympus BHRFL-W) with maximum excitation wavelength of 494 nm and maximum emission wavelength of 518 nm. The images were captured on a Panasonic Camera Control Unit (WVCU204) and Panasonic Camera (WVCL700) and stored as Joint Photographic Experts Group images (JPEGs). The images were analysed using a Video Pro 32® automated image analysis system (Leading Edge Pty Ltd, Marion, South Australia). Colour images were collected at a magnification of 20X. For each sample, images were collected in a standardized pattern as shown in Figure 19. This pattern aimed to achieve consistent coverage of skeletal muscle fibres or of lung tissue. In the kidney, an even selection of cortical and medullary sections were chosen. The edges of tissue were avoided in order to minimize the edge effects of fluorescence enhancement that occur with FITC immunohistochemistry.

For the image analysis, the *brightness* level was chosen for quantitative comparisons. The luminance component of the image has a digital resolution of 8 bits, giving a range of values from 0 to 255 as the value for *grey* without corrections. The *intensity* is the grey value, corrected for the response of the camera, according to a software add-in programme, CL700 that is specific for Panasonic Camera (WVCL700). The response of the video camera is not linearly related to the intensity of light, rather it is described by a logarithmic equation that has an exponent factor of Gamma. Gamma is found by plotting the response of the camera against the known transmittance of standard filters and fitting a curve to the data, the calibration programme C1700 is used for this purpose. The value recorded is equivalent to a measurement of transmittance or reflectance, but is on a scale from 0 (black) to 255 (white). The *brightness* variable is used to relate to the total amount of light emitted from an object. The brightness is the product of the area of the feature and its intensity. In order to relate brightness to a unit scale, the following formula is used for brightness:

Equation 5: Calculation of Brightness level in Quantitative Immunohistochemistry.

$$B = I \times A/255$$

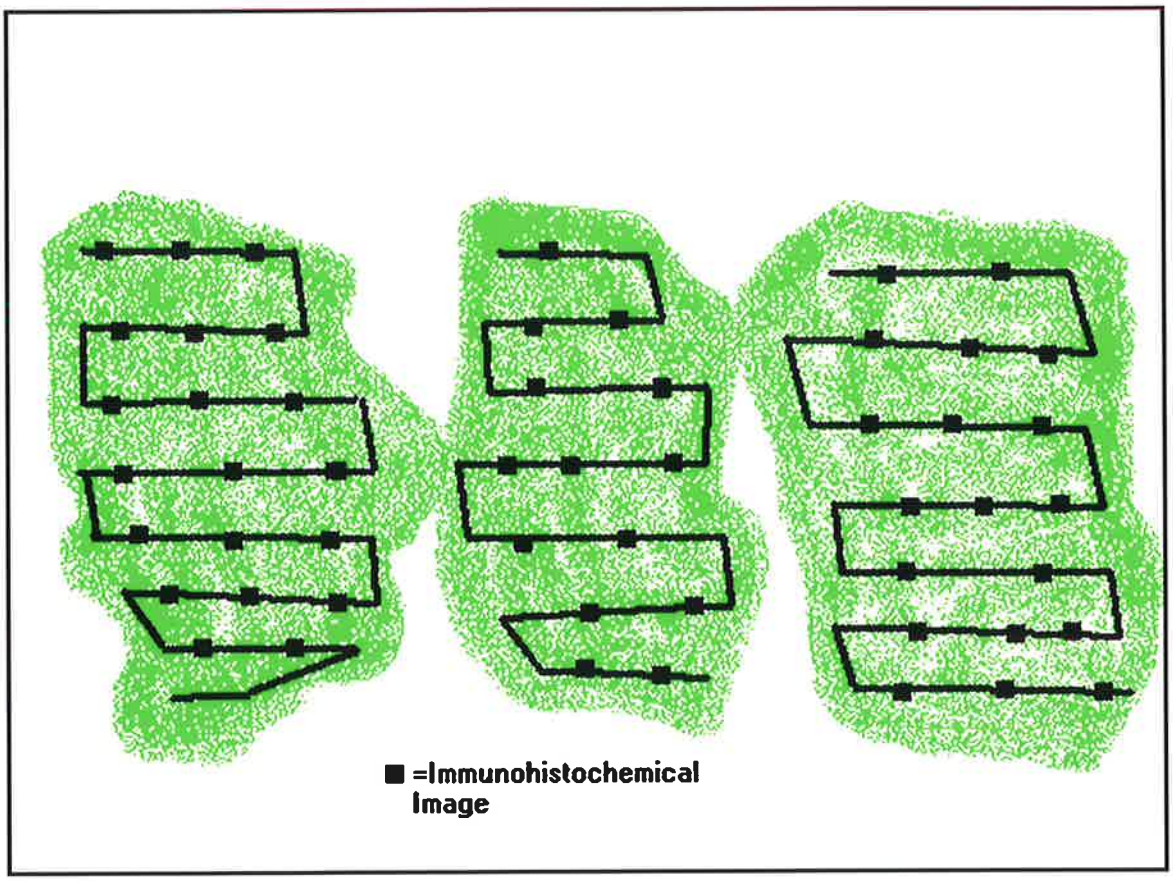
Where B = Brightness, I = Intensity and A = area.

For each set of images, a histogram level is set at a figure between 0 and 255. The grey level is measured in all pixels, but the discriminated value is only in those pixels between that histogram set figure and 255. Pixels with a value lower than the set histogram value are regarded as not emitting fluorescence and not containing significant levels of type IV collagen.

As an example, in image 8 of the quantitation images collected for left leg skeletal muscle, the histogram level was set at 160 so all pixels with a grey scale between 160 and 255 were measured and their average grey level was 192. This was then calibrated for the response of the camera (using software programme CL700), giving an intensity level of 158.05. The total area that was covered in pixels with grey values between 160 and 255 was 54312 units and hence using the equation stated above, this equates to a brightness figure of 33662 units for that image.

Figure 19: Diagrammatic representation of method of selection of Immunohistochemical images across tissue sections on one slide.

The edges of the tissue were avoided and images were taken at sufficient distance apart from each other to avoid the quenching effect from the preceding image.


■ =Immunohistochemical
Image

4.2.1 Pilot studies, Negative Controls and Histogram levels

The initial series of immunohistochemical staining experiments were performed to test the quality and suitability of the antibodies and to see if the was a difference in the level in the type IV collagen discernible between sham-operated and unilateral ischaemic rats. The first experiments were carried out using only 3 rats in each group, comparing sham-operated animals sacrificed at 24 hours to animals that underwent 4 hours of unilateral ischaemia and 24 hours of reperfusion. These first studies were carried out only in the left leg skeletal muscle and in the lung. In the skeletal muscle, a thickness of 10 microns per tissue section was initially chosen. In the lung, an initial tissue thickness of 3 microns was used. These thickness levels were further investigated in later experiments. The basic technique as described in section 4.2 was used.

A series of negative control slides were cut and stained in this investigation and throughout the remaining investigations. These followed the same technique as outlined above, however the primary antibody staining with anti-collagen type IV antibody was omitted and replaced with 100μl of PBS with 0.1% BSA.

For the analysis of images, an arbitrary histogram level was chosen which remained constant for each tissue within the one staining run. For one image, a series of data were collected at different histogram levels to demonstrate the nature of this arbitrary cut off level. Pixels with grey levels detected below this level were regarded as negative and pixels with grey levels above this cut off level up to the maximum intensity of 255 were regarded as positive and containing type IV collagen. Therefore, this represents the arbitrary cut-off between staining and non-staining of immunofluorescence. This histogram level was judged in each staining run for each tissue, by examining 10 images and choosing the appropriate histogram level to discriminate positive staining from background staining levels.

4.2.2 Determination of the Number of Images required for Reproducibility

This series of studies were performed to establish how many images were required in each run to achieve an appropriate number of images for statistical analysis. Two rats were selected; rat 36 (sham-operated animal, no ischaemia, sacrificed at 24 hours) and rat 46 (4 hours of unilateral ischaemia and 24 hours reperfusion). One section of each tissue was cut at

thicknesses of 10 μm for skeletal muscle, 3 μm for lung and 5 μm for kidney respectively. The slides were prepared as described in section 4.2. A series of 35 images were captured of each slide and the images were analysed with Video Pro 32® automated image analysis system to establish the individual Brightness level of each image. A cumulative mean brightness level was calculated in order to establish when the mean level reached a steady state and hence decide how many images were necessary for quantitative analysis of type IV collagen degradation during ischaemia/reperfusion. Cumulative mean was calculated as the mean for images 1 and 2; the mean for images 1, 2 and 3; the mean for images 1, 2, 3 and 4 and so on until the final mean is the average of all 35 images. The histogram limit was set at 160 for left leg skeletal muscle, 180 for lung tissue and 170 for kidney tissue.

4.2.3 Thickness of Tissue Sections

In order to establish the appropriate section thickness for each tissue, an immunohistochemical series of experiments was performed. If the tissue is too thick, it is possible that the antibody will not reach all levels of the tissue evenly and thus give a spurious result. If the tissue is too thin, an inaccurate result will also occur due to technical problems such tearing of the tissue whilst slicing and inaccurate representation of the antigens in the tissue.

For the skeletal muscle of the left leg, 1, 2, 5, 10 and 14 μm tissue sections sliced on the cryostat; for the lung, 1, 2, 3, 6 and 9 μm sections; and for the kidney, 1, 2, 5, 10 and 14 μm sections. Other than variation in the thickness of the tissue section, these slides were prepared as in section 4.2. All experiments were performed on rat 28, which was killed outright by stunning and cervical spinal dislocation. The histogram limits were 170 for left leg skeletal muscle, 180 for lung tissue and 170 for kidney tissues.

4.2.4 Quenching of Fluorescence by the Microscope Lamp

To investigate the rate of the quenching of the fluorescein isothiocyanate whilst the tissue slide is under the fluorescent lamp, a series of investigations were performed. The slide was placed under the microscope and multiple images taken at intervals from a few seconds to 30 minutes, without moving the slide. This was repeated for each tissue. All experiments were performed on rat 28, which was killed outright by stunning and cervical spinal dislocation. All

preparation methods were described in section 4.2. The histogram limits were 150 for left leg skeletal muscle, 165 for lung tissue and 170 for kidney tissues.

4.2.5 Antibody Saturation

The aim of this series of experiments was to establish the most appropriate concentration of primary antibody to be used. This creates the most reliable indication of actual level of type IV collagen, without saturating the protein level or failing to stain it adequately. The type IV collagen antibody dilutions that were chosen ranged from 1 in 1000 to 1 in 25 dilution. Each slide was incubated with the chosen antibody dilution and then processed as in section 4.2. Thirty-five images were captured for each tissue and the brightness level calculated. All experiments were performed on rat 28, which was killed outright by stunning and cervical spinal dislocation. The histogram limits were 170 for left leg skeletal muscle, 180 for lung tissue and 170 for kidney tissues.

4.2.6 Quantitative Immunohistochemistry

The main aims of this section were to quantify the changes in type IV collagen that occur during skeletal muscle ischaemia/reperfusion injury for the skeletal muscle itself and also for the remote organs of the lung and kidney.

Using the results of the thickness studies, the following tissue thicknesses were chosen: 10 microns for skeletal muscle, 5 microns for kidney and 3 microns for lung tissue. The primary antibody dilution was chosen as 1 in 100. Thirty-five images were taken of each tissue slide and analysed with the Video Pro 32® software.

The tissue slides were prepared as in section 4.2. Each set of samples to be compared were analysed together to avoid errors such as age of the antibodies, slight differences in concentration between batches, age of the fluorescent lamp, sampling differences and tissue differences. In all future analyses, only tissues that were analysed in one individual experiment were compared to one another to allow accurate comparisons.

For the left leg skeletal muscle, the histogram limit was set at 160 for the initial data set and set at 159 for the data set involving the bilateral 4 hour ischaemia and 72 hours of reperfusion.

The histogram limit for the right leg skeletal muscle studies was 190. The histogram limits for the lung were set at 190 for the main data set and 180 for the bilateral 4 hour ischaemia with 72 hours of reperfusion data set. The histogram limits for the kidney were set at 190 for the main data set and 170 for the Bilateral 4 hour ischaemia with 72 hours of reperfusion data set.

4.2.7 Descriptive Statistics

Data was analysed as a block of 10 cells for each tissue and these results are described in section 4.3.6.1, 4.3.6.2, 4.3.6.3 and 4.3.6.4. These statistical results were then spliced to give the comparisons between the individual cells and these results are described in sections 4.3.6.5 and 4.3.6.6.

Data was analysed using an unbalanced 2-way ANOVA 3x4 factorial design with interaction. ANOVA partitions the variation in variables values into variation between and within groups.

If the overall F-test is significant, this means that the model as a whole is significant, that is, there is evidence to suggest that the individual cell means are different.

The R-square is interpreted as the percent of variation in unit that can be accounted for by the model.

Type III sums of square are preferred in testing the effects of reperfusion, group and the interaction between them in unbalanced designs. An unbalanced design occurs when the number of observations per treatment combination are not equal or when there are missing cells.

The interaction term in the model tests the hypothesis that the effect of reperfusion does not depend on group and vice versa. If the interaction term is significant this means that the effect of reperfusion depends upon which group the animal is assigned.

4.3 Results

4.3.1 Pilot studies, Negative Controls and Histogram levels

Representative images of type IV collagen immunostaining of the sham-operated and unilaterally ischaemic animals are seen in Figure 20 for skeletal muscle and Figure 22 for lung tissue. These images show the marked loss of type IV collagen staining in the rat that underwent unilateral ischaemia, compared to the sham-operated animal. These figures are represented graphically in Figure 21 and Figure 23. The graphs show that there is a distinct difference in type IV collagen staining between the sham-operated animals and the unilaterally ischaemic animals. Given the demonstration that the immunohistochemical technique could be used to discern a difference in type IV collagen levels between sham-operated and ischaemia/reperfusion animals, further studies were performed. The summary of the brightness measurements for images is shown in Table IX on page 131. The complete set of raw data for brightness levels are shown in Appendix 7.6.1 Pilot studies, Table XXIII and Table XXV on pages 236 and 238.

There was very minimal background staining of fluorescence on the negative control slides. The level for brightness in each negative slide was small compared to the level for brightness on the equivalent positively stained slide. Hence, for the statistical analysis, it was accepted that there was no background staining and the negative control slides were not formally analysed. The complete set of raw data results for negative controls are shown in Appendix 7.6.1 Pilot studies; Table XXIV and Table XXVI.

The analysis of the histogram cut-off levels show that with increasing levels, then fewer pixels were included in the selected area, giving a lower figure for brightness, which would have been interpreted as less type IV collagen. The graphical representation is shown in Figure 24 and Figure 25 showing that if the histogram figure is too low virtually all of the image would be included and regarded as positive for staining, thus not allowing discrimination between images. If the histogram level is set too high, little staining is regarded as positive and the majority of the area that was actually staining for type IV collagen would be ignored. Hence, for future studies a value was chosen that selected most of the positive staining ensuring accurate interpretation of the level of type IV collagen in each image. As an example, in Figure 24, the histogram level would have been 150 units, and remained constant for that analysis. The level varies between experiments as the immunofluorescence level varies in intensity depending on error factors such as age of the fluorescent lamp, minor

variations in staining, and other technical considerations. These errors are regarded as constant for each set of data and hence the histogram level is set at a constant figure for each data set. The technical errors involved in immunofluorescence technique are discussed in detail in section 4.4.2. The histogram data set is shown in Appendix 7.6.1 Pilot studies, Negative Controls and Histograms, Table XXVII.

Table IX: Summary of Skeletal Muscle and Lung Pilot Data

Tissues were stained as described in section 4.2. Sham-operated 0/24 refers to rats with no ischaemia, 4 hour anaesthetic and euthanased at 24 hours. Uni 4/24 refers to rats that underwent 4 hours of unilateral ischaemia, then 24 hours of reperfusion. Brightness Positive indicates analysis of images in slides where the type IV collagen antibody was applied during the slide preparation. Brightness Negative, are the images of slides used as Negative Controls and had no type IV collagen antibody applied during the slide preparation.

Skeletal Muscle						**Lung**					
Sham 0/24			**Uni 4/24**			**Sham 0/24**			**Uni 4/24**		
Rat Number	**Brightness Positive**	**Brightness Negative**	**Rat Number**	**Brightness Positive**	**Brightness Negative**	**Rat Number**	**Brightness Positive**	**Brightness Negative**	**Rat Number**	**Brightness Positive**	**Brightness Negative**
19	25854	3.0	16	21228	230	19	93488	10.5	16	17602	19.7
33	37334	11.1	30	3920.2	41.0	33	78361	107.9	30	50818	11.1
34	46977	1365.9	32	33525	23.5	34	55470	52.5	32	16473	66.4
Mean	**36722**	**460.0**		**19558**	**98.2**		**75773**	**57.0**		**28298**	**32.4**
St Dev	**10575**	**784.5**		**14873**	**114.5**		**19141**	**48.9**		**19511**	**29.7**

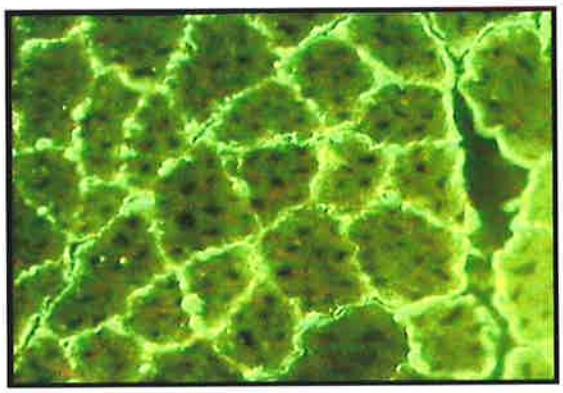
Figure 20: Representative Immunohistochemical Images of Skeletal Muscle from Pilot study.

Rats were subjected to a sham-operation with 4 hour anaesthetic and no ischaemia; or unilateral 4 hour ischaemia and reperfusion for 24 hours. Tissues were stained with type IV collagen antibody, FITC preparation, Magnification 20 X.

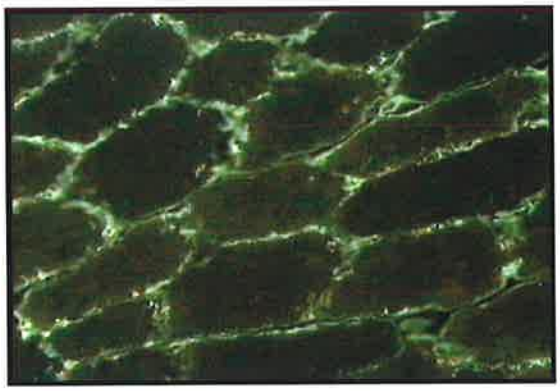
Figure 21: Analysis Graph of Skeletal Muscle Pilot Data.

Rats were subjected to a sham-operation with 4 hour anaesthetic and no ischaemia; or unilateral 4 hour ischaemia and reperfusion for 24 hours. Mean level for brightness is represented by the solid column with standard deviation bars.

Left leg skeletal muscle, 24 hours



Sham-operated



Unilateral ischaemia

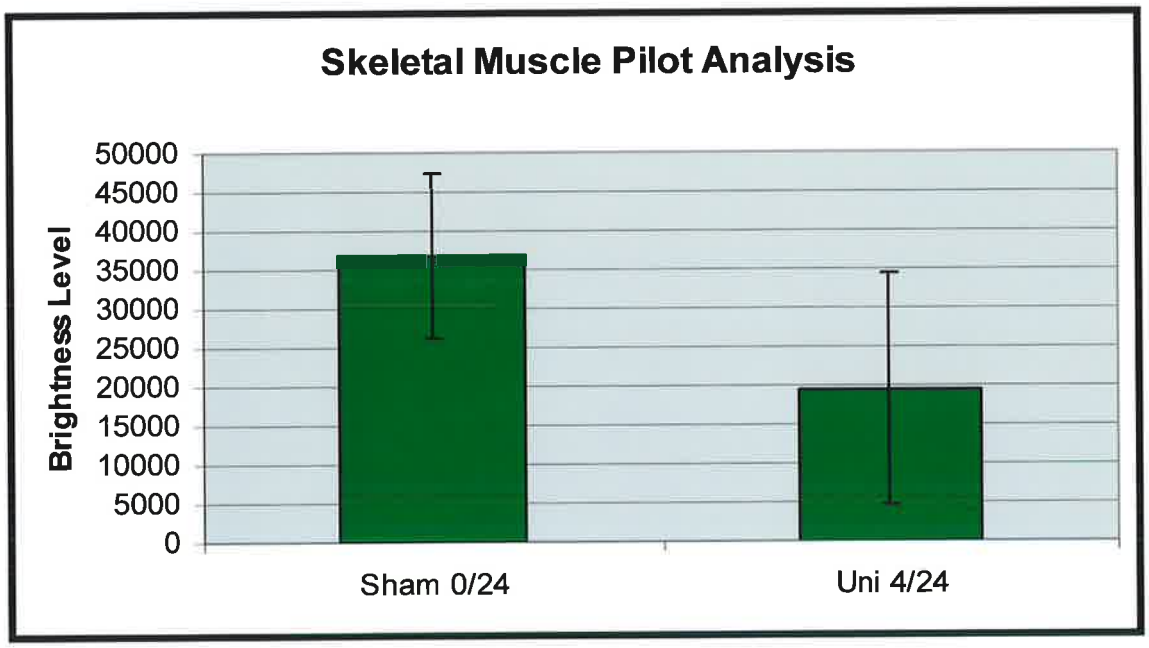


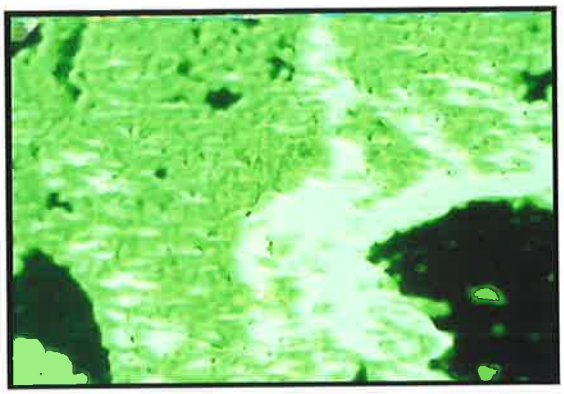
Figure 22: Representative Immunohistochemical Images of Lung from Pilot study.

Rats were subjected to a sham-operation with 4 hour anaesthetic and no ischaemia; or unilateral 4 hour ischaemia and reperfusion for 24 hours. Tissues were stained with type IV collagen antibody, FITC preparation, Magnification 20 X.

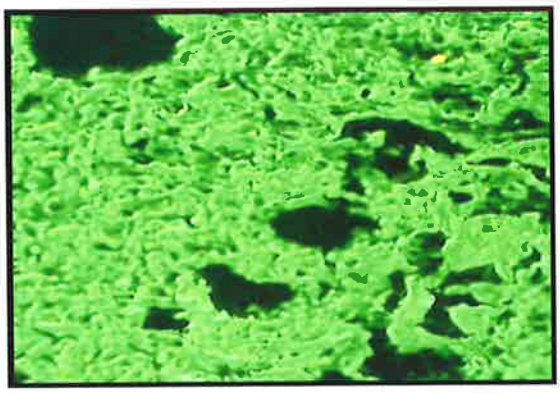
Figure 23: Analysis Graph of Lung Pilot data.

Rats were subjected to a sham-operation with 4 hour anaesthetic and no ischaemia; or unilateral 4 hour ischaemia and reperfusion for 24 hours. Mean level for brightness is represented by the solid column with standard deviation bars.

Lung, 24 hours



Sham-operated



Unilateral ischaemia

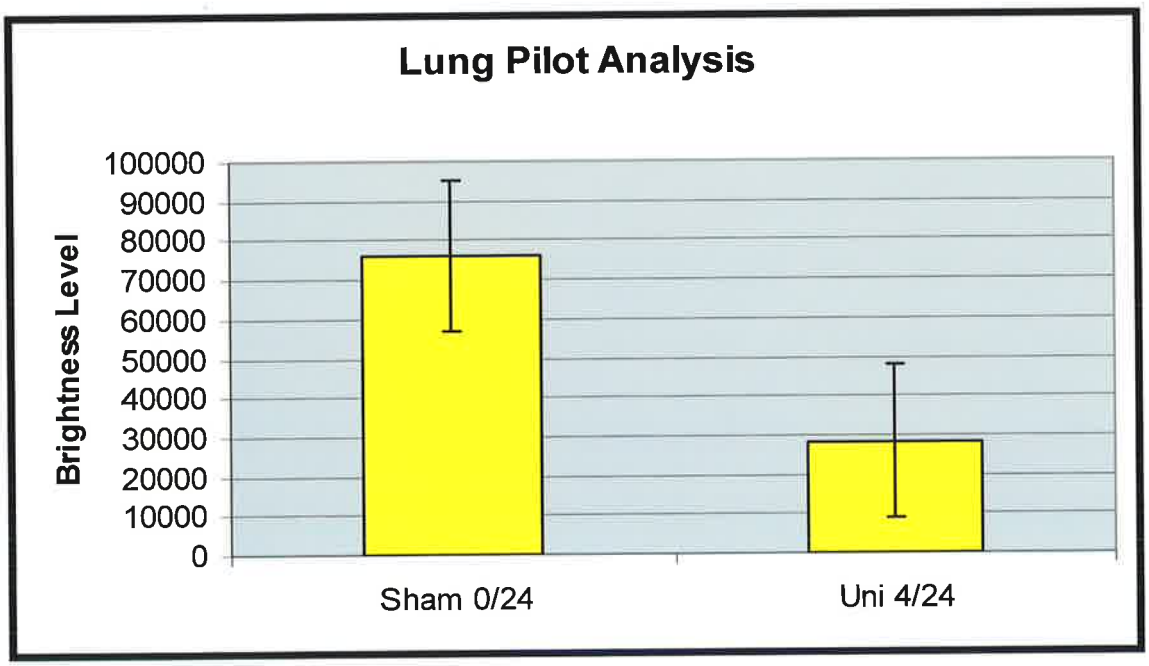
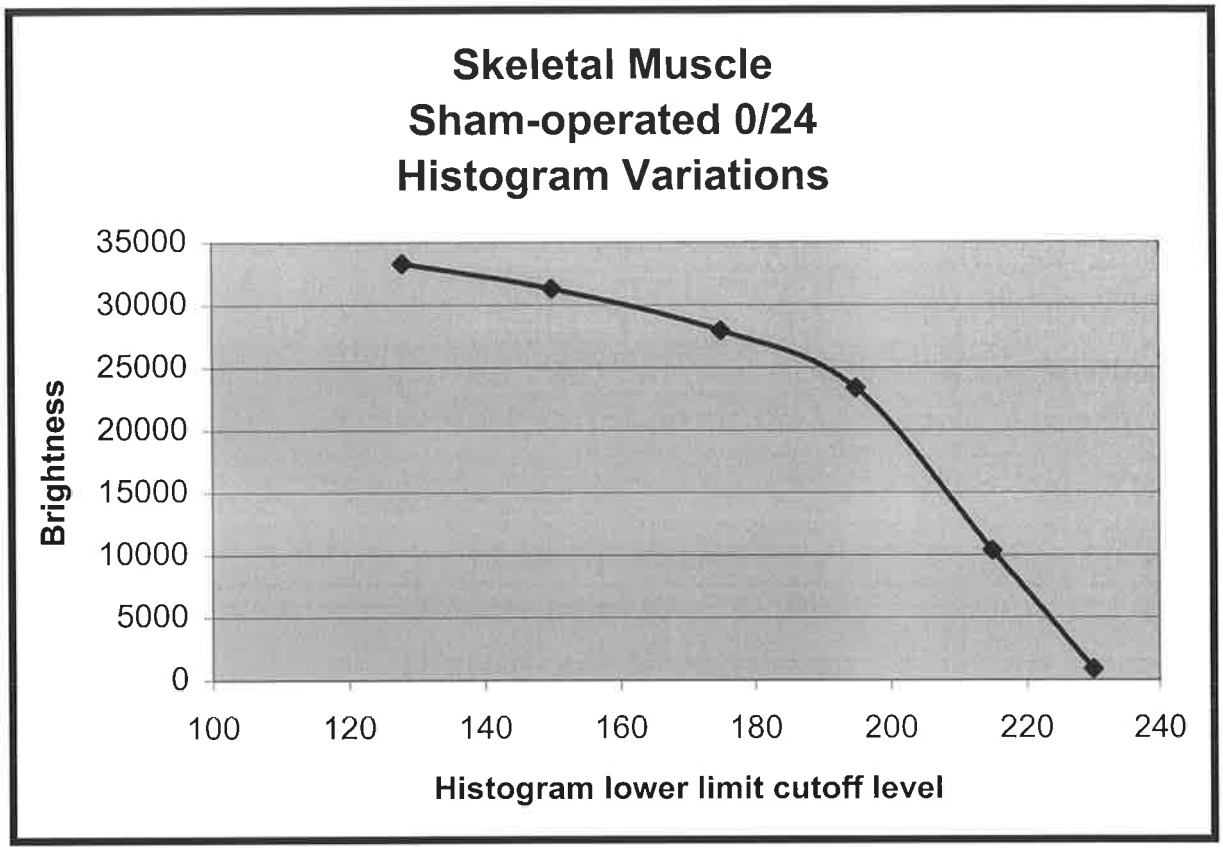


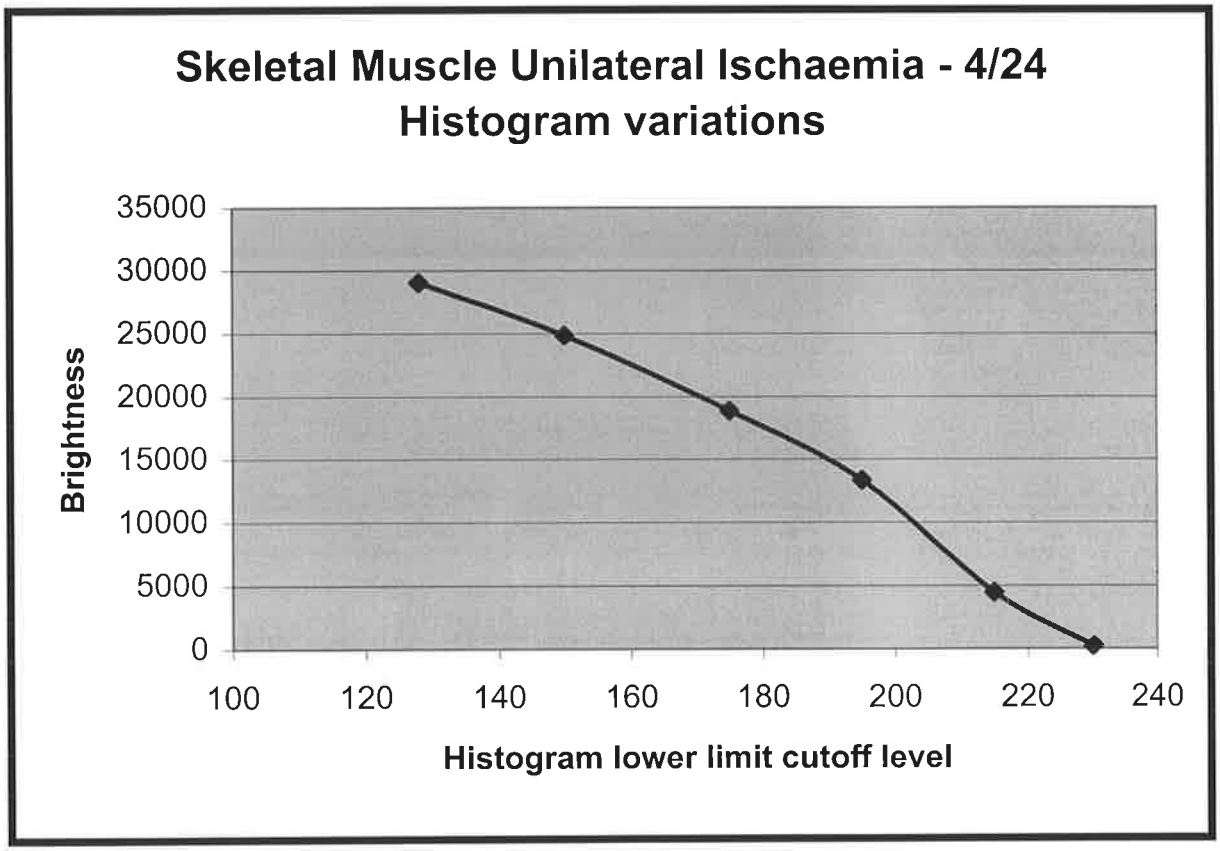
Figure 24: Skeletal Muscle of Sham-operated animals showing Brightness level at various Histogram cut-off points.

Sham-operated 0/24 refers to a rat with no ischaemia, 4 hour anaesthetic. Each point on the graph represents the lower limit of the histogram cut-off. The brightness level measured included all pixels with an intensity level between the lower cut-off level and 255. This is the area regarded as positive for type IV collagen staining.

Figure 25: Skeletal Muscle of animals that underwent Unilateral Ischaemia showing Brightness level at various Histogram cut-off points.

Unilateral 4/24 refers to rat that underwent 4 hours of unilateral ischaemia, then 24 hours of reperfusion. Each point on the graph represents the lower limit of the histogram cut-off. The brightness level measured included all pixels with an intensity level between the lower cut-off level and 255. This is the area regarded as positive for type IV collagen staining.


Skeletal Muscle
Sham-operated 0/24
Histogram Variations
Brightness
35000
30000
25000
20000
15000
10000
5000
0
100
120
140
160
180
200
220
240
Histogram lower limit cutoff level


Skeletal Muscle Unilateral Ischaemia - 4/24
Histogram variations
Brightness
35000
30000
25000
20000
15000
10000
5000
0
100
120
140
160
180
200
220
240
Histogram lower limit cutoff level

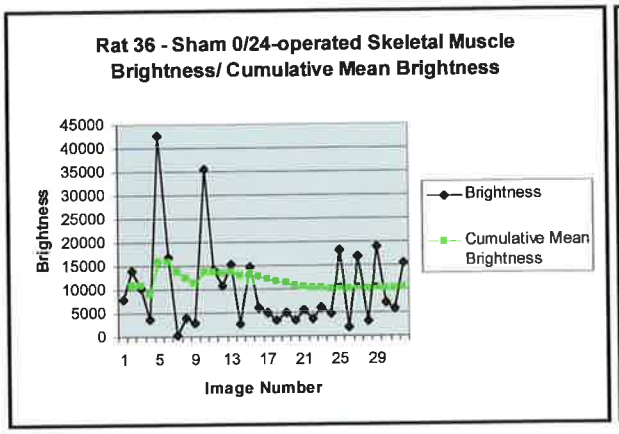
4.3.2 Determination of the Number of Images required for Reproducibility of Brightness Measurement

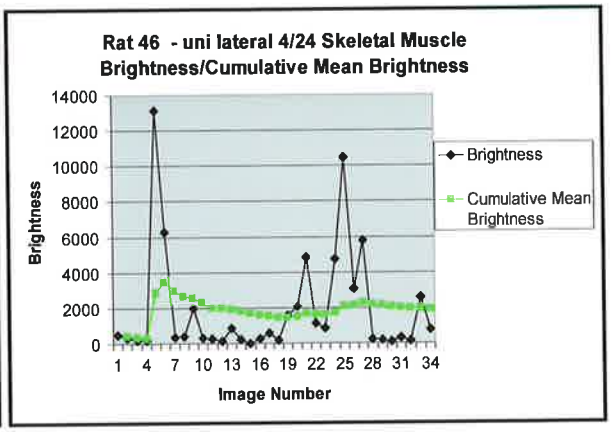
Figure 26 demonstrates graphically the result of the individual levels of brightness levels from the 35 images and the result of the cumulative mean brightness. As shown, for all three tissues the cumulative mean brightness reaches a steady state after 25 to 26 images have been included. This implies that after 25 images have been captured, any further images included would not change the arithmetic mean to be used in the statistical analysis. Therefore, for the future immunohistochemical quantitation analyses, 35 images were captured to ensure that the number of images reached a normal statistical distribution. This is illustrated in graphs of randomly selected rats showing the 35 images used in quantitation (Figure 27), which all showed a normal distribution of the brightness measurements, regardless of the type of experiment (sham-operated/unilaterally or bilaterally ischaemic).

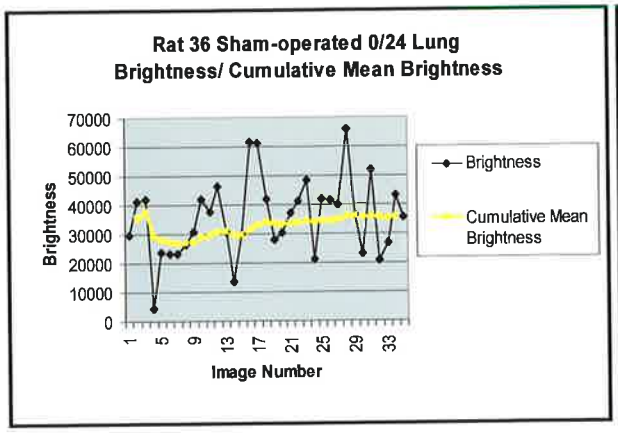
The complete set of raw data for the sampling analysis results are shown in Appendix 7.6.2 Number of images required for Reproducibility Studies, Table XXVIII.

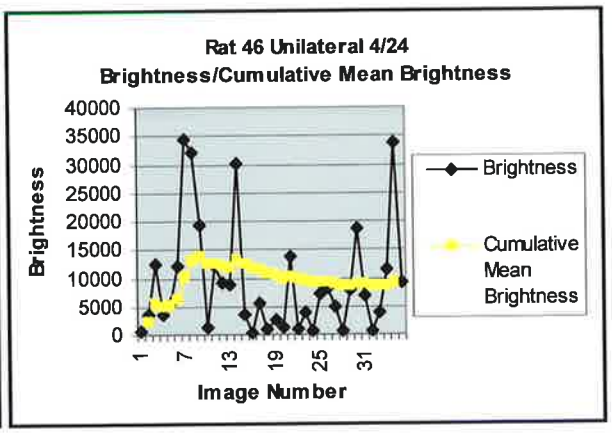
Figure 26: Graphs of number of images required for Reproducibility, showing Brightness/Cumulative Mean Brightness Levels.

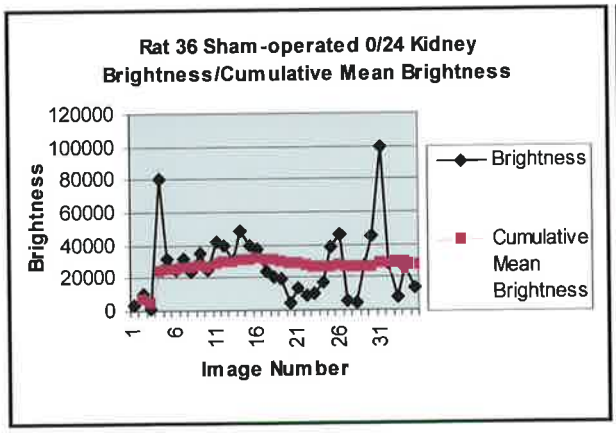
Sham-operated 0/24 refers to rats with no ischaemia, 4 hour anaesthetic and euthanased at 24 hours. Unilateral 4/24 refers to rats that underwent 4 hours of unilateral ischaemia, then 24 hours of reperfusion. The black lines refer to the individual level of brightness for each of the 35 images. The coloured lines refer to the level of the cumulative mean of the brightness for the images. Cumulative mean was calculated as the mean for images 1 and 2; the mean for images 1, 2 and 3; the mean for images 1, 2, 3 and 4 and so on until the final mean is the average of all 35 images.


Rat 36 - Sham 0/24-operated Skeletal Muscle
Brightness/ Cumulative Mean Brightness
Brightness
Image Number
Brightness
Cumulative Mean Brightness


Rat 46 - uni lateral 4/24 Skeletal Muscle
Brightness/Cumulative Mean Brightness
Brightness
Image Number
Brightness
Cumulative Mean Brightness


Rat 36 Sham-operated 0/24 Lung
Brightness/ Cumulative Mean Brightness
Brightness
Image Number
Brightness
Cumulative Mean Brightness


Rat 46 Unilateral 4/24
Brightness/Cumulative Mean Brightness
Brightness
Image Number
Brightness
Cumulative Mean Brightness


Rat 36 Sham-operated 0/24 Kidney
Brightness/Cumulative Mean Brightness
Brightness
Image Number
Brightness
Cumulative Mean Brightness

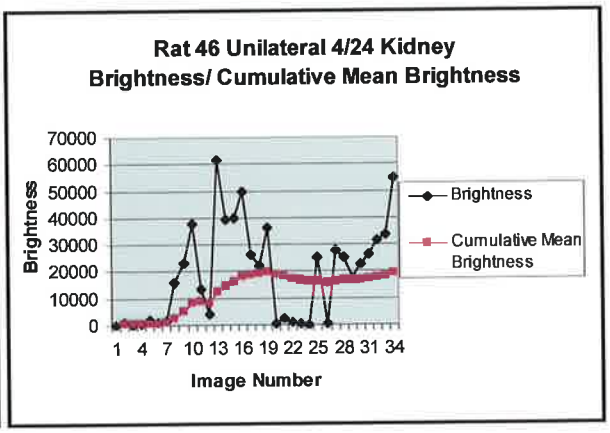
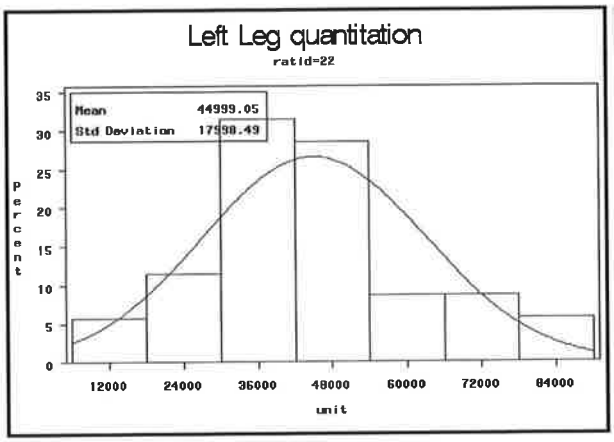
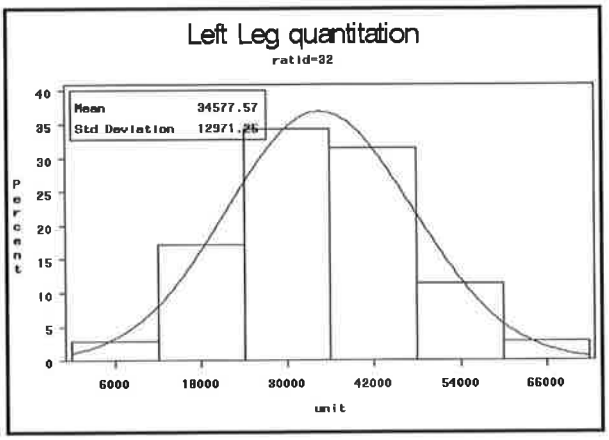
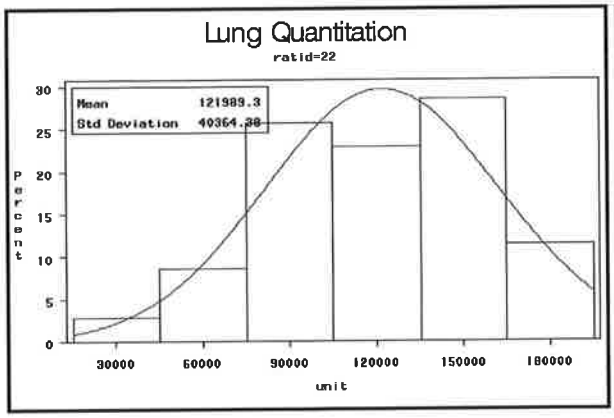

Rat 46 Unilateral 4/24 Kidney
Brightness/ Cumulative Mean Brightness
Brightness
Image Number
Brightness
Cumulative Mean Brightness

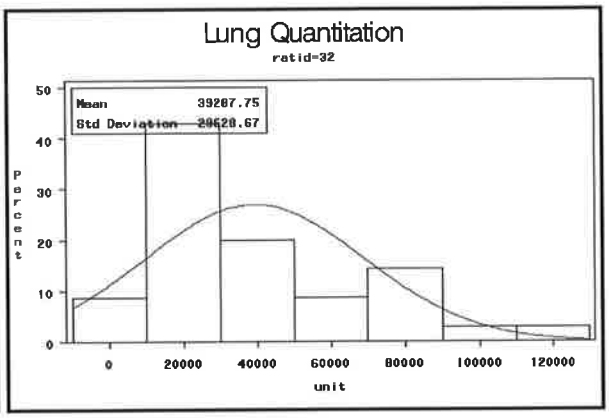
Figure 27: Normal distribution of Brightness measurements of Type IV Collagen in skeletal muscle, lung and kidney tissues

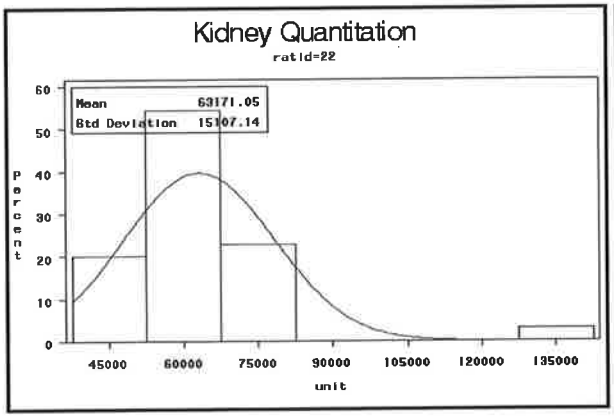
The x-axis refers to level of brightness and the y-axis refers to percentage of images with a given brightness. Thirty-five images were included in each graph, demonstrating that for each set of 35 images, there was a normal distribution.

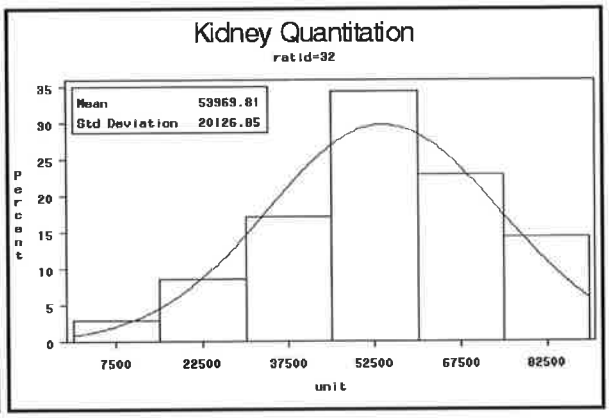

Left Leg quantitation
ratid=22
Mean 44999.05
Std Deviation 17598.49
Percent
unit
12000 24000 36000 48000 60000 72000 84000


Left Leg quantitation
ratid=32
Mean 34577.57
Std Deviation 12971.25
Percent
unit
6000 18000 30000 42000 54000 66000


Lung Quantitation
ratid=22
Mean 121989.3
Std Deviation 40364.38
Percent
unit
30000 60000 90000 120000 150000 180000


Lung Quantitation
ratid=32
Mean 39287.75
Std Deviation 20620.67
Percent
unit
0 20000 40000 60000 80000 100000 120000


Kidney Quantitation
ratid=22
Mean 63171.05
Std Deviation 15107.14
Percent
unit
45000 60000 75000 90000 105000 120000 135000


Kidney Quantitation
ratid=32
Mean 53969.81
Std Deviation 20126.85
Percent
unit
7500 22500 37500 52500 67500 82500

4.3.3 Thickness of Tissue Sections

Tissue sections were cut at 1, 2, 5, 10 and 14 μm for skeletal muscle, 1, 2, 3, 6 and 9 μm for lung and 1, 2, 5, 10 and 14 μm for the kidney. The graphs illustrating the results of the analysis of the 35 images for each tissue thickness are shown in Figure 28. In the left leg skeletal muscle, the brightness levels did not vary significantly for the different thicknesses from 2 to 14 microns. In the lung and kidney, there were similar findings with minimal variation from 1 to 3 microns and 1 to 10 microns in lung and kidney respectively. In the future immunohistochemical analyses, the thickness of tissue sections used was 10 μm for skeletal muscle, 3 μm for lung tissue and 5 μm for kidney.

A summary of brightness measurements for the thickness of tissue sections is shown in Table X on page 143. The complete set of raw data is shown in Appendix 7.6.3 Thickness of tissue sections studies, Table XXIX.

Table X: Summary of Brightness Measurements for the Thickness of Tissue Sections

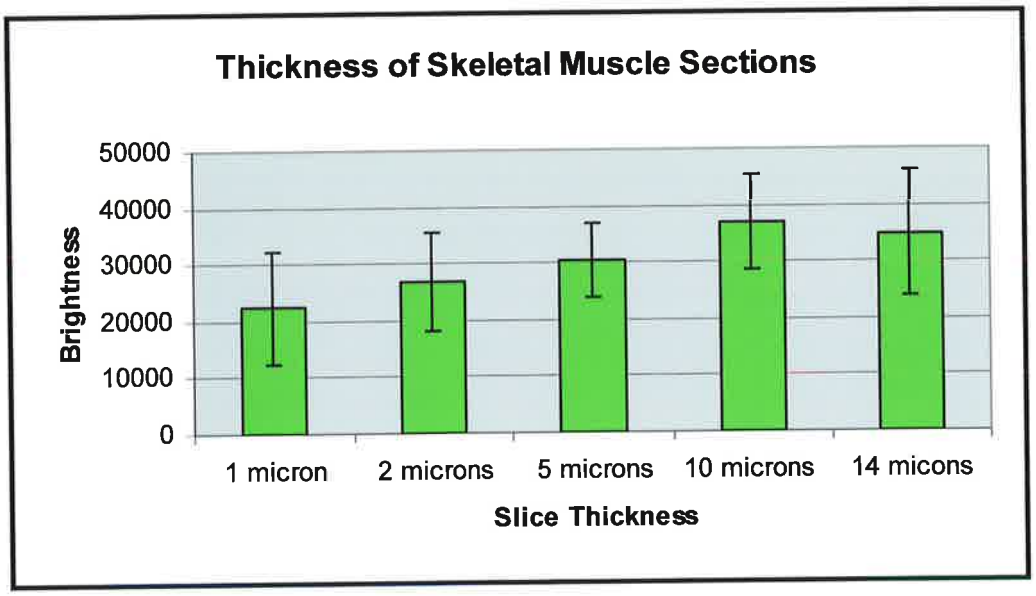
All experiments were performed on Rat 28, which was killed outright by stunning and cervical spinal dislocation. Mean refers to the arithmetic mean of the brightness measurements for the 35 images. Tissues were stained for type IV collagen antibody, FITC preparation. St Dev refers to the standard deviation.

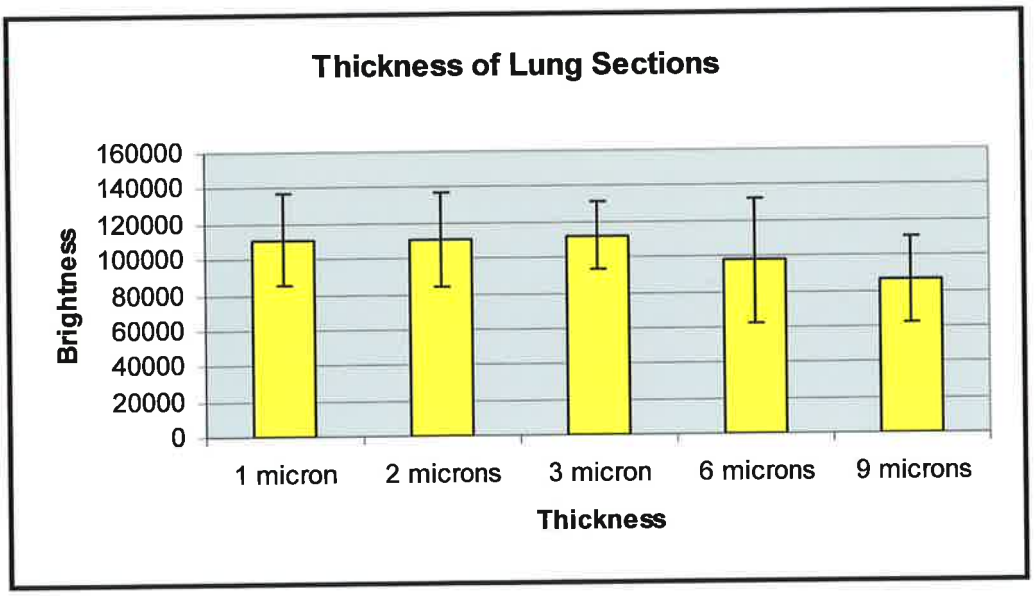
Tissue	Thickness	Mean	St Dev
Left leg Skeletal Muscle	1 micron	22324.87	9970.274
	2 microns	26946.99	8678.535
	5 microns	30519.6	6559.365
	10 microns	37022.52	8247.081
	14 microns	34927.67	11129.79
Lung	1 micron	111075.9	26411.53
	2 microns	110291.9	26268.9
	3 microns	112141.3	18854.78
	6 microns	97470.7	35175.61
	9 microns	86671.71	24131.16
Kidney	1 micron	57432.84	25608.64
	2 microns	75368.81	15612.09
	5 microns	68497.33	14650.49
	10 microns	60462.35	15365.55
	14 microns	64884.81	13397.48

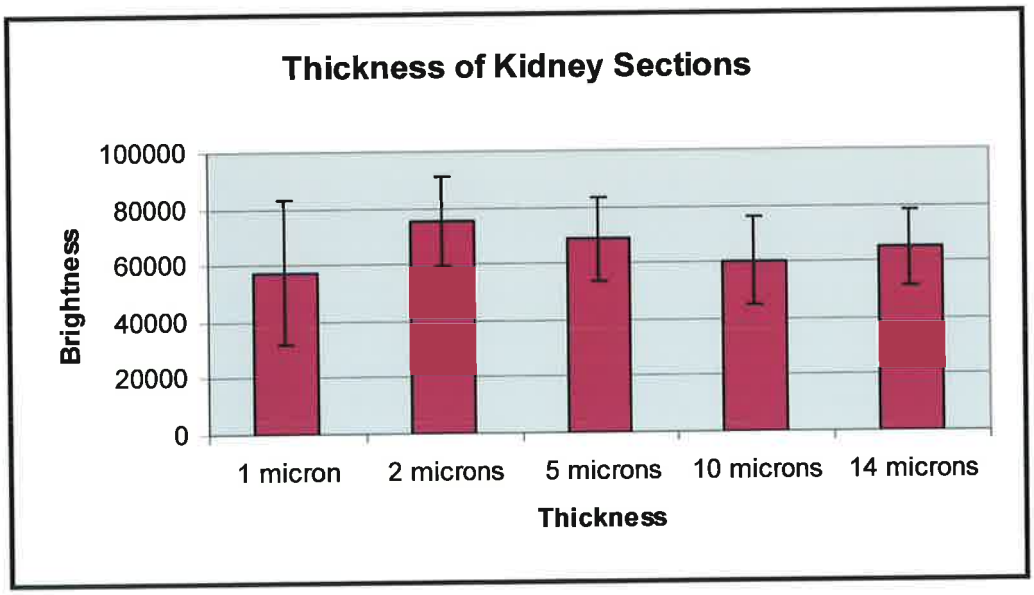
Figure 28: Graphs of Thickness of Tissue Sections

All experiments were performed on Rat 28, which was killed outright by stunning and cervical spinal dislocation. Tissues were stained with type IV antibody, FITC preparation as described in section 4.2. Tissue was sliced at the thickness shown on the graphs. Thirty five images were captured per section.

