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Abrogation of Ischemia Reperfusion Injury in Murine Hind Limb and Skin Flap Models of Injury

A Thesis Submitted to the National University of Ireland, Galway in the Fulfilment of the Degree of Doctor of Medicine

By

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Academic Supervisor: Professor Michael Kerin

February 2014
Table of Contents

Table of Contents ................................................................. ii
ABSTRACT .............................................................................. iv
Statement Related to this Work.................................................... vi

STATEMENT OF HYPOTHESIS AND OBJECTIVES .................. VII
List of Figures and Illustrations ................................................... ix
List of Symbols, Abbreviations and Nomenclature ............................. xiii

CHAPTER ONE: INTRODUCTION ............................................. 1
  1.1 ISCHAEMIA INTRODUCTION ........................................... 2
  1.2 CELLULAR RESPIRATION .............................................. 4
    1.2.1 MITOCHONDRIA AND AEROBIC METABOLISM ................. 4
    1.2.2 ANAEROBIC RESPIRATION ..................................... 8
    1.2.3 OXIDATIVE STRESS .............................................. 8
  1.3 ISCHEMIA REPERFUSION INJURY .................................... 9
    1.3.1 SYSTEMIC REPERFUSION INJURY ............................ 11
  1.4 THERAPIES FOR ISCHEMIA REPERFUSION INJURY .......... 12
    1.4.1 PRE-CONDITIONING IN ISCHEMIA REPERFUSION INJURY .... 12
    1.4.2 POST-CONDITIONING ......................................... 17
    1.4.3 STATINS ....................................................... 21
    1.4.4 HYPERBARIC OXYGEN ......................................... 21
    1.4.5 NITRIC OXIDE ................................................. 21
    1.4.6 ANTIOXIDANTS .................................................. 22

CHAPTER TWO: POLOXAMER 188 DECREASES MUSCLE INJURY AND
MORTALITY FROM ISCHEMIA-REPERFUSION INJURY IN A MURINE
HIND LIMB MODEL OF INJURY .................................................. 23
  2.1 INTRODUCTION .......................................................... 24
    2.1.1 POLOXAMER 188 STRUCTURE ................................ 27
    2.1.2 POLOXAMER 188 AS AN EMULSIFICATION AGENT .......... 28
    2.1.3 POLOXAMER 188 AND CELL SALVAGE IN VITRO .......... 29
    2.1.4 POLOXAMER 188 AS AN IN VIVO THERAPEUTIC IN ANIMAL MODELS .... 38
    2.1.5 POLOXAMER 188 IN HUMAN TRIALS ........................ 53
    2.1.6 POLOXAMER 188 AND LIPID MONOLAYERS .................. 57
  2.2 MATERIALS & METHODS .............................................. 59
    2.2.1 POLOXAMER 188 TREATMENT ................................ 60
    2.2.2 ANIMAL CARE PROTOCOL ................................... 61
    2.2.3 IN VIVO HINDLIMB ISCHAEMIA ............................. 62
    2.2.4 MUSCLE HISTOLOGY .......................................... 65
    2.2.5 MUSCLE INJURY SCORING ................................... 68
    2.2.6 TISSUE ADENOSINE TRIPHOSPHATE LEVELS .............. 70
      2.2.6.1 ATP EXTRACTION ....................................... 71
      2.2.6.2 STANDARDS PREPARATION .............................. 72
      2.2.6.3 ASSAY PROCEDURE ..................................... 72
    2.2.7 HINDLIMB MORTALITY ......................................... 75
### CHAPTER THREE: A NOVEL, NATURAL IGM-BINDING, PEPTIDE DECREASES MURINE SKIN FLAP INJURY FROM ISCHEMIA REPERFUSION INJURY