Each column represents the arithmetic mean with standard deviation of the mean shown as bars.


Thickness of Skeletal Muscle Sections
Brightness
50000
40000
30000
20000
10000
0
1 micron
2 microns
5 microns
10 microns
14 micons
Slice Thickness


Thickness of Lung Sections
Brightness
160000
140000
120000
100000
80000
60000
40000
20000
0
1 micron
2 microns
3 micron
6 microns
9 microns
Thickness


Thickness of Kidney Sections
Brightness
100000
80000
60000
40000
20000
0
1 micron
2 microns
5 microns
10 microns
14 microns
Thickness

4.3.4 Quenching of Tissue Fluorescence by the Microscope Lamp

The representative samples of images captured at time points from 5 seconds to 30 minutes are shown in Figure 29. This demonstrates that the deterioration in fluorescence occurs very rapidly over the period of thirty minutes. The graph in Figure 30 shows the brightness levels at each time point over the thirty minutes for left leg skeletal muscle, lung and kidney tissues. As can be seen in the images and in the graphs, there is very rapid quenching of the fluorescein isothiocyanate, which gives a rapid decline in measured brightness of the image. In order to minimize the effect of quenching the images were subsequently captured as rapidly as possible with each image taking 3-4 seconds to photograph.

The brightness measurements for quenching of fluorescent lamp are shown in Appendix 7.6.4 Quenching of Fluorescence Studies.

4.3.5 Antibody Saturation

The results are shown in graphical form in Figure 31. In both the left leg skeletal muscle and kidney, the brightness reached a peak at a concentration of 0.01 or 1 in 100 dilution of the type IV collagen antibody, beyond which there is minimal further increase in brightness with increases in antibody concentration. A similar result was observed with lung tissue, except there was a lower result for the concentration of 0.02 or 1 in 50, presumably due to technical error.

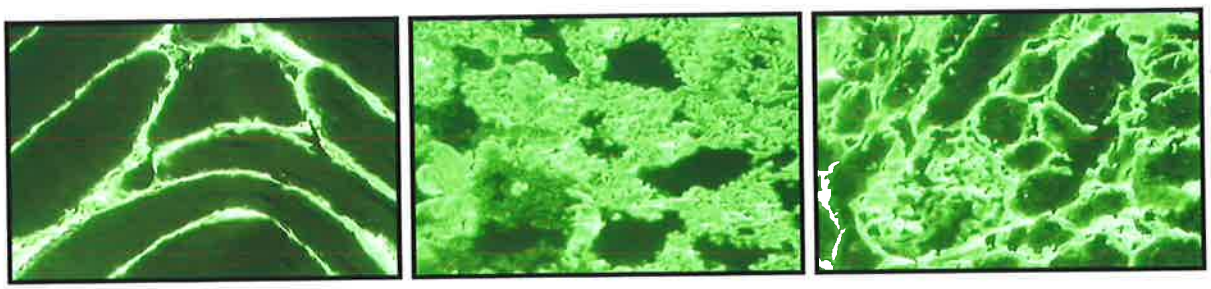
Therefore, in all further immunohistochemical analyses, a dilution of 1 in a 100 of type IV collagen was utilized.

The complete set of raw data is shown in Appendix 7.6.5 Antibody Saturation, Table XXXI, Table XXXII and Table XXXIII.

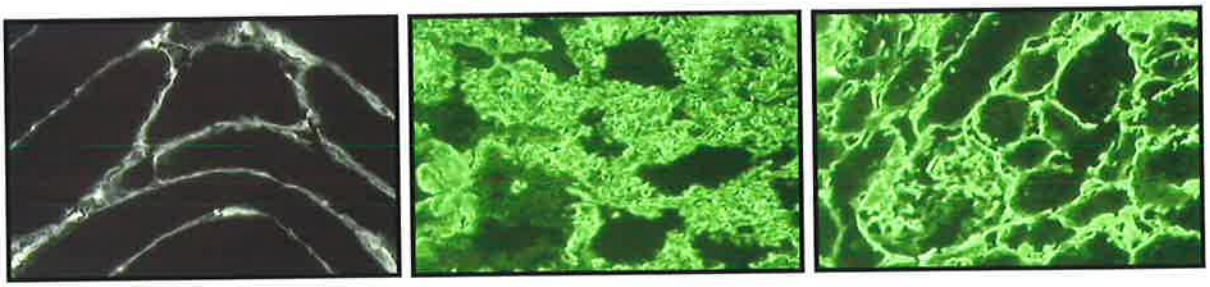
Figure 29: Quenching of Immunohistochemical Fluorescence over time, showing left leg skeletal muscle, lung and kidney

All experiments were performed on Rat 28, which was killed outright by stunning and cervical spinal dislocation. Tissues were stained with type IV antibody, FITC preparation as described in section 4.2.

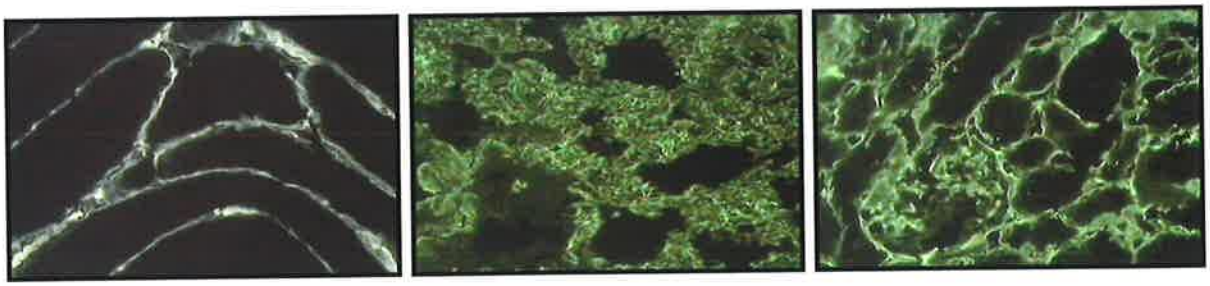
Left leg Lung Kidney



0.083 minutes=5 seconds



1 minute

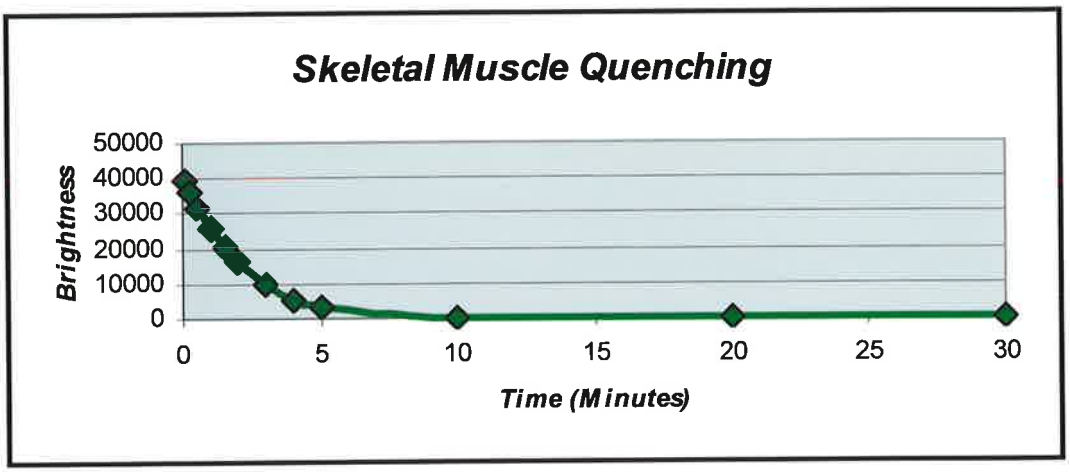


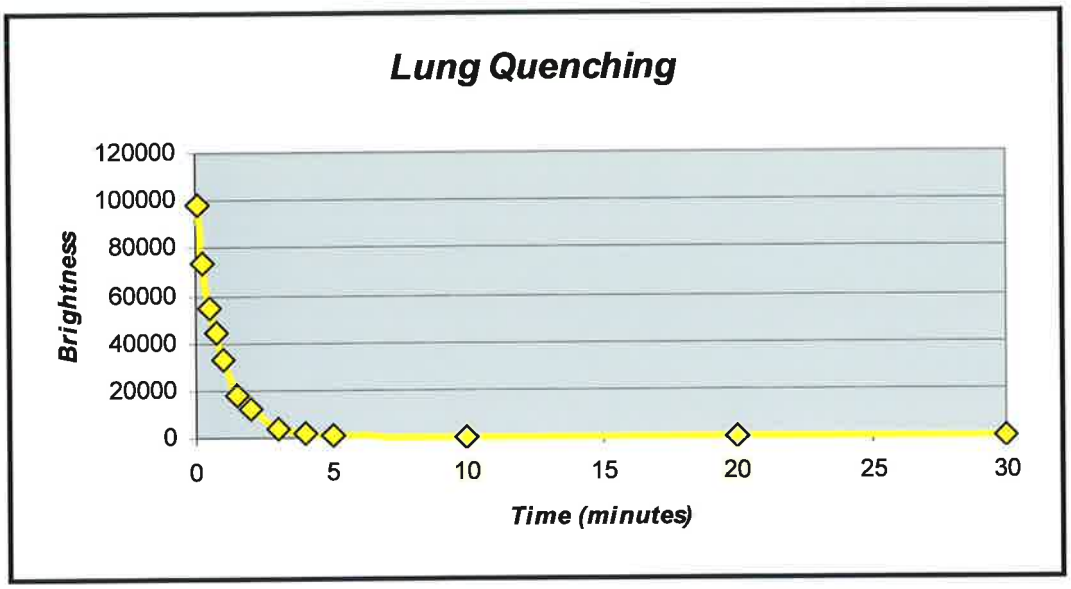
5 minutes

30 minutes

Figure 30: Graphs of Quenching of Immunohistochemical Fluorescence over time

All experiments were performed on Rat 28, which was killed outright by stunning and cervical spinal dislocation. Images were captured of the same microscope field at varying time points after the image was initially visualised. Each diamond on the graph represents an image that was captured for analysis at that time (x-axis) and the brightness level for that image. Rapid deterioration of the brightness level is shown with increasing time.


Skeletal Muscle Quenching
Brightness
50000
40000
30000
20000
10000
0
0
5
10
15
20
25
30
Time (Minutes)


Lung Quenching
Brightness
120000
100000
80000
60000
40000
20000
0
0
5
10
15
20
25
30
Time (minutes)

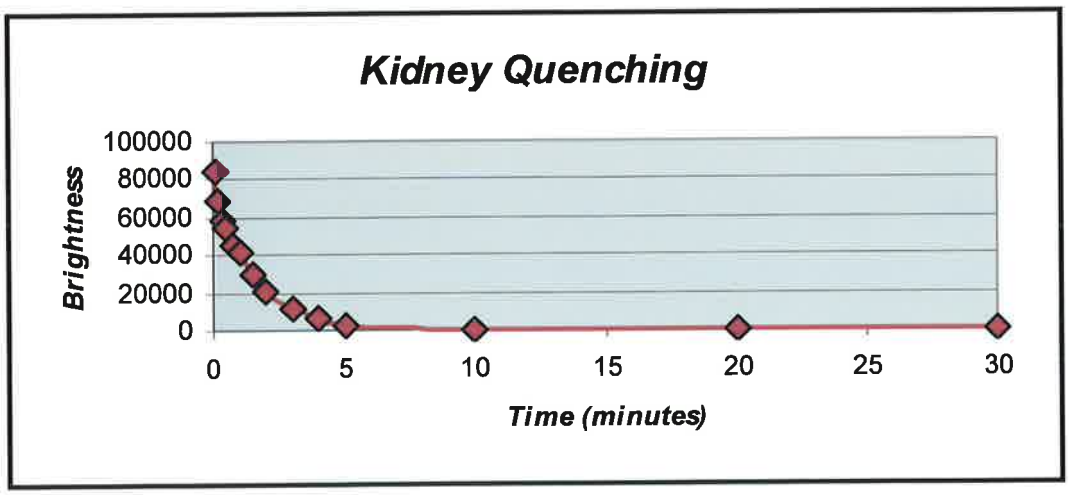
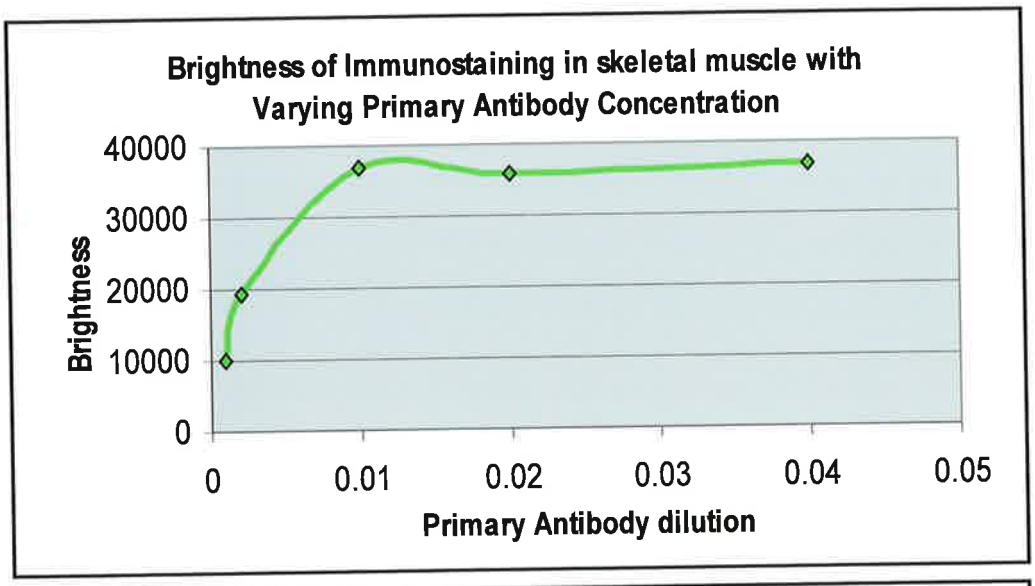
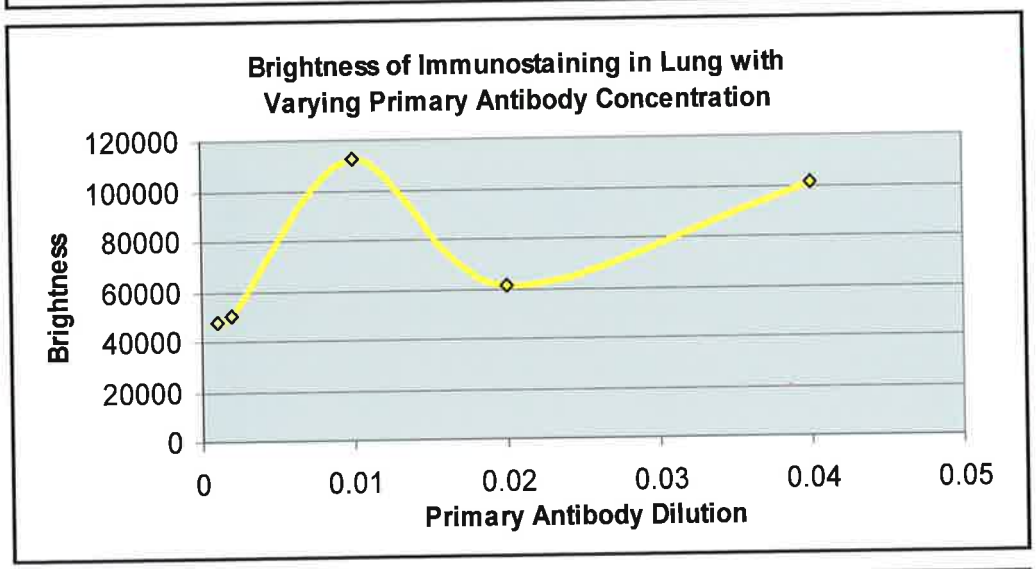
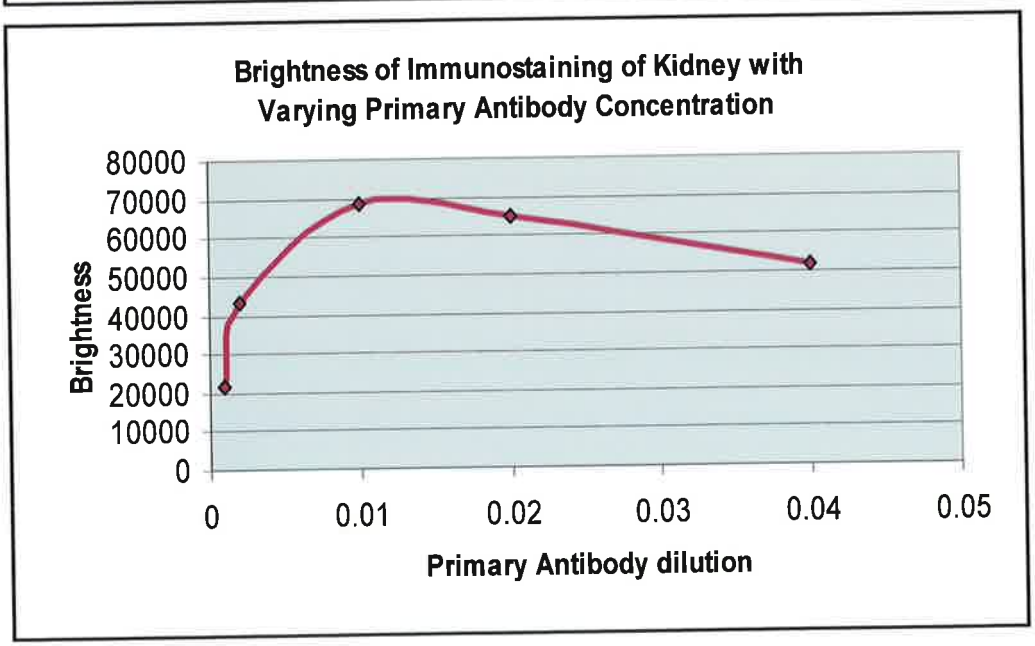

Kidney Quenching
Brightness
100000
80000
60000
40000
20000
0
0
5
10
15
20
25
30
Time (minutes)

Figure 31: Changes in level of Brightness with variation in Primary Antibody Concentration.

All experiments were performed on Rat 28, which was killed outright by stunning and cervical spinal dislocation. Primary Antibody was Type IV Collagen. FITC preparation as described in section 4.2.


Brightness of Immunostaining in skeletal muscle with Varying Primary Antibody Concentration
Brightness
40000
30000
20000
10000
0
0
0.01
0.02
0.03
0.04
0.05
Primary Antibody dilution


Brightness of Immunostaining in Lung with Varying Primary Antibody Concentration
Brightness
120000
100000
80000
60000
40000
20000
0
0
0.01
0.02
0.03
0.04
0.05
Primary Antibody Dilution


Brightness of Immunostaining of Kidney with Varying Primary Antibody Concentration
Brightness
80000
70000
60000
50000
40000
30000
20000
10000
0
0
0.01
0.02
0.03
0.04
0.05
Primary Antibody dilution

4.3.6 Quantitative Immunohistochemistry

The sections 4.3.6.1, 4.3.6.2, 4.3.6.3 and 4.3.6.4 describe the changes that occurred with each tissue type. The statistical analysis that is included here describes the *tissue specific* data as a whole for left leg skeletal muscle, right leg skeletal muscle, lung and kidney.

These statistical results were then *spliced* to give the comparisons between the individual cells (*separate animal groups,* for example: comparisons between sham-operated animal at 24 hours versus unilaterally ischaemic animals at 24 hours). Sections 4.3.6.5 and 4.3.6.6 describe firstly, the changes between ischaemia alone and increasing duration of reperfusion times and secondly, the changes between the group of sham-operated, unilaterally and bilaterally ischaemic animals and the detailed spliced statistical results.

4.3.6.1 Left leg Skeletal Muscle

Representative images of the immunohistochemistry of the left leg skeletal muscle can be seen in Figure 32, illustrating the changes in immunostaining that occur within the different experimental groups. In these representative images, there are no obvious changes in the type IV collagen staining in the sham-operated group with reperfusion times. However, after the completion of the anaesthetic there was a decrease in staining with the unilaterally ischaemic animals upon reperfusion and a further decrease in staining with the bilaterally ischaemic animals upon reperfusion. This indicates that the bilaterally ischaemic animals appear to have marked type IV collagen destruction.

The summary of the data is shown in Appendix 7.6.7 Quantitative Immunohistochemistry, Summary Data Sets Table XLIV and Table XLV on pages 271 and 273. The left leg skeletal muscle complete set of raw data is shown in Appendices 7.6.6 Quantitative Immunohistochemistry, Complete Data Sets; Table XXXIV, Table XXXV and Table XXXVI on pages 247, 252 and 253 respectively.

The grouped statistical data for the analysis of type IV collagen staining in skeletal muscle from the left leg is summarised in the Table XI. Tissues from animals that underwent bilateral ischaemia and 72 hours of reperfusion were analyzed separately and summarised in Table XII.

Table XI: Quantitation of Type IV Collagen levels in Left Leg Skeletal Muscle

The mean is the arithmetic mean of the brightness levels for the five animals in each group. (Thirty-five images per animal).

Reperfusion (Means)				
	0 hours	**4 hours**	**24 hours**	**72 hours**
Sham	61135.74	59484.76	46635.98	72101.37
Unilateral	35329.39	Not Done.	33776.39	26838.45
Bilateral	45572.36	19887.62	17211.75	Not Done.
Reperfusion (Standard Deviation)				
	0 hours	**4 hours**	**24 hours**	**72 hours**
Sham	10251.34	7009.17	11289.74	18079.48
Unilateral	12561.76	Not Done.	17269.97	17685.54
Bilateral	29788.11	14194.83	8921.70	Not Done.

The overall F test is significant (F=6.67, P<0.0001) indicating that there is evidence that the means for the 10 cells are different. The R^2 for this model accounts for 60.0% of the variation in unit. The Interaction term is not significant (F=2.26, P=0.0798). This means that the effect of group does not depends on the level of reperfusion and vice versa. The main effect of reperfusion (0/4/24/72) is not significant (P=0.0651). The main effect of group (Sham/Unilateral/Bilateral) is significant (P<0.0001). The statistics were further spliced and will be discussed below in sections 4.3.6.5 and 4.3.6.6.

Table XII: Quantitation of Type IV Collagen levels in Left leg Skeletal Muscle including rats undergoing 4 hours of Bilateral ischaemia and 72 hours of reperfusion

The mean is the arithmetic mean of the brightness levels for the five animals in each group. (Thirty-five images per animal).

Reperfusion (Mean)				
	0 hours	**4 hours**	**24 hours**	**72 hours**
Bilateral 4/72	37188.39	32313.53	25875.44	23903.10
Reperfusion (Standard Deviation)				
	0 hours	**4 hours**	**24 hours**	**72 hours**
Bilateral 4/72	8832.89	8625.32	11519.49	9338.82

The overall F test is not significant (F=1.55, P=0.1653) indicating that there is no evidence that the means for the 10 cells (cells not relevant for this section are not shown) are different. The Interaction term is not significant (F=1.19, P=0.3142). The main effect of the bilateral group is not significant (P=0.1165).

4.3.6.2 Right leg

Representative images of immunohistochemistry of skeletal muscle from the right leg can be seen in Figure 9. The sham-operated and unilaterally ischaemic animals (where the left leg is the ischaemic side, so the right leg is the contralateral limb) appear similar throughout with reasonable preservation of brightness and therefore type IV collagen. There is a decrease in intensity of immunofluorescence seen following bilateral limb ischaemia particularly at 24 hours of reperfusion. This showed again, that ischaemia leads to a decrease in the level of type IV collagen present. The statistical data, which illustrates this result for the right leg skeletal muscle immunohistochemistry analysis, is summarised in Table XIII.

The right leg skeletal muscle complete set of raw data was analysed and the results are shown in Appendix 7.6.6 Quantitative Immunohistochemistry, Complete Data Sets, Table XXXVIII and Table XXXIX on pages 255 and 259.

The summary of the brightness measurements for the right leg skeletal muscle is presented in Appendix 7.6.7 Quantitative Immunohistochemistry, Summary Data Sets, Table XLVI, on page 274.

Table XIII: Quantitation of Type IV Collagen levels in Right leg Skeletal Muscle

The mean is the arithmetic mean of the brightness levels for the five animals in each group. (Thirty-five images per animal).

Reperfusion (Mean)				
	0 hours	**4 hours**	**24 hours**	**72 hours**
Sham	30754.2	36710.71	31184.55	36054.69
Unilateral	36756.7	Not Done	38868.09	36662.77
Bilateral	32121.1	22395.18	25186.08	Not Done.
Reperfusion (Standard Deviation)				
	0 hours	**4 hours**	**24 hours**	**72 hours**
Sham	2304.06	6716.96	5443.85	9651.53
Unilateral	3024.83	Not Done.	11594.36	4430.98
Bilateral	11189.3	3857.39	5520.01	Not Done.

The overall F test is significant (F=2.92, P=0.0093) indicating that there is evidence that the means for the 10 cells are different. The R^2 for this model accounts for 39.7% of the variation in unit. The Interaction term is not significant (F=2.26, P=0.0798). This means that the effect of group does not depends on the level of reperfusion and vice versa. The main effect of reperfusion (0/4/24/72 hours) is not significant (P=0.9070). Main effect of group (Sham-operated/Unilateral/Bilateral) is significant (P=0.0027). The statistics were further spliced and will be discussed below in sections 4.3.6.5 and 4.3.6.6.

The levels of type IV collagen were not analysed in skeletal muscle harvested from right legs of rats subjected to bilateral ischaemia and 72 hours of reperfusion.

4.3.6.3 Lung

Representative images of the immunohistochemical staining for type IV collagen of the lung can be seen in Figure 10. There were decreases in the level of fluorescence as the degree of ischaemia increases, from sham-operated to unilateral ischaemia to bilateral ischaemia. This indicates that the level of type IV collagen decreases following a more profound ischaemic insult to the animal and subsequent reperfusion.

The complete set of raw data for brightness levels with type IV collagen staining in the lung was analysed and the results shown in Appendix 7.6.6 Quantitative Immunohistochemistry, Complete Data Sets, Table XL and Table XLI on pages 260 and 264. The summary of the data is presented in Appendix 7.6.7 Quantitative Immunohistochemistry, Summary Data Sets in Table XLVII on page 275. The lung immunohistochemistry that analysed the group involving animals that underwent bilateral ischaemia for 4 hours and 72 hours of reperfusion was performed at the same time as the doxycycline data in Chapter 5, so these results are in a different table and underwent separate analysis.

The results of bilateral 4 hour ischaemia with 72 hours of reperfusion are included with the doxycycline data sets in Table L, page 279, with the summary in Table LIII on page 289.
The grouped statistical data for the lung immunohistochemistry is summarised in Table XIV and Table XV below.

Table XIV: Quantitation of Type IV Collagen levels in Lung.

The mean is the arithmetic mean of the brightness levels for the five animals in each group. (Thirty-five images per animal).

Reperfusion (Mean)				
	0 hours	**4 hours**	**24 hours**	**72 hours**
Sham	89198.2	77072.93	96714.78	92611.45
Unilateral	131588.	Not Done	47034.02	35735.86
Bilateral	19284.0	26090.75	56545.40	Not Done.
Reperfusion (Standard Deviation)				
	0 hours	**4 hours**	**24 hours**	**72 hours**
Sham	21271.2	7975.73	44444.72	29672.08
Unilateral	21320.1	Not Done	12946.65	14807.26
Bilateral	5805.80	7040.88	29155.17	Not Done.

The overall F test is significant ($F=12.67$, $P<0.0001$) indicating that there is evidence that the means for the 10 cells are different. The R^2 for this model accounts for 74.0% of the variation in unit. The Interaction term is significant ($F=12.23$, $P<0.0001$). This means that the effect of group depends on the level of reperfusion and vice versa. The statistics were further spliced and will be discussed below in sections 4.3.6.5 and 4.3.6.6.

Table XV: Quantitation of Type IV Collagen levels in Lung including rats undergoing 4 hours of Bilateral ischaemia and 72 hours of reperfusion

The mean is the arithmetic mean of the brightness levels for the five animals in each group. (Thirty-five images per animal).

Reperfusion (Mean)				
	0 hours	**4 hours**	**24 hours**	**72 hours**
Bilateral 4/72	29123.37	24159.58	10863.00	13103.12
Reperfusion (Standard Deviation)				
	0 hours	**4 hours**	**24 hours**	**72 hours**
Bilateral 4/72	5051.01	6266.60	2637.28	4611.28

The overall F test is significant (F=27.09, P<0.0001) indicating that there is evidence that the means for the 10 cells are different. (Data not relevant for this section is not shown, with the other cells included in data in Chapter 5.3.3). The R^2 for this model accounts for 85.6% of the variation in unit. The Interaction term is not significant (F=1.24, P=0.3031). This means that the effect of group does not depends on the level of reperfusion and vice versa. The main effect of reperfusion is significant (P=0.0029). The main effect of group is significant (P<0.0001). When the data from the bilateral ischaemic group was spliced, there was a significant difference between reperfusion times which will be discussed below in sections 4.3.6.5 and 4.3.6.6.

4.3.6.4 Kidney

Representative images of the kidney can be seen in Figure 11. These representative figures show that there is decrease in immunofluorescence with both increasing levels of ischaemia and at increasing durations of reperfusion, indicating a decrease in the level of type IV collagen in the basement membrane.

The complete set of raw data from the kidneys was analysed and the results shown in Appendix 7.6.6 Quantitative Immunohistochemistry, Complete Data Sets, Table XLII and Table XLIII on pages 265 and 270. The summary of data is presented in Appendix 7.6.7 Quantitative Immunohistochemistry, Summary Data Sets in Table XLVIII on page 276. The bilateral 4 hour ischaemia with 72 hours reperfusion is included with the doxycycline data sets in Table LI on page 284, with the summary in Table LIV on page 290.

The grouped statistical data for the kidney immunohistochemical analysis is shown in Table XVI and Table XVII below.

Table XVI: Quantitation of Type IV Collagen levels in Kidney

The mean is the arithmetic mean of the brightness levels for the five animals in each group. (Thirty-five images per animal).

	Reperfusion (Mean)			
	0 Hours	**4 hours**	**24 hours**	**72 hours**
Sham	65714.45	70857.19	80819.17	94955.60
Unilateral	53000.99	Not Done	51632.51	44863.89
Bilateral	57361.43	43983.91	39376.01	Not Done
	Reperfusion (Standard Deviation)			
	0 hours	**4 hours**	**24 hours**	**72 hours**
Sham	9695.50	6783.12	14770.64	22512.51
Unilateral	12669.23	Not Done	8500.46	6245.81
Bilateral	10389.73	6539.80	21806.18	Not Done

The overall F test is significant (F=3.97, P=0.0084) indicating that there is evidence that the means for the 10 cells are different. The R^2 for this model accounts for 66.8% of the variation

in unit. The interaction term is significant (F=3.97, P=0.0084). This means that the effect of group depends on the level of reperfusion and vice versa. The statistics were further spliced and will be discussed below in sections 4.3.6.5 and 4.3.6.6.

Table XVII: Quantitation of Type IV Collagen levels in Kidney including rats undergoing 4 hours of Bilateral ischaemia and 72 hours of reperfusion

The mean is the arithmetic mean of the brightness levels for the five animals in each group. (Thirty-five images per animal).

Reperfusion (Mean)				
	0	**4**	**24**	**72**
Bilateral 4/72	59473.79	49421.32	47909.78	41622.23
Reperfusion (Standard Deviation)				
	0	**4**	**24**	**72**
Bilateral 4/72	6181.26	8915.39	4623.62	6481.52

The overall F test is significant (F=5.16, P=0.0001) indicating that there is evidence that the means for the 10 cells are different. (Data not relevant for this section is not shown, with the other cells included in data in Chapter 5.3.3). The R^2 for this model accounts for 53.7% of the variation in unit. The Interaction term is not significant (F=1.33, P=0.2763). This means that the effect of group does not depends on the level of reperfusion and vice versa. The main effect of reperfusion is significant (P=0.0008). The main effect of group is significant (P<0.0001). When spliced, the bilateral ischaemic group, there was a significant difference among reperfusion times and will be discussed below in sections 4.3.6.5 and 4.3.6.6.

Figure 32: Representative Images of Immunohistochemistry of Left Leg Skeletal Muscle showing levels of Type IV Collagen

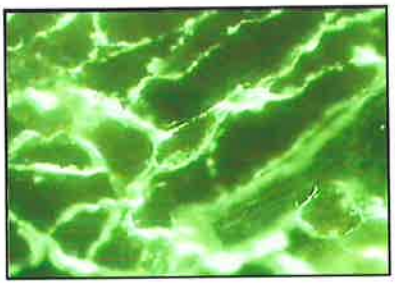
Magnification 20 X, Primary antibody was directed against type IV collagen and then secondary detection was carried out with Biotin-labelled antibody and Streptavidin-Fluorescein Isothiocyanate. Images were collected on Olympus BH2 microscope with Reflected Light Fluorescent Attachment (Olympus BHRFL-W) with maximum excitation wavelength of 494 nm and maximum emission wavelength of 518 nm. The images were captured on a Panasonic Camera Control Unit (WVCU204) and Panasonic Camera (WVCL700).

Sham - 0 hours refers to rats that underwent anaesthetic only and were sacrificed immediately following the anaesthetic. Sham – 4 hours, Sham - 24 hours and Sham – 72 hours refers to rats that underwent a four-hour anaesthetic and then were sacrificed 4, 24 or 72 hours respectively after the anaesthetic.

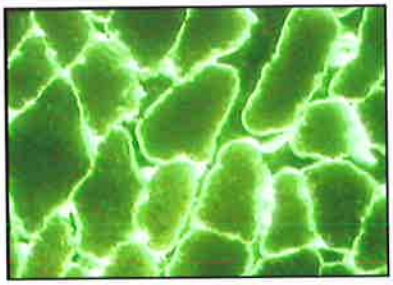
Unilateral - 0 hours refers to rats that underwent four hours of left leg unilateral ischaemia under four hours of anaesthetic and were then sacrificed immediately. Rats undergoing 4 hours of unilateral ischaemia and 4 hours of reperfusion were not performed.

Unilateral - 24 hours and Unilateral - 72 hours refers to rats that underwent four hours of left leg unilateral ischaemia under four-hour anaesthetic, the tourniquet was then released and the rat was sacrificed after 24 or 72 hours reperfusion respectively.

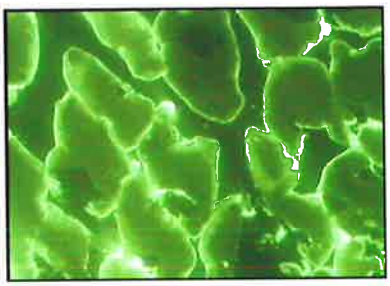
Bilateral - 0 hours rat underwent four hours of bilateral lower limb ischaemia and were then sacrificed immediately. Bilateral 4, 24 and 72 hours rats underwent 4 hours of bilateral lower limb ischaemia followed by 4, 24 or 72 hours of reperfusion and were then killed.



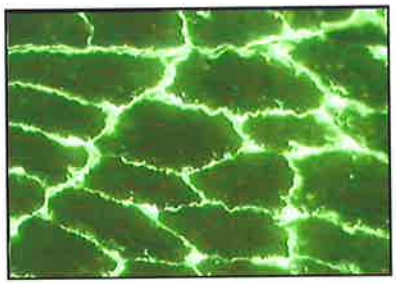
Sham – 0 hours



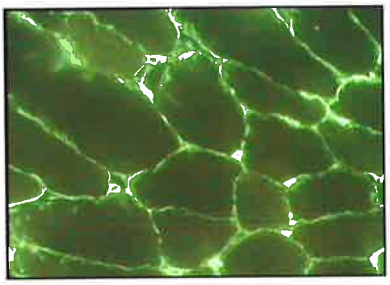
Unilateral – 0 hours



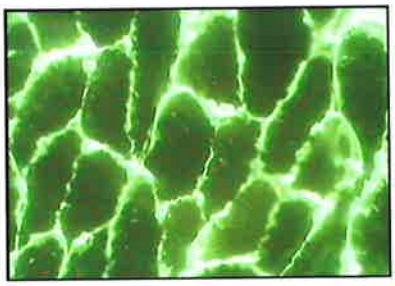
Bilateral – 0 hours



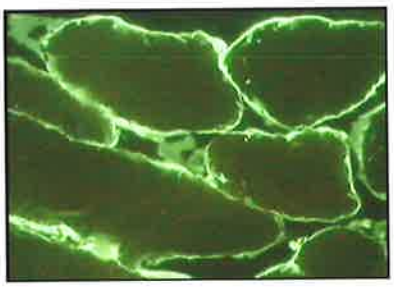
Sham – 4 hours



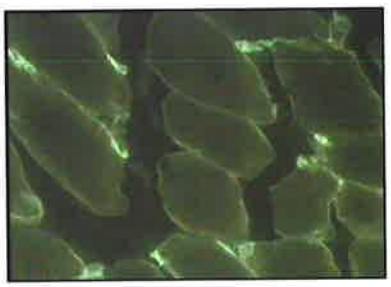
Bilateral – 4 hours



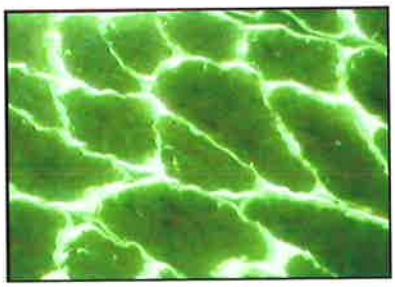
Sham – 24 hours



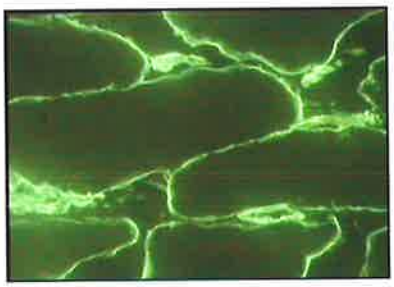
Unilateral – 24 hours



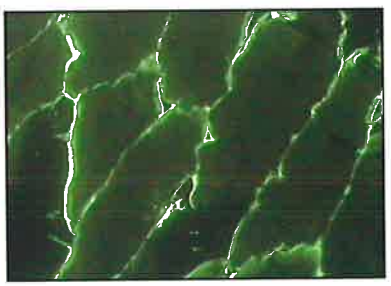
Bilateral – 24 hours



Sham – 72 hours



Unilateral – 72 hours



Bilateral – 72 hours

Figure 33: Representative Images of Immunohistochemistry of Right Leg Skeletal Muscle showing levels of Type IV Collagen

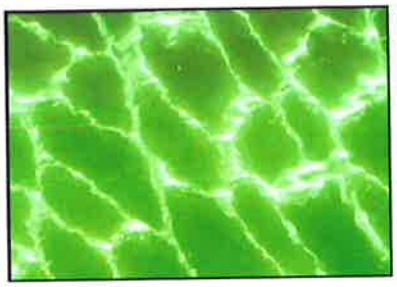
Magnification 20 X, Primary antibody was directed against type IV collagen and then secondary detection was carried out with Biotin-labelled antibody and Streptavidin-Fluorescein Isothiocyanate. Images were collected on Olympus BH2 microscope with Reflected Light Fluorescent Attachment (Olympus BHRFL-W) with maximum excitation wavelength of 494 nm and maximum emission wavelength of 518 nm. The images were captured on a Panasonic Camera Control Unit (WVCU204) and Panasonic Camera (WVCL700).

Sham - 0 hours refers to rats that underwent anaesthetic only and were sacrificed immediately following the anaesthetic. Sham – 4 hours, Sham - 24 hours and Sham – 72 hours refers to rats that underwent a four-hour anaesthetic and were then sacrificed 4, 24 or 72 hours respectively after the anaesthetic.

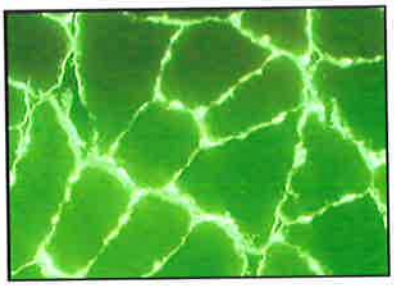
Unilateral - 0 hours refers to rats that underwent four hours of left leg unilateral ischaemia under four hours of anaesthetic and were then sacrificed immediately. Rats undergoing 4 hours of unilateral ischaemia and 4 hours of reperfusion were not performed.

Unilateral-24 hours and Unilateral-72 hours refers to rats that underwent four hours of left leg unilateral ischaemia under four-hour anaesthetic, the tourniquet was then released and the rat was sacrificed after 24 or 72 hours reperfusion respectively.

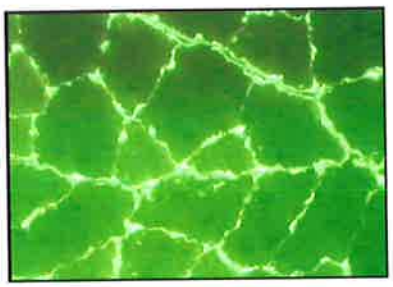
Bilateral - 0 hours rat underwent four hours of bilateral lower limb ischaemia and were then sacrificed immediately. Bilateral 4, 24 and 72 hours rats underwent 4 hours of bilateral lower limb ischaemia followed by 4, 24 or 72 hours of reperfusion and were then killed.



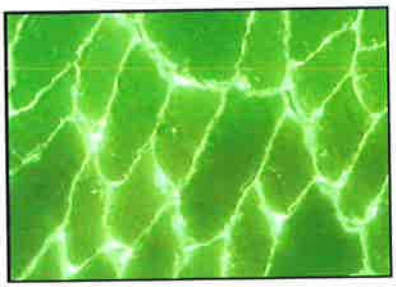
Sham – 0 hours



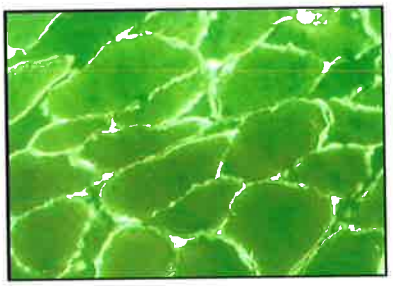
Unilateral – 0 hours



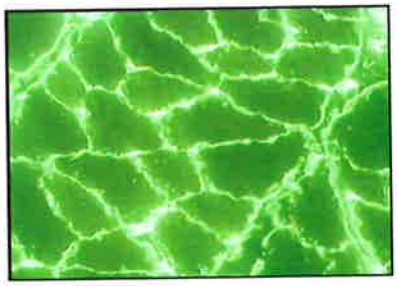
Bilateral – 0 hours



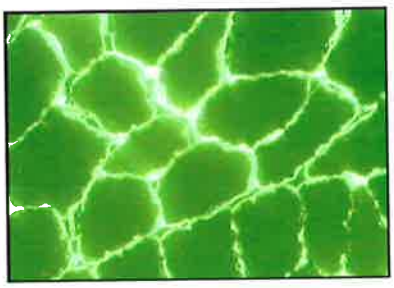
Sham – 4 hours



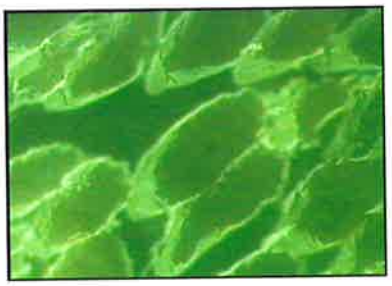
Bilateral – 4 hours



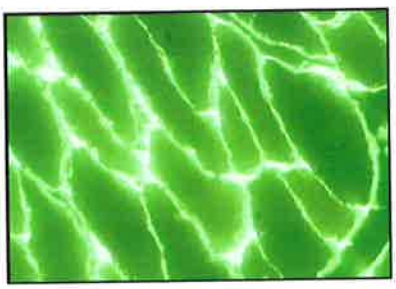
Sham – 24 hours



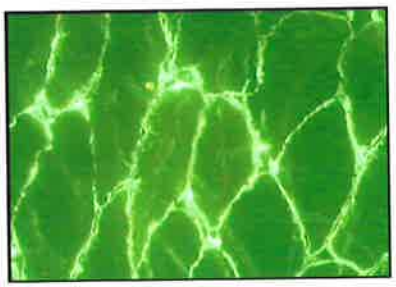
Unilateral – 24 hours



Bilateral – 24 hours



Sham – 72 hours



Unilateral – 72 hours

Figure 34: Representative Images of Immunohistochemistry of Lung showing levels of Type IV Collagen

Magnification 20 X, Primary antibody was directed against type IV collagen and then secondary detection was carried out with Biotin-labelled antibody and Streptavidin-Fluorescein Isothiocyanate. Images were collected on Olympus BH2 microscope with Reflected Light Fluorescent Attachment (Olympus BHRFL-W) with maximum excitation wavelength of 494 nm and maximum emission wavelength of 518 nm. The images were captured on a Panasonic Camera Control Unit (WVCU204) and Panasonic Camera (WVCL700).

Sham – 0 hours refers to rats that underwent anaesthetic only and were sacrificed immediately following the anaesthetic. Sham – 4 hours, Sham - 24 hours and Sham – 72 hours refers to rats that underwent a four-hour anaesthetic and were then sacrificed 4, 24 or 72 hours respectively after the anaesthetic.

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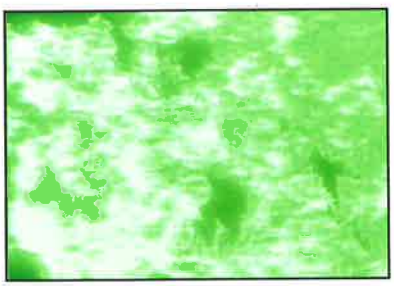
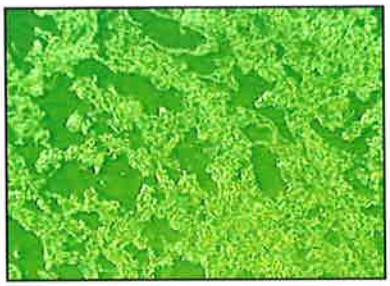
Unilateral - 24 hours and Unilateral - 72 hours refers to rats that underwent four hours of left leg unilateral ischaemia under four-hour anaesthetic, the tourniquet was then released and the rat was sacrificed after 24 or 72 hours reperfusion respectively.

Bilateral 0 hours rat underwent four hours of bilateral lower limb ischaemia and were then sacrificed immediately. Bilateral 4, 24 and 72 hours rats underwent 4 hours of bilateral lower limb ischaemia followed by 4, 24 or 72 hours of reperfusion and were then killed.

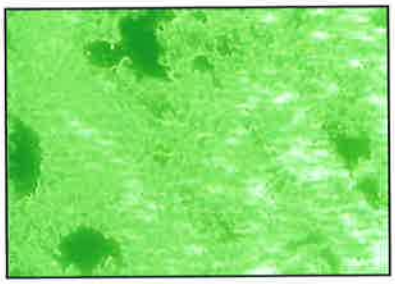


Sham – 0 hours

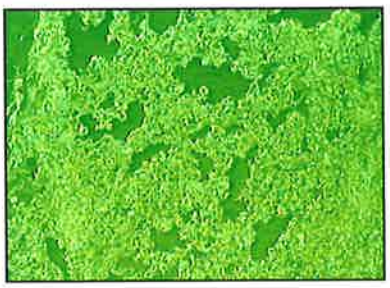
Unilateral – 0 hours



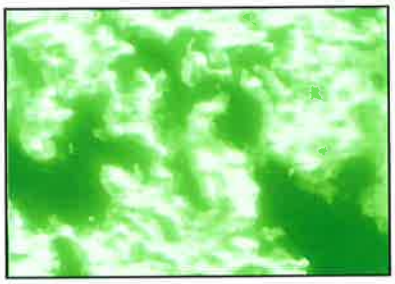
Bilateral – 0 hours



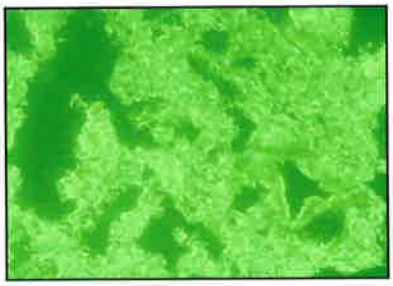
Sham – 4 hours



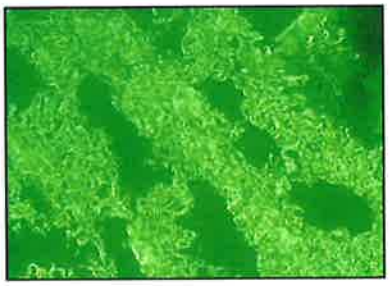
Bilateral – 4 hours



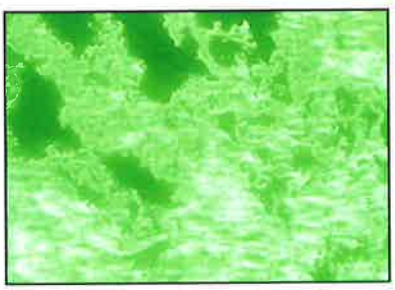
Sham – 24 hours



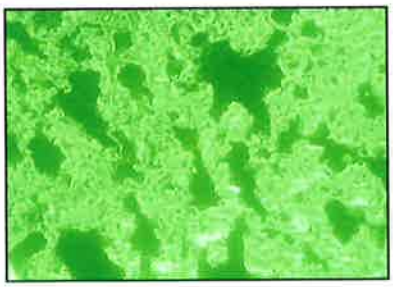
Unilateral – 24 hours



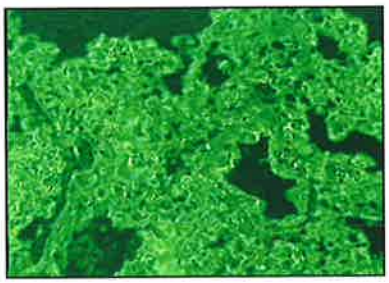
Bilateral – 24 hours



Sham – 72 hours



Unilateral – 72 hours



Bilateral – 72 hours

Figure 35: Representative Images of Immunohistochemistry of Kidney showing levels of Type IV Collagen

Magnification 20 X, Primary antibody was directed against type IV collagen and then secondary detection was carried out with Biotin-labelled antibody and Streptavidin-Fluorescein Isothiocyanate. Images were collected on Olympus BH2 microscope with Reflected Light Fluorescent Attachment (Olympus BHRFL-W) with maximum excitation wavelength of 494 nm and maximum emission wavelength of 518 nm. The images were captured on a Panasonic Camera Control Unit (WVCU204) and Panasonic Camera (WVCL700).

Sham - 0hours refers to rats that underwent anaesthetic only and were sacrificed immediately following the anaesthetic. Sham – 4 hours, Sham - 24 hours and Sham – 72 hours refers to rats that underwent a four-hour anaesthetic and were then sacrificed 4, 24 or 72 hours respectively after the anaesthetic.

Unilateral - 0 hours refers to rats that underwent four hours of left leg unilateral ischaemia under four hours of anaesthetic and were then sacrificed immediately. Rats undergoing 4 hours of unilateral ischaemia and 4 hours of reperfusion were not performed.

Unilateral - 24 hours and Unilateral - 72 hours refers to rats that underwent four hours of left leg unilateral ischaemia under four-hour anaesthetic, the tourniquet was then release and the rat was sacrificed after 24 or 72 hours reperfusion respectively.

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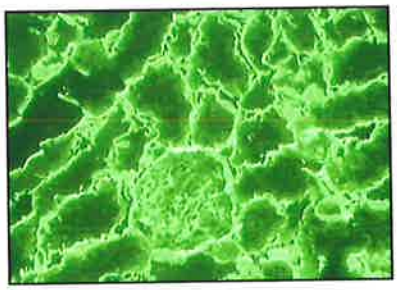
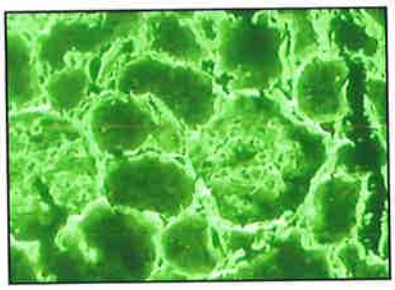
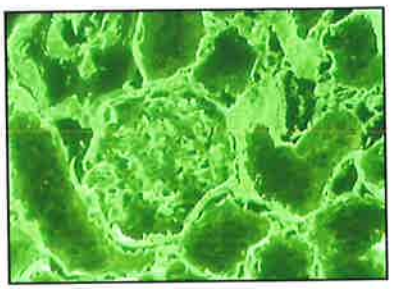


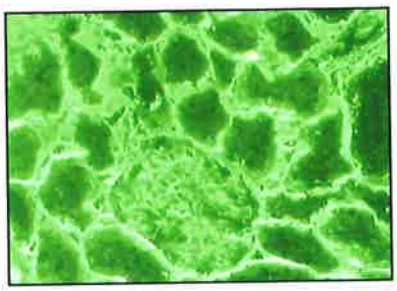
Sham – 0 hours



Unilateral – 0 hours

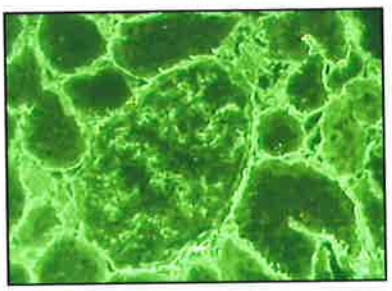


Bilateral – 0 hours

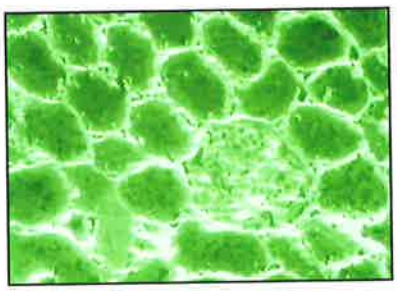


Sham – 4 hours

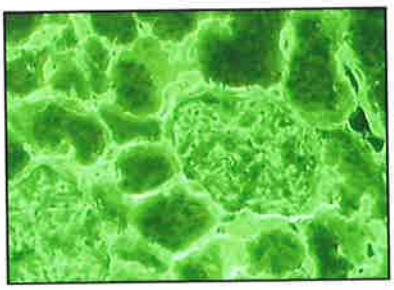
Unilateral – 4 hours



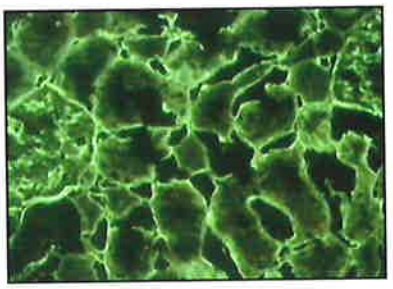
Bilateral – 4 hours



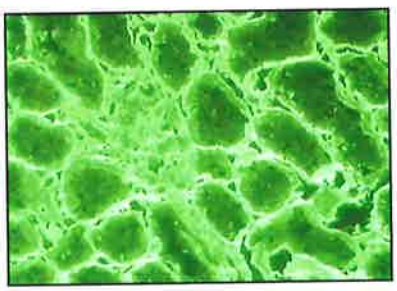
Sham – 24 hours



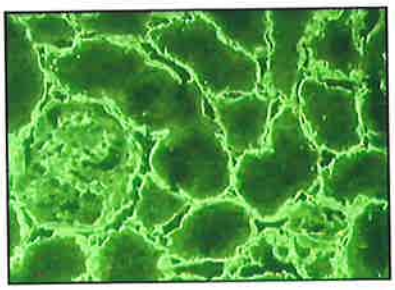
Unilateral – 24 hours



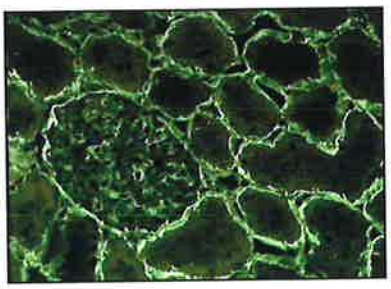
Bilateral – 24 hours



Sham – 72 hours



Unilateral – 72 hours



Bilateral – 72 hours

4.3.6.5 Effect of Increasing Duration of Reperfusion on Levels of Type IV Collagen

Ischaemia Only

These rats were all sacrificed at the end of the four-hour anaesthetic. These results are shown graphically in Figure 36. Hence, for sham-operated animals, any changes in type IV collagen level are purely the effect of the anaesthetic alone. For the unilaterally and bilaterally ischaemic animals, any changes in type IV collagen levels are due the effects of both the anaesthesia and the ischaemia. Levels of type IV collagen in the *left leg skeletal muscle* tended to decrease following unilateral or bilateral ischaemia, but due to large standard deviation bars, there was no statistical significance. The *right leg skeletal muscle* showed minimal changes in the levels of type IV collagen between sham-operated animals, unilaterally ischaemic animals and bilaterally ischaemic animals. The level of type IV collagen in the *lungs* increased between sham-operated and unilaterally ischaemic animals, indicating possibly less lung damage in the animals that had ischaemia versus anaesthetic alone but this difference was not significant. The significant differences in levels of type IV collagen immunostaining in the *lung* were between sham-operated animals and bilaterally ischaemic animals ($p=0.007$) and between unilaterally ischaemic animals and bilaterally ischaemic animals ($p<0.0001$). The *kidney* showed minimal changes in type IV collagen level between sham-operated animals, unilaterally ischaemic animals and bilaterally ischaemic animals.

Rats sacrificed at Four Hours

These rats were sacrificed at the end of a four-hour period after the anaesthetic. The results are shown graphically in Figure 37. As can be seen from the graph, all tissue showed that destruction in type IV collagen has occurred at four hours to a significant level when comparing sham-operated animals to bilaterally ischaemic animals. In the *left leg skeletal muscle* $p=0.0003$, *right leg skeletal muscle* $p=0.0029$, *lung* $p=0.0010$ and *kidney* $p=0.0027$.

Rats sacrificed at Twenty four Hours

These rats were sacrificed at the end of a twenty four hour period of reperfusion. The results are shown graphically in Figure 38. The *left leg skeletal muscle* showed a decrease in type IV collagen levels across the groups ($p=0.0208$), but in view of large standard deviation bars,

these findings were not significant between the individual groups after statistical splicing. Similarly, there was a decrease in brightness level across all groups together in the *right leg skeletal muscle* (p=0.0154), but significant differences between the groups did not occur, with moderately large standard deviation bars present, especially in unilateral 4 hour ischaemia and 24 hours of reperfusion group. In the *lung,* there was a significant decrease in brightness level between the sham-operated and the unilateral 4/24 ischaemic groups (p=0.0376). This did not continue onto the bilaterally ischaemic group which had an extremely large standard deviation bar. The *kidney* showed differences in immunofluorescence brightness between sham-operated and unilateral ischaemia at 24 hours (p=0.0363) and between sham-operated and bilateral ischaemia (p=0.0006). There was no difference between the type IV collagen levels in the kidney in unilaterally ischaemic and bilaterally ischaemic rats.

Rats sacrificed at Seventy two Hours

Rats in these groups were killed 72 hours after the completion of the anaesthetic in the sham-operated group, or after 72 hours of reperfusion in the unilateral or bilateral ischaemia groups. These results are shown graphically in Figure 39. There was a trend toward decrease in type IV collagen levels in the *left leg skeletal muscle*, but these changes were not significant. In the *lung* there was a marked decrease in type IV collagen level with sham-operated and unilaterally ischaemic animals being different (p<0.0001) and sham-operated and bilaterally ischaemic animals being significantly different (p<0.0001). Unilaterally ischaemic animals and bilaterally ischaemic animals did not have significantly different levels of type IV collagen when compared to each other. In the *kidney,* there were decreases in type IV collagen levels across the groups (p=0.0017), with sham-operated being significantly different from bilaterally ischaemic groups (p=0.0041) and unilaterally ischaemic rats being significantly different from bilaterally ischaemic rats (p=0.030).

4.3.6.6 Differences between Type IV Collagen Levels in Sham–operated, Unilaterally Ischaemic and Bilaterally Ischaemic Rats

Sham-operated rats

Rats in these groups underwent a four anaesthetic and were sacrificed either immediately or after 4, 24 or 72 hours. The results of the changes in brightness on immunofluorescence are seen in Figure 40. There were no significant differences seen in the left or right leg skeletal

muscle or the lung. However, the *kidney* showed an *increase* in the level of type IV collagen between the end of anaesthetic and 72 hours of reperfusion, when the brightness level increased from 65714 to 94955 ($p=0.0358$).

Unilateral Ischaemic Rats

Unilateral ischaemic rats underwent unilateral left leg skeletal muscle ischaemia and were then killed without any reperfusion or allowed to undergo reperfusion for 24 or 72 hours. These results are shown graphically in Figure 41. In the *left leg skeletal muscle*, there was a trend towards less brightness in immunofluorescence and a decrease in type IV collagen particularly after 72 hours, however this was not significant. There were no changes seen in the *right leg skeletal muscle*. In the *lung*, there was a marked decrease in the type IV collagen after 24 hours of reperfusion ($p<0.0001$) and between end of the anaesthetic (ischaemia only) and 72 hours of reperfusion ($p<0.0001$). There was no significant difference between levels of Type IV collagen in the lung after 24 hours or reperfusion compared with after 72 hours of reperfusion. There were no significant differences between the levels of immunohistochemical brightness detected in the *kidney*.

Bilateral Ischaemic Rats

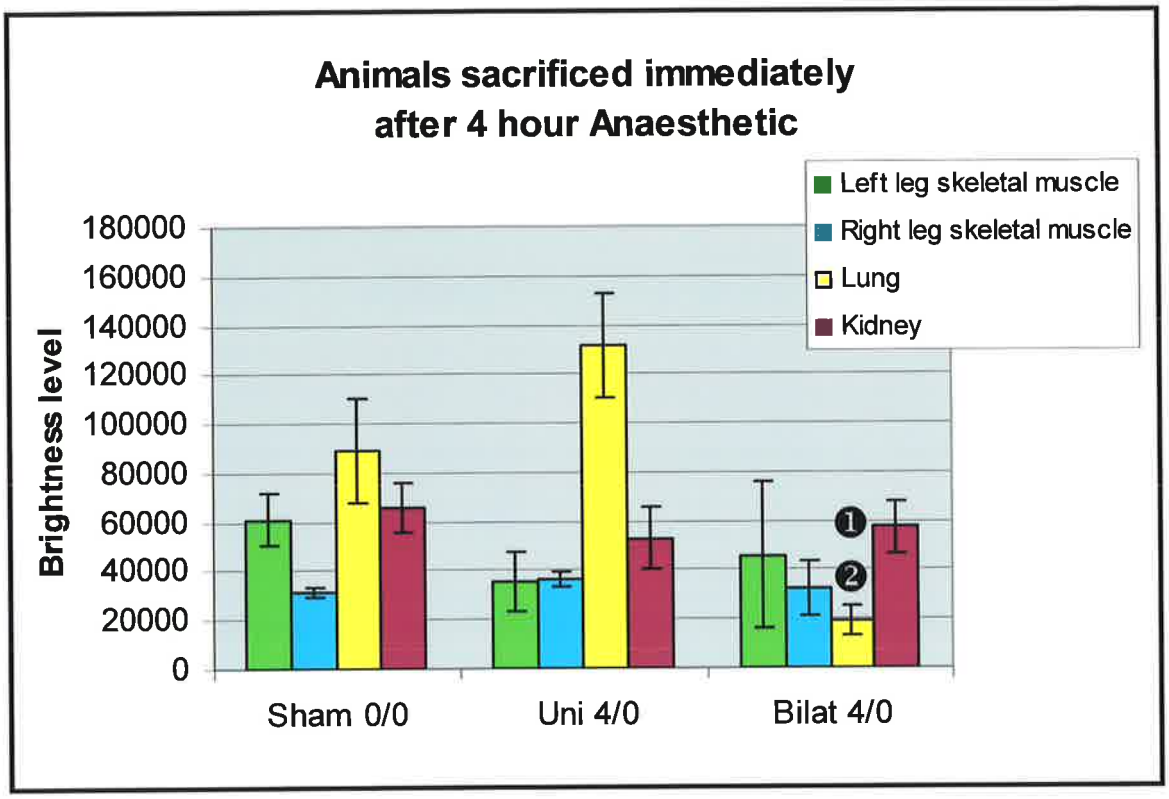
Bilateral ischaemic rats underwent bilateral lower limb ischaemia and were either killed without reperfusion or after 4, 24 or 72 hours of reperfusion. The result of the brightness levels of immunofluorescence after staining with type IV collagen antibodies are shown graphically in Figure 42. The *left leg skeletal muscle* showed a trend towards a decrease in level of brightness but these changes were not significant. In the *lungs*, there was no decrease in type IV collagen at four hours, but by 24 hours of reperfusion, a significant decrease in the level of type IV collagen had occurred ($p=0.0204$). The changes in the lung type IV collagen almost reached significance between end of the anaesthetic and 72 hours ($p=0.0643$). In the *kidney*, there was a decrease in type IV collagen level as the time of reperfusion increased, reaching significance between end of the anaesthetic (ischaemia only) and the 72 hours of reperfusion ($p=0.0017$).

Figure 36: Mean Brightness Levels of Type IV Collagen Immunofluorescence for Animals sacrificed immediately after 4 hour anaesthetic.

Tissues were labelled with type IV collagen antibody, prepared and analysed as in Section 4.2. Brightness refers to level of Type IV collagen seen with immunofluorescence. Sham 0/0 refers to animals that underwent four hours of anaesthetic only. Uni 4/0 refers to animals that underwent four hours of anaesthetic with unilateral ischaemia and were sacrificed at the end of the anaesthetic without release of the tourniquet. Bilat 4/0 refers to animals that underwent four hours of anaesthetic with bilateral lower limb anaesthesia and were sacrificed immediately without release of the tourniquet. Each column on the graph represents the mean brightness level of the five rats in that group with 35 images per slide; giving an arithmetic mean involving 175 images. The standard deviation bars are shown. ❶ LUNG: Significant difference in level of brightness between sham-operated 0/0 and bilaterally ischaemic 4/0 animals, P=0.007. ❷ LUNG: Significant difference in level of brightness between unilaterally ischaemic 4/0 and bilaterally ischaemic 4/0 animals, P<0.0001.

Figure 37: Mean Brightness Levels of Type IV Collagen Immunofluorescence for Animals sacrificed 4 hours after 4 hour anaesthetic.

Tissues were labelled with type IV collagen antibody, prepared and analysed as in Section 4.2. Brightness refers to level of Type IV collagen seen with immunofluorescence. Sham 4/0 refers to animals that underwent four hours of anaesthetic only and were then sacrificed after a further 4 hours. Bilat 4/4 refers to animals that underwent a four hour anaesthetic with bilateral lower limb anaesthesia and were sacrificed 4 hours after release of the tourniquet. Unilateral 4/4 animals were not performed. Each column on the graph represents the mean brightness level of the five rats in that group with 35 images per slide; giving an arithmetic mean involving 175 images. The standard deviation bars are shown. ❶ LEFT LEG SKELETAL MUSCLE: Significant difference between sham-operated 4/0 and bilaterally ischaemic animals 4/4, P=0.0003. ❷ RIGHT LEG SKELETAL MUSCLE: Significant difference between sham-operated 4/0 and bilaterally ischaemic animals 4/4, P=0.0029. ❸ LUNG: Significant difference between sham-operated 4/0 and bilaterally ischaemic animals 4/4, P=0.0010. ❹ KIDNEY: Significant difference between sham-operated 4/0 and bilaterally ischaemic animals 4/4, P=0.0027.


Animals sacrificed immediately
after 4 hour Anaesthetic
Left leg skeletal muscle
Right leg skeletal muscle
Lung
Kidney
Brightness level
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Sham 0/0
Uni 4/0
Bilat 4/0
1
2

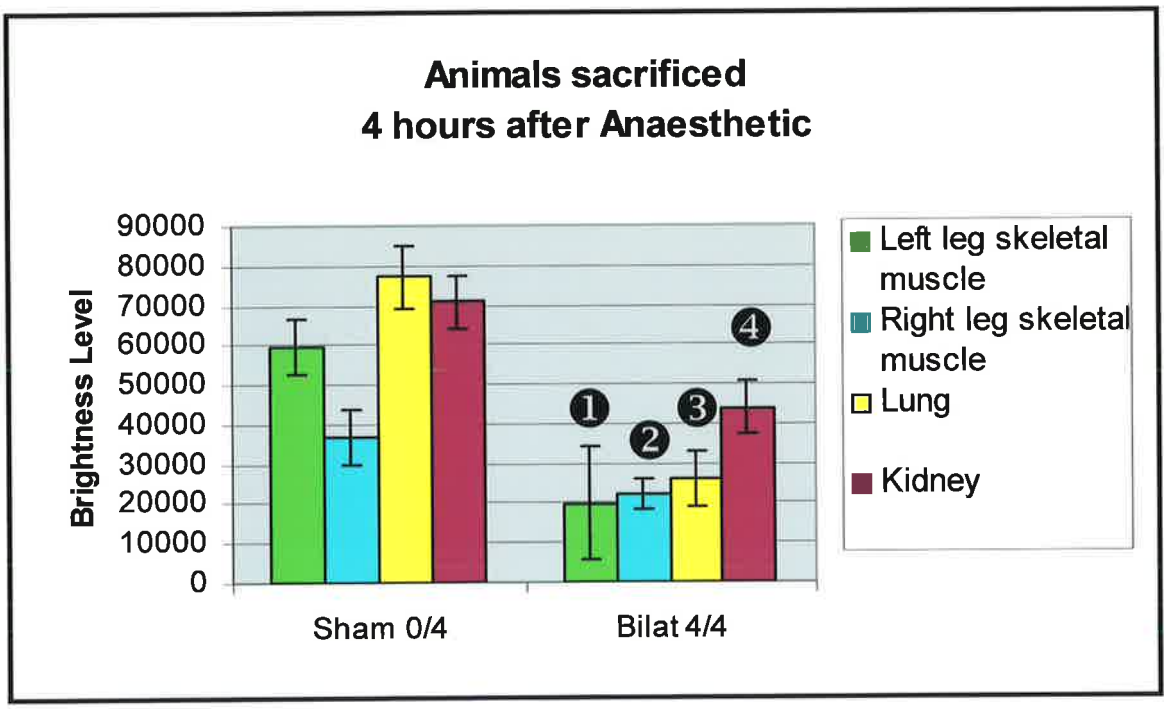
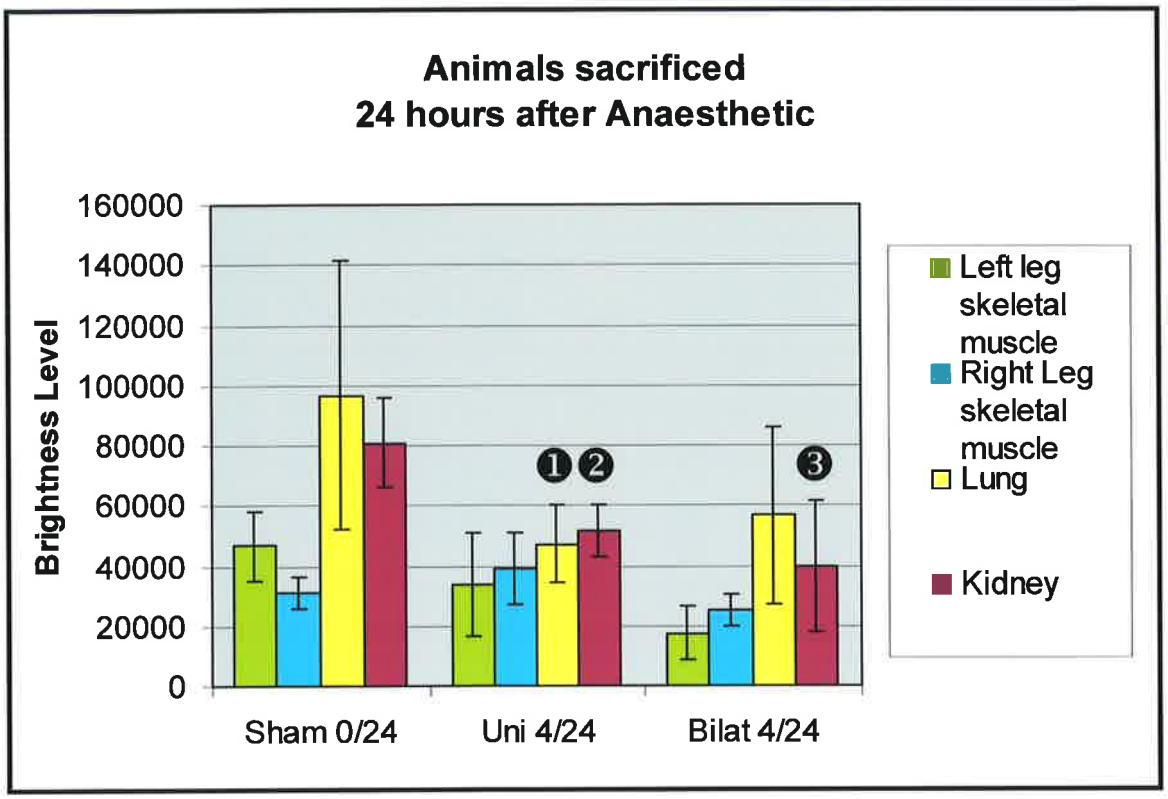

Animals sacrificed
4 hours after Anaesthetic
Left leg skeletal muscle
Right leg skeletal muscle
Lung
Kidney
Brightness Level
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
Sham 0/4
Bilat 4/4
1
2
3
4

Figure 38: Mean Brightness Levels of Type IV Collagen Immunofluorescence for Animals sacrificed 24 hours after 4 hour anaesthetic.

Tissues were labelled with type IV collagen antibody, prepared and analysed as in Section 4.2. Brightness refers to level of Type IV collagen seen with immunofluorescence. Sham 0/24 are rats that underwent 4 hours of anaesthetic followed by 24 hours before euthanasia. Uni 4/24 are rats that underwent 4 hours of unilateral left leg skeletal muscle ischaemia followed by 24 hours of reperfusion. Bilat 4/24 are rats that underwent 4 hours of bilateral lower limb ischaemia followed by 24 hours of reperfusion. Each column on the graph represents the mean brightness level of the five rats in that group with 35 images per slide; giving an arithmetic mean involving 175 images. The standard deviation bars are shown. ❶ LUNG: Significant difference between sham-operated 0/24 and unilaterally ischaemic 4/24 rats, P=0.0376. ❷ KIDNEY: Significant difference between sham-operated 0/24 and unilaterally ischaemic 4/24 rats, P=0.0363. ❸ KIDNEY: Significant difference between sham-operated 0/24 and bilaterally ischaemic 4/24 rats, P=0.0006.

Figure 39: Mean Brightness Levels of Type IV Collagen Immunofluorescence for Animals sacrificed 72 hours after 4 hour anaesthetic.

Tissues were labelled with type IV collagen antibody, prepared and analysed as in Section 4.2. Brightness refers to level of Type IV collagen seen with immunofluorescence. Sham 0/72 are rats that underwent 4 hours of anaesthetic followed by 72 hours before euthanasia. Uni 4/72 are rats that underwent 4 hours of unilateral left leg skeletal muscle ischaemia followed by 72 hours of reperfusion. Bilat 4/72 are rats that underwent 4 hours of bilateral lower limb ischaemia followed by 72 hours of reperfusion. Each column on the graph represents the mean brightness level of the five rats in that group with 35 images per slide; giving an arithmetic mean involving 175 images. The standard deviation bars are shown. ❶ LUNG: significant difference between sham-operated 0/72 and unilaterally ischaemic 4/72 animals, P<0.0001. ❷LUNG: significant difference between sham-operated 0/72 and bilaterally ischaemic 4/72, P<0.0001. ❸KIDNEY: significant difference between sham-operated 0/72 and bilaterally ischaemic 4/72 animals, P=0.0041. ❹KIDNEY: significant difference between unilaterally ischaemic 4/72 and bilaterally ischaemic 4/72, P=0.030.


Animals sacrificed
24 hours after Anaesthetic
Brightness Level
160000
140000
120000
100000
80000
60000
40000
20000
0
1 2
3
Sham 0/24
Uni 4/24
Bilat 4/24
Left leg skeletal muscle
Right Leg skeletal muscle
Lung
Kidney

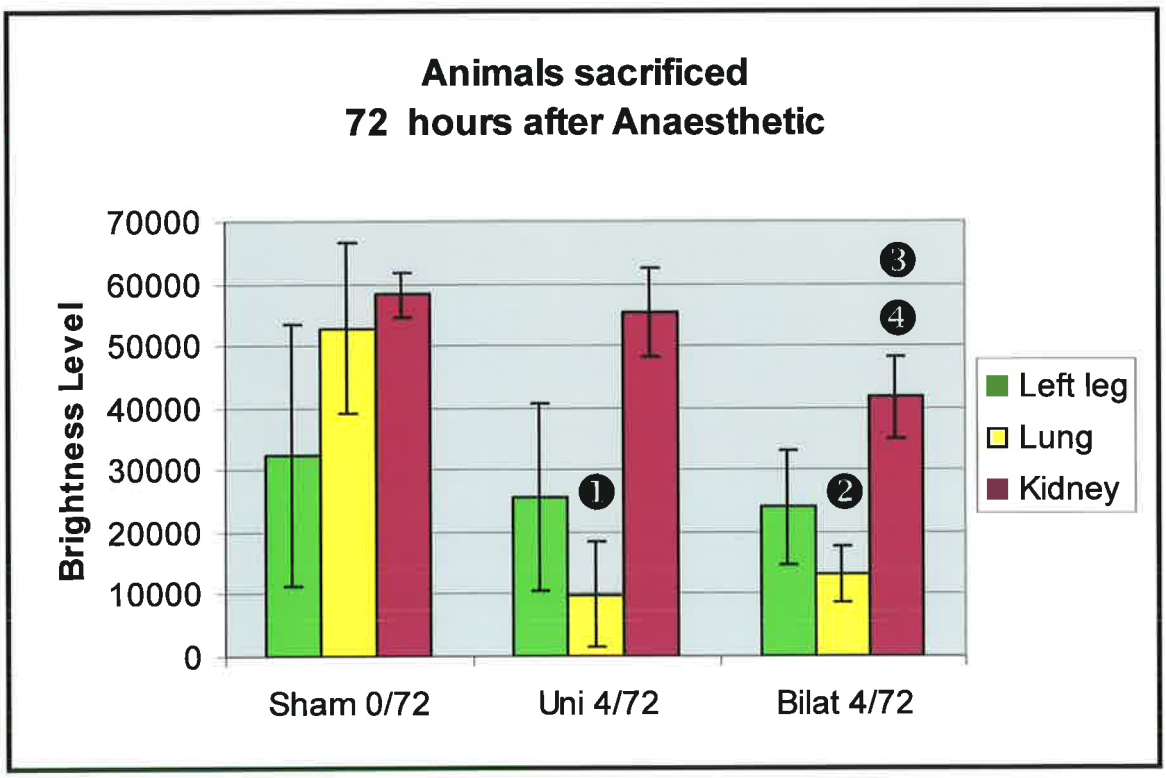

Animals sacrificed
72 hours after Anaesthetic
Brightness Level
70000
60000
50000
40000
30000
20000
10000
0
1
2
3
4
Sham 0/72
Uni 4/72
Bilat 4/72
Left leg
Lung
Kidney

Figure 40: Mean Brightness Levels of Type IV Collagen Immunofluorescence of Animals that underwent Sham-operations, 4 hour anaesthetic only, at different intervals between end of anaesthetic and Euthanasia.

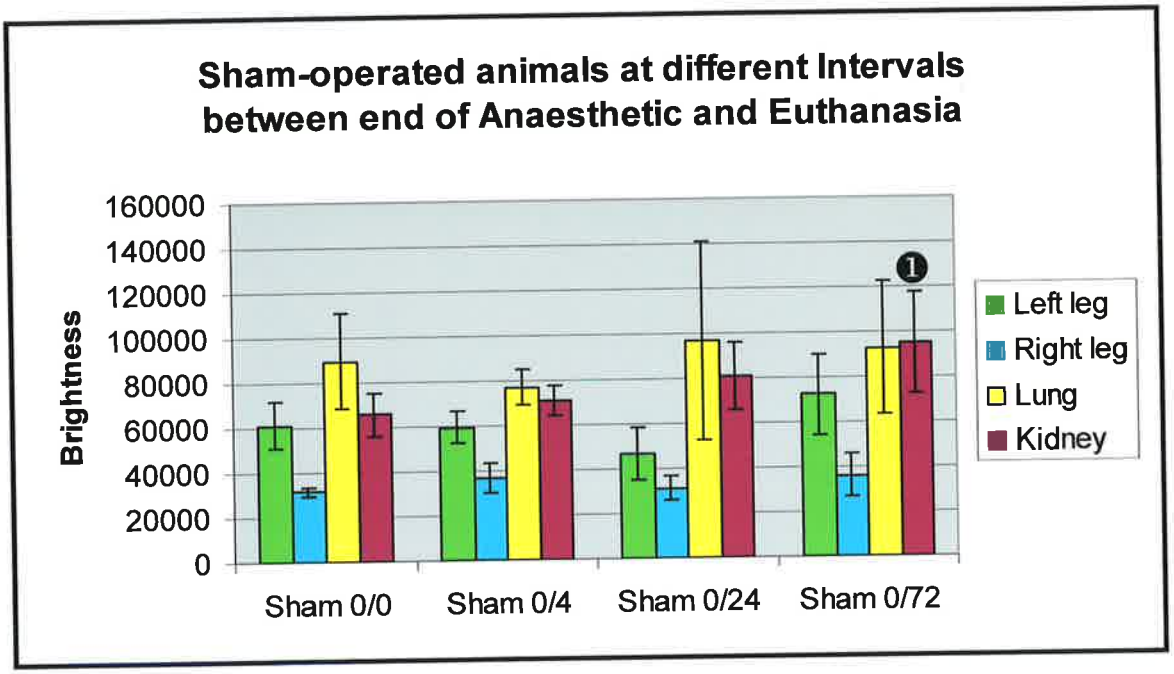
Tissues were labelled with type IV collagen antibody, prepared and analysed as in Section 4.2. Brightness refers to level of Type IV collagen seen with immunofluorescence. Sham 0/0 refers to animals that underwent a four-hour anaesthetic and were sacrificed immediately at the end of that procedure. Sham 0/4, 0/24 and 0/72 animals underwent a four-hour anaesthetic followed by euthanasia at 4, 24 or 72 hours respectively.

Each column on the graph represents the mean brightness level of the five rats in that group with 35 images per slide; giving an arithmetic mean involving 175 images. The standard deviation bars are shown. ❶ KIDNEY: Significant *increase* in brightness level between end of anaesthetic (Sham 0/0) and 72 hours of reperfusion (sham0/72), P=0.0358.

Figure 41: Mean Brightness Levels of Type IV Collagen Immunofluorescence in Animals that underwent Unilateral Left leg Skeletal Muscle Ischaemia under a 4 hour anaesthetic, then sacrificed at different Reperfusion times.

Tissues were labelled with type IV collagen antibody, prepared and analysed as in Section 4.2. Brightness refers to level of Type IV collagen seen with immunofluorescence. Uni 4/0 refers to animals that underwent four hours of unilateral left leg ischaemia under a four-hour anaesthetic and were sacrificed immediately at the end of that procedure. Uni 4/24 and 4/72 animals underwent four hours of unilateral left leg ischaemia under a four-hour anaesthetic followed by reperfusion for 24 or 72 hours respectively, before euthanasia.

Each column on the graph represents the mean brightness level of the five rats in that group with 35 images per slide; giving an arithmetic mean involving 175 images. The standard deviation bars are shown. ❶ LUNG: Significant decrease in brightness level between end of the anaesthetic (Uni 4/0) and 24 hours of reperfusion (Uni 4/24), P<0.0001. ❷ LUNG: Significant decrease in brightness level between end of anaesthetic (Uni 4/0) and 72 hours of reperfusion (Uni 4/72); P<0.0001.


Sham-operated animals at different Intervals between end of Anaesthetic and Euthanasia
Brightness
160000
140000
120000
100000
80000
60000
40000
20000
0
Sham 0/0
Sham 0/4
Sham 0/24
Sham 0/72
1
Left leg
Right leg
Lung
Kidney

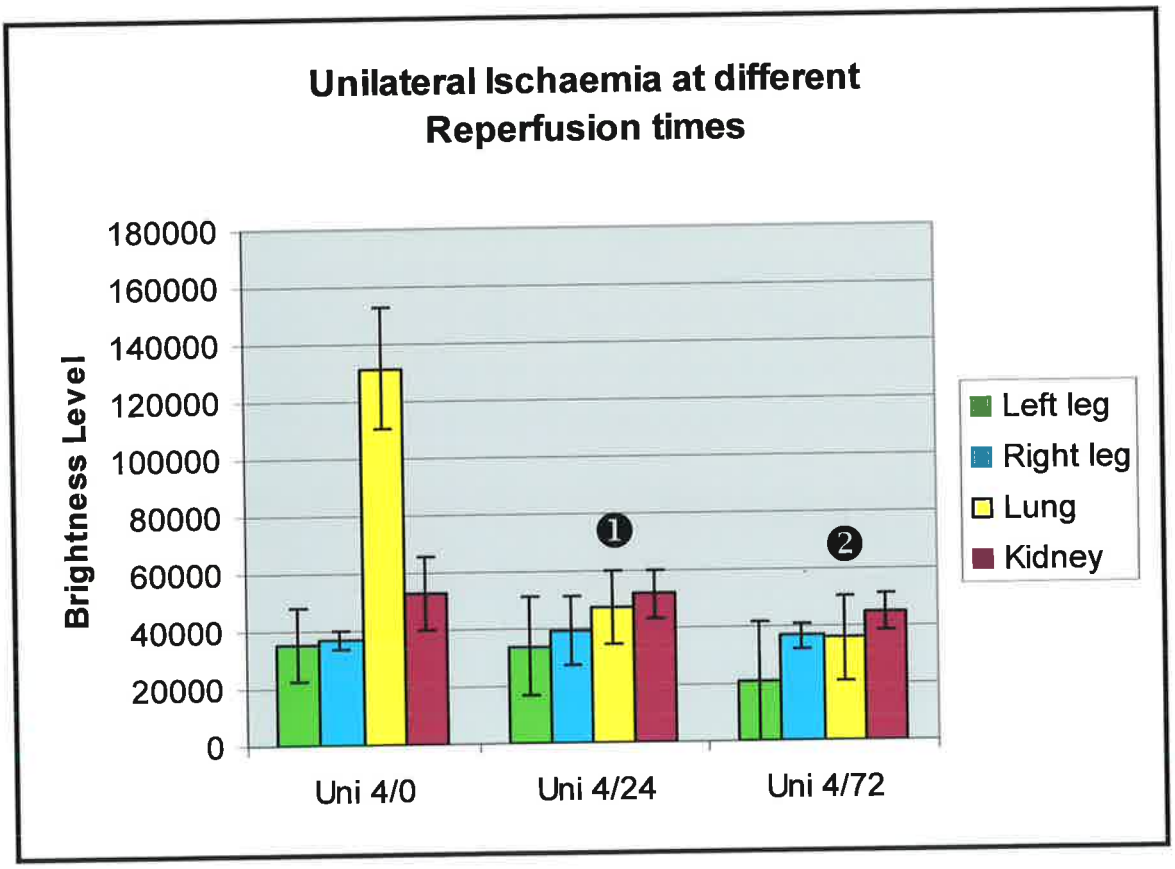
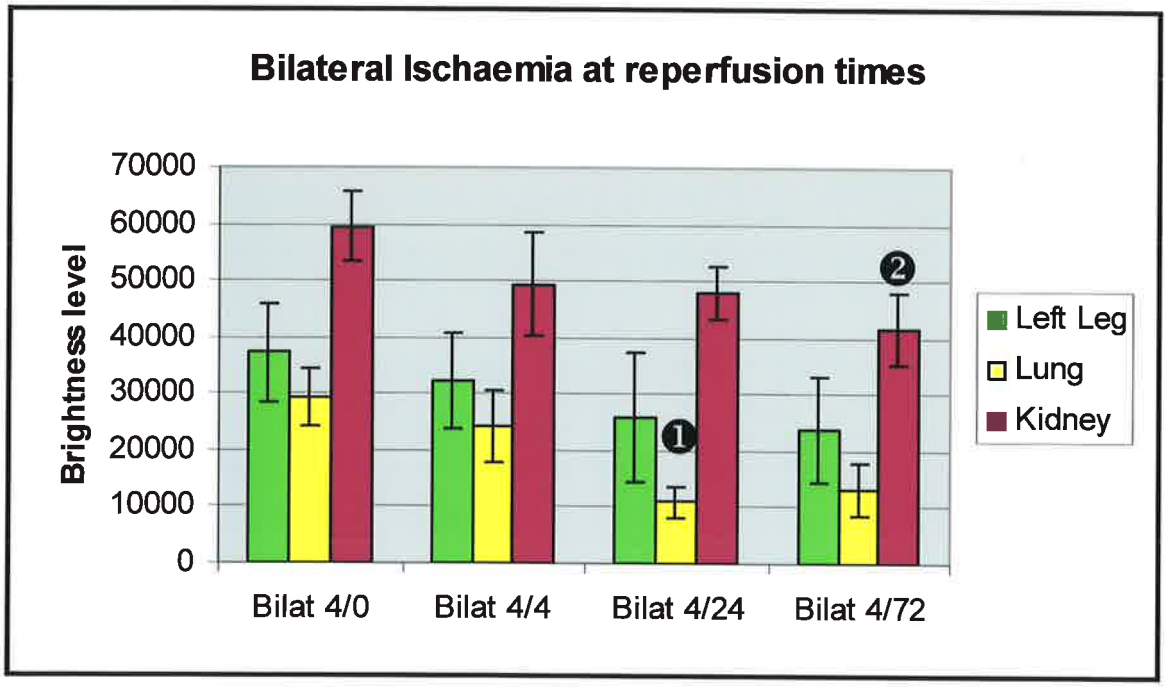

Unilateral Ischaemia at different Reperfusion times
Brightness Level
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Uni 4/0
Uni 4/24
Uni 4/72
1
2
Left leg
Right leg
Lung
Kidney

Figure 42: Mean Brightness Levels of Type IV Collagen Immunofluorescence in Animals that underwent Bilateral Lower leg Skeletal Muscle Ischaemia under a 4 hour anaesthetic, then sacrificed at different Reperfusion times.

Tissues were labelled with type IV collagen antibody, prepared and analysed as in Section 4.2. Brightness refers to level of Type IV collagen seen with immunofluorescence. Bilat 0/0 are animals that underwent four hours of bilateral lower leg ischaemia under a four hour anaesthetic and were sacrificed immediately at the end of that procedure. Bilat 0/4, 0/24 and 0/72 animals underwent four hours of bilateral lower leg ischaemia under a four hour anaesthetic followed by reperfusion for 4, 24 or 72 hours respectively, prior to euthanasia.
Each column on the graph represents the mean brightness level of the five rats in that group with 35 images per slide; giving an arithmetic mean involving 175 images. The standard deviation bars are shown. ❶LUNG: Significant decrease in brightness level between end of anaesthetic (Bilat 4/0) and 24 hours of reperfusion (Bilat 4/24), P=0.0204. ❷KIDNEY: Significant decrease in brightness level between end of anaesthetic (Bilat 4/0) and 72 hours of reperfusion (Bilat 4/72), P=0.0017.


Bilateral Ischaemia at reperfusion times
Brightness level
70000
60000
50000
40000
30000
20000
10000
0
Bilat 4/0
Bilat 4/4
Bilat 4/24
Bilat 4/72
1
2
Left Leg
Lung
Kidney

4.4 Discussion

4.4.1 MMP-2, MMP-9 and Type IV Collagen

There is some debate within the literature regarding the ability of MMP-2 and MMP-9 to cleave type IV collagen. The majority of reports indicate that MMP-2 and MMP-9 of leukocyte[316,594-596], bone[597,598], connective tissue[366,547] and tumour cell[599] origin degrade both pepsin solubilized and heat denatured forms of type IV collagen. However, some reports show that these enzymes degrade native full-length type IV collagen[366,547,594]. However, there are contradicting reports stating that tumour cell gelatinases of 65 kDa and 92 kDa show little or no ability to degrade native full-length Engelbreth-Holm-Swarm type IV collagen under conditions that preserve the tertiary structure of type IV collagen[600,601]. The controversy continues with human neutrophil gelatinase being relatively incapable of degrading native full-length Engelbreth-Holm-Swarm type IV collagen[316,602], but porcine neutrophil gelatinase of similar mass has been shown to degrade native full-length type IV collagen to produce fragments[594]. The 92 kDa type IV collagenase secreted by SV40-transformed human lung fibroblasts has been reported to be identical to a 92 kDa human alveolar macrophage gelatinase[366] capable of digesting native type IV and V collagen, although this was contradicted by another report showing that a 90 kDa human alveolar macrophage gelatinase was incapable of degrading full length type IV collagen. While the initial cleavage site of type IV collagen by MMP-2 has been found to be located one quarter of the distance from the amino terminus[594,603], the cleavage site of the full length native type-IV collagen remains to be determined[378].

In human studies in vivo, several workers have shown degradation of type IV collagen with MMP-2 and MMP-9. Zeng et al used immunohistochemistry to show increased MMP-9 correlating with marked degradation of type collagen in 100% and 23% of colorectal cancers with and without metastases[555]. Type IV collagen destruction has been seen in other colorectal carcinomas[604-606] in association with increased MMP-2 and MMP-9 expression in these tumours[607-609].

In the rat, degradation of basement membrane type IV collagen by MMP-2 and MMP-9 has been shown to occur in vitro, with purification of type IV collagenolytic activity using [3H]proline-labelled type IV collagen purified from Engelbreth-Holm-Swarm tumour as a substrate[610]. Koskinen et al showed an increase in MMP-2 and decrease in type IV collagen in quadriceps femoris muscle after extreme exercise in vivo in rats[611]. This reduction in type IV

collagen was associated with a reduction in TIMP-2 after exercise[611]. Frisdal et al showed a marked upregulation of MMP-2 and also an increase in MMP-9 associated with a dramatic decrease in type IV and laminin immunohistochemical staining following permanent muscle ischaemia in rats[549]. MMP-2 and MMP-9 were also induced after drug-induced muscle damage in normal and mutant mdx mice, the murine model of Duchenne muscular dystrophy[612] and in rat renal tissue, MMP-2 was associated with a decrease glomerular collagen IV post ischaemia[476]. Ng et al showed that reduction in MMP-9 was associated with type IV collagen accumulation in a model of rat experimental pancreatic fibrosis[613]. Hence, it appears that in rat tissue, MMP-2 and 9 both in vitro and in vivo degrade type IV collagen.

The α2(IV) chain specifically forms a high-affinity complex with proMMP-9[614] but α1(IV)/proMMP-9 complex could not be detected[615] unless under overexpression conditions of intracellular α1(IV) in recombinant mice[615]. In lysates of human cell lines, which contain α1(IV) and α2(IV), there is preferential binding of α2(IV) to proMMP-9, suggesting that the enzyme has a lower affinity for α1(IV)[615]. ProMMP-2 has a weaker affinity for α2(IV) compared with that of proMMP-9[614]. The precise sites of interactions of proMMP-9 with the α1(IV) and α2(IV) chains are yet to be defined, however, are of importance during degradation and turnover of type collagen molecules[615]. This process may result in the exposure of proMMP-9 binding sites in both chains allowing the bound enzyme to fulfil its function as type IV gelatinase[616]. It is possible that α2(IV) polypeptides act as a surface or matrix binding protein for proMMP-9[615].

One conceivable explanation for the differences between the in vitro and in vivo studies with type IV collagen and MMP-2 and-9 is that in vivo, there is partial degradation of the collagen IV network by a protease(s) other than the MMP-2 and MMP-9. Once partially degraded, proMMP-9 forms a very tight complex with the α2(IV) chain and proMMP-2 to a lesser extent[614]. After activation of the α2(IV)-bound proMMP-9, the enzyme would then contribute to the complete degradation of the collagen IV network consistent with its ability to degrade denatured collagens[616].

Basement membrane degradation is achieved by several MMPs and other enzymes. The other MMPs known to degrade components of the basement membrane include MMP-1/interstitial collagenase[547] and MMP-3/stromelysin[547,617,618].

The other enzymes that are reported to degrade native full-length type IV collagen include elastase[619,620], cathepsins B, D and G[621,622], trypsin[623,624], pepsin[625,626], plasmin[627,628] and MMP-7/PUMP-1[350]. However, MMP-2 and MMP-9 appear to be the most important basement membrane type IV collagen degradation[629-631].

4.4.2. Immunohistochemical Technique

Multiple potential errors are possible in using the current technique for quantitation of type IV collagen with fluorescein isothiocyanate. Some of these errors were discussed in the introduction. The aim of achieving accuracy with quantitation involves recognizing and minimizing the potential errors.

Solid tissues must be sectioned to avoid scattering of image-forming light. Fresh tissues are not rigid enough for sectioning and freezing the tissue in liquid nitrogen, storing at -80 degrees Celsius and slicing the tissue in a cryostat conferred this rigidity. Fixation with acetone also confers rigidity upon tissue by hardening the tissue and preventing dislocation of the constituents[632]. Tissue sampling errors can occur if the slicing of the tissue inadvertently selects an area of adipose cells or other tissue that was not the primary target. This selection bias was minimized by using 5 rats in each group, using multiple tissue slices per slide and the use of 35 images per slide. There could be variability due to section thickness, although as shown below, this had a minimal overall effect. Minor variations owing to the cryostat microtome variability can lead to an uneven cut tissue surface, but this did not appear to have a significant effect.

The primary antibody used to detect type IV collagen was commercially made and was claimed by the manufacturer to show minimum cross reactivity to Type I, II, III, V and VI collagens, as well as negligible non-specific cross reaction with other serum or non-collagen extracellular matrix proteins. This possibility of cross reactivity was not investigated in these studies and the manufacturer's confirmations were accepted.

The biotin/streptavidin system of amplifying the signal from the antigen/antibody complex was employed in these studies. Others have shown that this system has a higher signal to noise ratio than direct conjugation of monoclonal antibodies to peroxidase[633,634]. The biotinylated anti-rabbit IgG has <1% cross reactivity with rat IgG[635]. The streptavidin has four affinity binding sites for biotin and is much less prone to non-specific binding than avidin[592].

Excess labelling reagent was removed by gel filtration chromatography by the manufacturer[592], helping to minimize method non-specificity.

Apart from reactions between unconjugated fluorochrome and electrostatic and hydrophobic bonding of conjugate, other causes of loss of specificity include autofluorescence of tissue and non-specific reactions of second antibody and of conjugated proteins that contaminate the second antibody solution. Some unstained tissue components possess green autofluorescence of similar wavelengths as that of the emission of fluorescein-isothiocyanate. However, as was seen in Section 4.3.1 Pilot studies, Negative Controls and Histogram levels, when the primary antibody was omitted there was minimal background autofluorescence or cross reactivity of the biotin anti-rabbit IgG with the rat antigens.

During the actual image collection, a variety of errors can occur. Quenching of the fluorescence due to auto-absorption of light is discussed below. The mercury lamp itself can vary in its transmitted light wavelength[636]. It has a definite warm-up period and the lamp was turned on 30 minutes prior to use in these studies. The other errors include correcting for glare, out of focus error[637], camera correction, condenser aperture error[638], diffraction error[639], distributional error[640], out of range error[641], stray light[638] and chromatic aberration[639]. All of these have a lack of control and standardization in current techniques[642]. The net effect of all these optical errors has been found to be 1.2 - 2% as measured in quantitative immunohistochemistry[638,643]. Image segmentation is the process to select and discriminate meaningful objects in an image from the background. This includes the avoidance of the edge of tissue slices as there is marked enhancement of the immunofluorescence in this region[642]. This was avoided by selecting tissue images inside the edge of the tissue sample. The use of anti-fading agent (Dako® Fluorescent Mounting Medium) in the mounting medium helps prevent fading of tissue images[644].

All other variables on the microscope and attachments, such as excitation filters, barrier filters and magnification of the objective were kept constant throughout the quantitative immunohistochemistry measurements.

4.4.3. Degradation of Type IV Collagen during Ischaemia/Reperfusion Injury.

The pilot studies showed that there was a discernible difference between sham-operated animals and unilaterally ischaemic animals and hence that further analysis was feasible. As the immunostaining of the negative control values were negligible compared to the brightness values from the positive slides, they were not included in the statistical analyses.

The histogram graphs show the effect of changing the cut-off level selected for each group of images. The histogram level was chosen as a standard value for each group of analyses. The actual figure set as the lower cut-off for grey level was selected in order to show the majority of the positive immunofluorescence but not include background dark areas of image that had minimal immunofluorescence enhancement. This value varied between each batch of slides because the overall level of brightness in each batch varied for reasons such as age of the antibodies, slight differences in concentration between batches, age of the fluorescent lamp, sampling differences and tissue differences.

The sampling studies confirmed that a minimum of 25 images was required in order to achieve a normal statistical distribution. Thirty-five images were used in each study to ensure that the normal distribution would be maintained.

Given the similarity in the results for brightness of the different thickness slices, values of 10 microns for skeletal muscle, 3 microns for lung and 5 microns for kidney were chosen. However, if there had been a large variation in section thickness, it would have increased the variation in measured brightness levels.

The quenching level of the fluorescein isothiocyanate was extremely rapid. In order to minimize this effect, the image was selected and taken within approximately 3 to 4 seconds. Then the slide was moved to the next area for image capture and taken again within 3 to 4 seconds, hence aiming to keep the quenching effect to the lowest level technically feasible. The fading of stained preparations on exposure to fluorescent stimulating light has been an accepted feature of the immunofluorescent procedure since the definitive description of the technique[645]. Other investigators have also observed quenching of the image but not with the same rapidity as in these studies[556]. Using p-Phenylenediamine in the mounting medium, to

prolong and intensify fluorescence without any evident effect on antibody binding[646,647], can minimize this major disadvantage of fluorescence with fluorescein isothiocyanate.

The saturation studies show that the tissue is saturated with antibody at a concentration of 1 in 100 without further increases in levels of measured brightness for higher concentrations. Hence, this concentration was chosen for all future analyses in all three tissues.

The quantification studies showed that at the end of the anaesthetic, the bilaterally ischaemic animals had significant destruction of the type IV collagen in the *lung,* when compared to either the shams or the unilaterally ischaemic animals. Part of this effect may be secondary to the additive effects of the anaesthetic itself and either the ischaemia alone or partial reperfusion. It is possible that in spite of a flat trace on the photoplethysmography during the procedure that the animal is that underwent incomplete or partial ischaemia and allowing some venous return from the limb. This would send a variety of ischaemia products, including the possibility of MMP-2 and MMP-9 throughout the systemic organs.

After four hours from the end of the anaesthetic, all of the tissues all show degradation of type IV collagen when comparing sham-operated animals to bilaterally ischaemic animals. In this comparison both the *left* and *right leg skeletal muscle* studies consistently agree, as expected, as both legs are non ischaemic in the sham-operated group and ischaemic in the bilaterally ischaemic group. Unilateral ischaemia with sacrifice at 4 hours was not performed, as discussed in Chapter 2. Unlike the changes at the end of the anaesthetic, the *skeletal muscle* and the *kidney* showed damage, as well as the *lung*.

After twenty four hours from the end of the anaesthetic, there was a trend for the *left leg skeletal muscle* to show degradation of type IV collagen but this level was not significant. Both the *lung* and *kidney* continued to show significant decreases in the levels of type IV collagen that was observed immunohistochemically in these tissues. Similar findings have been seen after renal ischaemia/reperfusion with reduction in glomerular type IV collagen and an increase in MMP-2[476].

After 72 hours from the end of the anaesthetic, there was still a trend towards degradation of type IV collagen in the *left leg skeletal muscle* but this did not reach significance. The *lung* and *kidney* however continued to show a decrease in brightness levels, indicating marked degradation of type IV collagen.

When the contralateral limb was studied, there was no change between the sham-operated and the unilaterally ischaemic rat at 24 hours, indicating that the skeletal muscle in the contralateral limb was resistant to the damage of remote organ reperfusion injury. These changes have been confirmed by Summers et al, showing that the polymorphonuclear cells from the contralateral limb after ischaemia/reperfusion contained no differences in phagocytosis or chemotaxis compared with pre-ischaemia values[648]. However, Salm et al showed that there was a protective effect on the contralateral limb by using verapamil, indicating that there is at least some minor damage occurring in the contralateral limb during ischaemia/reperfusion injury[649]. The majority of authors use the non-ischaemic contralateral limb as an internal control[47,650-653]. This was specifically avoided in these studies in order to examine the effects on the contralateral limb itself. However, there were no significant changes as detected by immunohistochemical analysis with type IV collagen.

As predicted there were minimal changes occurring in all tissues in the sham-operated animal. However by comparison, the changes in the left leg were that of decreased levels of type IV collagen in both the unilaterally and bilaterally ischaemic animals, with associated degradation of type IV collagen with increasing reperfusion times. These trends only reached significance when comparing sham-operated animals with bilateral ischaemic animals at 4 hours of reperfusion. There were minimal changes immediately at the end of the anaesthetic, so this decrease in type IV collagen appears to due to the products of reperfusion.

The *left leg skeletal muscle* showed deterioration in type IV collagen between unilaterally ischaemic animals and sham-operated animals, but this did not reach significance. The bilaterally ischaemic animals showed a further decrease in type IV collagen but again, this did not reached significance. The only significant level was between bilaterally ischaemic animals and sham-operated animals four hours after the end of the anaesthetic. With increasing duration of reperfusion in the unilaterally and bilaterally ischaemic animals, there was also a decline in type IV collagen that did not reach significance (Figure 41 and Figure 42).