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 INTRODUCTION</td>
<td>100</td>
</tr>
<tr>
<td>3.1.1 The Complement System</td>
<td>101</td>
</tr>
<tr>
<td>3.1.2 Complement in Ischaemia Reperfusion Injury</td>
<td>103</td>
</tr>
<tr>
<td>3.1.3 Toll-like Receptors in Ischaemia Reperfusion Injury</td>
<td>106</td>
</tr>
<tr>
<td>3.1.4 Cytokines in Ischaemia Reperfusion</td>
<td>112</td>
</tr>
<tr>
<td>3.1.5 Cellular Mediators of Innate Immunity in Ischaemia Reperfusion.</td>
<td>113</td>
</tr>
<tr>
<td>3.1.5.1 Macrophages</td>
<td>115</td>
</tr>
<tr>
<td>3.1.5.2 Neutrophils</td>
<td>115</td>
</tr>
<tr>
<td>3.1.5.3 Dendritic Cells</td>
<td>117</td>
</tr>
<tr>
<td>3.1.5.4 T-Cells in Ischaemia Reperfusion</td>
<td>117</td>
</tr>
<tr>
<td>3.1.5.5 B-Cells in Ischaemia Reperfusion</td>
<td>121</td>
</tr>
<tr>
<td>3.1.6 Reactive Oxygen Species in Ischaemia Reperfusion</td>
<td>123</td>
</tr>
<tr>
<td>3.2 MATERIAL AND METHODS</td>
<td>128</td>
</tr>
<tr>
<td>3.2.1 Animal Care Protocol</td>
<td>129</td>
</tr>
<tr>
<td>3.2.2 Dorsal Skin Flap</td>
<td>129</td>
</tr>
<tr>
<td>3.2.3 Skin Flap Viability</td>
<td>136</td>
</tr>
<tr>
<td>3.2.4 Flap Pre-Conditioning</td>
<td>138</td>
</tr>
<tr>
<td>3.2.5 P8 Peptide Administration</td>
<td>138</td>
</tr>
<tr>
<td>3.2.6 Skin Flap Immunohistochemistry</td>
<td>139</td>
</tr>
<tr>
<td>3.2.7 Statistical Analysis</td>
<td>140</td>
</tr>
<tr>
<td>3.3 RESULTS</td>
<td>141</td>
</tr>
<tr>
<td>3.3.1 Statistics</td>
<td>142</td>
</tr>
<tr>
<td>3.3.2 Effect of Pre-Conditioning on Flap Necrosis</td>
<td>142</td>
</tr>
<tr>
<td>3.3.3 Effect of P8 IGM Blocking Peptide on Flap Necrosis</td>
<td>145</td>
</tr>
<tr>
<td>3.3.4 Immunohistochemistry</td>
<td>148</td>
</tr>
<tr>
<td>3.4 DISCUSSION</td>
<td>151</td>
</tr>
<tr>
<td>3.5 CONCLUSION</td>
<td>156</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>157</td>
</tr>
</tbody>
</table>
ABSTRACT

Introduction/Aim

Ischemia Reperfusion injury is a poorly understood entity with wide-ranging clinical implications touching on most fields of clinical medicine. Using skin flap and hind limb models of injury in the mouse we attempt to reduce ischemia reperfusion injury by targeting different parts of the ischemia reperfusion injury pathway.

Methods

Dorsal lateral thoracic artery island skin flaps (3.5x1.5 cm) were elevated in C57BL/6 mice and rendered ischemic for 10 hours by placing a 7 mm microclamp on the vascular pedicle followed by 7 days of reperfusion. Hind-limb ischemia was achieved with orthodontic rubber bands applied above the greater trochanter of male C57BL/6 mice using a McGivney Haemorrhoid Ligator. Limbs underwent ischemia for two hours followed by 24 hours reperfusion prior to euthanasia. Animals were treated with intravenous Poloxamer 188 and P8 IgM-binding protein to assess their effect on ischemia-reperfusion injury.

Results

Administration of P188 prior to ischemia gave an average injury score of 8% which was significantly lower than the control group, injury score 55% (p<0.001). When administered just prior to reperfusion 12% injury was seen in the P188 group compared to 33% in the control group (p<0.001). Similarly when given two hours post-reperfusion P188 treated animals had 14% injury, compared to 38% injury in the control group (p<0.001). There was, however, no protection seen when treatment was given four hours post-reperfusion.
In animals treated with P8 peptide, there was 14.61±2.77% flap necrosis. This represents a statistically significant 56% reduction in flap necrosis compared to controls (p<0.001).