There was markedly significant *lung* type IV collagen degradation. The level of type IV collagen differed from the sham-operated animal to the bilaterally ischaemic animal at the end of the anaesthetic, indicating that this reduction is not an effect of the anaesthetic alone (Figure 36). In the unilaterally ischaemic animal, there were no significant changes in lung type IV collagen levels at the end of the anaesthetic (Figure 36) but after 24 hours of

reperfusion, there was a significant deterioration in the lung level of type IV collagen (Figure 41). Hence, it is likely that this damage is commenced initially during the anaesthetic period, but deteriorates markedly particularly over the first 24 hours. The degradation of type IV collagen in the lung is likely to be an effect of the skeletal muscle reperfusion.

Although the *kidney* is not quite as sensitive to type IV degradation as the lung, there were certainly changes between the groups with destruction of renal type IV collagen occurring at all time points after reperfusion had commenced. Over the various time points, the destruction in type IV collagen reached a maximum in the bilateral ischaemic animals at 72 hours. Hence, the destruction appears to be slower than in the lung.

The initial sections of this chapter aimed to establish the technique, describe and minimize the known errors in quantitative immunohistochemistry. It was established that these techniques are feasible to measure the level of type IV collagen in tissue. As type IV collagen is the predominant substrate of MMP-2 and MMP-9, these techniques were then used to establish the level of destruction of type IV collagen in skeletal muscle ischaemia/reperfusion injury.

It was shown for the first time, that the level of type IV collagen deteriorates markedly in the ischaemic limb skeletal muscle and in remote organs with ischaemia/reperfusion injury. This is consistent with the changes in matrix metalloproteinase levels seen on zymographic analysis and these relationships will be discussed in Chapter 6.

CHAPTER 5:

THE LOCAL AND REMOTE EFFECTS

OF

DOXYCYCLINE

IN

SKELETAL MUSCLE REPERFUSION INJURY

5.1 Introduction

The primary aims of all investigations into ischaemia/reperfusion injury are to ascertain the aetiological factors causing this phenomenon in order to develop therapies that will prevent or ameliorate the pathological process occurring. Known intervention strategies that have been studied include limiting oxygen free radical-mediated injury, limiting leukocyte mediated injury, blocking complement activation, limiting calcium ion-mediated injury and modulating eicosanoid, coagulation, phospholipases, nitric oxide and cytokine systems[271].

The focus of the current studies is on the role of MMP-2 and MMP-9 in skeletal muscle ischaemia/reperfusion injury. There are many known inhibitors of matrix metalloproteinases, including naturally occurring tissue inhibitors of matrix metalloproteinases (TIMPs), α_2–macroglobulin and synthetic inhibitors such as MMP chelators and tetracyclines. TIMPs are secreted by most connective tissue cells and occur in plasma, synovial fluid and amniotic fluid[654]. These inhibitors were discussed in detail in Chapter 1.

5.1.1 Doxycycline Rationale

Tetracyclines are natural products derived from Streptomyces species. Their semi-synthetic derivatives include doxycycline and minocycline. Tetracycline antibiotics have been investigated extensively in their role as inhibitors of metalloproteinases, acting by mechanisms distinct from their antimicrobial properties[454,655-659]. Tetracyclines inhibit collagenases[660] and other MMPs in vitro and in vivo[661].

Doxycycline was chosen as the MMP inhibitor in these studies. The techniques of investigation used were histopathological examination, zymography and Type IV collagen immunohistochemistry. In order to try to maximize the level of difference achieved between sham-operated and ischaemic animals, the bilateral ischaemia and 24 hour reperfusion time point were chosen for all treatment rats.

The dosages used in these in vivo studies were 50 mg/kg twice daily or 200 mg/kg twice daily administered by gavage. Curci et al used doxycycline in a rat model to investigate the role of MMPs in the development of aortic aneurysms and found half maximal inhibitory effect at 6 mg/kg/day and maximal effects at greater than 30 mg/kg/day[447]. The recommended dose of doxycycline for human clinical practice is 50-100 mg orally twice daily (1.4 to 2.8 mg/kg/day

for 70 kg person), in the treatment of some conditions, doxycycline is administered in doses as high as 200 mg orally twice daily (5.7 mg/kg/day)[662]. The most important adverse effects of the tetracyclines relate to gastrointestinal intolerance, which is dose dependent[663]. Although the doses used in these studies were high, they were chosen in ensure that if a MMP inhibitory effect was present that it would not be missed due to administration of too low dose. There was no evidence of toxicity of doxycycline in the rats in the current studies.

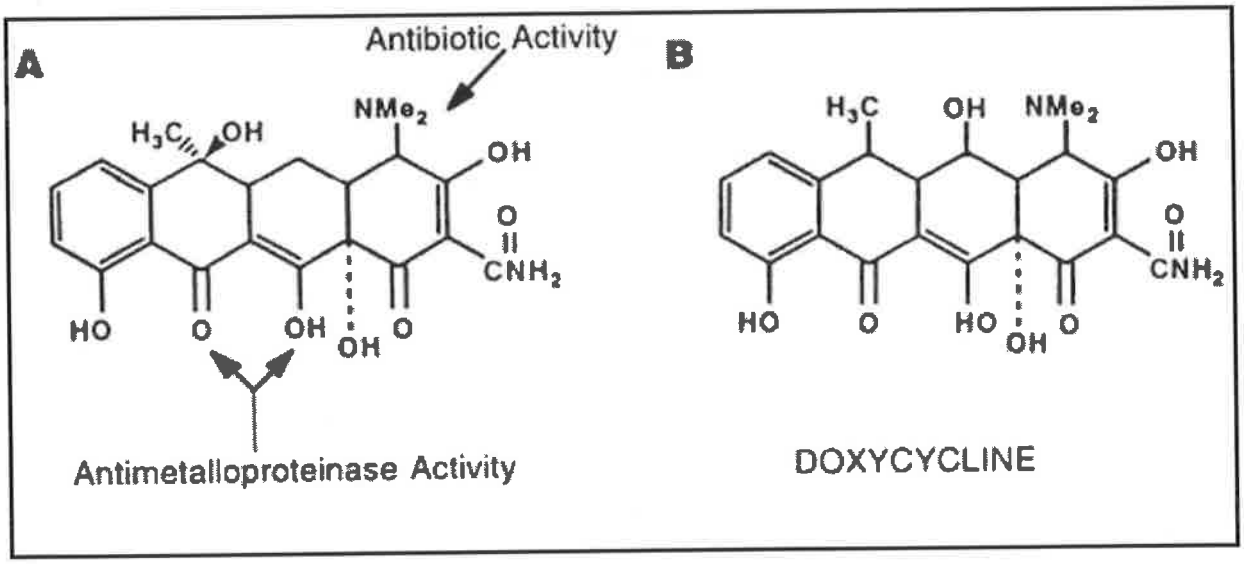
Tetracyclines inhibit collagenase and other MMPs in vitro and effectively prevent MMP-mediated tissue injury in animal models of gingivitis[454,655], arthritis[656,657] and in aortic aneurysm models[447-449,664-666]. They have been used in vivo in prevention of arthritis[667-669], ischaemic brain damage[670], animal models of aortic aneurysm growth[447] and human aortic aneurysm studies[665] periodontal disease in animals[671] and in humans[661]. Tetracyclines are known to inhibit MMP-2[659], MMP-8[660,672], MMP-9[660] and partially inhibit MMP-1[660]. Doxycycline is an attractive candidate for MMP suppression in skeletal muscle ischaemia/reperfusion as they are known to be efficient MMP inhibitors[660], they are low cost, have established safety for human use and are already in use for treatment of other human diseases.

Doxycycline is 6-deoxy-5-hydroxy tetracycline and its chemical structure is shown in Figure 43. Doxycycline interacts with a zinc cation in the MMP enzyme to mediate its inhibitory effect[660]. Using in vitro assays, it has been shown that it is the 4-dimethylamino group that is required for antibacterial activity, but MMP inhibition appears to require the presence of the 11-oxy and 12-hydroxyl groups[673-675]. Tetracyclines are thought to act primarily as direct pharmacological inhibitors, non-selectively inhibiting MMPs by binding to the active Zn site[660] and by binding to an inactive calcium site, which causes a conformational change[676] and loss of enzymatic activity. Secondary mechanisms have also been proposed including a reduction in MMP gene expression[659] and a reduction in activation[677].

Figure 43: Chemical Structures of Tetracyclines.

A = Chemical groups responsible for different biological activities within the tetracycline nucleus. The 4-dimethylamino group is required for antibiotic activity *(single arrow) and* the 11-oxy and 12-hydroxy groups are responsible for direct metalloproteinase inhibition *(split arrow)*.

B=Doxycycline (6-deoxy, 5-hydroxy tetracycline)[447].



5.2 Methods

The methods of anaesthesia, establishment and monitoring of ischaemia, euthanasia and tissue processing were all performed as outlined in Chapter 2.2.1 Animal Model Protocol on page 55.

Two doses of doxycycline were chosen. Low dose doxycycline was defined as 50 mg/kg twice a day. Hence, in the average 250mg rat, this dose equated to 12.5 mg twice a day. Doxycycline was dissolved in 2 ml of sterile water and given to the rat by oral gavage at 0730 and 1830 hours. The drug was commenced 7 days before the 4 hour bilateral ischaemia experiment. Following the experiment, the rat was woken and doxycycline treatment continued until the time of euthanasia 24 hours later. Hence, the rat received 2 doses of doxycycline after anaesthesia and before death. High dose doxycycline was defined as 200mg/kg twice a day. Hence, in the average 250 mg rat, this equated to 50 mg twice a day and was given in the same method as outline above. Three tissues were analysed, left leg skeletal muscle, lung and kidney.

5.2.1 Quantitation of Lung Oedema

As there was no significant results seen in the Wet/Dry weight lungs with the non drug treatment group between sham-operated animals and bilateral animals, it would not be possible to show a difference with the doxycycline treatment groups and hence these studies were not performed.

5.2.2 Histopathological Assessment of Tissue Damage

The tissues were embedded in paraffin and stained with haematoxylin and eosin by the same technicians in the Histopathology Department at The Queen Elizabeth Hospital. Both the qualitative and quantitative studies were again performed using the same methods as described in Chapter 2.2.3 Histopathological Assessment of Tissue Damage on page 57.

5.2.3 Zymographic Analysis of Matrix Metalloproteinase Activity

The tissues were processed and prepared, protein concentration was analysed by Bio-Rad Protein Assay and run in gelatin zymograms (40 μg protein/lane) as per the methods in 3.2 Methods.

5.2.4 Effect of Doxycycline on Degradation of Type IV Collagen during Skeletal Muscle Ischaemia/Reperfusion Injury

The immunohistochemical methods used were as described in Chapter 4.2, Immunohistochemical methods on page 121. Tissue sections were cut at 10 μm for skeletal muscle, 3 μm for lung tissue and 5 μm for kidney tissue. The primary antibody dilution was 1 in 100 and 35 images per slide were captured for image analysis. To allow valid comparisons between treatment groups, all tissues within that group were analysed together. (See discussion on errors in immunohistochemical technique in Chapter 4.1.3 and 4.4.2). Histogram limits were set at 140 for left leg skeletal muscle, 180 for lung and 170 for kidney tissue.

5.3 Results

5.3.1.Histopathological Assessment of Tissue Damage

5.3.1.1 Qualitative Analysis

Representative histopathological samples of each tissue; left leg skeletal muscle, lung and kidney in the sham-operated, bilateral ischaemia without doxycycline treatment, bilateral ischaemia with low dose doxycycline and bilateral ischaemia with high dose doxycycline groups are shown in Figure 44.

Left leg Skeletal Muscle

(Figure 44)

There were minimal pathological changes in skeletal muscle from the left leg of sham-operated rats. Left leg skeletal muscle from a bilaterally ischaemic animal without doxycycline treatment showed increased striated muscle destruction, oedema and cellular infiltration. There were some minor changes in the doxycycline treated groups compared to the bilaterally ischaemic and 24 hour reperfusion group that were not treated with doxycycline; with slightly less muscle fibre destruction and cellular infiltration.

Lung

(Figure 44)

In all the images of lung tissue, there was some cellular infiltration and focal areas of collapse. There were minimal difference seen in these representative images between sham-operated animals, bilaterally ischaemic animals without doxycycline and the doxycycline treated animals.

Kidney

(Figure 44)

The sham-operated animals showed preservation of renal tissue with normal glomeruli and minimal cellular invasion. Kidneys from bilateral ischaemic animals without doxycycline showed evidence of oedema, haemorrhage and congestion of glomeruli. In doxycycline treated animals, there was slightly less oedema, congestion and haemorrhage observed in the kidneys.

5.3.1.2 Quantitative Analysis

Table XVIII illustrates the comparison between histopathological scores in the bilaterally ischaemic animals without any drug treatment to the two groups receiving doxycycline treatment. The results of the overall statistics for the entire table are given in Table XIX.

Left leg Skeletal Muscle

After excluding Left Leg score for rat 56 in bilateral group (as discussed in Chapter 2), the results were ranked and analysed using analysis of variance. The spliced results are displayed in Table XX. The median score for sham-operated animals was 0; indicating no abnormal findings, with cigar shaped nuclei, muscle striations complete and no cellular infiltrate. In the animals subjected to bilateral limb ischaemia, the median score for the left leg skeletal muscle was 6.5. In the animals treated with low dose and high dose doxycycline, the median scores were 6 and 7 respectively.

There was a significant difference between the sham-operated animals and the animals subjected to bilateral ischaemia and low dose doxycycline($p=0.0276$). There was also a significant difference between sham-operated and the high dose doxycycline treated animals ($P=0.0234$). There were no significant differences between the bilateral ischaemia rats without doxycycline and those with low dose or high dose doxycycline.

Lung

There was no significant difference observed in the histopathological analysis between the sham-operated, bilaterally ischaemic animal without doxycycline and the animal treated with either low dose or high dose doxycycline. The median score for sham-operated animals was 2, bilaterally ischaemic animals was 3, low dose doxycycline treated animals was 3 and high dose doxycycline treated animals was also 3. This indicates that there was mild to moderate mononuclear cell infiltration with occasional to focal areas of collapse in all tissues.

Kidney

In the sham-operated animals the median damage score was 1, the bilaterally ischaemic animals without doxycycline had a median score of 3, whereas the low dose doxycycline and high dose doxycycline treated animals with bilateral ischaemia had median scores of 2 and 3 respectively. There was a significant difference between the bilaterally treated animals without doxycycline and the animals treated with low dose doxycycline ($p=0.0162$). There

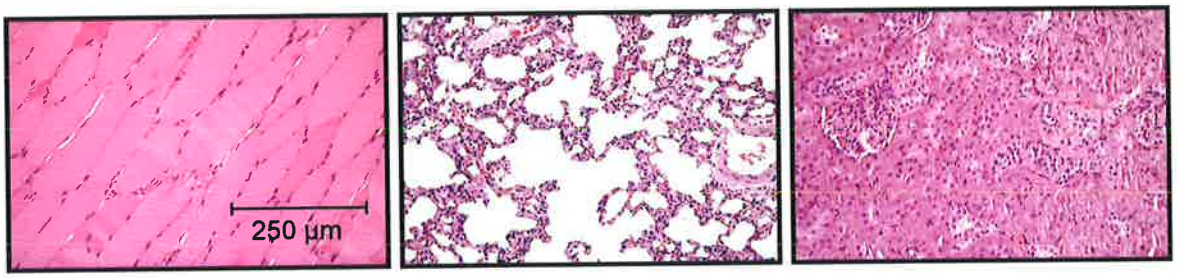
were also significant differences between sham-operated and bilateral ischaemia without doxycycline ($p=0.0276$), sham-operated and low dose treated animals with bilateral ischaemia ($p=0.0276$) and between sham-operated and high dose treated animals ($p=0.0372$).

Figure 44: Representative Images of Histopathology of Left leg Skeletal Muscle, Lung and Kidney Tissue including images of animals treated with Doxycycline.

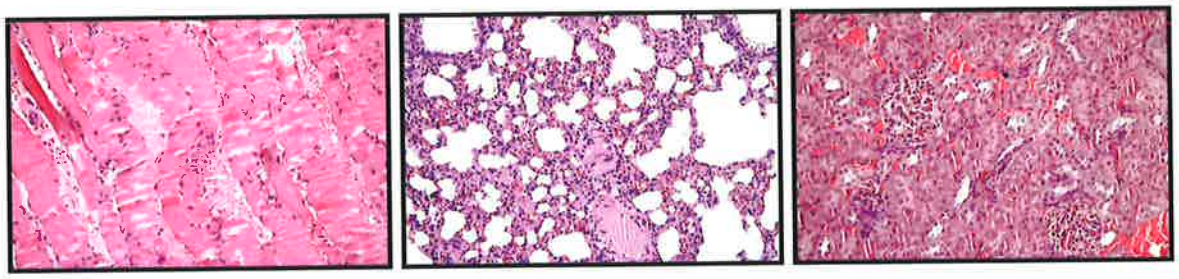
Shams, no Drug, 24 hours refers to animals that underwent a 4 hour anaesthetic and were sacrificed at 24 hours, with no doxycycline treatment. Bilateral, no drug, 24 hours refers to animals that underwent 4 hours of bilateral ischaemia, followed by 24 hours of reperfusion before euthanasia. Bilateral ischaemia + Low Dose Doxycycline, 24 hours refers to animals treated with Doxycycline 50 mg/kg twice a day for seven days before 4 hour anaesthetic with 4 hours of bilateral ischaemia and then reperfusion allowed for 24 hours. Bilateral ischaemia + High Dose Doxycycline, 24 hours refers to animals treated with 200 mg/kg twice a day for seven days before a 4 hour anaesthetic with 4 hours of bilateral ischaemia and then reperfusion allowed for 24 hours.

Paraffin Blocks. Haematoxylin and Eosin Stain. Magnification 20 X.

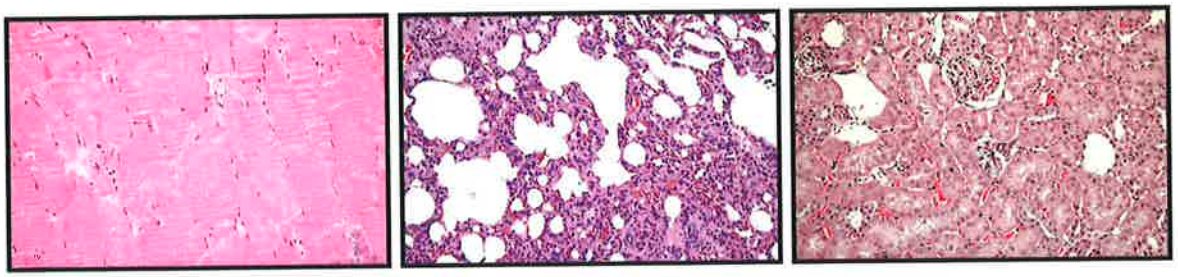
Left leg Lung Kidney



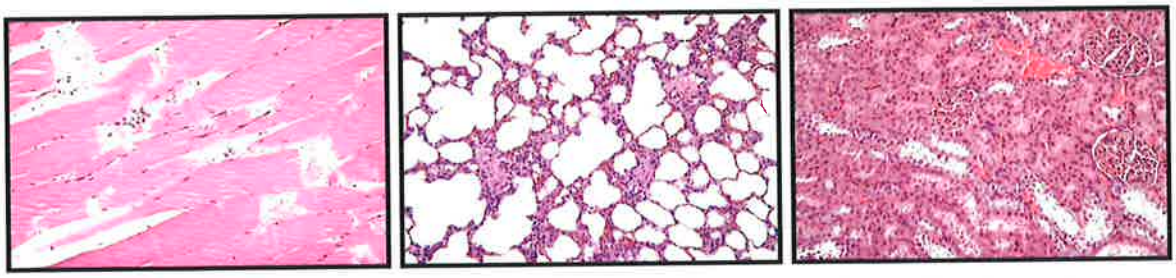
Sham, no drug, 24 hours



Bilateral ischaemia, no drug, 24 hours



Bilateral ischaemia + Low Dose Doxycycline, 24 hours



Bilateral ischaemia + High Dose Doxycycline, 24 hours

Table XVIII: Histopathological Score of Level of Tissue Damage – Doxycycline Treatment

Sham-operated refers to animals that underwent an anaesthetic only and were sacrificed at 24 hours after the end of the anaesthetic. Bilateral Ischaemia – 24 hours reperfusion refers to animals that underwent 4 hours of bilateral lower limb ischaemia and 24 hours of reperfusion before euthanasia. Low Dose Doxycycline, 24 hours reperfusion refers to animals treated with Doxycycline 50 mg/kg twice a day for seven days before 4 hour anaesthetic with 4 hours of bilateral ischaemia and then reperfusion allowed for 24 hours. Bilateral ischaemia + High Dose Doxycycline, 24 hours reperfusion refers to animals treated with 200 mg/kg twice a day for seven days before 4 hour anaesthetic with 4 hours of bilateral ischaemia and then reperfusion allowed for 24 hours. Histopathological Score is based on Carter et al for the left leg skeletal muscle and adaptations developed in Chapter 2 for the lung and kidney tissues[479].

RAT GROUP	RAT Number	Left leg Skeletal Muscle	Lung	Kidney
SHAM-operated – sacrificed 24 hours after end of Anaesthetic	19	0	2	1
	21	0	3	1
	33	0	3	0
	34	0	2	0
	36	0	2	1
BILATERAL ISCHAEMIA – 24 hours, reperfusion	52	4	4	3
	53	6	3	3
	54	7	3	3
	56	0	3	3
	60	7	2	3
LOW DOSE DOXYCYCLINE TREATMENT – 24 hours reperfusion	62	4	3	2
	63	4	3	2
	64	6	3	2
	65	6	3	2
	66	6	4	2
HIGH DOSE DOXYCYCLINE TREATMENT – 24 hours reperfusion	72	7	2	3
	73	7	3	3
	74	6	3	2
	76	7	4	3
	77	7	3	3

Table XIX Overall Statistical Analysis of Histopathological Scoring- Doxycycline Treatment for Left leg, Lung and Kidney.

Median score is the median of the 5 scores for rats. Sham-operated refers to animals that underwent an anaesthetic only and were sacrificed at 24 hours after the end of the anaesthetic. Bilateral refers to animals that underwent 4 hours of bilateral lower limb ischaemia and 24 hours of reperfusion before euthanasia. Low Dose Doxycycline, 24 hours reperfusion refers to animals treated with Doxycycline 50 mg/kg twice a day for seven days before 4 hour anaesthetic with 4 hours of bilateral ischaemia and then reperfusion allowed for 24 hours. Bilateral ischaemia + High Dose Doxycycline, 24 hours reperfusion refers to animals treated with 200 mg/kg twice a day for seven days before 4 hour anaesthetic with 4 hours of bilateral ischaemia and then reperfusion allowed for 24 hours.

	Group (median)				
	Sham-operated	**Bilateral**	**Low Dose Doxycycline**	**High Dose Doxycycline**	**P-value**
Left leg Skeletal Muscle	0	6.5	6	7	0.0044
Lung	2	3	3	3	0.1669
Kidney	1	3	2	3	<0.0001

Table XX: Analysis of Histopathological Scores of Skeletal Muscle of Left leg.

Comparison groups		Difference between Means	Simultaneous 95% confidence Limits *** = P<0.05
Sham-operated	**Low Dose Doxycycline Treated**	6.000	0.361 - 11.639 *** **P=0.0276**
Sham-operated	**High Dose Doxycycline Treated**	12.800	7.161 - 18.439 *** **P=0.0234**
Bilateral	**Low Dose Doxycycline Treated**	-3.750	-9.731 - 2.231
Bilateral	**High Dose Doxycycline Treated**	3.050	-2.931 - 9.031
Low Dose Doxycycline Treated	**High Dose Doxycycline Treated**	6.800	1.161 - 12.439 ***

Table XXI: Analysis of Histopathological Scores of Skeletal Muscle of Kidney

Comparison groups		Difference between Means	Simultaneous 95% confidence Limits *** = P<0.05
Sham-operated	**Low Dose Doxycycline Treated**	7.200	3.267 - 11.133 *** **P=0.0276**
Sham-operated	**High Dose Doxycycline Treated**	14.400	10.467 - 18.333 *** **P=0.0372**
Bilateral	**Low Dose Doxycycline Treated**	-9.000	-12.933 - -5.067 *** **P=0.0162**
Bilateral	**High Dose Doxycycline Treated**	-1.800	-5.733 - 2.133
Low Dose Doxycycline Treated	**High Dose Doxycycline Treated**	7.200	3.267 - 11.133 ***

5.3.2 Zymographic Analysis of Matrix Metalloproteinase Expression

The results of a representative gelatin zymogram for the skeletal muscle, lung and kidney are shown in Figure 45. This illustrates the marked reduction in gelatinolytic activity of MMP-2 and MMP-9 in *skeletal muscle* following pre-treatment of the rats with doxycycline before the actual 4 hours of bilateral ischaemia and 24 hours of reperfusion. The gelatinolytic activity of MMP-9 was virtually abolished and the levels of both proMMP-2 and the active MMP-2 were reduced. The lower dose of doxycycline (50 mg/kg twice daily) was sufficient to inhibit gelatinolytic activity, while increasing the dose to 200 mg/kg twice daily did not cause a further reduction in activity. In contrast, there was no significant change in MMP-2 and MMP-9 activity in *lung* tissue following administration of doxycycline (Figure 45B). The levels of gelatinolytic activity of proMMP-2 in the *kidneys* also did not change after doxycycline treatment (Figure 45C).

5.3.3 Effect of Doxycycline on Degradation of Type IV Collagen during Skeletal Muscle Ischaemia/Reperfusion Injury

Representative images of the results of the immunohistochemical staining for type IV collagen are shown Figure 46. These images show that the administration of doxycycline partially protected against degradation of Type IV collagen. The third row in Figure 46 illustrates immunofluorescence of representative tissue sections from animals pre-treated with low dose doxycycline compared to animals subjected to 4 hours of bilateral ischaemia and 24 hours of reperfusion without doxycycline (Row 2, Figure 46). The intensity of immunofluorescence in skeletal muscle, kidney and lung tissue from doxycycline treated animals was brighter than in animals without the doxycycline treatment, however, the intensity levels did not return to baseline levels as seen with the sham-operated rats (Row 1, Figure 46).

The complete data sets for quantitative analysis of type IV collagen levels in the left leg skeletal muscle, lung and kidney are shown in Appendix 7.7.8 Quantitative Immunohistochemistry with Doxycycline, Complete Data Sets In Table XLIX, Table L and Table LI on pages 277, 279 and 284 respectively. The summary of this data is shown in Appendices: 7.7.9 Quantitative Immunohistochemistry with Doxycycline, Summary Data Sets in Table LII, Table LIII and Table LIV.

The summary of the immunohistochemical values for brightness used for statistical analysis is shown in Table XXII.

Table XXII: Quantitation of Type IV Collagen Levels in Left leg Skeletal Muscle, Lung and Kidney, including rats pre-treated with doxycycline.

Sham-operated refers to animals that underwent an anaesthetic only and were sacrificed at 24 hours after the end of the anaesthetic. Bilaterally Ischaemic refers to animals that underwent 4 hours of bilateral lower limb ischaemia and 24 hours of reperfusion before euthanasia. Low Dose Doxycycline, 24 hours reperfusion refers to animals treated with Doxycycline 50 mg/kg twice a day for seven days before 4 hour anaesthetic with 4 hours of bilateral ischaemia and then reperfusion allowed for 24 hours. Bilateral ischaemia + High Dose Doxycycline, 24 hours reperfusion refers to animals treated with 200 mg/kg twice a day for seven days before 4 hour anaesthetic with 4 hours of bilateral ischaemia and then reperfusion allowed for 24 hours.

The mean is the arithmetic mean of the brightness levels for the five animals in each group. Thirty five images were captured for each tissue section.

	Left leg		Lung		Kidney	
	Mean	**Standard Deviation**	**Mean**	**Standard Deviation**	**Mean**	**Standard Deviation**
Reperfusion Time	**24 hours**		**24 hours**		**24 hours**	
Sham-operated	27348.2	5956.33	62504.4	13264.47	63037.1	8582.78
Bilaterally Ischaemic	17413.5	3582.31	10863.0	2637.28	47909.78	4623.62
Low Dose Doxycycline	30056.7	9243.98	16442.0	3261.91	56518.12	5841.34
High Dose Doxycycline	27784.7	3115.31	24857.9	2197.18	51691.56	5203.64

These results are shown graphically in Figure 47. As can be seen in the graph, the preservation of skeletal muscle type IV collagen in the left leg was significant when the animals were treated with low dose doxycycline. The higher dose of doxycycline did not confer any additional protection against collagen degradation. In both the lung and in the kidney, there was a trend towards preservation of type IV collagen with the doxycycline treatment but these changes were not statistically significant.

For the *left leg skeletal muscle*, there was a significant difference in the type IV collagen levels among the groups (P=0.0194), with bilateral 4 hours of ischaemia and 24 hours of reperfusion being significantly different from low dose doxycycline treated animals.

For the *lung*, the type IV collagen levels in the sham-operated animals were different to all other groups ($p<0.0001$), but there were no significant differences between the bilaterally ischaemic animals without doxycycline treatment and the doxycycline pre-treated groups.

For the *kidney*, the sham-operated animals were different to bilateral ischaemia and 24 hours of reperfusion without doxycycline animals ($p=0.0128$); there were no other significant changes seen.

Figure 45: Zymographic Analysis of effect of Doxycycline on MMP-2 and MMP-9

Rats in groups of five were given 50 mg/kg twice daily (low dose) or 200 mg/kg doxycycline twice daily (high dose) by oral gavage for seven days before being subjected to 4 hours of Bilateral ischaemia and 24 hours of reperfusion. Tissues were harvested and analysed by gelatin zymography.

Panel A: Left leg skeletal muscle

Panel B: Lung

Panel C: Kidney

The left side track is the molecular weight marker.

Track 1: 4 hours of unilateral left leg ischaemia and 24 hours of reperfusion.

Track 2: 4 hours of bilateral ischaemia and 24 hours of reperfusion

Track 3: 4 hours of bilateral ischaemia and 24 hours of reperfusion in rats treated with low dose doxycycline

Track 4: 4 hours of bilateral ischaemia and 24 hours of reperfusion in rats treated with high dose doxycycline

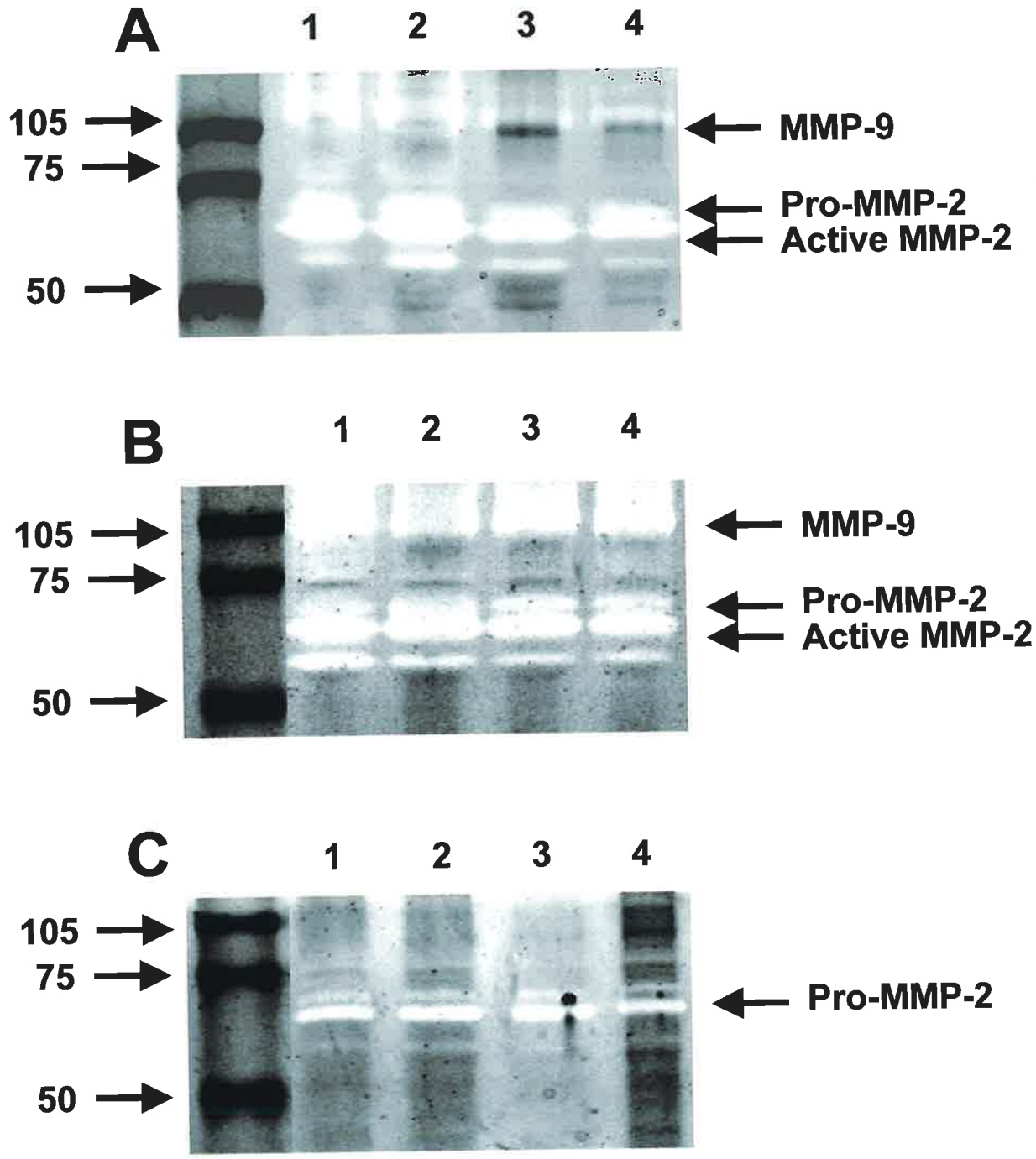
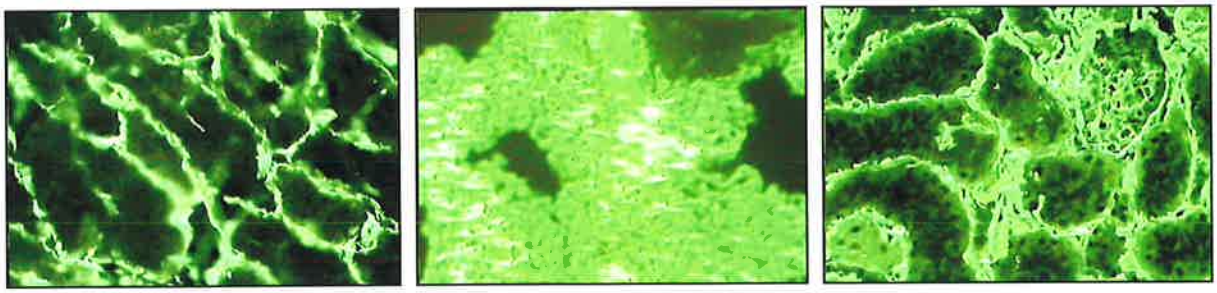

A
1
2
3
4
105
75
50
MMP-9
Pro-MMP-2
Active MMP-2
B
1
2
3
4
105
75
50
MMP-9
Pro-MMP-2
Active MMP-2
C
1
2
3
4
105
75
50
Pro-MMP-2

Figure 46: Representative Images of Immunohistochemistry of Left Leg Skeletal Muscle, Lung and Kidney showing levels of Type IV Collagen, including animals treated with Doxycycline.

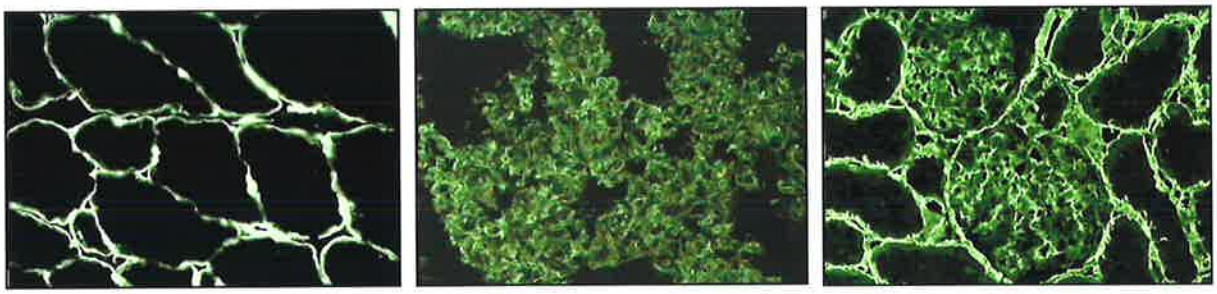
Magnification 20 X, Primary antibody is directed against Type IV Collagen, followed by a secondary detection with Biotin labelled anti-IgG and Streptavidin-Fluorescein Isothiocyanate. Images were collected on Olympus BH2 microscope with Reflected Light Fluorescent Attachment (Olympus BHRFL-W) with maximum excitation wavelength of 494 nm and maximum emission wavelength of 518 nm. The images were captured on Panasonic Camera Control Unit (WVCU204) and Panasonic Camera (WVCL700).

Shams, no Drug, 24 hours refers to animals that underwent a 4 hour anaesthetic and were sacrificed at 24 hours, with no doxycycline treatment. Bilateral, no drug, 24 hours animals underwent 4 hours of bilateral ischaemia, followed by 24 hours of reperfusion before euthanasia. Bilateral ischaemia + Low Dose Doxycycline, 24 hours refers to animals treated with Doxycycline 50 mg/kg twice a day for seven days before 4 hour anaesthetic with 4 hours of bilateral ischaemia and then reperfusion allowed for 24 hours. Bilateral ischaemia + High Dose Doxycycline, 24 hours refers to animals treated with 200 mg/kg twice a day for seven days before 4 hour anaesthetic with 4 hours of bilateral ischaemia and then reperfusion allowed for 24 hours.

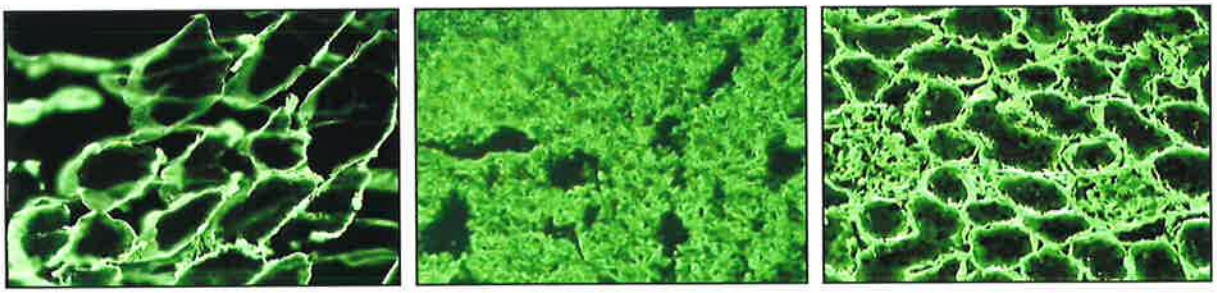
Left leg Lung Kidney



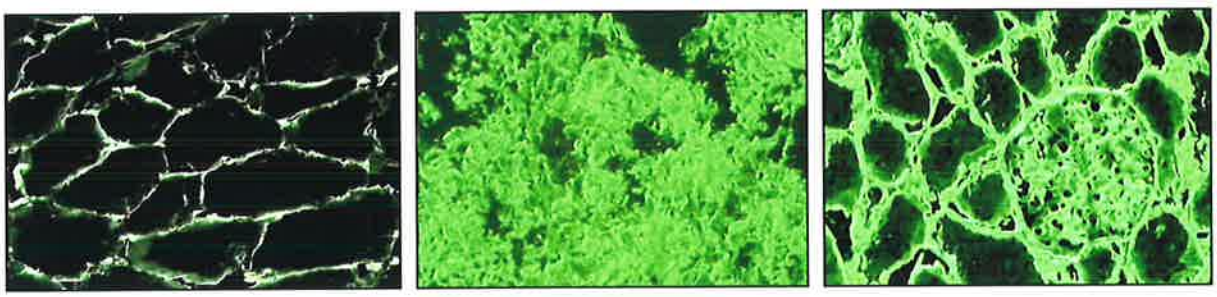
Sham, no drug, 24 hours



Bilateral ischaemia, no drug, 24 hours



Bilateral ischaemia + Low Dose Doxycycline, 24 hours



Bilateral ischaemia + High Dose Doxycycline, 24 hours

Figure 47: Mean Brightness levels for rats that underwent Sham-operations or Bilateral lower limb ischaemia, without or with Doxycycline treatment.

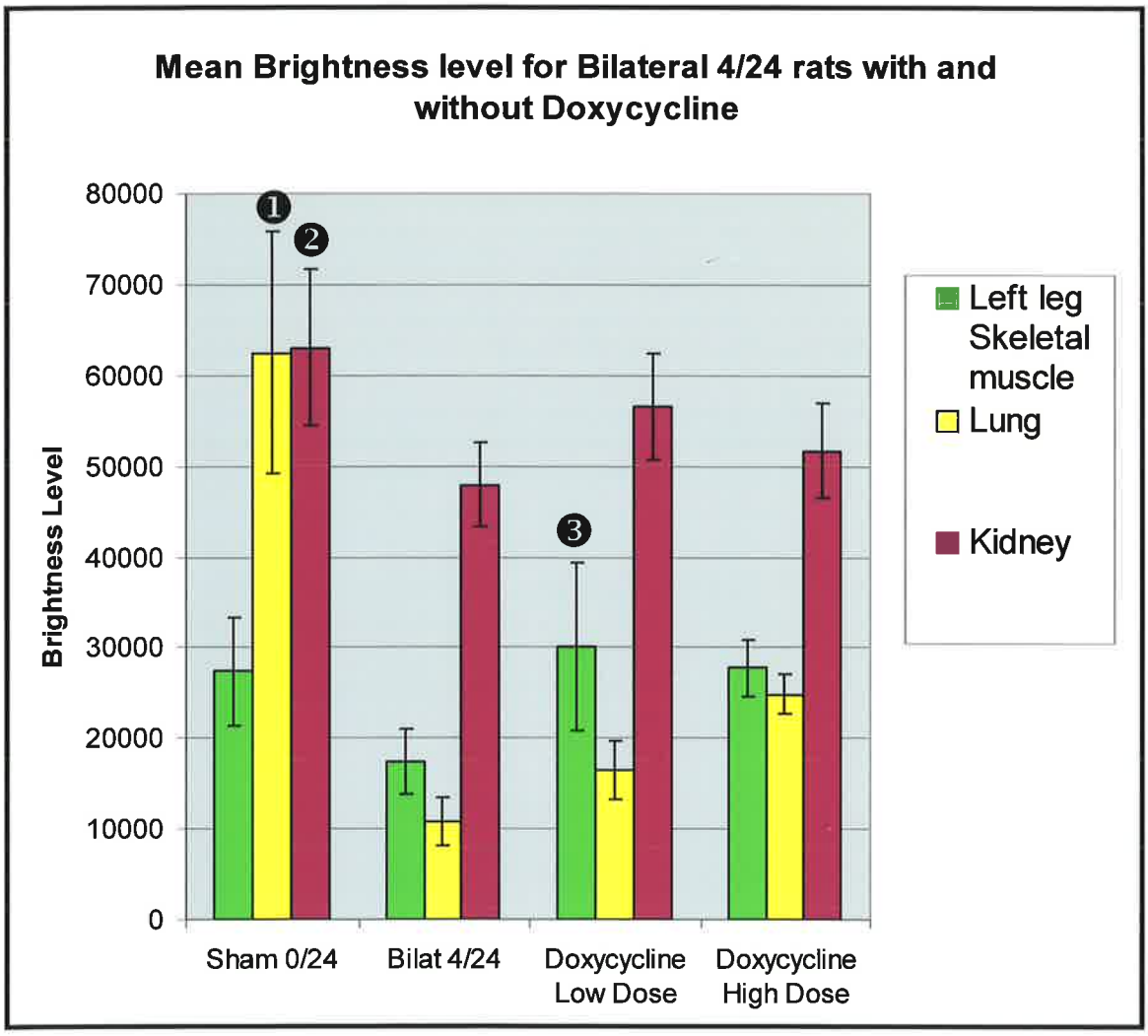
Sham 0/24 refers to animals that underwent four hours anaesthesia and killed after 24 hours. Bilat 4/24 rats underwent 4 hours of bilateral lower limb ischaemia, followed by 24 hours of reperfusion. Low dose treated rats were given 50 mg/kg twice-daily doxycycline by oral gavage for seven days before 4 hours of bilateral ischaemia and 24 hours of reperfusion. High dose rats were treated the same as the low dose except they were given 200mg/kg twice daily doxycycline.

Each column on the graph represents the mean of the type IV collagen brightness levels for the five rats in that group with 35 images per slide; giving an arithmetic mean involving 175 images. The standard deviation bars are shown.

❶LUNG: Sham – operated (sham 0/24) animals were significantly different from all other groups, $P<0.0001$ Sham-operated versus Bilateral 4/24, $P<0.0001$ for Sham-operated versus Low Dose Doxycycline, $P<0.0001$ for Sham-operated Versus High Dose Doxycycline.

❷KIDNEY: Sham-operated animals were significantly different to bilateral 4/24 $P=0.0128$

❸LEFT LEG SKELETAL MUSCLE: Significant difference between Bilat 4/24 without doxycycline and Low Dose Doxycycline, $P=0.0194$.


Mean Brightness level for Bilateral 4/24 rats with and without Doxycycline
Brightness Level
80000
70000
60000
50000
40000
30000
20000
10000
0
1
2
3
Sham 0/24
Bilat 4/24
Doxycycline Low Dose
Doxycycline High Dose
Left leg Skeletal muscle
Lung
Kidney

5.4 Discussion

5.4.1 Histopathological Assessment of Tissue damage

In agreement with the findings in Chapter 2, there was a significant difference in the levels of histological damage occurring between the sham-operated animals and all of the ischaemia treated animals. There was no detectable difference histologically in *skeletal muscle* and *lung* harvested from rats undergoing 4 hours of bilateral ischaemia and 24 hours of reperfusion and the rats undergoing 4 hours of ischaemia and 24 hours of reperfusion and treated with low or high dose doxycycline. This raises two possibilities; either the doxycycline had no effect or has an effect that was not able to be detected using Carter's scoring system of histopathological damage in skeletal muscle and lung. These theories were further investigated with the zymographic and immunohistopathological studies. However, in the *kidney* there was a significant difference between the bilaterally ischaemic animals without doxycycline and those treated with low dose doxycycline. There was less histological damage occurring in the doxycycline treated rats (P=0.0162).

5.4.2 Zymographic Analysis of Matrix Metalloproteinase Expression

There was a qualitatively significant decrease in the gelatinolytic activity in MMP-2 and MMP-9 in the skeletal muscle of animals that were treated with low dose doxycycline, with no further reduction inactivity with the high dose drug. There gelatinolytic activity did not detectably alter in the lung and kidney tissues with treatment with doxycycline.

5.4.3 Effect of Doxycycline on Degradation of Type IV Collagen during Skeletal Muscle Ischaemia/Reperfusion Injury

There was significant preservation of type IV collagen in the *left leg skeletal muscle* after low dose doxycycline treatment, compared to the animals that underwent bilateral ischaemia with no doxycycline treatment, without further preservation in the high dose doxycycline treated groups. These findings correlate with the zymographic results of changes in MMP-2 and MMP-9, which showed a decrease in gelatinolytic activity following doxycycline treatment. However, these changes did not alter the degree of gross histopathological damage.

The relationship between MMPs and skeletal muscle ischaemia/reperfusion has not previously been reported and hence there are no comparative studies of MMP inhibitors in this setting. However, in the myocardium, Lu et al[550] showed that the MMP-9 activity increased with ischaemia/reperfusion injury. Although they used a MMP inhibitor (GM-2487), they did not measure the levels of MMP-9 after the use of the inhibitor and found no functional change in contractile function of the myocardium, indicating that the use of the inhibitor had no demonstrable clinical effect. They found no change in collagen ultrastructure when assessed by cell maceration scanning microscopy[550]. Cheung et al[475] showed increased MMP-2 in myocardial reperfusion after global no-flow ischaemia. Like the present studies, the MMP-2 gelatinolytic activity was markedly decreased with doxycycline treatment in their in vitro experiment[475]. Hence, in the current study, MMP-2 and MMP-9 appear to be implicated in causing damage in the skeletal muscle in skeletal muscle reperfusion injury, with protection conferred by doxycycline treatment.

In the *lung*, degradation of type IV collagen was demonstrated following bilateral ischaemia/reperfusion with partial protection afforded by the treatment with doxycycline. There were no gross morphological differences seen in the histopathology between bilateral ischaemia/reperfusion without doxycycline and animals with doxycycline treatment. There was also no reduction in the gelatinolytic activity of MMP-2 and MMP-9 with the doxycycline treatment. There have been no reported studies on the role of MMPs in the lung as a remote organ in ischaemia/reperfusion injury. However, others have shown upregulation of MMP-2[551], MMP-9[470,471,551,678] and MMP–13[551] in various models of lung injury. Treatment with MMP inhibitors have been shown to attenuate lung injury due to mechanical ventilation[679] and in acute respiratory distress syndrome[680] with reduction in MMP-2 and MMP-9 and lung wet/dry weight ratios. Carney et al showed that the lung injury seen after cardiopulmonary bypass was reversed clinically with chemically modified tetracycline[681]. Hence, it appears that MMP inhibitors confer protection in decreasing lung tissue damage after injury. The increase in type IV collagen seen in the lung immunohistochemistry studies in the doxycycline treated groups, shows that doxycycline has a protective effect against lung injury, although it may be due to inhibition of MMPs other than MMP-2 and MMP-9.

In the *kidney,* considerable degradation of type IV collagen was demonstrated in the bilaterally ischaemic group without doxycycline, that was partially reduced by pretreatment with doxycycline. No detectable difference on histopathological morphology was found in these animals. There was no correlational decrease in activity of MMP-2 and MMP-9 seen on

the zymograms. These findings are in agreement with studies by Jain et al[477] who showed that in a rat model of renal ischaemia/reperfusion; MMP-2 levels were only elevated very late (8 weeks after 45 minutes of renal ischaemia) in the reperfusion phase. It therefore appears likely that early renal collagen loss observed in the current study is mediated by proteases other than MMP-2 and MMP-9. Only very low levels of leukocyte infiltration were observed in kidneys of rats subjected to ischaemia/reperfusion, correlating with the absence of MMP-9 in these tissues.

The mechanism of action of doxycycline in the skeletal muscle to decrease the collagen breakdown is unclear, however a direct inhibitory action on matrix metalloproteinase activity is likely given that doxycycline was effective within 24 hours of the ischaemia. The other possibility is that the gene expression of MMP-2 and MMP-9 was altered in the seven days before the ischaemic period. This could be investigated further by measuring the RNA levels of MMPs in serum and tissue. Another suggested mechanism of action of tetracyclines is that polymorphonuclear cell take up tetracyclines and concentrate them intracellularly, modifying the function of the polymorphonuclear cell[670,682]. The interaction of the tetracycline with MMP-9 intracellularly in polymorphonuclear cells could explain the reduction in MMP-9 in the skeletal muscle. The possible mechanism in this model is therefore that the orally administered doxycycline is concentrated within polymorphonuclear cells and reacts/bind with proMMPs. During degranulation, the proMMPs are released from the polymorphonuclear cells into the extracellular matrix, where doxycycline blocks the activation of proMMPs[683] and inhibits already active polymorphonuclear MMPs[684-686].

In summary, the administration of an MMP inhibitor, doxycycline, led to reduction in the level of upregulation of MMP-2 and -9 and protection from degradation of type IV collagen in skeletal muscle after ischaemia/reperfusion injury. In the lung and kidney, there was partial protection from type IV degradation but minimal changes in the levels of MMP-2 and MMP-9 suggesting that other proteases may be inhibited by the action of doxycycline.

CHAPTER 6:

SUMMARY,

FUTURE DIRECTIONS

AND

CONCLUSIONS

6.1 Summary of Chapters

Acute limb ischaemia is a common phenomenon in medical practice. Reperfusion is the pathological event that occurs when the blood flow to the ischaemic tissue is restored. The effects of reperfusion occur both in the primarily ischaemic tissue and in the remote organs. The morbidity and mortality of reperfusion injury is high, leading to local tissue oedema and necrosis, as well as remote effects of cardiac complications, renal failure and pulmonary failure. Multiple studies have helped to delineate the multifactorial nature of ischaemia/reperfusion injury. Pathologically, there is increased tissue permeability secondary to destruction of the basement membrane and infiltration of neutrophils into the ischaemic tissue and the remote organs. The known factors involved in this process include release of oxygen free radicals, activation of neutrophils leading to production of a variety of cytokines, activation of the complement pathway and production of nitric oxide, prostaglandins, thromboxanes and leukotrienes. No-reflow involving failure of capillary perfusion, occurs due to leukocyte plugging, swelling of the endothelial cells and development of tissue oedema. There have been a great many inhibitors of reperfusion injury investigated in the experimental setting, however apart from supportive measures, there are no therapies for ischaemia/reperfusion injury that are in routine clinical use.

Matrix metalloproteinases (MMPs) are a family of zinc dependent enzymes that have the ability to degrade the extracellular matrix in common. There are over 20 MMPs known at the present time and these are divided into groups dependent upon their structure and function. These studies concentrated on the gelatinase pair of MMPs: MMP-2 and MMP-9. MMP-2 is constitutive in many cells but is upregulated in variety of processes. MMP-9 is mainly secreted by neutrophils, macrophages and monocytes. MMP-2 and MMP-9 have the ability to degrade type IV and V collagen, gelatin, elastin, fibronectin; amongst other extracellular matrix components. Type IV collagen is the predominant structural component of the basement membrane of tissues. The control and activation mechanisms of both MMP-2 and MMP-9 are both extremely complex as discussed in Chapter 1.

Matrix metalloproteinases have not previously been studied in skeletal muscle ischaemia/reperfusion injury. Some studies in ischaemia/reperfusion in brain cardiac and pulmonary ischaemia/reperfusion injury have shown involvement of matrix metalloproteinases in the tissue damage.

The aims of these studies were to explore the role of MMP-2 and MMP-9 in skeletal muscle ischaemia/reperfusion injury. Type IV collagen degradation during skeletal muscle ischaemia/reperfusion injury was used to quantitate the level of damage that occurred. The correlations between changes in MMP levels and type IV collagen levels were explored.

The role of doxycycline, as a MMP inhibitor was investigated in skeletal muscle ischaemia/reperfusion injury.

Chapter 2

The aims of the second chapter were to establish the animal model and validate its use in the study of skeletal muscle ischaemia/reperfusion injury. Three methods of investigation were used to establish and validate the animal model. The animal model itself was explained and its rationale discussed. The animal model utilized rats, which underwent either sham operations, unilateral or bilateral lower limb ischaemia for 4 hours. The rat was then either killed immediately in order to study the effects of the anaesthetic and ischaemia only; or reperfusion was allowed to occur for 4, 24 or 72 hours. The level of lung oedema was quantified to demonstrate whether there was an element of pulmonary oedema caused by the reperfusion in this animal model. These results were not significant due to small sample size and insensitivity of the technique. Thirdly, a histopathological analysis of the quantitative and qualitative histopathological changes that occur in skeletal muscle ischaemia/reperfusion injury was performed. This showed that there was significant destructive damage seen in the skeletal muscle and in the kidney. The changes seen in the lung were not significant, primarily due to the complicating effects of an inhalational anaesthetic.

Skeletal muscle, lung and kidney were harvested to study the effects of reperfusion in the muscle and in remote organs. There is a body of literature discussing ischaemia/reperfusion injury in the skeletal muscle and lung and the remote effects. The effects on the liver, kidneys and myocardium after skeletal muscle ischaemia/reperfusion have been less widely studied. Hepatic tissue shows a high ischaemic tolerance due to high capacity of antioxidative mechanisms of liver tissue and the ability of a higher oxygen extraction ratio under nearly ischaemic conditions[532] and thus was not studied.

Chapter 3

The third chapter in these studies investigated the activity of matrix metalloproteinases in skeletal muscle ischaemia/reperfusion injury. The methods of zymography and western blot analysis were used to demonstrate matrix metalloproteinase 2 and-9. These studies showed an upregulation of MMP-2 and MMP-9 in the ischaemic skeletal muscle when compared with sham-operated animals. The maximal levels of MMP-2 and MMP-9 were seen after 24 hours of reperfusion. There was no discernible difference between the levels of MMP-2 and MMP-9 in skeletal muscle between the unilaterally ischaemic and the bilaterally ischaemic animals. The lung tissue showed moderate upregulation of MMP-2 and MMP-9 in all tissues and these effects were maximal immediately after the anaesthetic and for 4 hours of reperfusion. At 24 and 72 hours there was a decline in the levels of MMP-2 and MMP-9 in the lung tissue. The findings in the skeletal muscle and lung were confirmed with anti-MMP-9 antibody by western blot analysis. In the kidney, there were low levels of constitutive proMMP-2 seen by gelatinolytic activity in the zymogram, which did not increase either in the sham-operated or the ischaemic animals. These findings are consistent with others[476,477], showing that MMP-2 levels do not increase at an early stage in the kidney despite signs of damage occurring by rising creatinine levels[552].

Chapter 4

The fourth chapter described the immunohistochemical techniques that were used to identify and quantify the level of type IV collagen in tissues. As type IV collagen is one of the predominant substrates of MMP-2 and MMP-9, it was chosen to quantitate the level of damage to tissues following skeletal muscle ischaemia/reperfusion injury. Prior to performing the definitive quantitation of alterations in type IV collagen a series of other studies were performed to establish and validate the technique of type IV collagen quantification using fluorescein isothiocyanate immunohistochemistry.

There were minimal changes at the end of the anaesthetic in the changes to the type IV collagen levels in the *left leg skeletal muscle* between sham-operated, unilaterally ischaemic and bilaterally ischaemic animals. Similarly, there were minimal histological changes in these animals. The type IV collagen levels in the left leg skeletal muscle had deteriorated significantly after 4 hours of reperfusion, when bilaterally ischaemic animals were compared with sham-operated animals. This correlates with the same duration of reperfusion at which the gelatinolytic levels of MMP-2 and MMP-9 increased on zymography. After 24 and 72

hours of reperfusion, there were continued decreases in the level of type IV collagen in the left leg skeletal muscle of the unilateral and bilaterally ischaemic animals, again correlating with the marked upregulation of MMP-2 and MMP-9 on zymography. The histopathological changes showed significant tissue damage after 24 hours of reperfusion when comparing bilaterally ischaemic animals to sham-operate animals.

In the *skeletal muscle of the right leg*, there were minimal differences between the levels of type IV collagen levels between sham-operated, unilaterally ischaemic and bilaterally ischaemic animals at the end of the anaesthetic. At 4 hours after the anaesthetic, there was a significant fall in the type IV collagen level when comparing bilaterally ischaemic animals (where the right leg was ischaemic) to sham-operated animals. After 24 hours following the anaesthetic, there were no changes in the type IV collagen levels between the sham-operated animals and the unilaterally ischaemic animals (where the right leg was the non-ischaemic, contralateral limb). This shows that the right leg is resistant to changes in type IV collagen when the right leg is actually a remote organ. However, 24 hours after the anaesthetic, the type IV collagen level had decreased when comparing bilaterally ischaemic (where the right leg is ischaemic) animals to sham-operated animals. These findings correlated with the increase in gelatinolytic activity of MMP-2 and MMP-9 seen on zymography and the degree of the qualitative histopathological damage. The right leg skeletal muscle results following bilateral limb ischaemia corroborate the findings in the left leg skeletal muscle following ischaemia. The breakdown in type IV collagen leads to destruction of the basement membrane that occurs in ischaemia/reperfusion injury, leading to leakage of macromolecules into the tissues and oedema.

The *lung* showed a decrease in type IV collagen levels when comparing the animal groups at all time points following the inhalational anaesthetic. Immediately after completion of the anaesthetic there was a significant deterioration in the level of type IV collagen in the bilaterally ischaemic animals, showing that damage to the lung had occurred. There were increases in gelatinolytic activity in both MMP-2 and MMP-9 in the sham-operated, unilaterally ischaemic and bilaterally ischaemic animals at this point. An interesting observation is the elevation in the MMP-2 and MMP-9 following the anaesthetic only, in the sham-operated animals. A comparison of breakdown of type IV collagen was not performed between the sham-operated animals and animals killed without an anaesthetic and hence it is not possible to correlate these two findings. As the duration of reperfusion increased, the breakdown in type IV collagen in the lung continued, with significant deterioration in type IV

collagen levels in the unilaterally ischaemic animals compared to sham-operated animals both at 24 and 72 hours of reperfusion. The bilaterally ischaemic animals also showed a significant deterioration in levels of type IV collagen in the lungs after 72 hours of reperfusion. This was in contrast to the changes in MMP-2 and MMP-9, which remained high immediately after the anaesthetic and at 4 hours of reperfusion but decreased in level after 24 and 72 hours of reperfusion (see Chapter 3, Figure 16A). As the MMP expression was increased initially, the matrix metalloproteinases continued to degrade basement membrane even after the levels of MMP seen on zymography dropped off. Histopathologically, there were no detectable differences in the level of damage in the lung throughout the experimental groups. Nonetheless, the deterioration in type IV collagen levels is likely to be clinically significant and contributing to the level of increased lung permeability that occurs following skeletal muscle ischaemia/reperfusion injury.

MMP-2, MMP-9 and MMP-13 are known to increase following lung damage[551]. In the current studies, the levels of MMP-2 and MMP-9 were maximal immediately following the anaesthetic, implying that the anaesthetic itself caused elevation of these enzymes. Following halothane anaesthesia, lung histology reveals patchy atelectasis, dystelectasis and interstitial oedema[521], all of which could be pathological features resulting from upregulation of matrix degrading enzymes MMP-2 and MMP-9. However, the changes in type IV collagen occurred later. There are two possible explanations for these events; firstly, it could be a true delayed effect from the MMP-2 and MMP-9 breaking down the lung tissues. This theory could be further investigated by studying the levels of MMP-2 and MMP-9 in bronchoalveolar lavage and in venous effluent from the lung during the delayed periods of 24 and 72 hours of reperfusion. The second possible explanation is that another enzyme or agent is breaking down the type IV collagen levels during the period of 24 and 72 hours of reperfusion. As discussed in Chapters 1 and 4, there are many other known agents that can degrade type IV collagen, including elastase[619,620], cathepsins B, D and G[621,622], trypsin[623,624], pepsin[625,626], plasmin[627,628] and MMP-7/PUMP-1[350]. None of these other enzymes were studied in these experiments, and obviously would be an important avenue to further explore the reasons for the type IV collagen destruction in lung tissue.

In the *kidneys*, there were minimal changes in type IV collagen levels between sham-operated animals, unilaterally ischaemic animals and bilaterally ischaemic animals at the end of the anaesthetic. However, after 4 hours, there was a significant decrease in the type IV collagen level in the bilaterally ischaemic groups of animals compared to sham-operated animals. This

destruction continued with significant degradation of renal type IV collagen after 24 hours in both unilaterally and bilaterally ischaemic animals, compared to sham-operated animals. Again at 72 hours, significant destruction of type IV collagen was seen in the bilaterally ischaemic animals compared to the sham-operated and unilaterally ischaemic animals. In the histopathological studies, there was a significant difference between the degree of damage in sham-operated and bilaterally ischaemic animals after 24 hours of reperfusion (P=0.0276). The findings of both the degradation of type IV collagen and histopathological changes correlate well, showing marked damage was occurring to the kidney when subjected to skeletal muscle ischaemia/reperfusion injury. However, The MMP-2 was expressed at constitutive low levels in all tissues and did not change with reperfusion. There was no expression of MMP-9 detected in the kidney. Forbes et al showed destruction in glomerular type IV collagen following rat renal ischaemia/reperfusion injury from 2 to 16 days following ischaemia, but marked MMP-2 was not detected until day 8[476]. Ziswiler et al showed that following renal ischaemia/reperfusion injury in a rat, there was evidence of damage to the kidneys with an increase in serum creatinine but no elevation of MMP-2 or MMP-9. It appears that MMP-2 and MMP-9 do not play a role in the remote organ renal damage following skeletal muscle ischaemia/reperfusion injury. The kidney appears to behave differently to other organs like skeletal muscle and lung, during ischaemia/reperfusion injury. While neutrophils are recognized to play a significant role in ischaemia/reperfusion in many different extra-renal organs, their role in the kidney is still debated or even negated[687-689]. If white cells do not have major role in renal damage in this setting, this may explain the lack of upregulation in MMP-9.

Chapter 5

As an element of the current studies, doxycycline, a broad spectrum MMP inhibitor, was administered to rats in two different doses, prior to an experiment of bilateral limb ischaemia for 4 hours followed by 24 hours of reperfusion. The studies of histopathology, zymography, and type IV collagen immunohistochemistry were all repeated.

In the *left leg skeletal muscle,* administration of doxycycline led to a decrease in the expression of gelatinolytic activity of MMP-2 and MMP-9, which was associated with significant preservation of type IV collagen after doxycycline treatment.

In the *lung,* there was partial protection from damage with less type IV collagen degradation seen after pretreatment with doxycycline. There was no change in the gelatinolytic activity of MMP-2 or MMP-9 on zymography. This correlates with the above findings in the initial studies, that MMP-2 and MMP-9 are involved in the remote effects of lung injury following ischaemia/reperfusion, but there are probably other enzymes involved in the destruction of the basement membrane type IV collagen.

Administration of doxycycline protected *renal* tissue from histopathological damage and also partial protection from destruction of type IV collagen. Again, there were no correlational changes in MMPs seen on zymography. This implies that the damage that occurred in the kidney during reperfusion injury may be due to MMPs other than MMP-2 and MMP-9. MMP-7, which is produced mainly from epithelial cells, MMP-12 from macrophages and MMP-19 from lymphocytes are all known to degrade type IV collagen, and this could form the basis of further investigation in this area.

The use of this broad-spectrum MMP inhibitor showed protection from pathological damage in the skeletal muscle and partial protection in the remote organs of lung and kidney. Tetracyclines are known to be fairly weak inhibitors of MMP activity in vitro[674]. Nonetheless, tetracyclines are frequently as effective as other MMP inhibitors in vivo and their beneficial influence on connective tissue destruction can often be achieved at remarkably low dose schedules[690,691]. Further investigation into the possible role of involvement of other MMPs in reperfusion injury is required. This would then allow experimentation with more specific MMP inhibitors. Other synthetic MMP inhibitors that could be utilized include the hydroxamate drugs[452,692] and HMG CoA reductase inhibitors[457,458,464].

6.2 Future Directions

The molecular and cellular mechanisms, which activate MMP-2 and MMP-9 during ischaemia/reperfusion, remain to be elucidated. As discussed in Chapter 1, the known control mechanisms for both MMP-2 and MMP-9 are complicated, involving the TIMPs, MT1-MMP, MMP-3 and a variety of other molecules. In ischaemia/reperfusion, elevated levels of MMP-9 are presumed to derive from the degranulation of infiltrating neutrophils, which are a major feature of reperfusion injury, both in the skeletal muscle and in remote organs including the lung[508]. The mechanism inducing the elevated levels of MMP-2 and the nature of the cells

which synthesize and secrete MMP-2 are not yet clear. It has been reported that human skeletal muscle satellite cells in culture secrete MMP-2 and can be induced to express MMP-9 by treatment with phorbol ester[693]. It is known that the MMP-2 promoter contains a p53 consensus binding site and expression of p53 will cause transcriptional activation of MMP-2[694]. Elevated levels of p53 can be detected during ischaemia/reperfusion[695], suggesting a possible mechanism, which would result in the induction of elevated expression of MMP-2 in these tissues.

Other matrix metalloproteinases and their inhibitors, TIMPs, are also likely to participate in the cascade of events, which lead to the induction of tissue damage during ischaemia/reperfusion injury. The proteolytic activity of MMP-3 has been implicated in the breakdown of basement membrane proteins including laminin and fibronectin as well as displaying some proteolytic activity against type IV collagen[618]. MMP-3 is also one of the primary activators of MMP-9, making it highly likely to play a role in tissue damage in ischaemia/reperfusion injury. Further investigations are required into delineating the pathways leading to proteolytic damage of tissues during ischaemia/reperfusion injury.

There are some difficulties extrapolating the current studies to the human context. For identical periods of ischaemia, rat skeletal muscle exhibits a more rapid and severe metabolic deterioration and a slower recovery from tourniquet ischaemia than has been observed for human or canine muscle[511,696]. Although newer techniques for assessment of muscle metabolism such as ^{31}P nuclear magnetic resonance will make smaller muscles entirely suitable for metabolic studies[696], other methodologic difficulties with rat preparations remain. If MMP inhibitors that are safe in humans are investigated, such as HMG CoA reductase inhibitors, then these agents can be put to trial in humans, negating the difficulties of the rat model of ischaemia/reperfusion injury.

6.3 Conclusions

In an animal model of ischaemia/reperfusion injury, there was an increase in MMP-2 and MMP-9 expression in ischaemic muscle, which increased with increasing duration of perfusion. This correlated with marked decreases in the level of type IV collagen and histopathological evidence of significant tissue damage. The right leg skeletal muscle showed minimal changes in MMP levels, type IV collagen or histopathologically compared to sham-operated animals when it was the contralateral limb to the side of the ischaemia, showing that

the skeletal muscle is resistant to damage when it is a remote organ. When the right leg was ischaemic in the bilaterally ischaemic model, there were the same rises in MMP levels, falls in type IV collagen levels and qualitative histopathological damage as seen in the left leg skeletal muscle.

In the lung tissue following skeletal muscle ischaemia, there was marked destruction of type IV collagen. The levels of MMP-2 and MMP-9 were elevated immediately following the anaesthetic and after 4 hours of reperfusion, and then declined in levels after 24 and 72 hours of reperfusion.

In the kidney, there was marked destruction of type IV collagen over the 72 hours of reperfusion, associated with histopathological damage. However, there was no elevation in MMP-2 or MMP-9 during the study period, suggesting that these MMPs do not have a role in renal dysfunction following skeletal muscle ischaemia/reperfusion.

This is the first study showing elevation of MMP-2 and MMP-9 in skeletal muscle ischaemia/reperfusion injury and in the remote organ of the lung. These studies also showed for the first time parallel decreases in type IV collagen in ischaemic skeletal muscle and the remote effects in the lung and kidney. The MMP inhibitor, doxycycline showed a promising effects as a potential treatment of this condition, showing protection from pathological damage in the skeletal muscle, lung and kidney tissues.

As the ultimate aim in all studies in ischaemia/reperfusion is to decrease the morbidity and mortality of this important condition, further work is required to continue the delineation of the role of matrix metalloproteinases and their inhibitors in this pathological process.

CHAPTER 7

APPENDICES

7.1 Abbreviations

AAA	abdominal aortic aneurysm
ADP	adenosine triphosphate
AMP	adenosine monophosphate
APMA	4-aminophenylmercuric acetate
APS	ammonium persulfate
ARDS	adult respiratory distress syndrome
ATP	adenosine triphosphate
avg	average
bd	bis in die = twice a day
BSA	Bovine Serum Albumin
C	celsius
$CaCl_2$	calcium chloride dihydrate
cm	centimetres
CD	cluster determinant
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ELAM	endothelial–leukocyte adhesion molecule
GPI	glycosylphosphatidylinositol
HMG CoA	hydroxymethylglutaryl coenzyme A
HSP	heat shock protein
H_2O_2	hydrogen peroxide
5-HPETE	5-hydroperoxyeicosatetranoic acid
ICAM	intercellular adhesion molecule
IL	interleukin
IPC	ischaemic preconditioning
kDa	kilodaltons
LT	leukotriene
M	molar
ml	millilitre
mM	millimolar
mm	millimetres
MMPs	matrix metalloproteinases

MQ H_2O	Milli Q Plus H_2O
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAOH	sodium hydroxide
NK	natural killer
nm	nanometres
PAF	platelet activating factor
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PECAM	platelet-endothelial cell adhesion molecule
PG	prostaglandin
PMN	polymorphonuclear cells
PMSF	phenylmethanesulfonyl fluoride α-toluenesulfonyl fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
St dev	standard deviation
TEMED	N, N, N', N' - tetramethylethylenediamine
TIMPs	Tissue inhibitors of metalloproteinases
TNF	tumour necrosis factor
Tris base	tris [hydroxymethyl] aminomethane
Tris HCL	tris [hydroxymethyl] aminomethane hydroxychloride
µl	microlitre
µm	micrometre
uPA	u-plasminogen activator
VCAM	vascular cell adhesion molecule
W/v	weight per volume
%	percent

7.2 Chemicals and Reagents

Sigma Chemical Co Ltd., St Louis MO USA, supplied the following chemicals and reagents:

Ammonium persulfate (APS)

Bovine Serum Albumin – Fraction V

Brij® 35 solution 30% w/v. Brij is a registered trademark of ICI Americas, Incorporated

Coomassie Brilliant Blue - R

Ethylenediamine tetraacetic acid (EDTA)

Gelatin (Type A from Porcine skin)

Glycine

MMP Control-1 (M2928)

Phenylmethanesulfonyl fluoride α - Toluenesulfonyl fluoride (PMSF)

Poly-L-lysine Solution

N, N, N', N' - tetramethylethylenediamine (TEMED)

Tris [hydroxymethyl] aminomethane (Tris base)

Tris [hydroxymethyl] aminomethane hydroxychloride (Tris HCL)

Sodium dodecyl sulfate (SDS)

Urea

Ajax Chemicals, NSW, Australia supplied the following chemicals and reagents:

Calcium chloride dihydrate ($CaCl_2$)

Glacial Acetic Acid

Methanol

Sodium chloride (NaCl)

Sodium hydroxide (NaOH)

Triton – X 100

Other chemicals and reagents were purchased from the following sources:

Acetone	AnalaR® MERCK ltd, Kilsyth, Vic, Australia
30% Acrylamide/Bis Solution 37.5:1 (2.6%) Acrylamid: N, N Methylenbisacrylamid	Bio-Rad Laboratories, Hercules, CA, USA
Biotinylated Anti-Rabbit IgG (H+L)	Vector Laboratories, Inc., Burlingame, CA USA
Bromophenol Blue	May + Baker Ltd, Pakenham, England

Affinity purified Anti-Collagen Type IV [Rabbit]	Rockland, Gilbertsville, PA, USA
Dako® Fluorescent Mounting Medium	Dako Corporation, CA, USA
ECL	Amersham Pharmacia, Biotech UK Limited, Buckinghamshire, England
Foetal Calf serum	Gibco BRL LIFE technologies
Glycerol	Merck Pty Ltd, Vic, Australia
Milli Q Plus H_2O (MQ H_2O)	Millipore, Australia
Nitrocellulose membrane	Hyabond ECL-Enhanced Chemi-luminescence Amersham
PAP pen	Zymed Laboratories, Inc. San Francisco, California
Skim milk powder	Bonlac Food Pty Ltd, Melbourne
Tissue Tek® OCT	Sakura Finetek, Torrance, CA, USA
Streptavidin – fluorescein (RPN 1232)	Amersham Pharmacia, Biotech UK Limited, Buckinghamshire, England
Tween 20	Bio-Rad Laboratories, Hercules, CA, USA
Xray film Hyperfilm,	Amersham Pharmacia, Biotech UK Limited, Buckinghamshire, England

7.3 Equipment

Coverslip	Menzel GLÄSER, Gerhard Menzel, Glasbearbeitungswek, GmbH & Co. KG
Cryostat	Microm HM 505N, Microm Laborgeräte GmbH, Walldorf
Doppler machine	Parks Medical Electronics Inc. Oregon, USA
Homogeniser	B.Braun. Melsungen AG
Rectal probe	Kane-May Ltd, Welwyn Garden City, Herts
Spectrometer	Varian DMS 200, UV Visible Spectrometer

7.4 Buffers and Solutions

7.4.1 Homogenising Buffer for Zymography

Urea	2M
Tris-HCl	50mM
NaCl	1g/L
EDTA	1g/L
Brij 35	0.1%
PMSF	0.1 mM

Made with MQ H_2O.

Brought to pH 7.6 with NaOH

Filtered with 0.2µm filter (Point 2 Disposable Filter Holder 0.2µm).

7.4.2 Dialysis Buffer for Zymography

Tris-HCl	25mM
$CaCl_2$	10mM

Made with MQ H_2O

Brought to pH 8.5 with NaOH

Then autoclaved at 120°C for 20 minutes

Brij 35	0.1%
PMSF	0.1mM

7.4.3 Resolving gel for Zymography

MQ H_2O	6ml
0.10% Gelatin	2ml
30%, 37.5:1 Acrylamide/Bis	6.7ml
1.5M Tris HCL	5ml
10% SDS	200µl
10% APS	100µl
TEMED	10µl

7.4.4 Stacking gel For Zymography and Western blots

MQ H_2O	6.1ml
30%, 37.5:1 Acrylamide/Bis	1.3ml
0.5M Tris HCL	1.25ml
10% SDS	100µl
10% APS	50µl
TEMED	10µl

7.4.5 Zymogram Loading Buffer

Tris HCL	62.5mM
SDS	1.4M
Glycerol	45%
Bromophenol Blue	0.025%

Make up to pH 6.8

7.4.6. Western Loading buffer

Tris HCL	62.5mM
SDS	1.4M
Glycerol	45%
Bromophenol Blue	0.025%
β-mercaptoethanol	2%

Make up to pH 6.8

7.4.7 Zymogram and Western Blot Tank/Running Buffer

Tris Base	25 mM
Glycine	200 mM
SDS (Laurel SO_4)	3.5mM

Mix with ~ 900ml H_2O and stir with magnetic stirrer.

Measure pH and make up to pH = 8.3

Make up to 1000ml.

7.4.8 Zymogram Development Buffer

Tris base	50mM
NaCl	200mM
$CaCl_2$	5mM
30% Brij	0.02%
Made with MQ H_2O	
Made up to pH 7.5	

7.4.9 Coomassie Blue Stain for Zymography

Methanol	40%
Glacial Acetic Acid	10%
Coomassie Blue–R	0.5%
MQ.H_2O	50%

7.4.10 Destain for Zymography

Methanol	40%
Glacial Acetic Acid	10%
MQ.H_2O	50%

7.4.11 Resolving Gel for Western Blot Analysis

Water	6ml
30%, 37.5:1 Acrylamide/Bis	6.7ml
Tris Base 1.5M (ph8.8)	50ml
SDS 10%	200µl
APS (0.1g/ml) 10%	200µl
TEMED	10µl

7.4.12 Western Transfer Buffer

Tris Base	6.06g
Glycine	28.8g
Methanol	400ml
Milli Q H_2O	to make total of 2Litres

Stored at 4° Celsius.

7.4.13 Non-fat Powdered Milk Solution

Skim Milk Powder	5g
Phosphate Buffered Saline	100ml

Prepared fresh before use.

7.4.14 Tris Buffered saline for Western Blots

Tris-HCl	2.42 g
NaCl	29.22g
Milli Q H_2O	1000ml

pH adjusted to 7.5 and autoclaved.

7.4.15 Western Antibody Buffer

Phosphate Buffered Saline	45ml
Foetal Calf Serum	5ml
Tween-20	25µl

7.4.16 Phosphate Buffered Saline for Immunohistochemistry

Sodium Chloride	8g/L
Potassium Chloride	0.2g/L
Potassium di-Hydrogen Orthophosphate	0.2g/L
Di-sodium Hydrogen Orthophosphate	1.15g/l

Add MQ H20, pH to 7.2-7.4 and then added:

Calcium chloride	0.1g/L
Magnesium Sulphate	0.059g/L

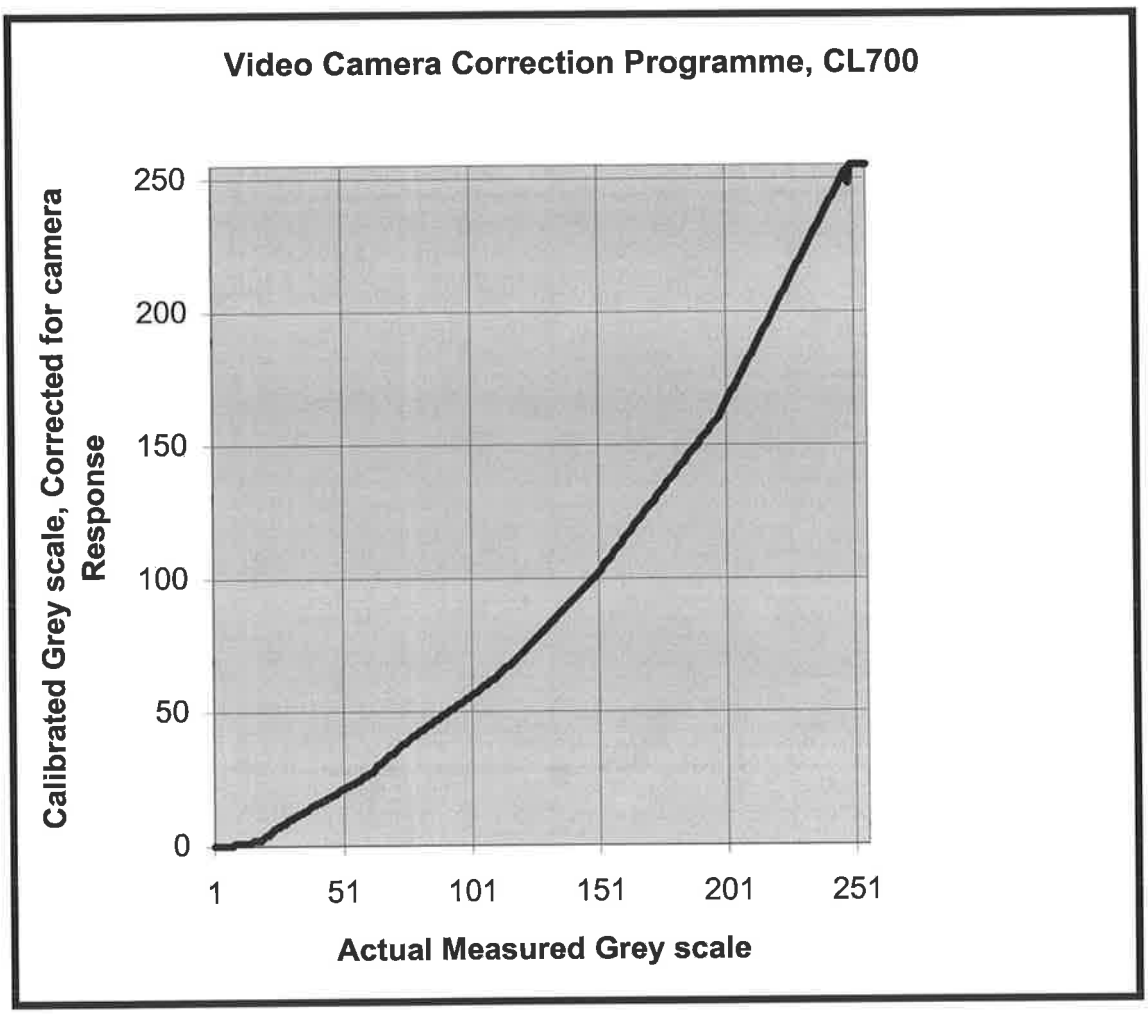
7.5 Immunohistochemical Methods

7.5.1 Slide preparation for Immunohistochemistry

Poly-L-Lysine Solution was diluted 1:10 with deionized water before coating slides. Clean slides were placed in diluted Poly-L-lysine solution for 5 minutes. Slides were drained and allowed to dry in 60 degrees oven overnight. They were stored until utilized.

7.5.2 Video Camera Correction Programme

The graph shows on X-axis, the actual measured grey value and on Y-axis the corrected grey value after calibration for the camera response using Programme CL700 in Video Pro 32® software.



7.6 Immunohistochemical Brightness results – Complete Data Sets

7.6.1 Pilot studies, Negative Controls and Histograms

Table XXIII: Left leg Skeletal Muscle Pilot Study

Slides were prepared as for standard method described in Chapter 4.2. Sham-operated 0/24 refers to rats with no ischaemia, 4 hour anaesthetic and sacrificed at 24 hours. Unilateral 4/24 refers to rats that underwent 4 hours of unilateral ischaemia, then 24 hours of reperfusion. Mean refers to the arithmetic mean of the brightness levels of the images.

	Sham-operated 0/24			**Unilateral 4/24**		
Rat Number	**19**	**33**	**34**	**16**	**30**	**32**
Brightness Level	31394.49	33293.89	47481.98	8921.63	9269.9	29111.5
	24057.25	41433.34	54416.79	29024.4	1700.85	18314.32
	23022.14	39171.28	30190.12	26590.31	789.75	37787.23
	27083.44	32595.96	35358.79	39130.93		33772.06
	23714.9	37183.41	60755.66	41053.48		44034.72
		35493.24	61217.18	2222.79		38128.53
		42169.55	51893.33	1653.03		
			34505.86			
Mean	**25854.44**	**37334.38**	**46977.46**	**21228.08**	**3920.167**	**33524.73**

Table XXIV: Left leg Skeletal Muscle Pilot Study - Negative control slides.

Slides were prepared as for standard method described in Chapter 4.2, with the primary antibody omitted. Sham-operated 0/24 refers to rats with no ischaemia, 4 hour anaesthetic and euthanased at 24 hours. Unilateral 4/24 refers to rats that underwent 4 hours of unilateral ischaemia, then 24 hours of reperfusion. Mean refers to the arithmetic mean of the 3 images.

Left leg Skeletal Muscle Pilot Study – Negative Control						
	Sham-operated 0/24			Unilateral 4/24		
Rat Number	**19**	**33**	**34**	**16**	**30**	**32**
Brightness	2.93	7.73	3473.34	17.44	28.34	5.06
	3.16	18.49	479.14	565.41	31.63	13.51
	2.94	7.2	145.31	107.14	63.07	51.97
Mean	**3.01**	**11.14**	**1365.93**	**229.9967**	**41.01333**	**23.51333**

Table XXV Lung Pilot Study

Slides were prepared as for standard method described in Chapter 4.2. Sham-operated 0/24 refers rats with no ischaemia, 4 hour anaesthetic and euthanased at 24 hours. Unilateral 4/24 refers to rats that underwent 4 hours of unilateral ischaemia, then 24 hours of reperfusion. Mean refers to the arithmetic mean of the brightness levels of the images.

Lung Pilot Study						
	Sham-operated 0/24			Unilateral 4/24		
Rat Number	**19**	**33**	**34**	**16**	**30**	**32**
Brightness Level	27544.89	52500.38	69212.71	3578.39	51946.98	25614.24
	151209.8	68756.58	40742.63	1032.76	44350.2	23336.13
	120200.1	67436.97	54426.61	28918.28	79699.37	9354.22
	105567.6	58630.63	80218.84	36879.36	40774.7	9311.41
	116655.1	144481.9	55978.16		37319.62	14747.99
	77022.23		32238.9			
	56218					
Mean	**93488.24**	**78361.29**	**55469.64**	**17602.2**	**50818.17**	**16472.8**

Table XXVI: Lung Pilot Control Study – Negative Control Slides

Slides were prepared as for standard method described in Chapter 4.2, with the primary antibody was omitted. Sham-operated 0/24 refers rats with no ischaemia, 4 hour anaesthetic and euthanased at 24 hours. Unilateral 4/24 refers to rats that underwent 4 hours of unilateral ischaemia, then 24 hours of reperfusion. Mean refers to the arithmetic mean of the brightness levels of the 3 images.

Lung Negative controls						
	Sham-operated 0/24			Unilateral 4/24		
	19	**33**	**34**	**16**	**30**	**32**
Brightness	0	298.44	124.08	1.38	0	72.29
	31.4	0	0	55.42	33.37	88.95
	0	25.3	33.55	2.29	0	37.93
Mean	**10.46667**	**107.9133**	**52.54333**	**19.69667**	**11.12333**	**66.39**

Table XXVII: Histogram Cut-off levels showing different levels of brightness.

Slides were prepared as for standard method described in Chapter 4.2; Sham-operated 0/24 refers to rats with no ischaemia, 4 hour anaesthetic and euthanased at 24 hours. Unilateral 4/24 refers to rats that underwent 4 hours of unilateral ischaemia, then 24 hours of reperfusion.

	Skeletal Muscle Sham-operated 0/24				Skeletal Muscle Unilateral 4/24			
Histogram Lower limit	**Area**	**Grey**	**Intensity**	**Bright-ness**	**Area**	**Grey**	**Intensity**	**Bright-ness**
128	54646	190.84	155.36	33293.89	55125	176.06	134.26	29024.4
150	47409	198.61	168.35	31299.8	41701	187.83	151.95	24848.27
175	39463	206.03	180.58	27946.16	28126	200.43	171.14	18876.25
195	31270	211.51	190.34	23341.1	18275	209.2	186.09	13336.13
215	12562	221.64	210.48	10368.98	5459	220.67	208.75	4468.82
230	954	233.87	231.93	867.68	247	232.99	230.26	223.04

7.6.2 Number of images required for Reproducibility Studies

Table XXVIII: Number of images required for reproducibility Studies

Slides were prepared as for standard method described in Chapter 4.2; Sham-operated 0/24 refers to rats with no ischaemia, 4 hour anaesthetic and euthanased at 24 hours. Unilateral 4/24 refers to rats that underwent 4 hours of unilateral ischaemia, then 24 hours of reperfusion. Mean refers to the arithmetic mean of the result for brightness for the 35 images. Cumulative mean was calculated as the mean for brightness of images 1 and 2; the mean for brightness of images 1, 2 and 3; the mean for brightness of images 1, 2, 3 and 4 and so on until the final mean is the average of all the brightness levels in each of 35 images.

	Rat 36 Skeletal Muscle Sham 0/24		Rat 46 Skeletal Muscle Uni 4/24		Rat 36 Lung Sham 0/24		Rat 46 Lung Uni 4/24		Rat 36 Kidney Sham 0/24		Rat 46 Kidney Uni 4/24	
	Brightness	Cumulative Mean Brightness	Brightness	Cumulative Mean Brightness	Brightness	Cumulative Mean Brightness	Brightness	Cumulative Mean Brightness	Brightness	Cumulative Mean Brightness	Brightness	Cumulative Mean Brightness
	7824.9		480.42		29565.8		803.38		3155.96		38.79	
	13957	10891	319.9	400.16	41346	35455.9	3595.95	2199.67	9845.98	6500.97	815.16	426.975
	10263.9	10681.9	174.69	325.003	41803.1	37571.6	12346.1	5581.8	802.51	4601.48	86.46	313.47
	3752.4	8949.54	169.84	286.213	4522.3	29309.3	3486.3	5057.93	80299	23525.9	472.5	353.228
	42521.2	15663.9	13124.3	2853.83	23517.5	28150.9	5471.5	5140.64	31226	25065.9	2008.8	684.342
	16760.2	15846.6	6307.27	3429.41	23193.1	27324.6	12063.6	6294.47	24519.4	24974.8	1074.59	749.383
	278.12	13622.5	383.16	2994.23	23257.9	26743.7	34285.7	10293.2	32261.9	26015.8	1802.08	899.769
	3832.09	12398.7	406.23	2670.73	26292.2	26687.2	32098.9	13018.9	23781.1	25736.5	16096.3	2799.33
	2812.13	11333.5	1990.87	2595.19	30889.2	27154.1	19459.9	13734.6	35044.1	26770.7	23350	5082.74
	35628.8	13763.1	294.98	2365.17	41960.9	28634.8	1337.26	12494.9	24872.7	26580.9	38077.6	8382.22
	14118.1	13795.3	278.3	1991.1	37660.1	29455.3	12165.7	12464.9	42308.7	28010.7	13402	8838.57
	10679.2	13535.7	143.58	2006.13	46290.7	30858.2	9020.36	12177.9	39332.8	28954.2	4030.55	8437.9
	15192.3	13663.1	872.85	1918.95	30705.2	30846.5	8910.36	11926.5	30752.7	29092.5	61923.1	12552.2
	2642.46	12875.9	243.75	1799.3	13624.9	29616.4	30193.7	13231.3	48699.6	30493	39404.6	14470.2
	14794.9	13003.8	19.05	1680.61	30079.4	29647.2	3507.41	12583.1	39435.7	31089.2	39999.6	16172.1
	6167.32	12576.6	261.57	1591.92	61529.5	31639.9	173.28	11807.5	36885.2	31451.5	49971	18284.6
	4913.79	12125.8	588.44	1532.89	61009.6	33367.5	5477.51	11435.1	23363.6	30124.4	26627.4	18775.3

	3294.37	11635.2	185.07	1458.02	41973.8	33845.6	1019.48	10856.5	19868.4	30358.6	22154	18963
	5086.74	11290.5	1583.97	1464.64	27812.2	33528.1	2700.06	10427.2	19622.7	29793.6	36272.4	19874
	3519.08	10901.9	2089.29	1495.88	30252.7	33364.3	1246.32	9968.14	4282.67	28518	397.53	18900.2
	5425.24	10641.2	4869.48	1656.52	37234.8	33548.6	13891.5	10155	13610.7	27808.2	2424.05	18115.6
	3590.41	10320.7	1131.58	1632.66	41079.8	33890.9	1032.65	9740.31	9418.53	26972.3	885.88	17332.5
	5975.23	10131.7	868.81	1599.45	48470.5	34524.8	4058.79	9493.29	9644.2	26218.9	554.28	16603
	4621.89	9902.15	4765.17	1731.36	21157.6	33967.9	556.15	9120.91	17471.4	25854.4	90.81	15915
	18199.2	10234	10465.5	2080.72	41923.3	34286.1	7337.21	9049.56	38693.2	26367.9	25481.9	16297.7
	1873.84	9912.49	3083.32	2119.28	41782.3	34574.4	8299.77	9020.72	46365.8	27137.1	461.61	15688.6
	16770.2	10166.5	5783.83	2255.01	39921.2	34772.4	5024.99	8872.73	5900.35	26350.5	27793.8	16136.9
	3045.68	9912.16	206.37	2181.84	65849.9	35882.3	578.37	8576.51	4753.74	25579.2	25652.5	16476.8
	18962.2	10224.2	186.64	2113.04	36430.7	35901.2	7767.29	8548.6	27075.8	25630.8	17563.8	16514.2
	7165.26	10122.3	87.28	2045.52	23371.6	35483.6	18689.7	8886.64	45809.6	26303.5	23019.8	16731.1
	5673.46	9978.76	316.84	1989.75	52188.7	36022.5	6846.26	8820.82	100051	28682.4	26585.7	17049
	15548.4	10152.8	121.82	1931.38	20872.7	35549	558.78	8562.63	27756	28653.5	31674.2	17506
			2592.4	1951.41	26861.6	35285.8	3993.08	8424.16	8203.93	28033.8	33706.3	17996.9
			775.18	1916.82	43123.9	35516.3	11592.4	8517.34	26316.6	27983.3	54787.2	19079
							33717.7	9237.35	14069.7	27585.8		
Mean	**10152.**		**1916.8**		**35516.**		**9237.3**		**27585.**		**19079**	

7.6.3 Thickness of tissue sections studies

Table XXIX: Thickness of Tissue Sections

All experiments were performed on Rat 28, which was sacrificed by cervical spinal dislocation. Tissues were stained for type IV collagen antibody, FITC preparation. Mean refers to the arithmetic mean of the brightness of all 35 images. St Dev refers to the standard deviation.

	Left Leg Skeletal muscle					Lung					Kidney				
	1 μm	2 μm	5 μm	10 μm	14 μm	1 μm	2 μm	3 μm	6 μm	9 μm	1 μm	2 μm	5 μm	10 μm	14 μm
	17061	39018	33405	29708	37193	52913	149205	76753	117665	70539	32385	93545	84979	41198	63278
	12923	35647	29807	28742	38750	133124	147326	99487	105949	107189	42642	100608	79078	81148	73017
	19292	22721	27997	39308	43222	98464	123089	73996	116165	107055	60521	85062	110870	70496	69621
	9921.1	31416	26723	29142	43238	80357	110336	96353	121279	82846	60911	79768	96515	64915	86651
	37616	24264	31420	35249	38243	66259	128294	124088	104230	98276	70535	66870	69583	65104	71031
	31627	23692	41710	35249	39805	108616	98105	99961	107936	80573	86785	74458	72358	69272	75442
	52932	30300	30221	42396	36125	80211	92172	120451	109486	86714	51212	53093	78871	62241	75488
	35920	30586	43084	30851	35863	110368	91640	111119	132028	77424	52882	57799	79645	62909	63721
	35005	35351	32765	34034	41291	119990	118848	99961	116669	101939	34053	85588	63122	54609	57634
	23564	46681	34671	23178	42663	132268	132960	121147	102906	170444	46701	56239	69302	48277	68488
	25312	49526	31195	38263	22235	118331	122050	103248	126144	101869	31435	80249	63816	44115	50635
	24156	39643	27659	30425	31101	167623	110404	129520	138507	47293	39300	52794	83798	48915	59624
	27611	21117	27501	37871	58837	121663	75413	127922	199392	48948	31965	36756	69926	62258	61182
	23360	41996	27927	32300	64455	122720	100144	138684	136054	66293	51105	73115	72760	86073	46005
	25470	19232	21504	32295	39209	113906	99881	89253	116712	68472	41053	74730	55183	65737	66324
	10411	17925	23429	45119	30623	123260	117852	114116	72551	69079	38557	66590	39511	66218	55809
	12451	16791	23336	50600	57892	107297	119022	142244	67030	62409	18436	76190	45988	64204	59299
	17798	28135	27641	45412	26329	70400	111334	127822	61659	92903	23021	75186	51844	52783	62080
	8043.3	19451	30140	45834	40193	91688	120128	112361	35826	68163	13734	77957	65515	61100	61426
	7782.4	25257	33383	38341	46492	130069	105342	120505	37156	58036	4905.5	63801	52580	48933	56297
	13708	27238	30389	31714	36135	121025	154193	151921	37148	57208	94491	61050	54443	56921	62698
	35182	27017	20248	56088	25746	87994	161856	137446	45604	68718	71103	59447	54443	53557	96536
	13735	32251	27833	34974	29757	123103	109174	107842	86149	80745	93780	63279	67125	59890	91920
	16859	20405	35466	37952	21935	125723	85199	92002	103661	87603	108510	99904	74800	90301	67857
	20008	18474	39506	63237	19416	145429	108291	119117	94834	104160	99416	108543	71700	99614	67486
	18834	20620	28945	32401	21042	158041	85043	90276	78795	77406	69392	95462	70179	77024	75485
	21988	20952	35490	28535	25584	127335	143133	122762	107747	94433	71625	91322	60028	66592	72291
	15374	21229	51434	27957	21678	139266	108947	144990	98364	91658	55833	82051	61675	60849	82273
	23867	31964	29938	35557	27215	124637	127439	102585	86304	78446	62971	82960	78292	76065	75859
	28856	17183	30962	38300	19982	88520	123089	114254	97045	99468	84301	88546	73377	48260	58713
	19050	21871	26580	34324	34155	93069	61864	115636	118094	91810	65490	68187	48878	53926	53765
	34504	20346	23026	33590	28410	111413	52044	92609	76935	121768	84496	88171	49498	42061	60945
	19065	21392	20172	45440	43316	76299	73408	93447	135093	79612	81778	69156	75322	40897	47447
	29489	17451	26895	34600	25835	135614	65042	106854	88760	121768	71994	82254	68543	45505	32062
	12597	26002	35784	36800	28505	80663	127950	104215	31598	112242	62832	67178	83858	24215	42582
Mean	**22325**	**26947**	**30520**	**37023**	**34928**	**111076**	**110292**	**112141**	**97471**	**86672**	**57433**	**75369**	**68497**	**60462**	**64885**
St Dev	**9970.3**	**8678.5**	**6559.4**	**8247.1**	**11130**	**26412**	**26269**	**18855**	**35176**	**24131**	**25609**	**15612**	**14650**	**15366**	**13397**

7.6.4 Quenching of Fluorescence Studies

Table XXX: Quenching of Fluorescent Lamp over time

All experiments were performed on Rat 28, which was killed by cervical spinal dislocation. Slides were prepared as in Chapter 4.2.

Rat 28 - killed outright					
Left leg		Lung		Kidney	
Minutes	Brightness	Minutes	Brightness	Minutes	Brightness
0.0833	39603.55	0.0833	98368.69	0.083	83855.8
				0.166	69305.4
0.25	35929.54	0.25	74087.32	0.333	59036.5
0.5	31567.48	0.5	55140.16	0.5	54731.84
		0.75	44648.08	0.75	46085.96
1	25546.58	1	33061.17	1	41586.81
1.5	20487.93	1.5	18010.36	1.5	29690.65
2	16751.38	2	11846.71	2	20788.95
3	10011.41	3	4211.95	3	11711.17
4	5302.39	4	1656.79	4	6156.01
5	3209.27	5	722.72	5	2628.23
10	91.76	10	17.68	10	281.02
20	1.08	20	2.19	20	6.51
30	0.97	30	1.24	30	1.68

7.6.5 Antibody Saturation

Table XXXI: Left leg Antibody Saturation Complete Data

All experiments were performed on Rat 28, which was killed by cervical spinal dislocation. Slides were prepared as described in Chapter 4.2. Concentration refers to the level of dilution of the type IV antibody. Mean refers to arithmetic mean of the brightness of all of the 35 images.

Concentration of Antibody	**1 in 1000**	**1 in 500**	**1 in 100**	**1 in 50**	**1 in 25**
	0.001	**0.002**	**0.01**	**0.02**	**0.04**
	14262.54	10465.69	29708.12	31260.71	35387.75
	14405.35	15569.93	28741.71	20555.55	32584.43
	7817.62	14947.76	39307.82	46915.17	45879.61
	12820.7	11001.82	29142.37	37835.79	50801.51
	13178.44	13233.68	35249.27	30284.77	48561.01
	11552.56	18570.07	35249.27	27103.9	35899.88
	8877.8	16372.18	42395.57	43220.95	42764.37
	10581.09	15029.63	30851.06	38250.3	37337.24
	22675.03	14815.25	34033.89	35370.56	54887.89
	7119.25	15919.74	23178.15	48683.37	30632.78
	11072.79	12493.39	38262.98	26018.19	36295.63
	11367.1	13682.94	30425.12	33530.21	44034.34
	7923.88	13703.68	37871.32	33113.05	44401.86
	15984.79	10747.32	32299.89	26436.04	44038.21
	17028.89	16693.36	32295.33	26786.74	45069.74
	10058.7	17586.63	45119.29	34763.54	24989.87
	9666.03	19340.11	50600.14	61968.25	26419.81
	3148.75	20003.35	45411.7	32825.86	35031.48
	11349.59	26429.64	45834.31	38682.33	28166.96
	11854.23	24408.18	38341.36	53521.68	29446.25
	14882.24	19839.26	31713.73	40062.99	44520.52
	13457.45	22681.9	56088.38	36325.32	26971.66
	16969.67	24657.44	34973.67	42915.91	38858.92
	8740.98	22067.2	37951.78	39650.71	27669.24
	11200.9	24723.14	63236.96	56372.23	24907.72
	7177.68	25415.58	32401.13	50585.09	30950.25
	10313.99	23279.38	28535.24	26398.67	37249.84
	4626.32	19379.06	27957.28	40935.29	34506.03
	7177.72	26074.85	35557.4	30785.28	40786.71
	3521.48	20325.29	38299.92	30552.15	32069.91
	7948.02	28267.74	34324.08	29494.96	42262.14
	2733.9	16348.7	33589.7	31211.56	30004.26
	6057.91	31303.43	45440.45	24503.72	42060.82
	2361.98	26237.38	34599.84	26254.02	28979.38
	2849.96	18816.14	36800	24036.76	33443.33
Mean	**10079.01**	**19155.17**	**37022.52**	**35920.33**	**36796.32**
Standard Deviation	**4647.23**	**5432.665**	**8247.081**	**9879.553**	**7836.343**

Table XXXII: Lung Antibody Saturation Complete Data

All experiments were performed on Rat 28, which was killed by cervical spinal dislocation. Slides were prepared as described in Chapter 4.2. Concentration refers to the level of dilution of the type IV antibody. Mean refers to arithmetic mean of the brightness of all of the 35 images.

Concentration of Antibody	**1 in 1000**	**1 in 500**	**1 in 100**	**1 in 50**	**1 in 25**
	0.001	**0.002**	**0.01**	**0.02**	**0.04**
	77021.82	51894.31	76753.01	88603.45	124766
	80071.05	65797.08	99486.73	71912.1	170192.1
	74410.93	54231.3	73996.26	64330.07	119881.5
	62338.38	46245.4	96352.85	89298.15	89056.72
	68428.12	34384.75	124087.9	65322.31	118169.8
	58469.36	34384.75	99960.69	68586.75	81593.02
	85909.52	72684.05	120451.5	63134.22	120061
	78990.77	59757.95	111119.4	72070.14	122316.4
	42093.97	53525.01	99960.69	68725.69	128708.2
	44780.05	42336.66	121147.3	56533.55	118103.9
	48447.54	47319.17	103248.2	67130.21	104938.9
	42711.12	52965.54	129519.6	79954.02	100634.6
	48471.05	35233.97	127921.9	77498.88	75272.34
	49325.36	52594.77	138684	70106.21	52131.1
	47583.82	83927.02	89253.26	75502.13	43141.78
	65577.11	744.22	114116.1	124584.2	112809.6
	68109.04	3224.23	142244.1	118492.2	99161.1
	44927.2	32412.82	127821.6	81559.56	101666
	48729.19	37505.93	112361	59305.2	150499.1
	48274	43115.61	120504.9	63174.2	135870.1
	42110.35	44529.31	151921.2	51800.55	137111.1
	48664.8	44628.02	137446.1	48222.35	95723.21
	45120.2	46882.5	107841.8	63613.15	57935.47
	43746.79	69657.36	92001.66	43778.31	76726.65
	37248.71	74814.96	119117	62409.32	46731.04
	42067.48	61206.62	90275.74	21362.22	111799.8
	38559.97	48277.75	122762	9071.12	120734.3
	31132.78	45951.54	144989.7	22318.88	95062.86
	31132.78	43859.43	102585.4	15928.02	77680.02
	18002.12	42429.57	114254.4	21031.41	122299.1
	10941.23	81524.92	115635.9	32700.78	84768.97
	18858.25	81524.92	92609.33	36111.38	80428.21
	37786.87	63444.29	93446.86	64438.06	98106.63
	11544.95	58166.68	106853.8	57074.11	71696.44
	30841.24	52674.87	104215.4	58578.36	95570.47
Mean	**47783.65**	**50395.92**	**112141.3**	**60978.89**	**101181.4**
Standard Deviation	**18955.3**	**18407.05**	**18854.78**	**25504.55**	**28934.61**

Table XXXIII: Kidney Antibody Saturation Complete Data

All experiments were performed on Rat 28, which was right by cervical spinal dislocation. Slides were prepared as described in Chapter 4.2. Concentration refers to the level of dilution of the type IV antibody. Mean refers to arithmetic mean of the brightness of all of the 35 images.