**Conclusion**

Single dose P188 administered to animals undergoing hind-limb ischemia results in an increased myocyte survival rate following IR injury. This therapy is effective in pre-treating animals as well as in a post-hoc, salvage role. We hypothesize that this effect is most likely due to P188 inserting into membrane defects and allowing membrane repair. This degree of protection from IR injury is comparable to the best results in other studies. P8 blockage of a specific IgM can protect against IR injury in an axial skin flap. This type of protection could be useful in improving results in free flap transfer and composite tissue transplantation.
Statement Related to this Work

This work was carried out in the Plastic Surgery Research Laboratories of the Massachusetts General Hospital, Harvard Medical School, Boston, MA. The author performed the work as outlined above, however there was significant contribution from many others including: Soner H Tatlıdexe MD, Michael C McCormack MBA, David A Bichara MD, Miguel A Medina MD, John T Nguyen MD, Kyle R Eberlin MD, Francis D Moore Jr. MD, Michael T Watkins MD, and Raphael C Lee MD. Their assistance and support was invaluable and greatly appreciated.

A special debt of gratitude must be acknowledged towards the input of my supervisors at the Massachusetts General Hospital, Dr. William G Austen Jr., MD and Mr. Mark Randolph. Their constant support and guidance made this work possible and without their unending patience it would never have reached a conclusion.

This study was supported and supervised by Professor Michael Kerin, Professor of Surgery at NUIG.

These animal studies were undertaken with the prior approval of the Massachusetts General Hospital’s Institutional Review Board and all procedures complied with “Principles of Laboratory Animal Care” (Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication No. 86-23, Revised 1996).
Statement of Hypothesis and Objectives

Ischaemia and reperfusion are common clinical entities encountered in acute and chronic disease processes and in their treatment. Multiple examples exist but common morbidity causing examples include: myocardial infarctions, cerebrovascular accidents, peripheral vascular disease, organ transplantation and tourniquet/clamp applications during surgery.

Restoration of blood flow to ischemic tissue is vital for tissue survival. This reperfusion, however, can trigger a cascade of acute inflammatory events leading to increased cellular death resulting in tissue dysfunction and necrosis. Ischemia-reperfusion injury is modulated by complex inflammatory and immunological signalling pathways that have to date not been fully elucidated.

Although ischemia reperfusion injury is multifactorial, one of the earliest events in the process is related to the binding of circulating natural antibodies and the activation of complement. The final common pathway is oedema, attraction of activated leukocytes, and the formation of membrane-attack complexes all of which lead to disruption of cell membranes and resulting cell death.

The aims of this research are to evaluate the efficacy of various treatments aimed at arresting or reversing ischemia reperfusion injury at various points along the injury pathway. Using an IgM binding peptide we hope to arrest IR injury at one of the earliest stages of initiation and prevent compliment activation. Using P188 we seek to prevent and/or reverse IR injury by targeting the final common pathway of IR, namely cell membrane disruption.
Aims:
1. To determine the optimal dose of P188 using previously published *in vivo* and *in vitro* data
2. To assess the affect of P188 on hind-limb ischemia reperfusion when given
   i. Prior to onset of ischemia
   ii. Prior to initiation of reperfusion
   iii. After initiation of reperfusion (salvage therapy)
3. To investigate the effect of a novel IgM binding peptide on skin flap ischemia-reperfusion injury.
List of Figures and Illustrations

Figure 1. Metabolism........................................................................................................7

Figure 2. Infarct size reduction by pre- and post conditioning. Coronary occlusion, followed by reperfusion is associated with infarct development in the ischaemic risk zone (Control). Brief periods of ischemia and reperfusion performed either before index coronary occlusion or immediately after index coronary occlusion can significantly reduce infarct size. From Zhao et al., Am J Physiol Heart Circ Physiol, 2003.................................................................19

Figure 3. Chemical structure of a poloxamer. Poloxamers differ through varying numbers and ratios for a and b.................................................................27

Figure 4. Fluorescent dye leakage following electroporation of rat muscle cells in vitro. From RC Lee et al. PNAS, May 1992.................................................................30

Figure 5. Cell viability assay following high dose ionizing radiation injury of rat muscle cells in vitro. From B Greenebaum at al. Burns, Feb. 2004 ..................32

Figure 6. Effect of P188 on fibroblasts subjected to heat shock. From Merchant et al., JSR, Feb. 1998.................................................................................................34