Concentration of Antibody	**1 in 1000**	**1 in 500**	**1 in 100**	**1 in 50**	**1 in 25**
	0.001	**0.002**	**0.01**	**0.02**	**0.04**
	21900.61	51129.78	84978.6	63999.89	63509.73
	30325.72	73279.35	79077.74	45861.88	88678.09
	22108.31	85707.13	110870.3	65554.17	73803.8
	39789.9	91368.67	96514.94	75665.38	65803.55
	23563	89505.6	69582.83	101592.6	60593.88
	10992.08	88024.81	72357.58	56853.5	59027.17
	15504.21	93825.02	78871.26	71808.19	59027.17
	41126.31	64120.32	79644.85	69785	51079.28
	57634.5	578.49	63122.39	60826.68	41324.68
	57958.35	38338.73	69301.95	65008.32	53123.19
	26048.33	15249.99	63815.77	71848.1	59371.96
	37094.77	34355.15	83798.4	64764.71	59371.96
	24997.02	38057.34	69925.69	55323.98	48014.05
	21692.84	30462.28	72759.82	54672.98	27924.42
	30331.82	21978.81	55182.74	54848	39280.8
	20588.63	29393.63	39511.44	59322.73	48381.3
	20332.86	66359	45988.39	64214.94	52192.37
	17803.82	43901.12	51844.47	67003.59	51486.55
	16091.07	67447.7	65515.47	71155.28	54465.28
	23111.52	50216.11	52580.13	85020.47	63078.61
	24988.81	54984.58	54442.92	65536.61	54042.48
	27858.6	51590.93	54442.92	61421.55	52300.52
	7921.32	36435.14	67125.23	83844.12	52300.52
	1793.35	39624.42	74799.53	78267.87	40783.57
	38861.2	31215.89	71699.91	68401.55	28013.21
	23310.42	49985.8	70179.49	62900.43	38456.54
	17384.85	37881.25	60027.92	54360.53	24991.44
	21783.6	25023.04	61674.89	43308.58	15134.14
	6294	25272.62	78291.89	50250.4	66919.66
	2796.04	29286.33	73377.05	63027.1	34954.54
	12813.89	21247.04	48877.88	65963.34	62377.36
	1972.98	13882.54	49498.17	56313.18	70900.05
	11183.19	6313.71	75322.44	58414.36	48923.23
	3068.88	13286.76	68543.49	69813.93	57962.67
	6330.21	11457.39	83858.02	57126	47642.6
Mean	**21924.49**	**43451.04**	**68497.33**	**64688**	**51864.01**
Standard Deviation	**14185.29**	**25944.21**	**14650.49**	**11332.06**	**14812.06**

7.6.6 Quantitative Immunohistochemistry, Complete Data Sets

For all tables in section 7.6.6 Quantitative Immunohistochemistry; 0/0 – refers to sham-operated rats with 4 hour anaesthetic and euthanased immediately, 0/4 refers to sham-operated rats with a 4 hour anaesthetic and sacrificed after a further 4 hours, 0/24 refers to sham-operated rats with a 4 hour anaesthetic and sacrificed a further after 24 hours, 0/72 refers to sham-operated rats with a 4 hour anaesthetic and sacrificed after a further 72 hours. Uni refers to an animal that underwent four hours of left leg unilateral ischaemia and then was either sacrificed immediately (Uni 4/0), after 4 hours of reperfusion (Uni 4/4), after 24 hours of reperfusion (Uni 4/24) or after 72 hours of reperfusion. Bilat refers to animals that underwent four hours of bilateral lower limb ischaemia and then was either sacrificed immediately (Bilat 4/0), after 4 hours of reperfusion (Bilat 4/4), after 24 hours of reperfusion (Bilat 4/24) or after 72 hours of reperfusion.

All slides were prepared as described in Chapter 4.2.

There were five rats in each different group as shown. Mean refers to the arithmetic mean of the brightness of all of the 35 images. St Dev refers to standard deviation.

Table XXXIV: Left leg Quantitative Immunohistochemistry

	0/0	0/0	0/0	0/0	0/0	0/4	0/4	0/4	0/4	0/4	0/24	0/24	0/24	0/24	0/24	0/72	0/72	0/72	0/72	0/72
Rat No.	**9**	**22**	**23**	**31**	**35**	**49**	**55**	**58**	**59**	**61**	**19**	**21**	**33**	**34**	**36**	**5**	**11**	**18**	**20**	**27**
	32525	69089	78479	48578	64735	50135	32586	4295.2	49478	44586	7075.6	187044	25467	209853	47526	62795	52698	78167	65141	98031
	27023	23440	56977	98826	157635	56839	27961	23309	82924	55212	23258	58913	29436	36567	44772	60151	36226	64138	67435	106428
	37848	13678	25973	161439	94010	60323	72289	23240	88968	51077	71522	114546	46939	33926	39927	50579	173958	77294	56960	87830
	44197	47913	49288	53365	148105	40784	59473	25180	69150	44212	12399	49561	46976	75107	29623	67690	43765	52124	58160	58895
	36083	68788	44710	40940	76749	41327	51988	35234	47178	52817	24263	47631	31828	33272	27768	85489	63859	113215	51607	70301
	10949	53075	30968	34956	43982	127502	43236	39815	40031	79613	18445	53781	36605	78125	34017	127413	77853	112910	45215	81054
	6469.8	70983	41252	40743	90402	84867	83685	47048	65716	94563	11153	63208	34599	35361	30475	107966	40383	66969	46966	69384
	33662	47696	52116	65226	32109	119981	87998	52129	85527	52159	19781	73065	38889	50147	26524	75173	35328	67331	60942	84435
	33313	41865	44628	93737	34407	50215	54485	43514	61847	70299	30541	102462	29478	56969	26368	63131	77275	45429	66600	88934

	43084	31028	47025	55111	59129	28252	49575	53726	60737	41070	39138	100307	44818	49152	33074	56765	74923	66609	38452	37983
	48411	50677	81700	107898	35736	61653	48756	60884	60737	40571	21721	116122	36498	36785	26397	77584	87588	77661	71839	138461
	49516	17207	76465	41880	12711	41755	43245	45667	69990	32685	22945	79787	43088	56710	35200	72386	53151	41876	51262	58626
	41353	22862	53721	103267	53227	66344	42970	47403	49277	63182	25596	54861	26363	57730	35734	58732	47758	55461	69555	76554
	51760	42237	52546	100353	43609	81316	25311	58388	36253	44946	22841	55260	32002	52871	33169	55980	35753	66428	42177	107317
	77803	53956	67236	77697	85959	59962	47761	64109	70204	55919	46132	52438	48460	57608	47012	55007	34960	66630	67850	57996
	79412	34072	44555	95000	41202	54248	77803	64848	39678	54310	32717	56059	32410	58746	52042	110268	47418	41714	43416	71451
	36764	65969	80314	64988	30591	106145	62260	38613	120601	56938	34875	49227	30755	70581	35442	115297	44828	39646	47123	49135
	68761	43473	56085	93475	35122	55210	97915	53693	57247	45190	31630	45409	31195	27021	44512	76146	55226	110064	70603	75696
	47990	85874	82197	99683	70677	73178	67686	44889	45978	62334	35521	33696	35129	52468	61983	103803	51866	61836	58565	205722
	80564	86841	44616	55173	51389	87687	41864	51367	52176	70501	21541	27967	34478	43270	49697	122189	32740	79270	80514	80881
	56082	49582	65229	50937	57707	76756	51904	49954	75818	45319	34817	48478	29006	42733	50273	151272	34767	49699	79923	78085
	75272	47045	57806	88781	24539	46468	43254	45384	40868	77378	29242	54817	42093	49696	46402	132072	41904	61568	94133	75857
	84938	27915	90610	59826	67909	62970	56273	52902	62217	33444	31197	46275	54090	46510	39164	99103	47055	59606	102336	55743
	72405	35127	88177	77161	56704	57468	61418	61228	139545	41246	40720	54163	66163	32723	40726	115805	33249	47432	31742	91356
	111769	34038	195765	36870	50835	58368	30302	65046	116594	101732	41067	31956	43245	42456	51144	84102	42079	54783	39210	71887
	107325	32117	51150	34010	56867	89605	35697	66814	94723	50144	87152	54004	53068	57787	72125	123468	32877	67984	70980	143065
	126022	35239	19813	68842	96754	68389	34300	47845	79088	95738	40794	38383	59466	49741	44636	116856	54342	50481	73526	62447
	128754	33930	193798	39340	44753	68324	95753	30231	62257	57134	32059	41151	30323	59975	66321	97397	59216	83101	86659	154790
	78005	28387	195369	58245	44595	57669	57322	27807	82993	85478	24308	56543	32138	41409	66417	102024	35630	60089	48702	79347
	33752	41186	49773	47180	55328	61005	41441	55408	108641	57963	39706	52118	43091	35755	47818	145138	27069	52371	66460	58451
	56080	31315	57999	58661	118253	48269	93117	145709	53762	56490	43408	85040	61514	40420	107990	126187	50131	51642	52148	58708
	48079	45521	86924	98675	33888	35772	25407	188212	38711	56439	30093	40566	32874	24889	35814	130428	54820	59099	63309	70470
	87540	61025	50311	90784	69781	65090	35206	27610	57590	53834	27537	38600	51662	32889	42754	121195	46252	60335	81780	40407
	57159	64553	72464	36789	83517	49908	38308	49836	113722	33887	26162	38730	46486	70596	61226	144691	25560	82977	85243	68935
	48001	37267	43783	84041	49910	58465	40285	94883	41358	32538	28686	30388	46377	56629	121142	144497	73767	45497	77239	52818
Mean	**58819**	**44999**	**69423**	**70356**	**62081**	**64350**	**53110**	**53892**	**69188**	**56884**	**31715**	**60930**	**40200**	**53042**	**47292**	**98251**	**52179**	**64898**	**63251**	**81928**
	Uni 4/0	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**					
Rat No.	**8**	**10**	**13**	**17**	**37**	**6**	**7**	**16**	**30**	**32**	**14**	**15**	**24**	**25**	**29**					
	24090	32243	54259	62895	5566	11269	63535	53623	27409	15334	12212	12212	30850	1270.4	9918.6					

	61485	31521	59354	48298	7938.3	15987	70869	26679	84052	34386	0.94	0.94	7423.1	657.51	18787
	27363	46702	55902	40350	16514	8155.4	71117	34204	105925	28883	26.25	26.25	52764	2072.8	11131
	22427	55078	34953	21551	12807	2420.5	62005	36654	74079	52687	48.18	48.18	67044	82.89	27626
	10368	44372	10240	17011	18570	2793.9	62331	38869	43536	31704	1345.8	1345.8	43701	4626.2	25111
	19492	35226	3632.7	18231	21565	2897.1	44276	36954	67942	42183	861.41	861.41	52539	9811.7	23891
	20743	38744	29257	50196	16832	6429.7	44220	37092	73040	44836	2178.1	2178.1	34122	25182	35359
	17924	47445	30163	46531	14952	2228.6	37750	44475	29059	45065	49.98	49.98	57867	22547	15181
	28096	41739	24639	48347	18078	8051.2	43669	42879	24273	41130	54.32	54.32	101622	19119	35931
	34075	42183	29848	65231	25677	1654.3	26175	35062	31900	34062	418.79	418.79	94203	6384.1	27825
	33015	31883	28992	41387	16860	824.5	17135	37661	31901	53769	1441.2	1441.2	65444	12915	26230
	57047	39000	31856	58691	9285.2	1426.4	31304	33084	42814	23473	957.13	957.13	29010	16622	67878
	32417	32590	31856	50340	13499	2066.5	31078	34623	37478	36296	424.92	424.92	69009	4770	26572
	28902	51200	53799	49296	4155.4	2913.1	52105	36509	17616	39544	511.93	511.93	56878	53270	15428
	57771	24247	46791	39559	6305.8	684.46	35159	40549	24775	34720	139.57	139.57	36408	26676	40131
	39653	41586	59075	40382	5305.6	441.7	51937	46392	33556	35333	474.1	474.1	48499	4853.2	43424
	16801	35664	62739	64738	8389.3	403.5	46968	43812	32009	17622	403.51	403.51	47851	25415	39546
	17441	27964	47276	67963	11477	543.56	47327	34925	49214	43370	1915.6	1915.6	102307	34907	59948
	15747	36515	104220	78800	18227	2138.6	43151	39110	32659	41165	260.08	260.08	103368	8221.9	98319
	24716	54927	67089	44280	18734	13684	42849	50890	35220	25859	10.83	10.83	13098	20340	88723
	15374	37362	48084	57516	17603	8832.8	49392	52302	50295	40060	6.39	6.39	47980	32787	24481
	31471	50364	49206	48644	10676	3624.5	75697	51210	43828	41267	13.54	13.54	69339	52974	27393
	29116	36367	44019	49112	12517	1605.3	48632	51822	41902	18878	486.19	486.19	54320	4122.2	24801
	36250	39366	50769	41519	6368.6	661.21	44136	38387	24049	37810	1149.6	1149.6	27114	37540	44174
	33482	32729	54561	56197	6484.7	409.84	50139	47739	35024	27171	1507.7	1507.7	4363.5	16737	34038
	33749	45251	44688	11753	16924	179.36	55549	46450	32567	66916	662.69	662.69	4638.2	54964	62303
	25412	42275	69750	10704	3233.9	452.98	1447.6	42360	37490	49055	1831.7	1831.7	30953	19175	64329
	49193	39748	63379	24526	7767.4	959.29	51563	46474	49533	52912	1870	1870	57264	15198	50098
	41844	40373	65466	48498	15990	153.98	47500	50884	63434	28439	2793.2	2793.2	13721	16696	72671
	19187	35318	62815	38440	12185	1507.7	48083	39094	50337	31549	776.36	776.36	5790	32077	66033
	23200	34970	53133	36168	18515	1219.6	31720	44026	56963	29040	1532.5	1532.5	40963	27997	48691

	38907	45821	51024	32924	44768	2755.4	34811	54978	50446	12965	365.7	365.7	149.67	8574.2	15814
	35040	34249	50872	25032	43048	7241.1	9795.7	36476	48428	26937	122.61	122.61	56726	6076.8	44514
	35426	41752	56813	42170	37447	11523	38283	24151	36316	21267	104.46	104.46	25586	11731	34805
	24801	33294	51580	19743	37164	4024.7	33321	33328	60664	4527.3	320.41	320.41	43922	10586	25474
Mean	**30344**	**39431**	**48060**	**42772**	**16041**	**3776.1**	**44144**	**41249**	**45135**	**34578**	**1065.1**	**1065.1**	**45624**	**18485**	**39331**
	Bilat 4/0	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**
Rat No.	**44**	**45**	**47**	**48**	**51**	**39**	**40**	**41**	**42**	**43**	**52**	**53**	**54**	**56**	**60**
	111176	11979	17849	23357	172.2	58.53	3583.4	38947	34783	16656	2317.4	6.13	1271.5	780.42	1805.3
	40724	8218.6	17455	29227	153.79	25.59	3289.4	44137	21862	40047	2320.3	10.92	351.88	363.17	23667
	21670	5854.2	27212	47724	377.27	192.81	4575.8	53799	34123	46802	19464	2680.7	876.33	22977	33430
	53292	20307	44628	19100	255.17	148.42	540.71	25526	40358	44318	14066	12165	1281.6	27559	23813
	75537	21313	33934	81027	142.47	139.35	1184.5	33787	28958	47760	8119.9	32247	1252.4	29761	33805
	57831	35583	37010	71456	71.35	374.45	115.79	22614	23751	54312	10267	21360	914.51	16134	26010
	53677	20969	23779	56415	4099.8	686.25	1092.5	34426	39859	66362	8456.3	3135.1	1823.3	29108	23255
	77953	34008	44433	109875	609.1	1355.9	535.81	40008	32989	58810	5556	25.12	960.24	9013.9	16162
	87533	30895	28327	49102	661.5	5.32	40.01	40324	25322	27066	2553.6	2136.5	1346.8	32980	8166.1
	107453	38970	41319	54353	1293.8	101.35	55.8	33267	22573	39886	102.66	3172.5	2353	46060	3706.6
	142531	22474	62751	70178	7028.5	4075.3	45.08	33283	40883	31706	963.09	9083.4	12197	36738	15283
	134028	48078	35889	72153	9683.6	12938	16.2	29851	17471	32997	4819.8	3824.9	695.43	31883	34070
	170982	54276	33170	69852	13055	1693.8	57.27	25820	35347	28672	8402.9	14868	5528	26081	28680
	157969	63660	28186	44067	22825	598.29	94.98	18886	29523	29434	11364	24042	2602.9	29129	27917
	156547	44972	29869	3898.4	9180	662.66	516.75	23675	26071	33570	9819.2	10743	22091	34155	14450
	132727	28542	19144	13755	9977.3	2299.9	1201.4	24866	24470	42996	21979	3125.1	4153.9	21915	47816
	120070	48485	31711	21427	142.55	2759.2	3384.1	22706	16516	32366	23941	31909	9668.5	21264	47332
	182002	29059	32477	42065	5923.5	1806.6	2762.3	21898	15406	54445	15020	20369	3175	4436.5	46400
	99613	104408	39140	24893	987.16	903.07	1703.1	19271	26778	52457	14316	47561	1332	513.21	35134
	119934	44326	60904	38634	20427	1884.1	3494.7	17101	32537	48699	4351.8	52058	1250.1	3615.3	36752
	120806	37471	22926	32394	11203	36079	1788.1	20961	32002	56040	1997.3	29959	2564.2	18777	50780
	39628	55038	49066	11380	15201	7880.7	6014.1	16771	25657	39676	21.36	20447	594.06	20485	44923
	71528	30360	26459	14364	15545	620.13	435.28	28986	29464	59413	211.14	21783	3688.6	18710	29013

	68386	54994	35809	27243	52725	4208.4	264.24	23137	21392	16158	10332	32672	1238.4	14303	33264
	39791	47025	24951	37960	33147	59003	565.53	24197	41173	3816.7	35440	6764.8	698.55	10389	31587
	74551	55296	13985	29565	61159	73329	658.83	28325	21252	13313	30150	493.49	1693.7	21757	24950
	139865	113449	21894	26297	46148	12498	134.82	31708	25311	24324	20399	39901	5012.4	26430	39910
	108369	46012	20039	46142	55122	10428	769.67	38096	17208	17816	18686	6697.3	16576	10483	41211
	128569	14178	10833	40403	49208	9095.5	4136	18017	28402	19105	18262	39244	2176.4	12763	36911
	51431	35479	16933	31292	45270	3807.1	157.06	21102	14574	17121	23343	20549	8649.8	5883.7	41125
	91446	39739	29696	41816	21421	7182.1	1407	8418.5	38545	33217	27485	26691	9635.3	34559	39158
	82607	39103	12048	53721	27191	7060.7	5.27	15997	41960	20105	36873	24011	3036.7	12182	29968
	67029	82931	9926.7	94856	13502	10001	285.11	5072.8	31436	21684	30790	32401	11296	9697.4	34082
	50703	100066	29790	48345	10.99	23590	475.7	73.82	37770	29160	20898	5.87	13272	35141	33962
	79434	46958	41248	53938	2312.8	14944	205.54	10404	17499	33313	21588	7.85	30128	29773	1547.8
Mean	**94783**	**43271**	**30137**	**43779**	**15892**	**8926.7**	**1302.6**	**25585**	**28378**	**35246**	**13848**	**17033**	**5296.7**	**20166**	**29716**

Table XXXV: Left leg Quantitative Immunohistochemistry, Negative Controls

	0/0	0/0	0/0	0/0	0/0	0/4	0/4	0/4	0/4	0/4	0/24	0/24	0/24	0/24	0/24	0/72	0/72	0/72	0/72	0/72
Rat No.	**9**	**22**	**23**	**31**	**35**	**49**	**55**	**58**	**59**	**61**	**19**	**21**	**33**	**34**	**36**	**5**	**11**	**18**	**20**	**27**
	2.43	0	7.86	0	8.83	0	0	0	62.52	2.19	19.75	1.02	0.5	34.61	0.47	26.31	6.03	72.02	30.51	0.47
	2.28	14.3	35.74	10.75	84.16	0	2.36	0.47	0.97	8.12	2.49	0.53	1.41	31.35	3.37	1.51	2.08	23.21	1.5	3.09
	2.37	0	0.97	3.42	0	0	0	0.47	0	0	2.92	2.54	1.44	4.86	0.94	18.05	0.94	2.09	1.96	1.96
	2.5	0.47	0	1880	20.89	194	0	0	5.58	0.47	30.69	1.05	0.94	36.33	11.39	2948	5.97	0.47	422.8	6.41
	2.43	0	0	625.1	0	226.7		0.94	2.98	0.94	1.98	10.79	0.99	0.47	1	33.03	2.01	1.98	2.18	21.56
Mean	**2.402**	**2.954**	**8.914**	**503.93**	**22.776**	**84.138**	**0.59**	**0.376**	**14.41**	**2.344**	**11.566**	**3.186**	**1.056**	**21.524**	**3.434**	**605.29**	**3.406**	**19.954**	**91.78**	**6.698**
	Uni 4/0	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**					
Rat No.	**8**	**10**	**13**	**17**	**37**	**6**	**7**	**16**	**30**	**32**	**14**	**15**	**24**	**25**	**29**					
	22.81	3	2	20.38	0	1.04	2987	1711	41.51	0.52	0.94	0.94	0.97	28.44	4.75					
	16.65	1.94	23.65	148.3	468.1	51.88	9468	3497	249.3	2.01	0	0	0.47	2.51	0.97					
	55.34	3.61	41.97	1.44	6.84	5.46	3682	24.32	347.8	0.94	1.47	1.47	1	5.35	6664					
	13.28	7.81	335.9	8.98	28.42	2.04	3925	43.11	4371	0	0	0	9.06	0.97	2.51					
	0.99	9.2	25.21	1.5	31.58	451	3.4	214.2	2993	0.97	26.19	26.19	2.62	3.57	2.03					
Mean	**21.814**	**5.112**	**85.754**	**36.11**	**106.99**	**102.28**	**4013.2**	**1098**	**1600.5**	**0.888**	**5.72**	**5.72**	**2.824**	**8.168**	**1334.9**					
	Bilat 4/0	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**					
Rat No.	**44**	**45**	**47**	**48**	**51**	**39**	**40**	**41**	**42**	**43**	**52**	**53**	**54**	**56**	**60**					
	13.34	2.48	478.2	581.7	2.71	1	2.42	6.72	4.21	2.35	2.37	2.42	2.45	30.66	2.48					
	2.04	49.23	8.21	47.46	3.29	2.53	2.7	2.37	2.42	2.26	2.29	113	3.77	12.91	3.52					
	2.17	2.04	3.04	137.5	20360	0	2.32	2.31	2.49	2.29	2.37	2.31	2.4	9.64	2.46					
	61.82	1026	0.97	23.95	2.65	0.47	2.42	2.36	2.35	2.31	2.34	2.34	3.14	2.46	7.52					
	1.51	2.96	0.97	48.29	7.29	0.5	2.34	2.46	2.45	2.37	2.39	2.42	2.46	2.35	2.56					
Mean	**16.176**	**216.51**	**98.28**	**167.79**	**4075.2**	**0.9**	**2.44**	**3.244**	**2.784**	**2.316**	**2.352**	**24.506**	**2.844**	**11.604**	**3.708**					

Table XXXVI: Left leg Quantitative Immunohistochemistry including Bilateral 4/72 Data

	Bilat 4/0	Bilat 4/0	Bilat 4/0	Bilat 4/0	Bilat 4/0	Bilat 4/4	Bilat 4/4	Bilat 4/4	Bilat 4/4	Bilat 4/4	Bilat 4/24	Bilat 4/24	Bilat 4/24	Bilat 4/24	Bilat 4/24	Bilat 4/72	Bilat 4/72	Bilat 4/72	Bilat 4/72	Bilat 4/72
Rat No	44	45	47	48	51	39	40	41	42	43	52	53	54	56	60	67	68	69	70	71
	19068.57	49052.39	29999.62	25406.74	31225.63	15774.42	23009.18	33143.02	32993.94	13190.44	22396.34	28289.91	4908.85	6769.51	40958.16	20559.44	12989.32	363.07	37154.7	19140.04
	26245.88	33507.88	20083.41	36445.52	22202.79	26005.23	34907.65	30885.31	17847.79	13293.87	18095.6	33279.39	4645.37	5427.43	45541.13	16654.43	9459.76	2230.38	53222.6	18387.56
	37056.74	44106.92	38388.14	43869.54	17465.1	30139.47	22089.78	35880.26	29066.77	22675.35	18550.19	34881.26	5085.32	8212.91	52535.48	13398.18	13686.48	2080.55	13551.02	18641.78
	33431.62	29490.62	20804.1	23619.79	28270.88	19141.85	32840.61	49945.41	63273.94	20308.46	17941.63	44362.52	4285.47	10744.56	49712.68	11283.54	27447.17	592.84	23198.5	29737.46
	38856.2	55394.38	31624	66193.01	15844.84	54237.61	22952.23	18931.03	18647.71	7359.94	12926.54	33550.08	3892.82	8626.54	42510.38	19266.38	13420.37	9342.57	18761.91	18539.5
	20613.32	32901.3	16985.64	54317.63	30022.81	28529.67	27046.87	74142.16	26613.83	10110.42	19933.39	48513.82	9194.6	21165.85	15861.78	16913.72	10478.81	13062.92	37693.21	27710.31
	23758.13	36117.78	44079.45	64547.45	19438.25	23464.46	32078.4	63126.76	28122.12	12651.37	23654.77	39138.59	5280.41	16341.25	33109.82	10132.19	16732.12	971.57	10936.38	23437.64
	36063.75	58451.54	40120.38	44050.05	15611.97	43565.02	32853.94	52621.71	37359	11347.91	20987.7	41998.15	2356.25	12595.57	53977.68	8183.57	17886.6	4176.97	11061.36	40988.5
	27820.54	58739.64	48459.45	60043.17	11236.4	33227	33468.32	31237.93	26650.86	10849.78	23809.86	30362.99	1255.18	28818.24	39354.15	16167.7	17049.71	4903.85	33015.02	25015.27
	36954.66	27455.97	58961.3	56985.42	11944.13	87365.46	34788.77	40368	29931.31	14936.9	20229.39	38264.38	4552.93	30521.72	39176.83	17495.4	28375.15	17425.5	32440.4	21739.87
	45008.36	12717.96	38900.52	42571.82	16202.63	66264.86	42019.55	18944.67	32228.88	17303.02	25188.75	28700.75	9283.22	22354.7	39263.34	11207.26	10980.24	31172.13	11236.98	16665.63
	30354.68	48270.59	81734.73	34192.44	26934.06	49386.79	29687.49	38410.93	44032.34	37513.46	23737.45	11568.58	9152.04	30027.06	39005.39	16373.55	17179.82	31646.51	30686.99	42463.32
	27372.04	72543.85	29157.3	35852.64	15163.04	47822.11	30505.81	25829.8	44339.02	20778.58	29280.44	26817.09	23795.87	27828.63	36967.84	11920.79	19741.58	22592.5	44009.53	26192.29
	19749.9	56098.57	25599.49	53429.62	27699.57	20770.81	20957.6	34137.46	37468.28	13228.19	15756.71	55558.48	23607.06	23250.11	41903.04	13147.81	29129.68	23116.37	26643.03	28977.34
	21915.48	44256.95	34882.49	27473.14	16842.64	58834.66	29965.04	22343.7	29514.71	30855.12	16586.39	26534.07	20875	15767.78	41444.88	15840.04	15853.08	23324.41	30555.44	33687.73
	25760.5	55100.07	30692.45	26846.94	20749.15	33390.46	41595.55	19518.98	21579.43	26915.2	10737.73	31818.46	17578.04	33004.3	23823.75	51705.06	22188.35	18435.93	42457.87	24944.6
	36412.32	48596.84	38160.65	34317.76	23392.2	66263.22	13676.25	24795.52	37224.6	19582.41	11540.22	25259.44	24857.3	19320.93	35987.96	69927.72	23831.82	20994	66166.59	32843.66
	26045.97	51188.52	51793.12	35703.31	18936.15	63434.09	28143.19	29043.1	16306.47	24302.23	12960.5	38765.57	10856.23	30729.13	40447.98	65729.79	5688.37	19239.66	72265.99	48590.97
	43358.06	48667.75	88756.44	35490.01	31173.4	72760.8	40950.07	40263.48	35680.57	23916.01	8654.45	28270.72	2617.53	24366.74	35702.21	15412.17	11096.18	42723.07	65669.1	43039.45
	49978.56	49622.3	35645.07	66947.63	73052.63	65624.35	59395.52	29186.87	50233.81	13666.46	20133.99	21518.66	2318.45	15541.32	24582	25984.56	12435.26	22722.46	56397.6	49928.46
	32899.02	57441.3	55939.95	49807.34	18691.94	60342.55	61878.93	26958.48	16534.23	12956.06	20450.82	20161.44	1290.78	19257.15	30316.05	4443.89	6410.85	34252.18	55194.85	45090.61
	28039.24	57594.02	42195.77	48410.69	12130	30291.36	57599.27	35209.48	55106.76	3091.28	24930.26	16278.67	6010.3	40976.8	37230.89	19285.57	21797.5	15319.79	17974.2	29135.05
	31940.91	33710.53	47049.32	48666.8	31787.44	32616.26	32110.72	29879.03	50959.41	12005.77	20948.9	31977.52	8803.28	15192.41	58345.29	9926.91	13995.87	35613.29	65344.54	33541.45
	36461.12	44456.64	62470.04	41051.38	12875.25	9204.51	15675.64	34004	34074	15666	26549.56	37470.02	15930.91	23436.5	55685.13	3349.11	10707.16	15958.74	52404.38	33816.29
	34960.67	23546.94	47340.55	38613.11	10345.2	24342.19	26713.64	30363.43	40175.88	13877.06	32557.79	28403.87	10665.34	29762.24	38359.4	2548.79	13628.3	25850.86	32829.81	78938.69
	22886.3	20431.92	29280.34	27519.69	17053.17	18752.91	34363.88	38643.14	65536.27	9055.68	24969.1	40488.44	26547	30709.5	59476.27	3327.13	9351.74	21122.66	39227.03	81506.48
	16308.47	48135.49	32155.94	35850.9	15600.19	36848.69	31934.72	43185.18	32054.24	15830.62	38833.27	44727.33	13523.1	18234.03	45061.96	11172.96	29586.43	15866.95	39439.21	26905.93
	30468.51	37960.41	85837.75	34315.52	26867.21	23454.74	22180.42	40495.23	28930.68	13839.67	39530.93	36281.14	11005.73	16061.82	35798.84	9725.41	11369.73	13199.95	22436.51	32422.47

	46311.02	51844.33	30703.05	52249.86	18611.17	13791.73	21591.97	75118.79	34282.05	12160.67	14728.33	44684.54	10092.29	26989.21	40415.88	6299.87	14583.13	16364.35	17936.67	28628.94
	28019.91	26181.14	25348.41	37519.26	28201.31	13663.29	24268.32	40510.82	38597.4	35805.66	18642.43	32803.59	10895.65	42666.52	35716.05	10731.1	15837.22	27397.82	36200.36	26810.58
	28954.25	39838.54	26750.75	48430.04	41692.71	65500.93	24658.46	29542.35	18885.78	33648.39	31187.5	25676.78	6731.67	32700.33	42689.2	15156.39	19064.24	21444.85	39878.45	23287.39
	37825.79	39938.42	41397.61	56736.71	34858.6	41661.75	28358.37	32608.93	14681.3	28784.79	28062.17	41019.65	2684.77	35530.28	50849.34	18176.55	9982.23	10281.36	21086.23	35526.63
	10069.27	35565.72	48572.03	45518.21	15284.44	38916	26043.64	43114.73	31438.52	37111.27	27235.51	51723.2	4068.43	7422.86	38263.03	7196.93	14486.39	16481.66	23340.99	18140.37
	35700.48	97323.81	39860.39	52146.89	60361.79	82412.36	31326.59	30374.71	24070.65	15724.82	35373.6	38291.32	26430.91	44323.07	25988.89	12547.63	12888.15	8024.92	36806.44	33476.69
	39386.31	34576.77	35943.18	53229.31	49272.64	61223.45	34817.27	32501.81	22645.95	22691.22	29506.34	40075.3	4072.25	33312.72	20874.37	47052.54	13901.02	31660.9	27390.1	19087.87
Mean	**31030.19**	**44595.08**	**41590.65**	**43953.41**	**24772.61**	**41686.43**	**31612.96**	**36436.18**	**33345.39**	**18486.69**	**22474.53**	**34214.73**	**9975.724**	**23085.42**	**39626.77**	**17949.83**	**15806.85**	**17713.07**	**35560.4**	**32485.34**

Table XXXVII: Left leg Quantitative Immunohistochemistry including Bilateral 4/72 Run, Negative Controls

	Bilat 4/0	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/72**	**Bilat 4/72**	**Bilat 4/72**	**Bilat 4/72**	**Bilat 4/72**
	44	**45**	**47**	**48**	**51**	**39**	**40**	**41**	**42**	**43**	**52**	**53**	**54**	**56**	**60**	**67**	**68**	**69**	**70**	**71**
	0	0	0	1831.1	0	0	0	0.97	0.89	2.89	0	0	0.86	0	0	12.13	0	0	0.42	0
	0	0	0	161.4	0.42	0	0	0	1.04	16.24	0	0	0.42	0.91	0	0	0	0	0	0
	19.27	0	0	4347.0	0	0	0	0.85	0.93	1.07	0	0	0.44	0	2.91	2	0	0	0.85	0
	4086.5	0	0	2005.4	0	123.12	0	0.91	29987.	0.42	0.42	0.85	95.3	0.89	1.31	0	0	0	0	0
	0	0	0	442.36	0	0	0	92.16	0.97	0	0	0.86	0	1.04	0	0	0.42	0	0	2.65
Mean	**821.15**	**0**	**0**	**1757.4**	**0.084**	**24.624**	**0**	**18.978**	**5998.3**	**4.124**	**0.084**	**0.342**	**19.404**	**0.568**	**0.844**	**2.826**	**0.084**	**0**	**0.254**	**0.53**

Table XXXVIII: Right leg Quantitative Immunohistochemistry.

	0/0	0/0	0/0	0/0	0/0	0/4	0/4	0/4	0/4	0/4	0/24	0/24	0/24	0/24	0/24	0/72	0/72	0/72	0/72	0/72
Rat No.	**9**	**22**	**23**	**31**	**35**	**49**	**55**	**58**	**59**	**61**	**19**	**21**	**33**	**34**	**36**	**5**	**11**	**18**	**20**	**27**
	32120	41109	22449	18639	27976	32038	14896	16228	94653	22479	25657	15510	19544	24619	24663	30122	24848	22004	45942	46429
	48552	38875	21778	19609	14875	26804	63534	13028	27829	19640	20356	21688	23442	26688	49386	22787	27838	26410	21009	39116
	39109	32731	8368.8	28653	21265	20463	26031	14470	31115	21259	30584	36778	38635	22866	33654	22933	28753	57238	26184	51018
	25461	71589	20587	24851	36974	36865	31426	26992	27678	31242	37819	41343	47821	21557	39253	28114	25636	41436	6181.9	31246
	24591	46254	28430	37998	31617	35813	33320	16411	30528	33886	55231	46310	36849	35338	53620	32411	21143	44165	34435	39897
	21843	31593	35725	51132	32714	37022	30293	13483	21866	65043	45219	26571	20041	37311	37087	14472	32727	55044	20860	48755
	22761	56981	16530	26086	36871	43574	44347	32603	33510	53617	34315	38740	38296	40410	50615	14276	26219	39431	57767	63304
	14950	35294	22297	31024	32687	36295	43421	26122	24275	20499	34782	34280	29916	36173	46416	23484	28620	45284	40580	44977
	28091	44736	28979	34772	56565	25658	79890	35617	67587	30860	26117	49112	34838	24044	41725	18050	20387	36954	44583	56753
	32503	16950	15612	30680	30674	25060	48091	52773	28371	22634	40101	38632	18661	31141	45214	15560	41568	35708	39316	86246
	23671	87162	25012	23477	20371	22156	69190	29499	21858	25646	23561	37497	25463	16128	36428	10999	27166	40918	26107	67668
	37191	29271	5583.2	38801	26796	28533	43687	27887	26992	23062	26966	41281	33328	33532	47525	22710	54926	33098	30047	53554
	34923	7492.1	14032	38467	53529	23478	63693	38220	28459	12560	34612	35226	35602	18117	46125	37176	23271	55156	49290	91633
	56335	20109	34281	49952	39826	27702	52412	26493	18250	37474	36753	37594	35116	13027	49046	21427	37593	40701	41893	75711
	45071	9237.5	17422	36596	17507	25407	46719	30482	26248	34782	31146	23413	35735	30714	21323	51422	36415	18209	37855	60145
	32646	655.06	15750	31647	16450	24647	50981	34571	29662	40074	40110	23069	50107	16963	30180	50789	33075	69336	44691	39939
	26427	28558	10531	26410	29791	40394	73689	29696	30192	41603	32328	22047	14136	14426	33699	45674	29783	46215	34331	61766
	27230	22058	19184	28716	30872	42180	69124	36848	16118	38458	18490	29852	18617	13326	37432	45129	17505	30106	35118	42242
	26597	32809	13778	22164	28775	32946	39020	29737	28109	16413	20194	38684	26049	19610	41209	20259	26528	35434	38208	35006
	27248	15673	20867	36225	18407	59721	84311	20982	41213	42389	35297	34366	27105	20018	50642	43824	28350	67045	38882	48696
	56184	6614.4	37029	28913	18913	86860	55657	30961	24372	21953	29752	30513	21120	17372	29811	34396	52982	53351	20259	25421
	35138	46295	28901	34170	20401	36009	72175	38282	57762	27366	13357	28692	27511	20791	21892	27150	16341	37582	34863	74805
	41215	50371	49455	18348	28171	15318	66063	28140	50412	50583	20899	51442	24386	18842	42377	30551	39923	41521	25315	41573
	28962	31164	101812	30742	19715	20206	51967	24699	31790	32492	11002	30361	22078	27206	54692	23158	29582	20013	40574	55377
	21487	69799	60404	46472	28528	40304	41755	32932	36046	23679	21512	26829	12434	18359	41548	21011	22699	23918	45854	53500

	28620	27849	11600	24118	32164	30722	26591	27987	27918	25001	16478	22654	30558	18929	42376	2440.8	26402	23990	53921	51596
	38692	32456	21803	14941	26959	39057	34049	32876	51863	18386	14729	56708	32814	53931	63750	7514.8	28253	16102	29350	50289
	55221	14061	34827	36609	37895	36280	36291	24765	34857	29883	29800	25087	21623	15474	29657	2093.6	18590	23558	15509	59512
	25342	40926	35186	28226	20459	58113	45220	46972	25427	38743	24209	26264	28909	40240	38750	22094	15552	37997	29190	36132
	44903	22105	19483	17459	32114	54721	56198	36118	20663	35210	23093	37550	15501	33669	26613	20414	12288	39797	37990	44485
	30056	37438	25591	35128	41100	52437	38983	32879	41641	33389	28194	30118	15203	22554	25919	54380	10564	38619	57423	37408
	36259	29138	61848	14941	19107	57089	30538	34693	58959	45575	53469	33120	30282	10171	38534	49617	28152	45314	51480	27306
	26469	29608	43942	31158	29082	42692	30614	19707	54980	23832	23549	36983	30418	23435	44465	35801	24358	27530	27998	19989
	32244	71383	49606	26490	17692	40202	37017	67709	25186	34123	34987	40296	36736	43032	31087	20829	19048	60893	19466	56498
	20867	32772	53845	59508	26958	56570	44068	79637	27802	27250	36707	20653	30972	26710	33377	18515	31561	55419	47067	46316
Mean	**32828**	**34603**	**29501**	**30946**	**28680**	**37524**	**47865**	**31729**	**34977**	**31460**	**29468**	**33408**	**28281**	**25335**	**39431**	**26902**	**27676**	**39586**	**35701**	**50409**
	Uni 4/0	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**					
Rat No	**8**	**10**	**13**	**17**	**37**	**6**	**7**	**16**	**30**	**32**	**14**	**15**	**24**	**25**	**29**					
	11487	37193	47536	30485	37169	9052.2	96502	26024	32120	42107	3168.1	18673	25024	64668	29595					
	19429	39947	52915	30840	47639	19491	76878	40892	48552	60835	41555	29098	29520	31262	36246					
	19437	34527	45144	43375	32534	17136	80129	37428	39109	66348	44833	58053	28391	22900	39638					
	36799	19196	35539	20384	38048	23747	96321	31069	25461	54809	35468	41310	23940	21783	15334					
	49833	62140	19123	31424	44972	34740	76869	19127	24591	59381	28796	32554	33503	32899	62047					
	39135	69528	27069	33003	39413	38811	76726	25155	21843	76176	20954	23099	57599	34249	19283					
	38319	27190	40299	26705	67829	22737	76848	20971	22761	41721	33622	46636	60895	29419	20732					
	40529	34936	31780	25574	41356	30066	64251	27842	14950	80987	22577	38744	25194	23951	28252					
	45790	43363	54771	30080	34441	15552	60024	20886	28091	32995	7400.2	48198	42287	25758	40134					
	31151	61337	23846	47953	34063	52408	84968	15364	32503	55308	5880.8	36307	37603	41992	51547					
	31151	29868	63055	35765	25808	54922	79438	22349	23671	41493	18372	23458	53567	39924	31274					
	26062	64181	43287	25106	10665	24436	58152	39976	37191	53402	35132	25904	34735	53196	35724					
	29022	68104	40859	31639	54518	45125	76981	42402	34923	42768	44494	41031	35584	25438	36566					
	43787	61725	35664	28288	53923	43394	61892	39871	56335	54183	65795	44074	47327	31861	59720					
	35213	33285	45365	24325	24129	12755	38671	24615	45071	47528	52331	58549	47573	27891	39743					
	28799	39696	66973	34049	52815	21467	46909	33099	32646	37690	66362	47772	77782	45280	33489					
	24295	27073	33422	40274	28202	19391	65030	29566	26427	26588	52569	49733	49865	39820	6315.1					

	30779	25409	46523	28134	20519	25032	42695	48662	27230	40831	59404	41239	33408	38972	27023
	15657	22368	48328	20121	20311	30397	57167	14303	26597	36570	70741	35241	38401	59245	35718
	34504	46033	48068	41070	50040	25292	56260	17791	27248	34833	46900	40994	20748	36874	29447
	32628	47476	37749	40986	45502	28179	52754	17546	56184	50757	45209	14365	15509	39876	23401
	27581	35711	34825	49577	49881	26800	65472	31673	35138	33498	28665	15327	40794	30049	19715
	21679	30503	21671	39684	34322	24831	37188	45776	41215	56855	54315	36220	29788	38000	27095
	36332	47009	30951	45640	51453	34044	22366	30488	28962	27671	59015	51384	26910	37950	32838
	22893	64463	22197	40930	44913	21800	42397	40995	21487	30141	53666	31448	37939	21260	24039
	32996	57220	48622	66295	51053	14385	34778	56116	28620	39184	50576	23585	61236	31262	28375
	38607	32249	38650	31021	25687	22751	38054	41007	38692	56362	50213	32025	53401	28250	17868
	30542	23897	34089	41897	23485	14602	32002	36027	55221	34479	56007	9218.3	46009	45864	18946
	23669	24356	27609	31257	35396	47836	29591	19256	25342	46263	46500	34714	23970	56937	25637
	30421	28998	38695	45163	43460	53180	49831	35395	44903	25015	42682	38648	58755	35872	27712
	29130	13954	23331	38650	35476	61219	34633	18043	30056	35285	50312	39267	33718	28629	26919
	42810	27165	32340	44504	25876	44767	52508	19637	36259	50647	38994	36140	35956	29294	26424
	56059	31514	45004	34936	39744	37332	38844	6472.3	26469	29461	49485	16709	35138	37203	29562
	28245	32857	58185	19995	28751	51795	38416	29905	32244	37205	43431	34965	45553	40254	28664
	42741	23601	39552	36559	24740	23557	63933	22248	20867	34042	42703	24674	30912	59645	27223
Mean	**32215**	**39088**	**39515**	**35305**	**37661**	**30658**	**57299**	**29371**	**32828**	**44955**	**41946**	**34839**	**39387**	**36792**	**30350**
	Bilat 4/0	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**
Rat No	**44**	**45**	**47**	**48**	**51**	**39**	**40**	**41**	**42**	**43**	**52**	**53**	**54**	**56**	**60**
	2813.1	32595	27020	16070	62734	5215.9	9439.3	34610	16165	64649	9150.2	9989.9	20457	31211	18038
	39281	32872	15820	16915	49192	6790	20842	19419	50892	56290	14822	9905.5	2800.1	37358	38635
	35291	32056	17263	18575	56049	8222.3	20633	30761	23800	36802	28307	14923	27833	33163	41273
	13882	39006	31967	20662	63007	21283	6965.2	23928	54034	13746	17762	25612	21923	22441	37310
	14524	37105	20567	22033	58908	19556	30290	41323	38841	47731	35889	30317	16712	28676	38744
	38978	36875	18626	30311	79495	18752	28203	34143	34986	23031	30967	24565	29373	27724	15752
	23902	20006	23111	17408	68137	28121	19668	29817	17122	31430	40478	30831	45376	23272	38054
	37023	26614	12928	25748	44194	41242	20292	33690	11574	43017	22035	48945	27905	32592	60485
	36236	51699	22302	33657	52041	33270	31720	29520	11471	40065	27288	15070	27643	10663	40821

	21210	36030	28864	32468	51441	19308	20109	27906	19667	27158	37822	10242	28223	14964	16587
	24988	29091	20209	34717	59360	26036	16697	35342	19787	19299	22602	5022.1	49228	38490	31929
	26920	28520	35511	24924	42958	49093	4862.3	30722	28902	19411	30536	25164	55601	40574	19215
	26250	33622	11805	30674	59769	27926	8326.2	26439	6513.3	25734	23076	23890	19287	29918	15822
	21543	33008	25237	31424	59788	27282	15564	20464	41516	25500	27419	23877	33084	34059	8829.9
	21471	20880	30621	25269	62007	26834	16584	15075	58338	24910	28620	15742	31342	18769	1673.1
	18062	23254	40317	26014	92324	27008	9647.2	25262	41689	11948	28071	7623.1	68041	23516	14506
	18613	29375	25586	31220	66053	17134	15238	25511	73985	17653	23189	3610.4	69308	20376	49996
	40720	30963	36286	26577	49756	17558	19074	26979	18166	8132.2	26447	7026.3	52540	5969.9	24422
	34988	30049	28998	46804	29024	16457	14263	17777	20039	25108	50622	24381	44730	9702.2	30758
	47658	18842	28563	47458	35942	15254	25954	10332	16231	14008	29761	24340	14362	17941	38489
	21273	20020	21348	34336	40066	10112	19995	10716	14145	15757	30433	21407	11898	29522	15442
	5856.3	24176	34080	30103	38276	21686	20708	16639	6024.1	28875	30545	9024.3	12876	22496	21277
	3057.3	44983	34270	31648	36192	14292	13480	39328	5406.2	35761	26614	9024.3	18194	18889	23474
	3763	37750	38430	32358	66144	8015	12109	52634	5060	24314	30134	12442	6246.7	18773	13828
	9240.7	30205	32663	41767	56474	9073.6	7214.2	26191	4339.8	18525	29380	20710	20237	13372	8214.4
	39283	52559	16918	25612	25075	10623	14480	26484	16171	17796	22107	9769.8	18760	23712	20083
	12471	35961	25248	31942	56238	34610	19549	18394	15697	22550	30172	13531	29242	32451	33930
	14909	28761	33274	25478	65077	34095	15077	49309	15131	23136	35866	4246	15942	32659	14991
	12172	36370	26999	26139	50541	24145	11918	24611	7977.2	26680	23841	11222	21403	22387	54773
	11270	15382	15426	35255	33496	19065	24544	12562	10311	12223	38247	13363	30682	16355	12522
	27206	33009	31413	26711	40259	34760	14028	6244.5	9792	11684	45721	18058	41540	29634	56.38
	36747	27894	13467	24232	37294	25469	9603.6	13829	15142	10459	60737	23294	26746	14624	15802
	31310	25485	25132	24294	50239	19648	22754	23797	604.99	16611	52858	16122	19598	8943.6	27632
	27044	22260	25751	25783	36219	26131	23231	49617	192.4	27210	50045	12571	22691	33975	12397
	16684	25740	40620	28324	28226	19455	14148	30477	2019.6	19641	34239	12473	26563	28728	11386
Mean	**23333**	**30943**	**26190**	**28655**	**51486**	**21815**	**17063**	**26853**	**20907**	**25338**	**31309**	**16810**	**28811**	**24226**	**24776**

Table XXXIX: Right Leg quantitative Immunohistochemistry; Negative Controls.