Figure 7. Schematic diagram of membrane-targeted mechanisms causing NMDA-induced neuronal death and the actions of P188 on the plasma membrane. From Marks et al., FASEB J, Feb. 2001 ......................36

Figure 8. Normalized resistivity for flaps immediately post electroporation, 1h post IV saline, 1h post IV dextran, 1h post IV P188, and immediately after injury in animals pre-treated with P188. From Lee et al., PNAS, May 1992. 39

Figure 9. CMAP amplitudes in control, shocked lactated Ringers (LR) treated, and P188 treated rats. From Collins et al., Biochem & Biophysics Acta, Jan. 2007........................................................................................................41


Figure 11. Comparison of cyst formation between P188 and vehicle-treated spinal injuries. 3D reconstructions of injured spinal cord segments. The dark red regions are cysts (labelled c). From Borgens et al. J Neurosci Res. April, 2004........................................................................................................48

Figure 12. Photomicrographs of coronal sections of rat brain stereotactically injected with quinolinic acid and stained with immunohistochemical marker Neu-N. A; lesion after intrathecal injection of control. B; lesion after intrathecal P188 injection. From Curry et al. Neurosurgery, Oct. 2004. .......50
Figure 13. Effect of P188 on coagulation area 24h after the burn. Fluorescein/dextran was injected 24h after the burn, and fluorescent images were obtained. From Baskaran et al. JSR, Nov. 2001.

Figure 14. Kaplan-Meier analysis of proportion of patients remaining in crisis over time. From Orringer et al. JAMA, Nov. 2001.

Figure 15. Time points for different P188 administration.

Figure 16. Murine hind limb with orthodontic rubber band in situ and corresponding laser Doppler image confirming complete ischemia of the limb.

Figure 17. Mason trichrome stained section of murine gastrocnemius showing a mix of injured and uninjured muscle fibres as well as a neurovascular bundle.

Figure 18. Sample of Mason trichrome stained murine gastrocnemius muscle following IR injury. Each muscle fibre is deemed injured (I) or uninjured (U). A percentage injury score is generated once 600 individual fibres are scored for each muscle.

Figure 19. Example of an ATP Standard curve used to extrapolate the concentration of unknown values.

Figure 20. Dose response curve when P188 given 10 minutes prior to reperfusion.

Figure 21. Microscopic Skeletal Muscle Morphology: A. Mix of injured and uninjured muscle fibres in a control animal (60% injury). B: Uninjured Fibres in a P188 treated animal.

Figure 22. P188 protects against IR injury in muscle fibres when given prior to onset of ischemia (p<0.001).

Figure 23. P188 protects against IR injury in muscle fibres when given prior to onset of reperfusion (p<0.001).

Figure 24. P188 protects against IR injury in muscle fibres when given 2 hours following onset of reperfusion (p<0.05).

Figure 25. No difference is seen in muscle injury scores when P188 is given 4 hours after the onset of reperfusion.

Figure 26. Cellular ATP levels after 3 hours of reperfusion. Treatment administered 10 minutes prior to reperfusion beginning. Animals treated with P188 had 6.23 ng/mg ATP vs. 2.53 ng/mg in those treated with Dextran (p<0.05).

Figure 27. Cellular ATP levels after 6 hours of reperfusion. Treatment administered 10 minutes prior to reperfusion beginning. Animals treated
with P188 had 6.15 ng/mg ATP vs. 2.83 ng/mg in those treated with Dextran (p<0.05). .................................................................87

Figure 28. Cellular ATP levels after 24 hours of reperfusion. Treatment administered 10 minutes prior to reperfusion beginning. Animals treated with P188 had 5.55 ng/mg vs. 2.41 ng/mg in those treated with Dextran (p<0.05). .................................................................88

Figure 29. Cellular ATP levels after 24 hours of reperfusion. Treatment administered 2 hours after initiation of reperfusion. Animals treated with P188 had 2.2. ng/mg vs. 0.49 ng/mg in those treated with Dextran (p<0.05). .................................................................90

Figure 30. Individual animal ATP levels. Concentration in µM is extrapolated from a standard curve based on luminescence readings........................................90

Figure 31. Kaplan-Meier survival curve when treatment given 10 minutes prior to the start of reperfusion. 75% of P188 animals survived for 7 days compared to 25% of control animals (p=0.0077).................................................................92