	0/0	0/0	0/0	0/0	0/0	0/4	0/4	0/4	0/4	0/4	0/24	0/24	0/24	0/24	0/24	0/72	0/72	0/72	0/72	0/72
Rat No.	**9**	**22**	**23**	**31**	**35**	**49**	**55**	**58**	**59**	**61**	**19**	**21**	**33**	**34**	**36**	**5**	**11**	**18**	**20**	**27**
	4.47	1.27	0.61	0	1.27	1.3	0.61	0.64	2.59	1.26	112.2	1.29	0.64	0	1.3	0.66	0.66	0.61	0.64	1.97
	3.78	2.7	0	1.22	1.27	1.33	7.02	1.3	0	2	0.66	0.64	0.61	1.32	0.69	0.66	1.94	2.51	0.64	88.3
	2.79	17.25	1.33	0.66	0.61	1.33	2.68	1.26	0.61	2.48	0.61	0.69	2.73	20.03	0.64	1.26	1.33	0.61	0.66	2.56
	0	1.3	2.89	0	0	2.65	46.38	0	1.27	1.33	0.61	20.34	0.66	9.13	1.22	0	2.8	0.61	1.3	3.25
	2.89	0.61	2.53	1.27	1.29	2.62	18278	1.3	0.66	1.3	1.29	1.29	2.73	1.38	1.27	0.64	34.7	1.93	0.64	1.91
Mean	**2.786**	**4.626**	**1.472**	**0.63**	**0.888**	**1.846**	**3666.9**	**0.9**	**1.026**	**1.674**	**23.072**	**4.85**	**1.474**	**6.372**	**1.024**	**0.644**	**8.286**	**1.254**	**0.776**	**19.598**
	Uni 4/0	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**					
Rat No	**8**	**10**	**13**	**17**	**37**	**6**	**7**	**16**	**30**	**32**	**14**	**15**	**24**	**25**	**29**					
	0.64	57.6	1.26	1.27	0.61	0	326.6	0	2.65	1.27	0	5.61	0.61	1.88	2.05					
	1.27	0.64	1.26	1.29	0.66	0	497.2	0	836	2.05	1.27	0	0.61	1.33	1.3					
	1.26	0.61	1.26	0	1.3	0	266.5	0	13.76	1.99	1.26	0	1.94	0	1.93					
	0.61	48.99	0	0.61	1.22	0	1682	0	108	1.36	2.04	0	49.99	0.61	1.29					
	1.94	245.8	0.64	0	0.64	0	40.06	0	3.01	1.29	2.57	0.64	0	0.64	0.64					
Mean	**1.144**	**70.724**	**0.884**	**0.634**	**0.886**	**0**	**562.48**	**0**	**192.68**	**1.592**	**1.428**	**1.25**	**10.63**	**0.892**	**1.442**					
	Bilat 4/0	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**					
Rat No	**44**	**45**	**47**	**48**	**51**	**39**	**40**	**41**	**42**	**43**	**52**	**53**	**54**	**56**	**60**					
	0	0.64	0.64	3.23	3.87	2.93	1.57	3.19	3	1525	2.95	3.17	5.29	11.97	3.2					
	3.34	0	0.64	3.02	22.62	1.48	2.83	8.57	3.04	29.27	3.24	3.26	3.11	3.07	1023					
	0	0	0	25.53	3.13	9.1	37.53	3.28	2.82	26.83	2.91	2.98	3.26	3.1	3.31					
	1.22	1.33	0	3.14	3.2	2.94	11025	2.91	2.98	475.4	3.02	3.14	3.01	2.97	3.2					
	80.8	1.3	1.38	3.31	3.11	2.87	21306	2.8	126.7	66.36	2.98	3.17	3.1	3.04	3.14					
Mean	**17.072**	**0.654**	**0.532**	**7.646**	**7.186**	**3.864**	**6474.5**	**4.15**	**27.706**	**424.59**	**3.02**	**3.144**	**3.554**	**4.83**	**207.15**					

Table XL: Lung Quantitative Immunohistochemistry

	0/0	0/0	0/0	0/0	0/0	0/4	0/4	0/4	0/4	0/4	0/24	0/24	0/24	0/24	0/24	0/72	0/72	0/72	0/72	0/72
Rat No	**9**	**22**	**23**	**31**	**35**	**49**	**55**	**58**	**59**	**61**	**19**	**21**	**33**	**34**	**36**	**5**	**11**	**18**	**20**	**27**
	111884	28761	48586	59710	96372	5497.9	52307	50485	89981	151167	186136	72266	77751	18608	149737	109977	77127	4587.8	184373	52906
	97294	194530	42771	55268	68182	8381.8	12314	78666	46435	89575	184062	59324	17007	3729.1	127554	116172	11689	14384	113604	80219
	101322	156223	2601.7	19191	94224	92561	142181	18777	58215	55876	176204	50169	13898	53380	136851	166996	28307	15285	144813	47242
	95384	101969	16657	45716	97173	156580	130767	23481	88440	78805	119322	28170	19532	27393	90227	244473	41686	50158	164806	22319
	143136	106377	30389	80227	86967	134777	39133	67061	58059	67755	136943	48451	98439	14251	73104	119587	39771	61470	151248	39862
	105239	147205	16862	68202	38542	96971	56537	79093	80843	69543	134711	34386	63610	5291.4	142019	46933	31207	13649	117570	61579
	74475	152316	9582.3	104642	104827	163476	65846	91167	45750	41465	112154	34115	58920	2657	140657	5376.7	18239	3569.6	76261	70280
	94396	144511	10577	79218	56206	36410	35009	49205	50287	47479	81691	25938	39511	13357	146372	63713	24953	423.79	57683	57526
	80490	181134	8155.1	70109	29223	31195	9350.4	101159	74311	37094	69670	26143	50864	40700	127751	19512	36620	6514.7	17052	971.33
	19150	125794	24872	46740	9638.7	49957	7007.9	129197	116894	20537	67759	34513	32251	35707	135391	83837	36667	5924.5	26706	40586
	23339	113768	57167	78196	43677	84463	41150	146921	142543	10179	61575	50025	63936	27515	156021	130415	42416	13058	133913	107102
	14295	86099	58335	89715	66209	64916	41338	117488	125548	37112	117434	19583	92518	34169	144328	114693	37986	64138	117821	169723
	49760	186815	34289	86702	48028	56512	6717.1	102299	75040	47026	144532	35021	86385	8692.2	135138	184542	49153	27870	154131	99627
	78634	131708	64412	68402	72991	45771	30814	45414	118125	49534	130746	75924	81991	2793	148648	147447	33464	36689	207638	155990
	82387	154904	99361	88111	106152	79690	34904	61737	98198	41346	133834	69874	39561	21755	89496	194575	27672	57821	223977	131612
	88909	76938	124478	88696	101231	92917	15089	52679	60182	45910	117746	86874	60869	18700	75267	156380	25298	54752	226362	108318
	65913	73848	73965	50529	151890	38546	34593	118494	61123	49254	120546	110031	20445	29154	111273	81055	12191	13774	187494	58312
	70421	76314	60885	62588	163722	2038.4	147925	90944	78834	92052	165715	140490	16737	1472.1	144841	132124	10196	1470.2	133172	65024
	49283	116630	71628	87355	190761	115699	34413	33129	65992	144041	210347	97981	19052	37368	159775	141359	34823	28640	90496	131322
	58690	102886	65388	93894	97872	126307	36613	42287	41533	136877	240492	134444	14058	126292	141708	106877	74928	56761	88603	45006
	55026	53119	86134	126287	59708	175789	21503	6223.8	113389	107484	163954	111120	8311.2	204355	96754	112091	97867	54042	71289	100820
	157645	74685	91626	75811	65228	79177	27887	25107	104728	63441	220234	160138	92272	72226	111052	76807	74538	53398	89801	73377
	164430	165851	124899	57620	42659	86071	17854	12084	60202	35009	221780	145170	53802	87374	130110	85120	127547	101309	157895	86678
	124498	92985	125216	45811	172970	73070	5424.4	34914	82226	146184	222754	195581	60251	56211	110336	72482	116649	193810	140380	174018
	73796	118099	57442	146716	93752	49120	35080	101030	81708	68613	132129	183015	47795	22152	148174	165083	93236	161597	179239	76497

	126395	134295	39515	94447	159560	194580	148175	20715	50289	92298	129702	202590	51056	42171	141780	166152	69497	179544	185683	79569
	99487	158946	35605	78049	140804	87934	43532	104767	45128	65776	115771	148643	22385	72824	101374	214530	19773	173793	95799	85313
	92141	163375	66295	89959	96277	105491	188897	101748	85113	79575	140569	131300	31329	78373	117193	68099	29277	199393	160214	134523
	89484	91269	118412	66512	105625	67267	160417	105403	75481	71306	164459	171790	54308	119499	126775	51306	48860	168270	120274	111469
	80849	162905	118113	81642	120074	71694	119814	135807	69403	130703	114208	155934	9620.6	110494	259136	100154	38477	163537	111977	97820
	93201	155165	108529	63289	144412	197004	138024	109808	90750	105238	204388	157669	12303	129160	198280	178311	49563	273254	106822	61404
	56383	153912	78064	105442	138871	147764	138024	60047	109496	97768	167556	243120	56553	184378	208525	78805	126818	178448	154049	64627
	52422	115563	139924	80068	58574	87720	120029	74284	99786	112020	94703	143952	40536	105175	81082	52294	181040	85498	180118	46999
	56859	81233	165345	88467	83801	77431	101738	82077	153853	64647	136636	85437	60010	82956	43417	50055	120183	82487	181789	59633
	49127	89492	81914	78668	197724	50244	71416	131817	96867	89969	132508	135517	57074	65743	216257	44639	191075	112479	141174	41943
Mean	**82175**	**121989**	**67371**	**77200**	**97255**	**86658**	**66052**	**74443**	**82707**	**75504**	**144942**	**102991**	**46427**	**55888**	**133326**	**110913**	**59394**	**77480**	**134121**	**81149**
	Uni 4/0	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**					
Rat No	**8**	**10**	**13**	**17**	**37**	**6**	**7**	**16**	**30**	**32**	**14**	**15**	**24**	**25**	**29**					
	187686	135469	154530	149863	78125	6698.8	960.66	13494	1.29	16154	24127	115590	67451	40175	32975					
	209608	154001	186901	155411	86752	34542	5892.7	11098	47586	2233.7	68769	4983.6	95075	31852	10106					
	118889	232585	137185	185876	114384	82173	17214	11031	217.14	14751	59509	23112	11052	17287	4142.1					
	51784	139212	138608	172210	111880	70780	36325	6619.5	508.15	21174	72524	72360	5919.7	26900	4286.8					
	47790	131158	109285	156494	221183	20667	62119	34369	11357	17588	84318	5572.1	22480	10975	14249					
	129341	86443	200479	188401	162412	1199.6	62638	52759	50662	14170	29851	4043.7	34414	18963	10240					
	192908	104797	196360	199180	129147	37328	46484	97680	36901	25137	10798	14310	23246	13807	16959					
	138975	122249	146581	176789	79896	81592	60616	117458	925.26	4023.4	22521	34561	42159	35490	8049.2					
	182892	156719	159795	100335	43535	95671	86656	86357	3927.8	13357	4698	41389	55865	54151	15430					
	213544	151255	205241	149270	17201	63558	92161	64379	14306	40857	52250	54805	42436	33284	7352.7					
	200158	138416	138713	124260	94615	42243	110498	73966	3176.6	47655	63974	67252	53124	43141	3252.9					
	111528	86300	235491	179569	141896	30237	61451	79926	188.59	33768	59140	7552.7	56649	47185	1327.8					
	103809	154535	150707	155105	166304	44751	33000	55286	6388.9	29348	70441	15045	95750	28105	34516					
	66547	153928	169754	134474	142271	87271	101507	91529	18721	4260	130473	74519	57132	28183	17690					
	169491	116143	148900	109824	84981	120863	83294	120804	82933	21226	64917	103050	63779	22013	21221					
	149285	182527	200586	100928	89989	67552	83294	43514	134508	94013	14401	87511	46299	10691	25495					
	88116	91898	160821	179874	62969	101810	23511	25013	113964	85036	60948	27258	23063	10887	46186					

	90457	105633	165701	196433	93490	112821	18210	28048	81277	120106	70284	25789	32110	34640	22409
	106752	136911	144178	142468	107333	21732	2084.7	42893	47586	41473	92256	26748	18225	45084	41619
	78322	154123	152941	159882	109990	8534.1	451.99	47400	15591	81185	64868	44669	40816	37797	19514
	72832	138775	108184	203997	127166	25909	165.79	39875	33572	54140	35191	40687	18640	26380	33417
	74460	186718	47535	253504	104901	45316	1461.3	35269	2896.8	46352	61048	67624	53320	20185	11285
	87687	180287	25596	203757	99067	20329	4760.4	78563	18773	11575	9669.1	128120	27905	5201	13076
	75173	183403	77706	206055	86004	12773	32198	69632	82917	18911	26109	91048	33747	484.03	2675.7
	82425	111159	45688	184895	75073	27939	34576	69170	82689	76808	21542	82585	26211	1221.4	23003
	53196	72904	80421	174211	104756	308.32	60429	116305	108215	22509	31860	62880	109795	9369	10006
	149097	95316	102721	199021	123314	1511.5	60.28	72964	76097	15250	53468	78771	58708	25491	22750
	163025	82726	117053	193904	135276	1670	16.4	126530	70802	64264	17939	69117	44835	23641	14121
	150727	76866	150044	187952	122231	20737	139.4	116465	92608	37626	4547.8	78000	36226	23151	3543.9
	171165	110467	129801	93833	112552	7779.5	130.37	132215	144636	85589	56.19	29917	71213	17845	30.6
	114505	120170	115313	168485	116646	13330	17.87	153700	133062	37142	1146.4	71961	52437	20025	27191
	151536	153554	124702	124175	81775	10377	11.89	35265	164587	52770	4410.2	84822	35925	24859	14832
	155804	143351	134896	76364	36692	57546	550.96	28963	96424	77692	76026	6855.1	10120	19484	16878
	102997	192392	70568	85040	55923	73957	325.64	27434	126942	24739	62539	54438	4419.9	15536	9618.7
	99044	155276	110776	160483	52971	75288	1144.4	46475	47337	22190	22443	19342	2.85	8109.5	26835
Mean	**124044**	**135362**	**135536**	**160923**	**102077**	**43623**	**32124**	**64356**	**55780**	**39288**	**44259**	**51894**	**42016**	**23760**	**16751**
	Bilat 4/0	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**
Rat No	**44**	**45**	**47**	**48**	**51**	**39**	**40**	**41**	**42**	**43**	**52**	**53**	**54**	**56**	**60**
	402.02	12348	104.21	33091	5551.3	6430.8	1036.1	37.88	19741	16144	14530	163656	122824	2109.1	33307
	2360.2	6414.1	5758.4	39468	11757	7897.8	553.48	32.54	28973	1839.6	74593	109529	131919	2124.3	23214
	16192	2439.9	3270	31924	16174	5590.2	3022.5	27	20922	745.16	70421	113538	100288	811.97	11109
	13941	644.79	3035.3	12026	17488	7568.6	12129	10293	31198	20506	93530	188036	133916	488.03	6329.4
	11817	31.23	1851.9	27474	12696	13512	55012	25384	24717	36635	60857	87469	115661	342.9	22325
	20522	67.09	1806.2	37050	13972	20992	28194	43025	35423	36747	27313	16060	100190	194.91	27511
	8176.6	1173.5	12458	35850	29436	30643	41698	36032	1546.5	4582.8	134824	16710	65754	0.61	29918
	25959	17.26	680.13	15523	29436	13535	65729	31947	75654	22352	129436	11519	27474	26.08	25508
	26914	15.65	7513.5	7991.2	17411	5409	30319	23411	48975	61823	184188	20893	19695	38681	57534

	33303	342.43	11977	7212.6	26098	8416.8	38863	10173	18346	47200	156742	51151	18861	11400	66954
	31957	117.94	22133	8339.2	29574	10910	18261	14135	27820	16500	162271	84419	16889	26389	61297
	41777	253.47	23123	26244	40617	10258	49487	9826.8	58710	16365	94219	105551	100573	51767	27775
	16591	9630.7	18251	49226	32427	12325	66299	92821	47319	7511.5	84662	64228	110610	11619	7320
	7103.7	21791	4328.5	16251	31494	25299	71939	78518	24060	5800.3	124800	43357	133490	8194.5	35394
	8701.7	5918.2	63.68	12345	31179	22303	95532	40861	29088	908.97	70101	50938	153781	5501.6	50929
	3396.8	13092	33.55	12673	30615	6347.5	93948	18187	5994.8	55.52	54990	45076	159934	651.86	60079
	14750	20008	13539	33276	9838.4	5718.5	47857	25888	8019.4	6049	60378	61277	136498	53091	35644
	18741	21314	22532	31113	14601	4869.2	36391	26536	14457	15578	23593	95756	69238	36295	39737
	14354	41154	5940.4	18074	13299	29065	41190	41565	18456	19135	56912	78281	33350	4323.4	67914
	23221	21809	10522	23065	40666	26444	11816	53546	46221	3602.8	86194	74615	23881	12428	91629
	57515	20210	23217	29819	34630	12060	22124	48691	45351	1132.5	90445	69604	24268	7303.6	109277
	66593	32665	11626	306.98	13300	14780	55193	41188	30351	1132.5	86362	46295	27101	10731	63934
	75174	10539	13943	32578	3811.1	12252	37451	21141	44512	24419	127513	35914	12685	7681.9	97024
	33950	18731	7131.3	12959	5767.2	16125	24691	8173.6	55234	6499.3	79618	67083	6569.4	981.62	92994
	17256	18767	14768	5629.5	16250	42361	14979	3195.9	61732	15041	44135	57426	17314	7298	68818
	103502	6208.2	13028	5651.4	1795.6	35157	19416	16650	58246	8805	1638.9	51427	7685.2	10.41	98415
	81566	1312.7	12214	19694	47619	25089	12081	21171	56780	10259	149626	48690	17988	65.69	111905
	51725	72885	13868	3111.2	26712	24267	8723.8	53834	43243	20631	167478	58344	31623	10706	150795
	26436	46084	25430	13033	36164	30430	7884.2	64775	27539	54519	167478	12235	14235	6831.1	111352
	13070	32935	43330	8383.9	6572	15765	11611	43054	9009.5	68434	110226	14446	18312	10150	64767
	26720	22064	15546	6678.6	10119	7816.9	8491.3	45516	8049.3	49959	148705	20306	23366	4329.4	32634
	16729	29691	16830	30310	19595	11090	20972	3479.4	4316.5	29983	104728	36543	5301.6	10748	74214
	6630.2	39516	4560.9	25360	35968	9028.6	26746	20696	13454	49560	44719	59482	11633	21127	72956
	6405	19656	560.52	39814	18451	9307.8	24434	17982	6704.3	43170	30080	74658	61372	3216.6	71531
	3638.8	30114	587.38	36053	3405.9	23424	36329	27314	40491	29606	88452	72689	594.44	223.82	57724
Mean	**26488**	**16570**	**11016**	**21360**	**20985**	**16071**	**32583**	**29117**	**31161**	**21521**	**91593**	**63063**	**58711**	**10510**	**58851**

Table XLI: Lung Quantitative Immunohistochemistry; Negative Controls

	0/0	0/0	0/0	0/0	0/0	0/4	0/4	0/4	0/4	0/4	0/24	0/24	0/24	0/24	0/24	0/72	0/72	0/72	0/72	0/72
Rat No	**9**	**22**	**23**	**31**	**35**	**49**	**55**	**58**	**59**	**61**	**19**	**21**	**33**	**34**	**36**	**5**	**11**	**18**	**20**	**27**
	0	0	0	0	0	311.3	0	0	0	0	0	217.8	4.72	0	53.18	0	144.3	6.9	286	0
	0	0	0	0	0	40.35	0	3.29	0	0	0	0	133.8	0	1.48	45.56	0	27.23	559.1	0
	0	0	0	0	1.32	0	0	1934	0	0	0	0	217.8	0	1.22	0	0	0	84.16	2.51
	0	0	0	0	0	0	0	8262	0	0	2.57	0	6.51	0	122	2757	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	14.57	104.4	0	262.3	0	0	0	2.25	0
Mean	**0**	**0**	**0**	**0**	**0.264**	**70.336**	**0**	**2039.9**	**0**	**0**	**0.514**	**46.468**	**93.442**	**0**	**88.034**	**560.49**	**28.858**	**6.826**	**186.3**	**0.502**
	Uni 4/0	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**					
Rat No	**8**	**10**	**13**	**17**	**37**	**6**	**7**	**16**	**30**	**32**	**14**	**15**	**24**	**25**	**29**					
	2.14	110.1	0	96.51	0	15.43	0.61	0	0	0	1677	0	0.61	150.1	16.7					
	13.75	3.37	0	0	0	80	6920	0	0	0	0	0	0	26.62	0					
	28.04	16.7	0	0	0	0	4.71	0	0	0	0	47.6	1.3	15.52	77.07					
	2.45	217.4	17.41	0	0	140.7	175.3	1.3	3.26	108.5	0	0	0	33.6	0					
	1.87	24.9	0	0	0	0	2.59	0	2.57	0	0	0	0	37.75	0					
Mean	**9.65**	**74.488**	**3.482**	**19.302**	**0**	**47.228**	**1420.7**	**0.26**	**1.166**	**21.704**	**335.31**	**9.52**	**0.382**	**52.712**	**18.754**					
	Bilat 4/0	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**					
Rat No	**44**	**45**	**47**	**48**	**51**	**39**	**40**	**41**	**42**	**43**	**52**	**53**	**54**	**56**	**60**					
	0	10.32	154.6	0	199	0	2.71	3.96	0	28.39	19.11	0	10.37	0	0					
	1.22	65.56	0	205	58.1	0	6.23.	13.6	0	10.41	0	0	22.63	13.94	154.2					
	0	155.3	54.32	12.79	28.9	0	0	0	3.34	0	0	22.76	96.21	0	3.77					
	0	327.8	0	33.44	0	0	0	0	7.38	2.71	0	0	4.12	0	0					
	39.93	86.66	0	145.8	0	39.94	1.96	0	37.92	0	0	0	642.1	24.96	0					
Mean	**8.23**	**129.14**	**41.792**	**79.414**	**57.196**	**7.988**	**1.1675**	**3.512**	**9.728**	**8.302**	**3.822**	**4.552**	**155.08**	**7.78**	**31.602**					

Table XLII: Kidney Quantitative Immunohistochemistry

	0/0	0/0	0/0	0/0	0/0	0/4	0/4	0/4	0/4	0/4	0/24	0/24	0/24	0/24	0/24	0/72	0/72	0/72	0/72	0/72
Rat No	**9**	**22**	**23**	**31**	**35**	**49**	**55**	**58**	**59**	**61**	**19**	**21**	**33**	**34**	**36**	**5**	**11**	**18**	**20**	**27**
	28999	130164	121054	43860	58169	93705	80757	81289	35436	91324	90926	62262	74368	52953	57814	105490	119342	127981	112196	33710
	38590	71515	104665	47902	59852	69597	98534	58447	43895	74040	103901	48765	70517	55379	73569	57689	111190	150339	89580	54673
	28999	55986	104665	33013	58929	88531	89928	67351	55865	98806	122466	54775	88003	68850	75267	114328	97027	110181	59179	44828
	28795	42359	133089	41922	44642	81340	52638	83937	68538	61484	80705	72009	88672	52932	66261	75123	98087	133914	84375	52996
	20263	62162	98565	38524	49143	115298	48671	66997	67684	63423	85539	105851	66945	46937	64870	107697	79845	90599	90543	62750
	15453	61292	91977	40167	55737	88804	68870	54890	65317	83621	78448	70001	77584	64376	58181	87801	111951	143604	94666	60199
	15015	71526	134433	48094	57152	86015	70759	65443	66985	90073	105992	63949	92337	84729	65468	88553	84877	98287	68173	56553
	24434	55293	82533	69022	60860	59371	72778	80113	61014	93760	85215	76623	81373	63734	48009	119582	104869	138263	78938	130585
	44002	61971	56113	56337	72584	81102	79449	61110	116766	70778	82350	90318	55928	78492	66334	134580	84069	95840	81127	135453
	73072	50897	41954	49139	68155	66297	92418	62972	71649	54177	85770	79995	84777	64253	47664	145472	88145	133459	80500	93034
	83514	55642	53446	76470	47633	51148	71055	63252	67312	92858	110947	77806	127651	65209	82439	127896	106507	167382	89470	51815
	43603	57384	48716	56924	59286	64160	49010	72702	92844	103062	90255	81632	142866	107165	71384	157914	117488	108848	110735	58696
	38876	57051	19382	60663	54933	58312	51523	46889	87617	120210	117189	76247	142866	82434	63579	178418	125458	76929	89520	76801
	58723	60583	55488	70220	60189	61529	49149	66104	84237	112584	103853	90334	118211	69059	56545	132324	111673	74819	60073	76584
	50833	79665	97844	80091	61003	83020	58855	61062	73051	87135	123277	78625	159299	55328	61439	128275	120720	70087	73569	72131
	54728	59787	50868	105155	114259	57940	58456	74831	59719	80117	97320	78244	110021	82986	65447	149773	112962	93355	82590	65326
	59374	75108	51721	89052	130317	55795	52507	56823	99719	52105	90234	85766	108195	82548	76137	191805	111578	141424	92262	56787
	77201	59011	63882	83230	137063	62353	32559	63583	70738	68148	100014	93633	91117	73417	72434	194401	114195	102508	90677	52320
	50996	64159	62916	87536	138880	61113	53289	51592	78829	82587	130952	77262	103151	72783	66266	223916	111157	65831	61209	83698
	53817	63330	53427	70195	58104	44262	52940	64708	100144	66853	101219	75317	75728	72151	95123	119474	101482	74014	54159	92412
	48453	58822	63772	99058	80972	25006	51211	69279	104628	77630	173817	83941	73026	66910	42441	133797	111903	94363	78010	76343
	56237	62606	59391	70295	75352	37960	57074	86915	83909	74043	169113	85278	47239	59317	60083	115928	86114	56544	68992	77472
	61384	59277	48727	79102	62115	40141	63093	70451	38116	98234	103958	96189	94526	71097	49794	76959	56454	70022	83476	71714
	49608	75494	70398	74164	71611	57822	133375	65736	67704	81245	81322	84183	101079	96530	59344	118216	128859	84943	79634	58279

	41213	71561	79740	67298	57433	46035	52524	53281	71283	87704	101633	61840	96286	71274	67181	131185	63860	88299	89895	127243
	45989	65459	127162	89417	80559	47700	50387	60473	75017	75724	190003	57706	75373	75657	69008	150116	51994	101519	78041	115370
	49499	58394	85484	79112	78844	46632	52602	54161	111629	83161	74556	56322	73983	68284	58499	147804	50357	77671	69840	73139
	49418	79994	59164	75786	80064	49680	83135	53609	98750	83009	82201	62418	72056	83619	58452	128654	45098	91149	39870	81254
	64066	78492	64514	101441	80442	39704	65259	55491	96926	61214	77608	67193	85411	91275	60662	164372	99291	83755	83813	89054
	81438	61980	62980	73844	85929	23762	68950	59163	83092	56003	67819	74570	70242	77819	88229	144687	95510	82049	76726	71277
	66191	47353	59328	74197	83135	75562	49856	121268	125801	61449	84735	90996	74652	82072	75672	141110	87114	75713	53301	66515
	65101	49830	38030	77467	68023	62562	59590	77813	83558	60432	81233	68774	90714	62977	81844	105482	64251	89174	39830	66172
	51153	51637	51464	64115	86999	92404	88420	186085	70460	50786	78544	59566	64552	56981	86177	133929	77124	68618	33674	71480
	56479	47527	59278	62165	111121	85971	52901	67586	74838	47031	91228	63815	66891	49200	80415	90512	68921	69219	94305	85268
	80118	47674	39352	78483	74936	65986	59487	71479	75799	70813	121730	76134	108001	52134	62415	168806	84069	78087	82853	95098
Mean	**50161**	**63171**	**71301**	**68956**	**74984**	**63618**	**64915**	**70197**	**77968**	**77589**	**101888**	**75095**	**90104**	**70310**	**66698**	**131202**	**93815**	**97394**	**77023**	**75344**
	Uni 4/0	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**					
Rat No	**8**	**10**	**13**	**17**	**37**	**6**	**7**	**16**	**30**	**32**	**14**	**15**	**24**	**25**	**29**					
	88144	54357	74993	44711	14282	33007	51678	32036	31481	54125	33165	44284	43445	54514	33467					
	57602	59706	68166	61654	48715	34787	67874	42220	1700.3	49102	23536	34264	39336	47833	206.61					
	77202	64810	79283	53225	54043	35684	66332	20263	27193	57338	59296	24521	44487	45252	16878					
	101612	51266	74533	53516	55537	25589	77430	52211	39385	56443	10059	9828.5	20290	63464	29796					
	92397	44537	60674	73835	41455	10273	71901	74368	55322	45354	18283	57861	31776	51856	31902					
	95143	49565	74665	74202	47070	32351	74299	54199	44657	50226	29911	49919	24302	47386	23868					
	61398	61113	74555	60837	64317	32079	40825	52339	37931	45683	85650	49776	20059	52103	46064					
	105162	63933	64304	62736	57097	26019	60723	53672	39183	44338	58721	27219	34568	49229	52758					
	92131	84221	45153	75515	75129	36183	63517	55500	72640	57591	70889	41779	33705	51459	56428					
	103888	79725	41025	56091	35620	37677	63117	5924.5	79269	69411	74964	48256	30593	54345	58149					
	75621	73794	42410	53453	25510	53842	67058	17733	63217	68596	79257	63127	41100	52290	54565					
	62547	79403	63155	67657	15000	40864	71953	33583	68425	69324	43389	42963	40878	61821	59585					
	49462	93660	50185	46611	3136.4	25677	75264	10387	61115	64676	59980	60880	27741	49836	75235					
	55845	63405	70084	37538	23443	3061.2	70168	27641	54821	81918	52085	75106	30092	57496	52936					

	49960	47229	55695	33774	23776	15019	60057	40281	57235	84895	57351	60137	35788	47415	49573
	55229	45684	62245	38713	28410	29481	76101	8185.3	39935	67959	76365	51055	35972	55831	49742
	49072	51112	62138	48898	27500	36531	71099	12277	32269	54939	63656	53340	36134	55945	50060
	71572	61310	60183	54546	47879	74562	65534	23649	29485	49263	41730	59995	38510	57207	58110
	38815	76423	58780	50941	38812	65720	71611	48129	38747	56575	32359	57603	60482	48010	49654
	52302	52614	53897	57690	34257	86391	83624	64922	62189	79059	53128	58310	48691	32825	47861
	51107	43459	46965	54426	39648	77867	79801	70330	65815	84599	29913	32401	53036	46733	69446
	51305	27049	41091	46348	33767	63665	82432	30276	57984	75201	43780	2015.2	9852.9	52147	69423
	65846	47820	38411	65867	43137	77766	51826	35282	45247	74152	40220	27359	21143	43348	63773
	65936	51500	42947	58374	57688	61484	77476	43592	48574	74116	35042	45397	32508	45168	55986
	69738	37263	32377	56334	34010	85162	53908	63748	65812	70518	46977	56972	24792	55605	59500
	79063	53221	51429	42615	42152	74564	49899	86198	57035	43260	69614	67712	19593	48415	67423
	69893	33371	42353	43035	43998	78074	38383	32592	63559	37374	78849	19614	29587	40105	45713
	68406	26347	32606	52965	57395	56502	59547	22635	74739	28152	43240	43338	34948	43282	58458
	67526	13787	10454	68783	36017	68676	56998	4291.9	78281	44294	52164	55840	35202	39885	66406
	74333	27987	22427	60600	14431	66428	63061	26554	72612	350.92	38366	43022	34815	40168	49013
	62447	49204	38572	49699	5574.9	68771	64418	61381	58324	16334	52418	28527	30112	27352	65511
	83715	62816	47561	48187	168	61558	79696	54998	37424	17546	33119	36617	18033	27787	46786
	62111	59802	39909	44012	18241	71209	53987	31567	32406	47724	52059	52089	46612	24448	45767
	83396	56320	41100	65630	7682.6	68313	28615	685.3	51850	31713	36647	51210	45467	68165	31488
	75599	25796	42038	50134	21620	80482	6290.9	71532	43881	36792	35569	38613	38229	42875	3471.1
Mean	**70444**	**53532**	**51610**	**54661**	**34758**	**51295**	**62757**	**39005**	**51136**	**53970**	**48907**	**44884**	**34054**	**48046**	**48429**
	Bilat 4/0	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**
Rat No	**44**	**45**	**47**	**48**	**51**	**39**	**40**	**41**	**42**	**43**	**52**	**53**	**54**	**56**	**60**
	95054	91197	57459	5755.3	72554	20156	38406	15224	35711	45099	198.01	27258	34058	17956	5182.7
	46177	95204	69130	49186	59867	21716	43769	45563	46151	32807	2495.2	38302	10156	12970	4531.5
	74103	82836	81378	19314	63595	22549	69083	45430	37899	56134	4830.1	37602	44971	43677	34983
	84754	73767	79537	26108	70217	28177	64781	61869	37355	39844	548.26	32064	36126	43544	35549

	84684	107547	59086	63135	84843	25590	54989	61438	39148	34404	3435.1	42767	58990	47146	13307
	77571	100174	60086	33248	81920	37087	53727	56385	49414	65892	11.6	62274	37795	60601	8437.2
	62179	84473	64410	42449	80676	36466	55157	61421	82299	52941	413.03	56988	33012	61840	33942
	51961	85293	80371	49063	74521	53773	62777	58557	46665	51410	337.65	33896	50154	55112	44128
	59780	67597	73239	83533	87281	50065	55268	45931	53508	50661	358.37	25338	72641	63502	47771
	72175	65439	85915	80450	74703	40988	54626	51744	58034	68067	625.96	6321.4	43779	62798	46500
	63258	82697	68941	69950	56016	30747	44714	48584	70136	44626	321.66	57032	44777	63209	56354
	80964	72556	114175	72036	35042	34795	48385	74673	66562	49603	1060.6	65040	58644	51269	51602
	75466	75836	59648	70424	11965	22403	48935	55974	51739	52562	4930.2	58771	32525	21199	41969
	51614	74241	67176	36554	0	66337	65711	70073	41195	68782	3937.5	66661	63969	42382	57299
	65162	88289	50153	21742	8576.7	53378	36680	48198	39450	56062	569.92	52368	60420	41567	58498
	47495	68419	36109	22585	21063	62498	8060.9	37784	63468	50040	515.88	61686	55770	59745	52450
	52711	64802	34589	32453	38602	60470	40079	29720	53584	45895	173.04	17087	73085	66495	51191
	71366	55848	32098	49136	39330	61296	45173	14629	53730	45799	147.05	11627	57319	73145	51191
	78152	44832	48617	68951	37783	44665	49862	2043.3	49871	50078	1702.4	17409	57365	51443	71424
	47072	48275	54580	45640	43648	47167	36245	70022	11471	42588	3022.1	41464	65062	60148	61037
	45529	49991	60877	64249	39800	18317	11058	46702	19864	47488	3834.2	48623	59589	47995	65027
	47153	59139	46841	66987	54711	47150	43400	52058	51662	47037	2515.9	18588	63463	54585	57526
	60611	63172	77229	18244	63234	75553	7919.1	38763	55957	45630	2858.7	70761	51840	74488	53152
	76474	52653	54580	32334	54629	64476	9565.5	51215	74981	49922	411.77	71302	77851	74488	34379
	68689	65396	55148	48237	62657	53264	16449	53763	60716	44598	2859.3	26610	69450	59342	26578
	79484	49632	61958	10779	29472	71573	12375	55514	60708	37900	7843.6	307.03	55988	66300	29306
	92921	31325	57019	49876	16118	87812	7227.5	62021	61779	38787	9611.9	1.38	67468	74087	37669
	103614	27447	86517	36253	4760.6	33408	5454.7	41676	46966	21563	5736.4	29463	63017	61456	50483
	89554	55692	32075	64322	20001	24070	19214	50883	52605	24388	6674.4	23863	68210	73350	52901
	104547	58502	38885	63260	40283	38542	30919	47468	32815	31216	3490.3	55796	85702	78556	39726
	80960	45329	6567.1	49410	48879	56288	9858.3	44624	45914	33014	2369.9	75937	46881	62131	21719
	57772	66225	9319	41584	47504	48242	22630	48921	50653	14479	1386.3	74506	64579	67462	3176.4

	55516	27676	12795	52087	50878	44468	27388	45245	58372	2873.1	229.73	64771	63235	45831	31286
	56498	63966	82769	61497	39600	13021	1510.7	54206	57942	18065	606.86	68382	60998	48190	47396
	64393	57220	65163	63648	6503.6	19606	516.78	55934	75207	21120	137.3	75595	60489	36112	42969
Mean	**69298**	**65791**	**57841**	**47557**	**46321**	**43317**	**34340**	**48693**	**51244**	**42325**	**2291.4**	**43328**	**55697**	**54975**	**40590**

Table XLIII: Kidney Quantitative Immunohistochemistry, Negative Controls

	0/0	0/0	0/0	0/0	0/0	0/4	0/4	0/4	0/4	0/4	0/24	0/24	0/24	0/24	0/24	0/72	0/72	0/72	0/72	0/72
Rat No	**9**	**22**	**23**	**31**	**35**	**49**	**55**	**58**	**59**	**61**	**19**	**21**	**33**	**34**	**36**	**5**	**11**	**18**	**20**	**27**
	1.29	0	0	0	0	80.27	0	0.64	0	0	0	11.98	0	0	9.83	0	0	0	0	56.46
	6.71	7.27	0	2.53	2.45	35.21	0	0.61	21.23	0	0	0	13.93	0	0	370.8	14.5	311.1	0.61	0
	1.38	0	0	11.6	10.42	2.48	4.52	6.56	4.08	14.87	115	2.53	0	0	0	149.8	0.61	39.88	1.41	1.88
	153.2	0	0	88.02	1172	0	0.61	16.6	0	0	239.9	3.8	21.06	0	1.26	4773	162.2	10.91	0	2.45
	4.31	32.32	0	156.7	0	0	5.28	7.39	0	111.4	0	2.72	0	0	0	3.19	29.67	8.53	1.41	1.38
Mean	**33.376**	**7.918**	**0**	**51.772**	**237**	**23.592**	**2.082**	**6.36**	**5.062**	**25.254**	**70.974**	**4.206**	**6.998**	**0**	**2.218**	**1059.4**	**41.402**	**74.076**	**0.686**	**12.434**
	Uni 4/0	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/0**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/24**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**	**Uni 4/72**					
Rat No	**8**	**10**	**13**	**17**	**37**	**6**	**7**	**16**	**30**	**32**	**14**	**15**	**24**	**25**	**29**					
	12.6	20.85	4.06	20.57	1.27	4829	1.45	3.33	1.29	1.44	1.38	29.11	1.26	1.35	1.3					
	233.7	8.86	14.14	39.55	0	2560	1.3	1.41	1.3	85.61	1.38	116.1	213.1	3.96	3.89					
	85.48	38.11	476	1.33	0	1340	1.22	1.3	1.35	3.31	1.38	1.32	1.41	1.45	1.27					
	84.68	27.85	256.6	32.6	1.29	1.35	112.6	1.29	1.51	1.41	1.35	1.33	1.35	3.31	1.3					
	5.78	512.8	288.1	180.5	1.38	9139	13.61	54.78	14.28	1.38	14.45	0.61	67.3	1.29	1.3					
Mean	**84.448**	**121.68**	**207.79**	**54.906**	**0.788**	**3573.9**	**26.036**	**12.422**	**3.946**	**18.63**	**3.988**	**29.7**	**56.878**	**2.272**	**1.812**					
	Bilat 4/0	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**					
Rat No	**44**	**45**	**47**	**48**	**51**	**39**	**40**	**41**	**42**	**43**	**52**	**53**	**54**	**56**	**60**					
	0	183.1	1.27	78.81	2.91	1.41	1.33	1.33	1.29	1.38	1.26	1.38	7.31	1.41	1.29					
	0	1.35	77.42	1.48	3.3	2.52	6.31	1.41	1.32	34.75	53.5	56.85	3455	1.35	6.71					
	0	4.74	4.77	22.19	104.8	1.29	2.61	1.29	1.27	1.32	1.27	93.39	22.75	3.94	1.38					
	0.61	1.29	132.6	1.32	1635	23.1	1.35	1.35	1.38	18.27	0	7.03	1.3	1.33	153.2					
	1.48	81.03	19.96	1.48	91.96	1.27	40.17	1.29	1.38	3.38	0	36.65	2.57	1.29	4.31					
Mean	**0.418**	**54.302**	**47.21**	**21.056**	**367.64**	**5.918**	**10.354**	**1.334**	**1.328**	**11.82**	**11.206**	**39.06**	**697.76**	**1.864**	**33.376**					

7.6.7 Quantitative Immunohistochemistry, Summary Data Sets

For the section 7.6.7: Sham 0/0 – refers to sham-operated rats with 4 hour anaesthetic and euthanased immediately. Sham 0/4 refers to sham-operated rats with a 4 hour anaesthetic and sacrificed after a further 4 hours. Sham 0/24 refers to sham-operated rats with a 4 hour anaesthetic and sacrificed a further after 24 hours. Sham 0/72 refers to sham-operated rats with a 4 hour anaesthetic and sacrificed after a further 72 hours. Uni refers to an animal that underwent four hours of left leg unilateral ischaemia and then was either sacrificed immediately (Uni 4/0), after 4 hours of reperfusion (Uni 4/4), after 24 hours of reperfusion (Uni 4/24) or after 72 hours of reperfusion. Bilat refers to animals that underwent four hours of bilateral lower limb ischaemia and then was either sacrificed immediately (Bilat 4/0), after 4 hours of reperfusion (Bilat 4/4), after 24 hours of reperfusion (Bilat 4/24) or after 72 hours of reperfusion.

All slides were prepared as described in Chapter 4.2.

The value for brightness for each animal is the mean of the 35 images brightness levels as shown in Section 7.6.6.

Mean refers to the arithmetic mean of the brightness levels of the 5 rats in each group. St Dev refers to standard deviation.

Table XLIV: Left leg Quantitative Immunohistochemistry, Summary of Data

Rat Number	Sham 0/0	Rat Number	Uni 4/0	Rat Number	Bilat 4/0
9	58819.05	8	30343.54	44	94782.66
22	44999.05	10	39430.5	45	43270.69
23	69423.42	13	48060	47	30136.93
31	70356.46	17	42772.15	48	43779.25
35	62080.74	37	16040.78	51	15892.29
Mean	**61135.74**	**Mean**	**35329.39**	**Mean**	**45572.36**
St Dev	**10251.34**	**St Dev**	**12561.76**	**St Dev**	**29788.11**
Rat Number	**Sham 0/4**	**Not Done.**		**Rat Number**	**Bilat 4/4**
49	64349.97			39	8926.722
55	53109.53			40	1302.62
58	53891.89			41	25584.52
59	69188.22			42	28377.88
61	56884.2			43	35246.35
Mean	**59484.76**			**Mean**	**19887.62**
St Dev	**7009.174**			**St Dev**	**14194.83**
Rat Number	**Sham 0/24**	**Rat Number**	**Uni 4/24**	**Rat Number**	**Bilat 4/24**
19	31715.48	6	3776.107	52	13847.89
21	60930.18	7	44143.63	53	17032.83
33	40200.3	16	41249.35	54	5296.735
34	53042.16	30	45135.28	56	20165.7
36	47291.81	32	34577.57	60	29715.59
Mean	**46635.98**	**Mean**	**33776.39**	**Mean**	**17211.75**
St Dev	**11289.74**	**St Dev**	**17269.97**	**St Dev**	**8921.701**
Rat Number	**Sham 0/72**	**Rat Number**	**Uni 4/72**	**Not performed in this Section**	
5	98250.84	14	1065.083		
11	52179.21	15	1065.083		
18	64898.19	24	45623.82		
20	63250.66	25	18485.05		
27	81927.98	29	39330.88		
Mean	**72101.37**	**Mean**	**21113.98**		
St Dev	**18079.48**	**St Dev**	**20877.11**		

Table XLV: Left leg Quantitative Immunohistochemistry Including Bilateral 4/72, Summary of Data

Bilat 4/0	
44	31030.19
45	44595.08
47	41590.65
48	43953.41
51	24772.61
Mean	**37188.39**
St Dev	**8832.886**
	Bilat 4/4
39	41686.43
40	31612.96
41	36436.18
42	33345.39
43	18486.69
Mean	**32313.53**
St Dev	**8625.32**
Bilat 4/24	
52	22474.53
53	34214.73
54	9975.724
56	23085.42
60	39626.77
Mean	**25875.44**
St Dev	**11519.49**
Bilat 4/72	
67	17949.83
68	15806.85
69	17713.07
70	35560.4
71	32485.34
Mean	**23903.1**
St Dev	**9338.824**

Table XLVI: Right leg Quantitative Immunohistochemistry, Summary of Data

Mean refers to the arithmetic mean of the 35 images. St Dev refers to the standard deviation.

Rat Number	Sham 0/0	Rat Number	Uni 4/0	Rat Number	Bilat 4/0
9	32827.99	8	32214.6	44	23332.52
22	34603.32	10	39087.76	45	30943.29
23	29500.77	13	39515.31	47	26189.67
31	30946.26	17	35305.38	48	28654.55
35	28680.08	37	37660.93	51	51485.59
Mean	**31311.69**	**Mean**	**36756.79**	**Mean**	**32121.13**
St Dev	**2422.414**	**St Dev**	**3024.832**	**St Dev**	**11189.38**
Rat Number	**Sham 0/4**	**Not Done**		**Rat Number**	**Bilat 4/4**
49	37523.86			39	21814.94
55	47864.54			40	17063.08
58	31728.56			41	26852.94
59	34976.92			42	20906.57
61	31459.67			43	25338.36
Mean	**36710.71**			**Mean**	**22395.18**
St Dev	**6716.964**			**St Dev**	**3857.394**
Rat Number	**Sham 0/24**	**Rat Number**	**Uni 4/24**	**Rat Number**	**Bilat 4/24**
19	29467.84	6	30657.93	52	31308.56
21	33407.51	7	57299.35	53	16809.55
33	28281.28	16	29370.82	54	28811
34	25334.94	30	32827.99	56	24225.65
36	39431.16	32	44954.7	60	24775.64
Mean	**31184.55**	**Mean**	**39022.16**	**Mean**	**25186.08**
St Dev	**5443.852**	**St Dev**	**11944.95**	**St Dev**	**5520.007**
Rat Number	**Sham 0/72**	**Rat Number**	**Uni 4/72**	**Not Done**	
5	26902.32	14	41946.45		
11	27675.6	15	34838.75		
18	39585.63	24	39386.72		
20	35701.11	25	36792.13		
27	50408.8	29	30349.82		
Mean	**36054.69**	**Mean**	**36662.77**		
St Dev	**9651.527**	**St Dev**	**4430.982**		

Table XLVII: Lung Quantitative Immunohistochemistry, Summary of Data

Mean refers to the arithmetic mean of the 35 images. St Dev refers to the standard deviation.

Rat Number	Sham 0/0	Rat Number	Uni 4/0	Rat Number	Bilat 4/0
9	82175.49	8	124044.4	44	26488.29
22	121989.3	10	135361.9	45	16570.23
23	67371.21	13	135536.1	47	11016.11
31	77200.03	17	160923.5	48	21360
35	97255.03	37	102077.2	51	20985.46
Mean	**89198.21**	**Mean**	**131588.6**	**Mean**	**19284.02**
St Dev	**21271.23**	**St Dev**	**21320.14**	**St Dev**	**5805.797**
Rat Number	**Sham 0/4**	**Not Done**		**Rat Number**	**Bilat 4/4**
49	86657.7			39	16071.13
55	66052.02			40	32582.85
58	74443.25			41	29117.36
59	82707.24			42	31161.48
61	75504.46			43	21520.91
Mean	**77072.93**			**Mean**	**26090.75**
St Dev	**7975.729**			**St Dev**	**7040.882**
Rat Number	**Sham 0/24**	**Uni 4/72**	**Uni 4/24**	**Rat Number**	**Bilat 4/24**
19	144942	6	43622.66	52	91593.09
21	102991.4	7	32124.44	53	63062.9
33	46426.85	16	64355.72	54	58710.68
34	55887.89	30	55779.54	56	10509.8
36	133325.8	32	39287.75	60	58850.51
Mean	**96714.78**	**Mean**	**47034.02**	**Mean**	**56545.4**
St Dev	**44444.72**	**St Dev**	**12946.65**	**St Dev**	**29155.17**
Rat Number	**Sham 0/72**	**Rat Number**	**Uni 4/72**	**Not performed in this section**	
5	110913.5	14	44258.86		
11	59394.1	15	51894.03		
18	77479.94	24	42015.72		
20	134120.7	25	23759.76		
27	81148.99	29	16750.92		
Mean	**92611.45**	**Mean**	**35735.86**		
St Dev	**29672.08**	**St Dev**	**14807.26**		

Table XLVIII: Kidney Quantitative Immunohistochemistry, Summary of Data

Mean refers to the arithmetic mean of the 35 images. St Dev refers to the standard deviation.

Rat Number	Sham 0/0	Rat Number	Uni 4/0	Rat Number	Bilat 4/0
9	50160.94	8	70443.64	44	69297.52
22	63171.05	10	53531.74	45	65791.01
23	71300.62	13	51610.39	47	57841.1
31	68956.04	17	54661.49	48	47556.56
35	74983.61	37	34757.68	51	46320.95
Mean	**65714.45**	**Mean**	**53000.99**	**Mean**	**57361.43**
St Dev	**9695.501**	**St Dev**	**12669.23**	**St Dev**	**10389.73**
Rat Number	**Sham 0/4**	**Not Done**		**Rat Number**	**Bilat 4/4**
49	63617.67			39	43317.42
55	64914.55			40	34340.34
58	70196.73			41	48693.06
59	77967.7			42	51243.73
61	77589.32			43	42324.98
Mean	**70857.19**			**Mean**	**43983.91**
St Dev	**6783.118**			**St Dev**	**6539.796**
Rat Number	**Sham 0/24**	**Rat Number**	**Uni 4/24**	**Rat Number**	**Bilat 4/24**
19	101887.8	6	51294.8	52	2291.432
21	75095.34	7	62757.21	53	43327.5
33	90103.95	16	39005.23	54	55696.54
34	70310.35	30	51135.51	56	54974.88
36	66698.4	32	53969.81	60	40589.68
Mean	**80819.17**	**Mean**	**51632.51**	**Mean**	**39376.01**
St Dev	**14770.64**	**St Dev**	**8500.457**	**St Dev**	**21806.18**
Rat Number	**Sham 0/72**	**Rat Number**	**Uni 4/72**	**Not performed in this Section**	
5	131202	14	48907.2		
11	93815.47	15	44884.27		
18	97393.99	24	34053.69		
20	77022.91	25	48045.73		
27	75343.67	29	48428.58		
Mean	**94955.6**	**Mean**	**44863.89**		
St Dev	**22512.51**	**St Dev**	**6245.807**		

7.7.8 Quantitative Immunohistochemistry with Doxycycline, Complete Data Sets

For all tables in Section 7.7.8: 0/24 refers to sham-operated animals with a four-hour anaesthetic and sacrificed after 24 hours, Bilat 4/24 refers to animal that underwent 4 hours of bilateral lower limb ischaemia and sacrificed after 24 hours of reperfusion. Low Dose Doxycycline was defined as 50 mg/kg twice a day for 7 days before the ischaemia/reperfusion experiment. High Dose Doxycycline was defined as 200 mg/kg twice a day for 7 days before the ischaemia/reperfusion experiment. Both the Low Dose and High Dose groups of animals underwent 4 hours of bilateral lower limb ischaemia followed by 24 hours of reperfusion before being sacrificed. Mean refers to the arithmetic mean of the brightness of all of the 35 images. St Dev refers to standard deviation.

Table XLIX: Left leg Skeletal Muscle Quantitative Immunohistochemistry with Doxycycline

	0/24	0/24	0/24	0/24	0/24	Bilat 4/24	Bilat 4/24	Bilat 4/24	Bilat 4/24	Bilat 4/24	Low Dose	Low Dose	Low Dose	Low Dose	Low Dose	High Dose	High Dose	High Dose	High Dose	High Dose
Rat No	19	21	33	34	36	52	53	54	56	60	62	63	64	65	66	72	73	74	76	77
	35574	39924.2	27903.8	26191.6	22831.7	13669.8	20267.8	22425.6	24145.9	19811.4	39774.9	24197.6	36281	67717.4	35891.4	38054.9	26213.7	22701	9533.15	30625.4
	64531.8	23728.8	49600.8	19974.5	20246.2	11704.9	18604.6	20346.5	27079.7	27708.9	51684.9	18126.2	26234	75461	41669.3	59073.5	46393.8	15016.3	12244.9	27747.5
	35510.4	24477.6	32502.9	17824.7	16466.3	17301.3	9544.41	16966.9	19490.7	28300.3	41522.2	14289.3	40914	47419.5	34639.9	43547.2	33734.4	17154.8	16021.5	29389.1
	26018	26523.6	33284.4	29494.6	25182.9	19998.2	23244.4	22086.9	15190	24475.1	29881.8	21281.1	32891	27272.1	39607.4	30682.2	26799.9	20547.6	13421.9	28115.8
	23500.9	25827.8	33949.2	14493	24240.2	16183.5	21566.6	10825.7	22534.1	25322.6	19144	16678	31322	25114.7	15195.9	28698.2	17478.1	42706.6	53651.3	47274.4
	33934.5	25400.3	34975.5	25780.9	25828.7	29487.3	27044.3	11663.5	23414.2	20546.2	25138.7	30258.9	20824	19594.7	15653.5	36054.1	30094.8	27996.7	49080.9	51117
	44650.6	6702.4	26692.1	39087.8	25236.7	13160.6	5365.31	15508.9	15525.2	28544.4	35062.8	3889.97	23616	23130.6	28782.2	12193.4	42285.4	28264.9	18415.1	23624.9
	28439.6	22975.7	32221.7	21087.6	26629.4	24375	16860.4	12590.6	15931.2	21852	26860.2	62824.5	22324	21410.7	20114.1	35002.8	18753.4	23783.4	28876.9	40724.8
	20536.9	12036.7	36963.8	22740.5	20427.8	21137	12548.3	15924.2	15367.8	24447.5	38792.4	25369.5	19869	12482.4	11801.3	44897.4	32065.9	11615.3	24234.7	38170.9
	67616.4	23471.1	35061	16241.6	24989.5	21747.5	13843.5	14130.5	19630.9	20794.4	13956.4	16184.9	40932	15709.9	23356.2	39583.8	31548.8	23582.4	29745.5	26600.3
	16539.5	24142.5	35425	27878.4	36417.5	20161.7	17393.4	13220	12806.9	11861	30779.6	25477	43841	13456.1	30305.1	17552.3	14225.4	10911	15204.3	30531.8
	19947.7	18560.5	31205.9	14199.8	24019.1	16387.5	20236.1	15888.6	17690.3	6480.71	44205.4	19584.9	41951	15961.5	33375.7	28796.2	15234.7	24069.8	21975.1	25552.4
	17634.1	27179.3	51430.5	13288.4	20397.5	12071.5	27292.7	10219.4	22220.5	8263.07	79577.5	26518.5	48193	29423	35638.1	26352.5	33528.8	23522.8	17137.7	31672.5
	21737.6	20028.2	28056.5	31221.1	24992.3	18091.3	15383	5075.92	25782.2	18354.3	64526.4	22788.3	12170	29594.8	22382.1	20217.9	34096.7	20771.3	29910.2	37583.6

	26390.7	21243.6	27083.3	16744.1	22398.7	23332.4	11274.5	8843.87	21954.2	9800.18	69850.5	36091.1	30215	27566.2	17036.4	22556.3	18797.5	13809.1	21522.5	21517.4
	38875.4	20898.9	34648.9	18152	29145.8	30086.1	6713.11	5183.71	28290	19629.8	51411.9	23686.4	19306	20723.3	14860	18170.5	42262.4	23425.2	33926.7	26789.7
	25937.7	22140.2	39524.5	12196.5	30553.6	22126.8	15359.8	9465.67	17948.7	19219.3	59068.9	20348.7	14051	22026.9	6146.05	17037.9	16793.1	29738.7	18971.7	44313.1
	41853.3	27848.4	38176	24379.5	28698.7	21710.2	21281.1	9013.42	15371.3	19262.3	58040.6	20989.9	46220	21609.3	37814.3	27063.3	43287.1	23602.8	24423	28610.5
	34209.5	23894.9	38843.1	20113.2	22628.2	18259.2	27583.9	7240.68	16654.6	26469.8	69838.7	28256	30011	18164.3	17541.6	21266.5	32517	22673.9	27039.7	20168.5
	31836.3	28824.7	33218.5	19713.2	22239.6	10626.6	15102	10650.4	18095.3	28410	63666.5	20516.9	25695	27357.8	28484.3	25859	53013.5	31432.2	43514.7	38149.9
	27358.1	27521.5	32741.1	12648.8	14979	28808.9	12701.2	6128.82	21222.6	37785.2	70380	13690.5	40531	23266.2	24139.1	20914.3	29249.9	35697.2	25529.7	32576.5
	35813.7	33391.3	39303.1	27366	26764.8	20070.4	17292.7	9056.38	13294.4	23072.8	65399	15612.4	34853	35586.9	13207.1	7893.35	37060.2	23465.9	36323.4	31692.8
	25028.9	38800	28461.4	16335.7	21168.6	10355.9	20905	9426.7	13004.7	21669.3	61123.8	27256	25398	21983.7	15956.4	23527	26128.3	21314.9	44850.5	50247
	30736.5	39668.2	46081.8	24142.3	17741.1	26185.3	20536.5	7862.44	14956.3	28589.1	70037.9	23389.3	29625	25969.5	14434.8	14463.2	18393.7	31155.7	40322.2	36393.4
	28429.8	50638.7	35771	14342.8	26167.5	21639.8	12087	13952.5	9235.17	20364.3	67424.6	24494.1	29477	13812.8	2958.92	14352.6	32538.3	19481.1	28062.9	30570.9
	26350.6	39866.1	33152.6	17462.9	21121.8	11619.7	24130.7	10335.8	17507.1	30396.2	62308.5	49885.9	30473	26002.6	19773.6	26602.4	25693.3	29533.8	29134.3	28282.2
	24813.9	35873.5	37334.3	27230.1	29398.8	16073.5	23873	7226.79	20638.1	938.93	32406.6	32033.1	25544	26480	23830.8	26693	23731.6	24254.8	41999.6	42663.1
	30945.2	36262.3	24213.8	14372.1	28039.5	18617.5	22641.5	7114.15	19511.2	7735.54	27216.8	58288.7	22368	20402.2	14273.2	51457.4	28543	24204.2	30154.6	42663.1
	23325.1	28338.5	33899.1	15756.4	20855.4	20651	32185.1	7114.15	17902.9	16852.7	23942	19782.9	10093	30454.9	16178.2	16944.8	15749.3	25073.6	26511.7	29309.2
	29351.6	50571.2	28589.1	18357.9	17091.8	14976.7	22851.2	9791.79	23741.6	11705.6	37675.8	8457.36	39427	25065.7	17181.6	12812.8	18866.6	21183.9	26349.3	27281.8
	22031.8	32722.9	27756.4	14819.1	15499	8206.62	33905.1	8869.92	17465.6	23118.3	36469.6	13906.9	27308	34728	6327.94	33775.7	20127.9	26386	26567.5	22393
	24757.4	34401.2	47157	17937.9	16902.3	23180.2	16407.2	10336.2	3614.24	24253.5	28829.7	48976.7	16617	23293.8	29619.4	17545.9	23661.9	18270.1	31833.8	19352.8
	17944.9	33342.6	39553.2	10306.6	19759	15257.4	8424.85	8209.88	15521.6	23588.5	28489.8	32685.4	23825	32360.3	25439.5	23232	21587.8	21996.6	25236.6	18609.8
	17944.9	22800.9	30072.4	16294.3	18157.3	15553	13275.3	9265.99	13677.1	19040.8	39515.6	23543.4	28489	32360.3	8480.94	17441.4	48305.5	22194.7	23526.4	29883.9
	28818.1	19926.2	33641.9	15045	17041.1	5937.52	20645.6	11732.2	18749.2	21822.4	40003.1	19763.4	33321	22542.4	17225.6	17505	21074.3	17641.7	22621.5	31231.5
Mean	**29969.3**	**28284.4**	**34814.2**	**19806.3**	**22978.8**	**18250.1**	**18524.9**	**11248.4**	**18147.6**	**20585.3**	**45828.8**	**25403.5**	**29263.1**	**27300.2**	**21694.9**	**26223.5**	**28566.7**	**23405.1**	**27653.6**	**32040.9**
St Dev	**11324**	**9414.12**	**6420.73**	**6391.6**	**4866.97**	**5912.51**	**6773.22**	**4427.16**	**5011.11**	**7702.5**	**17935.2**	**12764.9**	**9642.03**	**13119.1**	**10140.6**	**11628**	**10220.9**	**6381.77**	**10402.7**	**8521.77**

Table L: Lung Quantitative Immunohistochemistry with Doxycycline

	0/24	0/24	0/24	0/24	0/24	0/72	0/72	0/72	0/72	0/72	Uni 4/24	Uni 4/24	Uni 4/24	Uni 4/24	Uni 4/24	Uni 4/72	Uni 4/72	Uni 4/72	Uni 4/72	Uni 4/72
Rat Number	19	21	33	34	36	5	11	18	20	27	6	7	16	30	32	14	15	24	25	29
	47152.4	52938.1	65565.4	116086	50104.6	96299.2	114234	48367	34974.1	10809.6	13190.4	2684.91	50670.6	667.58	11755.9	18696.9	11344.6	7249.6	1207.3	8066.18
	47951.3	38195.5	59744.5	81571.7	68412.1	82851.8	73143.5	77746.8	48110	7351.38	6815.61	2298.55	50309.7	19564.5	1691.66	9486.52	1030.83	14149.2	5496.57	1480.43
	41379.9	51615.6	72130.3	51131.7	108732	68105.3	60179.8	65657.8	56381.9	2969.98	6070.53	16391.3	50038.2	6046.13	1459.48	5383	12285.2	9072.62	4135.94	1175.22
	79316.8	54101.5	49652.9	80085	77299.1	95472.9	63143.2	51656.2	53530.6	693.52	4896.4	32602.4	9078.91	6249	2044.11	9033.61	1474.9	12251.7	10934.6	7436.61
	55175.5	43067.2	42828.2	66099.8	76266.7	87005.9	65625.3	16060.5	50824.6	32356.1	23988.6	44371.8	32614.1	25386.2	188.03	1219.05	370.97	6002.61	5237.97	1642.99
	46684.3	38122	47250.3	72661.3	67955.2	73369.2	77455.4	20295.6	69646.9	38194.5	15070.3	42583.4	9113.42	8040.4	423.44	6028.34	1498.58	8352.26	2173.63	1055.07
	32335.2	45014.1	48306.7	29974.1	124641	36709.1	75383.3	22217.2	54660.4	35183.9	21751.9	48566.9	1868.55	14305.6	896.89	24666.2	257.4	10540.3	3381.61	25.56
	40643.5	48520.8	22789.1	34589.3	80536.5	64201.7	52298	23614.7	59420.1	42358.1	35191.3	20248.3	9332.57	25865.9	416.36	2127.14	292.07	8461.04	10600.6	5586.03
	49774.6	39587.6	29342.8	71720	125502	122074	64897.9	30161.8	39412	49166.4	18687.3	31919.7	5346.74	8799.2	618.65	6486.72	254.52	8055.6	18768.9	8058.91
	57523.6	34457	51591.4	69758.2	104493	102638	43719.2	58787.6	39675.8	30500	21177.3	34255.8	788.71	19196.8	1108.01	9304.07	159.46	8057.55	19652.8	22039.5
	78048.3	54578	44668.1	123987	98323.7	63592.7	74073.5	17123.5	47346.5	39509.4	8705.37	39552.9	1414.65	30374.3	449.75	6064.73	258.63	7408.06	20994.9	14617.6
	67402.4	38254	64056.6	114087	115474	36548	46551.7	56149.7	59492.1	30396	20082.9	28967.5	9930.12	2553.33	610.11	20392.5	263.85	6671.42	12776.6	12264.8
	57039.9	54929.1	42856.3	127400	65110.8	57768.1	60585.4	59471.3	46486.1	17497.5	17272.6	56976.4	17301.5	7755.47	3240.58	2865.15	486.84	8837.68	13505	16545.1
	43992.1	31499.2	5796.45	174568	53094.7	81945.5	45912.1	52614.8	36924.6	48337.7	13332.1	27398.8	560.72	45.42	865.71	1069.95	391.94	8006.59	14187	8148.79
	30172.9	35871.1	19061.2	115064	65704.4	94320.6	47209	52384.5	52364.3	42675.8	8201.04	30349.1	3247.27	2754.23	293.49	771.81	3065.09	2395.15	17594.5	16519.5
	19118.8	50193.2	33149	113159	74183.5	81260.8	37026.7	45509.2	56869.2	80780.9	3526.4	41918.6	9332.57	17185.8	632.96	774.58	1050.65	4161.85	53068.7	15197.4
	72203.9	60822.9	25408.9	98009.9	81458.4	60360.4	16982.2	36987.2	49205.2	36377.9	9453.69	40369.6	8093.84	38216.2	3236.22	12436.2	117.28	3829.3	44632.8	13758.5
	59326	57790.7	61650.3	100595	82117.5	88813.9	48529.5	60972.8	44108.9	19465.6	3907.01	35219.1	28817.1	26544.6	1858.61	6079.76	183.03	8817.33	31188.5	15699.8
	57959.6	95398.2	35732.9	52825.4	53588.8	54456.4	25841.5	59089.7	35340.4	66270.5	3651.07	58256.8	12385.1	8645.69	3172.51	12400.7	148.4	5292.97	22573.8	18534.2
	72550.9	124437	48196.7	67926.7	20770	62731.9	41679.4	60854.1	23933.2	61389.1	6056.98	39390.7	4944.8	16247.8	16752.3	10695	154.81	17674	49680.6	24864
	59932.2	117367	29163.2	49433.7	68188.2	59643.6	50425	61346.1	123357	36206.4	3323.48	32328.2	2002.57	24916	2958.04	1921.93	115.96	10158.5	57180.9	23978.4
	75882.2	81366.3	31057.2	69883.2	10546.7	45805	44311.3	66417.5	57395.5	73852	1385.63	24522.9	3730.19	1044.36	8615.33	1954.03	241.12	2902.82	16479.4	23605.8

	55348.5	66000	26999.5	47546.3	28728.4	60670.9	46070.7	47121.8	47520.6	33933.2	27116	22152.9	32419.7	43035.7	1707.61	11278.4	353.8	15441.7	18515.9	11222.9
	102939	57527.5	67052.5	45032.4	17645.5	67760.2	72488	38993.9	41807.9	20125.1	834	27977.3	6271	26824.9	24746.4	1457.02	701.1	5945.28	25349.9	8261.6
	48253.5	61325.1	99193.5	42327.6	101180	47670.9	77247.1	40602.6	51584.2	16999.5	637.73	15862.5	56949.4	37129.7	10968.9	1409.45	1099.09	7872.19	27591.2	3302
	58569	61202.9	61769.8	106593	62122.3	66930.6	60259.7	36782.6	33092.7	51462.3	754.46	27648.6	40807.4	12712.2	22287.9	268.69	417.79	14957	35271.7	1971.31
	69144.1	79644.6	92235.6	84894.3	93900.7	63478.2	72319.1	38216.3	34833	18080.6	24.04	26614.8	8167.06	3437.75	35765	20.3	1017.22	6483.76	28896.9	1562.13
	62057	107581	60211.3	134972	65166.8	50924	98499.4	33863.8	52480.1	33274.2	84.87	38731.9	17914.6	204.69	32741.6	8.53	1016.92	21465.3	34274.8	654.06
	73467.5	76878.2	53039.6	94067.8	87701.2	50351.1	101756	37318.9	26619.1	97616.7	430.7	18765.2	10409.3	3.87	26297.3	6617.2	3162.52	14986.3	35971.7	9646.57
	63414.1	80494.4	43032.2	66165.5	36842.4	57317.6	110222	94195.1	40013.2	44201.7	393.89	15329.6	12274.7	154.6	20498.3	20932.2	558.44	8482.44	26829.5	8204.16
	52704.3	62305.4	22963.7	66333.2	9557.4	38372.9	125562	97798	34119.8	5325.92	1161.42	17145.1	20972	40.05	28847.1	4567.02	330.95	4796.62	34205.7	13012.8
	72158.8	57998.9	32837.9	101181	19739.9	34150.6	107290	73554.4	29758.4	5639.66	118.99	8463.31	20465.2	8564.91	27967.7	45.06	103.73	1937.21	35995.4	10036.6
	68187.8	53750.3	29574.1	65532.1	15917.8	27149.1	108350	73554.5	31163.3	30810.9	214.83	15078.6	17407.6	7344.14	31102.1	83.43	76.82	1170.98	38726.6	5713.12
	69895.3	69746.5	35481.6	74997	14545.7	33985.2	87718	102447	21602.5	11328.9	141.73	21208.9	10795.4	39.92	29186.7	0	5.1	0	28669	6439.98
	83935.1	66051.3	36506.8	76120.8	15181.4	20209.5	89619.1	46682.7	50404	9761.62	811.86	11019.2	15287.4	1547.25	20949.2	24.82	3.36	32	35718.1	3444.57
Mean	**59189.7**	**61389.5**	**45454.2**	**82468**	**64020.9**	**63855.6**	**68188.8**	**51560.5**	**46698.8**	**33740.1**	**8527.5**	**28490.6**	**16887.5**	**12898.4**	**10753**	**6187.71**	**1265.51**	**8054.83**	**24042**	**9822.07**
St Dev	**16674.5**	**22694**	**20079**	**32046.6**	**34516.1**	**23303.1**	**26488.2**	**21760.4**	**17475.5**	**22988.4**	**9377.46**	**13792.1**	**16011.8**	**12527.6**	**12212.4**	**6760.98**	**2739.33**	**4936.89**	**14859.2**	**7274.4**
	Bilat 4/0	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/72**	**Bilat 4/72**	**Bilat 4/72**	**Bilat 4/72**	**Bilat 4/72**
	44	45	47	48	51	39	40	41	42	43	52	53	54	56	60	67	68	69	70	71
	50380.9	27676.4	48431	12093.7	44651.9	41463.7	16075.5	3351.66	45549.7	5272.2	9977.83	10770.3	32.39	5229.38	3071.88	14208.8	4235.98	590.09	292.02	17784.5
	44834.4	19237.4	39856.8	20597.1	31511.1	17697	12301.1	6546.14	22959.2	25866.9	7791.95	12904.3	44.93	5593.14	6015.79	8943.69	2262.42	1163.45	3779.01	19380
	31376.2	17828.6	12275.8	33677.4	41333.7	19737	33427.2	12602	18138.9	25196	7456.62	6460.59	67.18	12428.5	2393.94	13938.4	1457.61	4461.04	2517.26	15874.9
	36046.8	20670.3	32420.8	36172.7	53422.1	26785.9	20404.3	9629.9	34652.9	18173.7	3964.58	30114.2	698.79	14610.7	11673.6	9719.35	2600.16	4335.43	5184.86	5946.51
	43063.6	20660	30319.5	33278.6	34874.1	35560.7	17717.1	22287.5	34052.5	11201.5	3273.03	7568.95	313.54	18116.9	7760.98	6849.62	2713.82	9293.6	4242.17	4454.89
	42894.6	25715.5	48297.7	37993.1	19120.2	33286.8	11668.8	9964.26	23873.8	14355.1	9472.92	15918.8	21212.1	20360.8	10074.2	19089.9	669.06	9317.28	677.15	11094.7
	31012.8	32753.7	21315.7	26852.2	24186.1	14252.1	9995.31	1662.95	21881	22452.9	7274.29	9950.22	10700.5	22078.4	10932.7	13535.8	1394.16	3156.25	7936.73	3479.81
	36300.5	21492.3	21956.1	18193.5	33849.7	18091.3	31747.1	38878	15796.8	20622.9	1239.53	7003.07	8124.62	16816.1	15311.9	7186.83	5.6	4801.52	3004.86	9667.81
	32625.6	22926.6	14292.4	12491.7	20445.1	19045.6	31636.2	21259.8	6897	9666.79	1244.07	9575.89	7198.26	9324.98	15713.1	15085.4	415.79	5037.84	4358.4	6485.09
	32115.9	27949.9	20340.7	14434.2	19589.2	41423.9	24087	25133.8	2136.46	14072.4	883.85	15119.4	5280.38	12639.5	26342.3	18677.2	2518.98	2375.75	6436.1	12817.6
	18477.3	18523.1	26987.7	17125.3	11205.3	52833	8915.32	17503.5	8712.32	38343.6	236.76	6577.57	3755.28	6484.17	20159.4	12608.2	2291.05	1420.33	6424.25	9178.41

	22176.1	30958.3	31419.4	12834.7	20438.5	38858.6	33504.7	20820.3	20784.4	39516.6	1200.39	4845.54	22358	9324.69	18008.8	21081.4	8898.41	2020.72	8389.52	3041.52
	44340.6	26536.4	69290.3	12318	17428.6	33131.2	23515.9	34632.6	19202.7	10111.1	4493.15	4416.89	27316.4	10391.2	13850.9	24128.3	14110.9	13316.7	6627.28	18695.8
	51763.5	36262.4	14726	23193.8	61323.4	23284.4	7756.04	36168.2	28366.7	5225.55	7323.25	4643.41	34671.4	17858.6	24378.8	24730.2	2311.75	2118.22	9751.44	10277.7
	25933.6	39950.1	23912.1	20905	20332.6	14393.9	4827.88	44242.4	44109.6	2903.5	2359.5	17820.1	18995.6	28095.4	6850.78	20796.5	14528.6	1155.41	6102.57	8137.21
	17184.1	29114.3	25652.1	14586.6	25211.1	25783.5	6492.07	21210.1	46690.2	5005.82	152.2	7215.46	21852.1	25340.7	2377.66	22857.4	16167.5	24549.8	11465.7	4967.58
	18982.6	31468.6	27601.8	11891.2	24378.9	23789.9	11075.1	24731.1	39607.4	4128.28	830.86	6791.46	6184.41	18298.9	1185.96	26090.4	15793.7	18731.7	14166.1	8531.93
	21612.9	13116.9	52285.6	21076.4	60445.3	38186.2	28244.6	24804.9	16585.1	13023	31212.4	4515.39	9763.14	11586.1	651.53	15076.4	7622.33	29773.2	12257.3	5014.99
	14095.5	39548.1	31284.2	24362	38473.3	52393.7	24646.7	28343.2	13657.4	12913	21464.6	12856.7	6255.14	1154.28	289.09	15085.8	8679.78	37323.5	12450.7	6417.15
	14387.6	25222.1	20395.5	29705.8	55191.5	53758.1	15242.6	27450.2	19088.3	12571.9	20323.7	15867	16619.6	3052.1	864.42	11476.7	6116.09	20229.5	16487.6	4020.58
	14036.2	28550.6	9874.82	25562.7	21040.5	37209.1	22893.7	31393.2	17067.3	8562.97	17092.3	7675.7	17187.3	3052.1	3061.37	13197.8	11439.7	27507.3	18207.6	1530.79
	54275.7	34135.1	8635.6	18535.2	19070.5	19352	30676	15447.4	7405.2	16414.4	27346.7	3791.86	7944.81	1361.05	875.55	17192.3	8391.07	17136.3	30017.5	19361.5
	50718.1	25656.4	13945.3	19220.6	30842	26695.8	41684.6	25458.2	11795.1	26995.7	33669.5	3499.48	13152.7	8078.1	2048.04	34953.5	499.75	19292.8	29659.1	19361.5
	56685.2	27065.5	17906.3	18289.7	34637.3	39377.4	31187.3	46010.2	3771.14	16052.1	21031.5	30911.2	4999.6	18125.3	5185.43	17536.4	2131.92	11346.6	19065.4	29273.3
	67530.3	19191	22455.6	31965.3	49703.1	30556.5	24866.1	33084.3	1620.7	18046.3	11837	31342.5	7179.73	17668.7	3905.18	9172.58	5698.58	20473.3	9742.5	39759.9
	41515	16517.4	30960.1	22880.7	22508.4	35477.7	34010.6	42933.5	644.55	13113.3	13440.4	13477.7	29682.5	12739.6	1296.34	11.07	4999.85	20887.5	10908.6	42898.3
	46431.9	19314.1	36741.5	63185.4	20173.3	46808.5	41659.1	42061.5	4957.23	16300.9	10928.1	7803.42	30785.1	13551.4	589.52	30321.6	14441.2	16472.2	14190.1	46248.1
	47697.4	27056.6	38210.7	33591.7	27284.9	53219.7	36350.1	38558.3	1768.62	17732.9	9949.12	17687.3	22275.9	21921.1	211.85	4772.31	8937.38	1909.64	16854.3	53901.2
	31033.8	17878.4	29048.6	7140.15	26239.7	32934.8	13202	45329.4	9047.02	14224	23272	15395.5	34148.8	17321.9	524.15	5.55	15248.9	8966.92	24358.3	37676.4
	37829.6	35594.3	27842.4	35016.7	35828.6	25173.2	42357.8	55768.4	5379.95	30098.6	40260.8	16286.3	19990.3	6250.63	1113.85	2732.49	13136.2	11258	21186.4	34175.1
	45404.9	21493	53941.2	19505.9	47632.5	18974.8	50872.6	41290.2	7189.55	41165.9	3045.3	597.98	4862.23	828.11	4532.32	7793.98	6416.64	11420.2	26641.5	39039.9
	32104	31408.3	43522.4	9794.63	31128.4	12910.2	25609.1	39384.9	3365.96	36567.3	236.35	714.68	6056.85	5873	42.9	27091.5	8935	7760.22	35114.8	37333.5
	39284.5	26891.1	28558	12251.3	29128	28911.5	38933.7	38873.4	26948.4	19253.9	26954.7	3339.97	8319.02	4163.62	181.91	14871.5	4863.67	381.93	26599.5	25153.4
	30737.6	34761.2	19262.3	16050.3	31488.2	41680.6	17520.4	47946.5	37523.2	12786	27122.7	6893.34	9456.94	7488.73	168.37	16689.1	5149.35	8825.51	34318.9	41037.5
	30689.7	39261.1	20495.9	20346.7	22928.3	37501.1	14277	40890.8	35646.9	7544.47	27939.9	3802.87	11003.4	11896.5	1330.09	20935.7	5808.94	11778.2	12198.1	9489.79
Mean	**35873.7**	**26639.6**	**28984.6**	**22489.1**	**31629.9**	**31729.7**	**23982.3**	**29018.6**	**18767.8**	**17299.5**	**12465.8**	**10690.1**	**12814**	**11974.4**	**6370.7**	**15384.1**	**6596.91**	**11159.7**	**13474.7**	**18900.3**
St Dev	**13393.4**	**7122.11**	**13700.2**	**10954.7**	**12993.4**	**11905.2**	**11930.1**	**13880.9**	**13958.2**	**10267.6**	**11439.6**	**7908.38**	**10221.4**	**7210.61**	**7432.48**	**8215.24**	**5054.63**	**9466**	**9754.29**	**15061.4**
	Low Dose	**Low Dose**	**Low Dose**	**Low Dose**	**Low Dose**	**High Dose**	**High Dose**	**High Dose**	**High Dose**	**High Dose**										

	62	63	64	65	66	72	73	74	76	77
	25047.2	48992.8	38228.1	59850.3	19755.8	43371.6	56212.2	9144.32	45326.4	35011.7
	23490.7	20566.3	6329.28	25572.3	6052.11	11417.8	28627.4	58767.6	25556.6	23117.9
	22265.1	13969.3	3332.74	40099.8	8686.64	6204.49	36364.3	52368.2	24151	29854.1
	13351	15632.8	21059.8	8545.18	13128.5	2351.72	29868.6	36672.5	36890.6	17783.2
	7789.41	8661.94	3730.04	16607.4	4157.53	20206.6	20793.9	31186.2	13204.1	35776.1
	29383.4	22487.3	25623	10017.6	18852.8	18875.8	31119.6	39675.2	30231.6	39425.9
	23095.7	9262.08	6636.91	3162.97	9475.76	18168.7	40194.4	33803.3	40394.5	19503.2
	20469.8	8168.77	45123.7	4552.16	20711	19747	19854.9	28285.5	24227.9	29044.8
	16725.5	7076.79	19359.6	6584.3	3541.13	17620.6	38186.8	33577	21371.9	33214.1
	36727.2	19259.3	34062.6	3423.36	18458.3	8839.81	17244.3	38520	31105.1	47104.3
	28827.4	24390.6	24670.9	4770.82	11167.5	7294.06	17557.4	38104.5	13534.3	60372.5
	18645.2	32172.6	16512.3	9948.41	8700.81	9436.66	20322.9	17865.4	4115.22	54449
	26325.5	26875.7	26083.6	7799.04	12041.4	5308.65	15583.8	18922.2	6221.9	53627.2
	24595.5	23365.7	33926.9	16194.1	26104.9	24697.2	18620.9	18925.3	3826.6	34842.6
	14472.9	30397.6	13942.7	3193.57	28828.1	31764.6	28998.3	31731.2	11324.7	33599.3
	13080.4	21723.3	14929.5	8690.13	28648.2	23365.7	10254.4	29023.2	7551	46592.4
	16831.5	14214.7	38752.1	8828.12	22915.6	21497.7	11352.4	42205.7	5244.31	29248.5
	26707	18173.2	11326.3	9443.24	5100.91	15030	23502.6	37797.1	13780.3	12556.8
	27044.5	18055.4	21314.3	11959.8	31725.4	30428.4	37752.5	35793.4	28088.3	4255.21
	21706.2	13225.8	41302.3	17080.6	11558.1	20302	38267.5	8498.73	36585.5	20635.5
	9461.02	4913	29197.2	8291.63	8704.54	29082.4	22798	16048.8	47076.1	4101.15
	61.61	7872.38	40309.4	8718.71	30349.2	38627.9	22605.1	28122.5	13089.3	1632.25
	9340.04	20374.8	25740.3	13084.1	13440.1	45162.3	21695.8	33394.5	24864.6	3289.34
	9454.23	15083.4	19768.8	12840.5	5481.49	51696.4	22974.4	26603.9	29707.4	3101.68
	7261.98	14304.8	5889.35	14857.2	19258.8	41968.8	11246.9	34081.3	31928	2630.93
	19740.9	13442.7	6843.36	10479.1	12822.9	38510.5	21832	20093.4	21863.3	6141.9
	18730.9	21466.4	5571.74	17955.6	9250.95	32932.9	44636.7	18086.8	11315.3	4351.55

	6288.98	18684.9	4763.14	16304.7	7268.55	16854	32991.6	25992.6	23998.3	4026.78
	13815.7	9767.3	1181.5	22441.4	6197.78	14067.4	34459.5	30920.6	51815.3	6093.3
	12141.2	13460.1	5280.3	5077.32	8960.42	20091	33845.8	20845.7	43092.4	13116.3
	6368.64	13522	1746.63	3525.23	12052.4	17588.1	7474.5	23313.5	13164.7	76671.7
	7639.49	7357.23	13569.3	4731.71	26359.3	21666.2	3216.07	15642.6	8976.88	11639.4
	9240.9	14601.2	54931.1	11961.3	29937.7	33790.9	3053.65	15054.4	8847.86	14745.9
	18359.5	15330.5	27504.5	6256.17	9523.8	32245.9	30303.9	9694.62	57353.3	5359.8
	3861.18	10882.9	54109.3	2362.38	4189.56	35888.9	14307	44912.7	17791.3	7702.74
Mean	**16809.9**	**17078.2**	**21218.6**	**12434.6**	**14668.8**	**23602.9**	**24803.4**	**28676.4**	**23646.2**	**23560.5**
St Dev	**8571.61**	**8643.95**	**15327.8**	**11182.1**	**8749.46**	**12491**	**12005.3**	**11880.9**	**14518**	**19572.1**

Table LI: Kidney Quantitative Immunohistochemistry with Doxycycline

	0/24	0/24	0/24	0/24	0/24	0/72	0/72	0/72	0/72	0/72	Uni 4/24	Uni 4/24	Uni 4/24	Uni 4/24	Uni 4/24	Uni 4/72	Uni 4/72	Uni 4/72	Uni 4/72	Uni 4/72
Rat Number	19	21	33	34	36	5	11	18	20	27	6	7	16	30	32	14	15	24	25	29
	71755.4	82227.32	46777.87	65321.57	94990.77	48585.27	47079.71	47494.52	70864.41	65690.66	53334.04	20055.16	64848.79	6275.99	53466.89	63882.07	311.73	64643.04	76714.96	10048.73
	4978.75	73905.04	45734.46	59201.07	54107.04	57811.81	27962.09	41421.91	43868.43	63830.17	65197.88	35546.37	71076.52	22568.06	55326.08	46021.21	10078.23	42480.64	55281.11	3509.42
	81980.6	74324.88	67504.76	61019.09	84373.05	60330.01	20024.02	57187.05	47662.58	54069.27	48768.61	60097.03	53325.84	53075.77	51778.66	50249.88	6943.56	45011.63	72869.09	35188.05
	72980.71	98050.36	42393.35	47894.27	51267.34	67724.54	43265.6	65648.59	47757.52	53132.39	62306.92	68020.04	61404.93	41211.34	57650.13	55897.7	23550.32	60308.5	64785.53	44857.46
	66489.77	71534.05	90958.02	100553.9	59533.45	50371.75	38306.15	45582.4	60617.23	56903.39	37249.63	64296.99	53178.46	51726.98	53673.8	47488.14	23334.33	76022.68	56346.36	45033.44
	60445.25	53039.36	85222.67	73261.89	58617.23	64526.52	42468.99	52197.29	53336.66	46942.93	47993.87	58730.38	57505.54	59783.87	40491.23	43848.77	33536.27	63238.86	56222.79	31364.5
	64430.12	57599.66	82773.56	86196.63	55142.13	63054.93	75138.7	66058.34	77850.25	53901.07	55340.21	71963.49	67091.23	52674.16	45306.61	53478.07	61405.81	45066.97	67664.23	49386.42
	69086.55	54186.17	73539.48	84494.16	66438.47	60518.46	65477.08	55065.89	88594.48	52639.82	59380.29	64801.39	57945.72	60452.09	43570.87	56764.95	50332.04	48751.54	46309.66	78964.38
	51380.81	73042.05	77595.41	86623.45	91690.17	67408.13	62153.69	54684.74	65926.39	51576.54	60673.55	53776.32	72692.96	44277.79	40158.31	64718.89	50922.6	43128.2	56686.2	65188.2
	55185.95	52999.43	57930.49	97674.19	67463.36	44772.43	63207.24	45193.77	71165.76	62064.38	67439.81	57679.44	56544.49	80653.88	36297.79	61844.7	49519.2	53448.69	69562.75	67753.35
	52119.36	51437.44	42199.59	88294.86	56966.15	43474.45	47754.82	41819.07	63325.95	54381.44	52313.28	42841.28	59981.63	29353.47	40407.27	59501.26	49255.75	68779.7	72889.34	66019.21
	54864.32	87337.45	50056.5	62430.93	63496.7	49843.98	64397.73	67993.74	56763.19	63372.13	47481.58	33699.05	47071.29	52166.03	44258.27	56102.3	46910.33	55190.63	70202.44	58521.36
	70732.34	82292.91	62137.57	60022.7	62222.67	60389.16	84231.08	55626.54	55015.11	59841.25	48072.2	55663.02	53718.71	60100.72	42826.33	46324.69	50553.08	91098.81	61514.18	56595.34
	50478.83	79485.23	53217.34	88387.09	56914.32	51911.94	84362.34	74851.1	80857.64	66396.98	47164.52	49887.98	67643.02	67342.09	43569.8	49601.94	52226.52	67553.71	55450.81	67011.92
	50456.3	57639.34	53217.34	74351.98	57056.7	54752.27	66217.81	86094.28	58947.68	69879.01	51164.79	42866.39	51341.34	64502.06	44191.75	61185.86	57137.82	73213.45	46213.69	61562.45
	55602.78	74345.88	47626.07	68808.59	52666.36	46206.66	70011.06	58158.86	82707.66	55327.11	37460.25	53436.49	52096.5	63872.24	46761.19	54721.04	68605.27	86533.32	56725.22	81925.82
	58811.04	77931.31	33604.55	82963.73	51714.58	55792.02	75705.68	52931	62886.95	43872.32	52841.25	54035.82	43276.63	56785.5	51544.98	52075.58	39507.09	71836.6	79099.26	72987.38
	57634.62	62214.14	41896.35	89985.69	53347.16	42866.2	72144.9	75021.51	64780.92	45075.54	50184.78	56891.5	55544.59	62749.77	43941.91	60981.13	42694.68	86310.33	75440.93	65066.25
	47798.34	69525.7	44601.25	137976.3	45133.67	58415	78551.58	62868.17	62539.22	48631.64	58031.02	64498.97	49856.46	57329.06	56289.5	57861.2	38740.82	41919.91	47879.28	73578.21
	58768.37	70254.34	60260.68	70874.27	53297.01	56976.74	78177.68	52090.65	66796.68	53485.27	52669.66	76980.6	51875.4	52103.68	61114.07	69617.31	50293.96	29632.29	86018.83	88043.07
	56489.04	59322.09	47920.99	65061.6	61673.79	48723.89	74449.79	54139.5	79293.88	59163.38	41211.39	74236.69	43889.2	51344.52	72299.99	68742.43	48273.34	20223.19	67064.78	72852.02
	50314.91	61774.3	45668.78	63340.93	51294.4	68932.28	76624.13	58346.67	83472.74	53738.32	57191.15	57853.81	54382.48	60560.68	62757.99	44719.38	56677.7	31938.76	81496.59	69470.26
	51057.6	54478.89	53658.54	52969.72	48322.69	58625.73	61400.8	61271.02	51679	49972.69	51568.96	68102.53	45632.75	56129.82	57539.44	42866.18	53110.07	29514.78	80220.77	70883.72
	39637.63	86795.63	54572.8	76196.77	45822.72	66088.2	73871.82	59120.07	58507.88	54777.2	45859.07	54066	49907.53	49283.38	59586.08	39627.86	55746.43	25552.74	75209.2	73092.85
	57411.84	66485.3	61456.67	73873.79	40963.42	57778.29	77458.78	52335.11	57320.16	41840.34	55906.79	49602.98	53663.05	61264.61	69447.96	39113.18	50442.08	53422.37	77473.81	64778.86
	53104.12	72635.98	58245.88	73371.38	33684.05	54835.74	55640.62	51209.57	52467.49	50223.5	61076.42	47998.82	35050.35	72329.84	51887.42	46115.64	51626.63	56641.6	82825.76	60984.22
	51985.99	65679.41	56129.42	65211.71	71750.65	67007.21	48577.91	43759.26	64718.61	49582.91	53436.9	53380.64	48095.28	51049.5	58990.93	14845.34	51324.05	50608.13	68825.09	69974.04
	51985.99	69349.83	58793.82	88106.7	63585.7	62945.92	47741.17	60217.83	63639.57	70231.57	55930.24	52938.95	61056.22	49354.98	76406.77	42422.64	56709.12	44899.29	59712.73	64678.7
	37712.32	74745.45	69881.74	55757.9	49000.41	53019.16	53987.98	39953.78	68179.67	76592.51	49364.44	53941.61	53834.89	34910.14	69721.95	52833.32	57250.04	36505.4	58558.76	62024.19

	49214.48	59813.98	67929.25	75492.88	48073.32	57741.79	47022.21	51401.26	68960.4	73400.23	50893.8	40857.5	54945.14	24725.23	62792.68	38630.73	53620.07	48493.07	58105.93	62021
	43974.16	47121.97	71762.04	68476.26	72711.55	53495.96	59788.22	40153.25	57950.13	69354.92	50604.23	50843.86	48825.44	50503.14	64319.69	46766.46	52610.91	48144.82	64545.17	55007.18
	45405.55	73345.3	56612.77	73510.31	57535.9	63364.7	68455.61	41356.85	65289.5	61474.95	55331.78	52112.95	63061.77	62929.75	69293.88	52183.45	49964.55	80112.57	41479.98	72080.99
	47628.44	57273.86	53721.35	73700.9	68317.98	61731.43	58700.77	32729.12	56009.41	57611.5	63138.21	45271.11	51591.57	48514.36	60839.14	45939.73	51359.12	62073.24	42873.56	61577.66
	62794.85	50868.43	60034.41	82075.57	73318.75	53499.06	64470.32	37483.26	47740.1	53594.65	58824.35	66411.8	57904.89	47476.86	7724.22	38628.63	68799.46	69412.52	62300.98	63885.39
	44261.04	62350.22	50072.99	75168.49	70269.77	63448.68	44094.17	41809.6	51071.12	53886.53	50423.52	67453.09	43063.23	44520.76	41630.61	44098.79	74512.95	68113.22	68688.23	56117.82
Mean	**54255.94**	**67583.22**	**57877.39**	**75675.75**	**59793.24**	**57056.29**	**60540.63**	**53807.87**	**63101.83**	**57041.67**	**53023.69**	**54881.14**	**54654.96**	**51540.06**	**52510.40**	**50562.87**	**46796.74**	**55423.57**	**64664.51**	**60058.11**
St Dev	**12994.77**	**12041.77**	**13301.19**	**16526.56**	**13214.25**	**7242.722**	**15816.76**	**11731.82**	**11227.48**	**8510.453**	**7012.188**	**11987.24**	**8240.157**	**14561.96**	**13140.63**	**10639.84**	**16486.79**	**18063.22**	**11913.77**	**17809.08**
	Bilat 4/0	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/0**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/4**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/24**	**Bilat 4/72**	**Bilat 4/72**	**Bilat 4/72**	**Bilat 4/72**	**Bilat 4/72**
Rat Number	**44**	**45**	**47**	**48**	**51**	**39**	**40**	**41**	**42**	**43**	**52**	**53**	**54**	**56**	**60**	**67**	**68**	**69**	**70**	**71**
	77139.6	60144.02	54568.98	30729.5	48942.34	47088.76	64595.71	49723.11	8583.33	38518.31	52822.86	51301.67	33322.15	72922.31	2774.32	29628.06	30603.34	40416.21	36437.71	294.4
	52467.97	47931.14	54269.85	43163.69	55115.16	55121.92	50972.71	51161.37	10496.18	41761.25	41336.61	51315.92	39355.2	69711.05	3504.7	41657.95	47777.77	36757.09	32854.84	8.09
	51485.36	53480.74	44635.86	52360.96	59645.51	55340.39	63474.04	61159.12	32704.97	31826.82	38835.49	36949.83	77907.61	72196.99	3260.61	37062.29	43289.16	36922.41	44722.65	7003.63
	39355.74	50507.41	74270.88	51597.37	59241.36	58695.01	56634.16	46046.29	74118.16	37658.4	48362.83	23252.6	64026.18	79734.73	1550.51	46621	48500.16	28449.19	37855.66	2537.41
	69664.85	52826.77	53675.01	38657.63	47323.84	40269.13	55146.19	63639.89	37586.68	32420.7	47383.47	9977.76	63862.14	61596.76	2750.59	48416.28	36669.87	30129.74	39713.55	8382.5
	60545.88	47699.3	76386.84	39872.58	60271.94	58311.46	51119.15	61358.47	26680.82	41087.85	62391.14	35211.16	62567.11	62368.02	5684.53	36708.91	49454.68	37342.3	35283.57	6588.87
	39794.84	57879.76	60322.84	52117.35	43625.55	69133.74	59111.37	57760.51	33061.55	41272.64	59383.91	43421.08	56910.71	64468.02	8980.72	34459.97	60515.81	54204.44	21596.28	10312.51
	80405.93	50979.09	55754.6	47964.86	48933.51	32033.74	59914.04	55201.46	57942	41427.6	56021.63	49225.23	56888.41	54330.69	64486.54	51068.57	19312.21	52420.68	28357.96	14360.73
	61200.47	46064.72	65520.63	53741.65	56738.34	29495.2	60643.93	55725.59	56853.1	28519.55	59197.91	20219.92	73123.99	50674.6	64456.95	58438.85	34845.53	52493.63	32999.22	7064.53
	62417.05	47255.92	66664.02	47977.91	56608.55	30423.33	49553.75	50805.93	67701.81	28914.11	58114.34	7408.36	61179.22	58554.17	56462.99	51330.65	27285.36	50563.55	31219.12	11315.36
	62796	54930.22	58241.45	54130	58616.04	48565.38	45723.58	46958.02	65145.48	24910.54	60960.25	27287.02	28918.91	37811.21	58073.88	56214.51	38070.16	58547.83	52949.61	8157.44
	49614.68	68842.94	65710.88	52701.24	45886.42	59505.39	56318.07	50413.41	65265.1	31042.06	52827.27	58100.66	24976.45	42872.71	56967.95	64317.66	34149.77	45149.78	40074.32	37866.43
	89022.05	50417.85	64978.47	55872.09	48314.16	24274.16	55518.92	43836.02	73361.85	26920.64	54568.2	57626.23	55829.71	48914.62	56713.45	55255.32	35604.77	40618.11	39976.83	27179.82
	86006.4	51759.46	71800.02	53707.86	39915.76	31895.25	64758.18	55036.03	57137.1	34422.76	54647.45	53629.43	81956.75	44931.42	53964.61	57645.05	51068.58	56704.81	38975.43	36556.7
	109513.2	58344	53999	47732.3	43383.66	39941.41	75356.57	55977.77	57345.96	33135.87	56629.89	65105.75	61472.47	50732.26	47778.26	49126.68	50112.26	46575.13	32546.65	34388.44
	79743.88	70035.76	60672.24	48369.52	56375.39	40587.8	50586.98	48908.12	48574.78	36429.63	46114.72	54457.65	66790.04	48078.09	38312.34	39428.45	45723.83	50338.58	41793.59	55220.48
	73902.93	62739.29	51438.61	45375.33	63673.91	45810.09	59121.29	47362.24	52383.86	43178.19	42904.08	78920.73	68321.45	51505.72	37794.47	55347.91	43222.2	49286.13	42232.86	29019.39
	57770.21	80364.02	46353.88	45122.48	78205.82	36588.7	52343.7	47727.37	64341.52	46247.74	50764.49	78657.31	72289.12	48035.33	54972.45	55872.88	46147.96	55520.6	51466.14	32890.61
	62688.18	106251.4	51913.54	50881.99	49955.9	43338.2	50797.94	55658.46	65329.34	47326.64	56881.18	69469.2	11105.9	48122.69	63414.95	37386.2	52580.66	46249.07	32978.82	32832.22
	66297.74	58769.37	57173.97	54469.32	62626.77	41957.23	66496.73	50238.05	56636.29	41748.32	60654.38	65973.3	3822.03	41630.79	60236.02	34461.96	53788.14	46227.61	43079.94	33131.94
	69430.59	58898.25	66534.8	58846.2	43647.16	21148.26	47182.14	59797.5	59503	45060.78	58435.98	59444.68	5965.68	45057.29	67677.9	39508.23	64731.48	49857.7	56964.87	34413.97
	69109.63	52786.89	71599.95	46882.28	66619.3	16597.52	50435.7	59396.01	56642.23	54447.6	54763.13	64618.36	4904.94	37753.5	64557.59	4853.27	62192.05	55860.98	63523.38	27913.72
	67954.9	60415.27	65095.88	51523.54	65033.69	6331.41	59349.02	76252.44	48712.82	53720.88	57296.65	64618.36	7187.89	45176.94	58385.26	12878.37	61494.24	52681.95	59378.66	31339.72
	65325.49	71555.47	70346.91	51549.11	46712.94	22178.7	72212.83	60662.61	50113.58	41952.71	63331.93	58928.04	6284.02	48633.64	49351.16	33564.96	57147.9	44241.19	50510.07	50020.72
	54356.97	59287.18	71254.68	42956.9	59308.23	35253.21	49186.49	72143.9	84009.32	46358.9	63138.53	62237.95	13230.8	53025	47856.54	47067.66	32399.55	40637.22	50406.09	57745.06
	45672.79	66743.43	74460.41	70323.09	58108.02	48216.52	57450.77	68267.24	75912.72	42068.32	61912.96	60693.5	16663.97	48603.37	55843.58	53972.02	54818.69	52387.46	59696.49	51162.09

	60040.57	60353.88	66826.26	62820.92	52859.75	26835.2	63562.85	67239.46	70209.22	42682.96	68035.94	49447.17	19639.84	49349	51109.17	54170.63	49533.52	48366.9	57526.05	47836.75
	61811.2	59357.31	76014.81	69136.46	63706.69	28707.91	61636.33	65279.42	44284.83	40850.65	66552.31	54819.09	27912.32	46329.48	51973.7	42767.28	55437.6	44794.94	57330.58	60462.74
	61005.04	65420.11	66441.63	53078.55	60728.52	24302.35	45796.18	73140.69	46280.93	41083.26	61967.69	52638.35	16399.4	57941.33	49604.9	48765.17	28924.68	54984.77	46288.12	15803.84
	79893.38	46890.42	70570.74	55213.94	51149.58	30459.4	51519.14	67721.82	55396.77	47614.36	61198.48	50863.51	3731.73	57057.21	61044.09	50956.34	7330.35	48126.12	43134.65	60252.92
	97685	58096.44	69885.44	47958.12	61978.72	23831.19	60005.27	58835.41	49183.23	48432.82	24086.03	43373.97	6274.84	66676.68	58313.5	64290.84	9324.22	52699.18	44096.35	52528.12
	81809.47	63819.88	62621.25	50640.43	58428.3	43056.93	60895.23	63368.31	39225.24	46438.66	21565.37	36581.69	44215.32	54232.73	65466.47	67011.62	41669.36	41198.46	41786.42	46823.46
	75401.62	62819.67	56155.58	67904.18	57513.97	28287.09	59574.29	53950.39	53889.95	68400.7	19723.71	41976.69	60262.55	42375.94	72254.52	50104.19	45014.95	47292.77	47856.13	46396.48
	71450.39	74954.26	50252.39	50104.14	56303.27	39218.95	59897.79	53587.89	37051.87	61078.72	7891.8	40775.79	68300.28	53110.73	70937.67	46731.32	19178.31	48843.16	36845.7	43819.73
	66983.31	81169.23	60713.14	61013.75	49238.63	43486.3	58753.05	57565.82	42550.22	71716.46	403.46	37003.3	63688.55	46670.24	60584.94	47971.46	31145.34	38761.8	31511.68	48511.03
Mean	**67421.80**	**60565.45**	**62489.29**	**51614.20**	**55278.19**	**37893.92**	**57304.11**	**57483.03**	**52120.45**	**42305.06**	**50031.60**	**49015.17**	**40836.79**	**53176.72**	**46488.62**	**45858.92**	**41973.27**	**46732.9**	**42970.57**	**30575.48**
St Dev	**15128.21**	**12096.37**	**8699.330**	**8239.578**	**8091.017**	**13814.95**	**7020.087**	**8234.293**	**16991.6**	**10560.84**	**16470.84**	**17190.28**	**26558.68**	**10217.13**	**22830.39**	**13134.15**	**14432.55**	**7398.003**	**10104.14**	**19134.6**

	Low Dose	**Low Dose**	**Low Dose**	**Low Dose**	**Low Dose**	**High Dose**	**High Dose**	**High Dose**	**High Dose**	**High Dose**
Rat Number	**62**	**63**	**64**	**65**	**66**	**72**	**73**	**74**	**76**	**77**
	66155.52	58168.34	63596.69	46345.96	55485.59	61299.08	69077.22	50835.86	55625.93	63974.66
	54706.76	37971.2	52104.7	35425.6	52599.21	52807.48	51020.75	56287.59	44232.57	63266.29
	63244.93	69875.6	49561.01	59024.2	56528.96	56603.86	49842.79	45347.89	39031.96	56923.47
	62109.02	69741.42	33683.57	50683.98	44641.63	53491.2	66047.76	47331.57	44247.35	42244.68
	60712	57411.8	29947.61	66591.63	60364.33	51052.15	50955.54	48102.64	49667.55	32952.54
	56900.84	68253.93	43886.97	45664.09	54099.12	71787.11	50692.88	45448.31	57809.6	43989.76
	59715.34	61446.62	41236.71	57106.33	58510.53	75121.62	45284.55	39212.5	40314.75	55698.85
	65342.86	59254.25	42549.19	51804.59	60499.14	75287.35	40761.18	38071.43	30096.19	43834.25
	69676.98	67007.53	40896.29	41968.25	68446.59	62457.02	68898.65	15972.44	28161.1	52003.2
	62380.79	64213.26	36336.22	52949.46	58062.79	61700.46	67492.41	49015.14	31807.6	56415.01
	57948.69	63020.01	46122.79	45154.91	56464.87	62516.43	59925.11	50983.66	35385.34	48838.02
	62833.01	63631.51	42438.02	52067.48	58718.73	67281.36	70475.69	48686.36	49962.86	47597.89
	60382.22	75992.57	35857.04	45591.38	58718.73	64401.83	62830.17	40286.53	60209.13	49578.7
	61661.07	68912.76	49734.22	76247.62	41564.49	50162.34	55768.93	49855.65	49022.13	60173.44
	61747.13	68258.59	50884.39	54472	48389.77	46105.91	61010.46	38479.21	42101.81	66494.73
	47085.73	59944.52	52200.82	62853.38	50993.34	45984.71	54947.83	37981.44	50907.01	60827.36
	75977.46	53043.79	46430.96	64734.7	55934.67	61143.62	63927.18	28177.97	41194.65	59486
	61343.39	79132.43	46359.98	60139.5	48343.41	61435.09	56761.72	24368.29	52559.69	57987.56
	51421.41	67838.03	58858.36	63059.47	63172.84	68173.86	59376.16	43481.83	60590.01	53053.35
	58795.15	60482.57	70130.85	69230.92	57176.27	80406.48	64565.88	44735.56	57935.87	52748.57
	59856.93	78106.41	58073.14	71189.91	42242.3	65903.31	57873.85	44734.63	55565.38	51442.78

	60311.94	85240.51	52338.33	54026.6	42872.37	57415.68	55568.61	41610.27	51554.21	56386.85
	56401.82	65731.38	42351.29	56511.4	69373.62	60735.62	62969.97	42846	49701.43	53437.16
	52629.28	60262.24	45251.55	47329.93	53017.44	68913.99	60989.64	55499.84	51368.5	54962.91
	69456.65	54022.97	52351.32	41832.61	58798.05	60433.85	46261.99	65563.38	58253.79	47901.42
	54307.27	55738.7	47918.13	46117.51	61657.44	64696.38	44006.59	57931.84	64095.54	44286.57
	59170.32	68084.3	44833.11	47550.26	58019.75	48331.79	30223.58	49998.47	61160.4	44440.76
	56286.41	48655.51	71477.34	46543.16	71448.95	48678.49	39151.29	63420.3	57450.98	44554.14
	72174.48	57563.3	57308.18	53440.5	46699.74	60785.02	35593.3	52411.51	64433.27	35321.39
	68082.09	41272.98	53790.97	55220.13	57327.23	48260.45	36567.26	43826.11	60486.92	40208.55
	66805.03	51907.2	46551.86	43110.04	50585.69	47409.13	59319.65	46058.09	49198.11	51151.71
	79719.25	26624.32	57610.22	47082.07	55479.02	40570.29	38711.88	42949.32	48057	43465.19
	77774.63	69284.39	58591.08	42279.15	55634.64	51532.77	47023.65	46902.7	42296.55	45552.54
	92664.21	55939.13	60804.8	38992	52751.34	59438.92	68283.41	49646.2	53513.39	37692.54
	68471.37	68388.65	70282.73	23590.94	63092.2	60785.02	42219.36	46469.64	60137.86	18926.54
Mean	**63264.34**	**61726.36**	**50067.15**	**51883.76**	**55648.99**	**59231.70**	**54126.48**	**45500.86**	**49946.75**	**49651.98**
St Dev	**8861.900**	**11603.49**	**10107.67**	**10811.11**	**7222.895**	**9367.866**	**11043.31**	**9665.141**	**9698.044**	**9788.042**

7.7.9 Quantitative Immunohistochemistry with Doxycycline, Summary Data Sets

Sham 0/24 refers to sham-operated animals with a four-hour anaesthetic and sacrificed after 24 hours, Bilat 4/24 refers to animals that underwent 4 hours of bilateral lower limb ischaemia and sacrificed after 24 hours of reperfusion. Low Dose Doxycycline was defined as 50 mg/kg twice a day for 7 days before the ischaemia/reperfusion experiment. High Dose Doxycycline was defined as 200 mg/kg twice a day for 7 days before the ischaemia/reperfusion experiment. Both the Low Dose and High Dose groups of animals underwent 4 hours of bilateral lower limb ischaemia followed by 24 hours of reperfusion before being sacrificed.

All slides were prepared as described in Chapter 4.2.

The value for brightness for each animal is the mean of the 35 images brightness levels as shown in Section 7.7.8

Mean refers to the arithmetic mean of the brightness levels for the 5 animals in each group. St Dev refers to standard deviation.

Table LII: Summary of Left leg Quantitative Immunohistochemistry with Doxycycline.

Rat Number	Sham 0/24	Rat Number	Bilat 4/24	Rat Number	Low Dose Doxy-cycline.	Rat Number	High Dose Doxy-cycline
19	29969.3	52	18250.05	62	45828.78	72	26865.19
21	29172.67	53	18836.28	63	26196.88	73	28566.73
33	34814.15	54	11248.42	64	29263.12	74	23405.15
34	19806.3	56	18147.58	65	27300.15	76	28045.72
36	22978.78	60	20585.32	66	21694.9	77	32040.87
Mean	**27348.24**	**Mean**	**17413.53**	**Mean**	**30056.77**	**Mean**	**27784.73**
St Dev	**5956.328**	**St Dev**	**3582.306**	**St Dev**	**9243.979**	**St Dev**	**3115.313**

Table LIII: Summary of Lung Quantitative Immunohistochemistry with Doxycycline.

Rat Number	Bilat 4/0
44	35873.6
45	26639.5
47	28984.6
48	22489.1
51	31629.8
Mean	**29123.**
St Dev	**5051.0**

Rat Number	Bilat 4/4
39	31729.7
40	23982.3
41	29018.6
42	18767.8
43	17299.4
Mean	**24159.5**

Rat Number	Sham 0/24	Rat Number	Uni 4/24	Rat Number	Bilat 4/24	Rat Number	Doxy Low Dose.	Rat Number	Doxy High Dose
19	59189.7	6	8527.49	52	12465.7	62	16809.9	72	23602.9
21	61389.4	7	28490.6	53	10690.1	63	17078.1	73	24803.4
33	45454.1	16	16887.5	54	12813.9	64	21218.6	74	28676.4
34	82467.9	30	12898.4	56	11974.4	65	12434.5	76	23646.1
36	64020.9	32	10752.9	60	6370.70	66	14668.7	77	23560.5
Mean	**62504.45**	**Mean**	**15511.4**	**Mean**	**10863**	**Mean**	**16442.02**	**Mean**	**24857.9**
St Dev	**13264.47**	**St Dev**	**7883.512**	**St Dev**	**2637.279**	**St Dev**	**3261.905**	**St Dev**	**2197.179**

Rat Number	Sham 0/72	Rat Number	Uni 4/72	Rat Number	Bilat 4/72
5	63855.5	14	6187.71	67	15384.1
11	68188.8	15	1265.51	68	6596.90
18	51560.5	24	8054.82	69	11159.6
20	46698.8	25	24041.9	70	13474.6
27	33740.0	29	9822.06	71	18900.2
Mean	**52808.7**	**Mean**	**9874.41**	**Mean**	**13103.1**
St Dev	**13793.8**	**St Dev**	**8540.17**	**St Dev**	**4611.27**

Table LIV: Summary of Kidney Quantitative Immunohistochemistry with Doxycycline.

Rat Number	Bilat 4/0
44	67421.8
45	60565.4
47	62489.3
48	51614.2
51	55278.1
Mean	**59473.7**
St Dev	**6181.264**

Rat Number	Bilat 4/4
39	37893.9
40	57304.2
41	57483.0
42	52120.4
43	42305.0
Mean	**49421.3**
St Dev	**8915.394**

Rat Number	Sham 0/24	Rat Number	Uni 4/24	Rat Number	Bilat 4/24	Rat Number	Low Dose Doxycycline	Rat Number	High Dose Doxycycline
19	54255.9	6	53023.7	52	50031.6	62	63264.3	72	59231.7
21	67583.2	7	54881.1	53	49015.1	63	61726.3	73	54126.4
33	57877.3	16	54654.9	54	40836.7	64	50067.1	74	45500.8
34	75675.7	30	51540.0	56	53176.7	65	51883.7	76	49946.7
36	59793.2	32	52510.4	60	46488.6	66	55648.9	77	49651.9
Mean	**63037.11**	**Mean**	**53322.05**	**Mean**	**47909.78**	**Mean**	**56518.12**	**Mean**	**51691.56**
St Dev	**8582.782**	**St Dev**	**1425.72**	**St Dev**	**4623.623**	**St Dev**	**5841.338**	**St Dev**	**5203.643**

Rat Number	Sham 0/72	Rat Number	Uni 4/72	Rat Number	Bilat 4/72
5	57056.2	14	50562.8	67	45858.9
11	60540.6	15	46796.7	68	41973.2
18	53807.8	24	55423.5	69	46732.9
20	63101.8	25	64664.5	70	42970.5
27	57041.6	29	60058.1	71	30575.4
Mean	**58309.6**	**Mean**	**55501.1**	**Mean**	**41622.2**
St Dev	**3584.23**	**St Dev**	**7157.03**	**St Dev**	**6481.52**

CHAPTER 8:

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