Figure 32. Kaplan-Meier survival curve when treatment given 2 hours after the start of reperfusion. 50% of P188 animals survived for 7 days compared to 8.3% of control animals (p=0.032).................................................................93

Figure 33. Schematic of the complement system........................................105

Figure 34. Deposition of CM22 IgM, C4, and C3 on injured intestinal tissues. Representative cryosections of intestinal tissues were harvested after intestinal IR from RAG-1/-/- mice pre-treated with either IgM from CM31 (a, d, g, j, m, and p) or CM22 (b, c, e, f, h, i, k, l, n, o, q, and r). All sections were stained with anti-IgM-biotin followed by streptavidin-Alexa 568 (red) and counterstained with 4_, 6-diamidino-2-phenylindole (violet). Cryosections were costained with anti-C4-FITC (green in a–i) and anti-C3-FITC (green in j–r). The co-localization of IgM and C4 (red _ green _ yellow) is represented in g–i. Co-localization of IgM and C3 is represented in p–r (red _ green _ yellow). High-magnification (X400) images of CM22 treatment (c, f, i, l, o, and r) were taken from the same region as X100 magnification marked by white boxes. From Zhang et al., PNAS, March 2006. ........................................................................................................110

Figure 35. The major sources of superoxide radicals (xanthine oxidase system and nicotinamide adenine dinucleotide phosphate system) and the fate of superoxide......................................................................................126

Figure 36. The dorsal skin flap marked out after depilation. The medial border of the flap is midline of dorsum, the lateral border is right axillary line, and the cranial and caudal borders are on the infrascapular line and the iliac crest, respectively.................................................................131

Figure 37. The flap is raised on the dorsal lateral thoracic vascular pedicle. Flap ischemia is achieved by placing a non-crushing 7mm microvascular clamp across both artery and vein. .................................................................132
Figure 38. Intravenous fluorescein is seen throughout the mouse skin except for the skin flap with vascular clamp in situ, confirming flap ischemia. .......................... 133

Figure 39. Within minutes of clamp release fluorescein is seen filling the vascular tree of the flap, confirming reperfusion................................................................. 134

Figure 40. Within 30 minutes of clamp release the entire flap is filled with fluorescein, confirming complete flap reperfusion. .................................................. 135

Figure 41. Flap viability assessment is carried out at Day 7 following IR injury. The non-viable area is marked within the heavy black line. This area is expressed as a percentage of the total flap area...................................................... 137

Figure 42. Bar-graph demonstrating % necrosis of murine skin flaps after 7 days. Group III subjected to 10 hours of ischemia following preconditioning with two twenty-minute cycles of intermittent ischemia and reperfusion. Group I flaps were not clamped. Group II treated with IV saline. Results expressed as mean +/- SEM. P<0.001 for comparison of Group III to all controls........ 143

Figure 43. Statistical analysis of results of flap ischemia experiments with Tukey’s Multiple Comparison Test................................................................. 144

Figure 44. Bar-graph demonstrating % necrosis of murine skin flaps after 7 days. Group I flaps were not clamped. Group II treated with IV saline. Group III treated with a random peptide and Group IV administered the CM22 binding P8 peptide. Results expressed as mean +/- SEM. P<0.001 for comparison of Group IV to all controls.................................................. 146

Figure 45. Statistical analysis of results of flap ischemia experiments with Tukey’s Multiple Comparison Test................................................................. 147

Figure 46. 200X photomicrographs of IgM deposition on medium vessels of murine skin flaps after 10 hours ischemia and 3 hours of reperfusion, as assessed by anti-IgM immunoperoxidase reaction. Panel A: Group II, no pre-treatment. Panel B: Group III, pre-treatment with IV saline. Panel C: Group IV, pre-treatment with IV P8 peptide. Panel D: Group V, pre-treatment with IV random peptide. P8 treated animals showed little or no IgM deposition compared to Groups II, III, or V............................................... 149

Figure 47. 200X photomicrographs of C3 deposition on medium vessels of murine skin flaps after 10 hours ischemia and 3 hours of reperfusion, as assessed by anti-C3 immunoperoxidase reaction. Panel A: Group II, no pre-treatment. Panel B: Group III, pre-treatment with I.V. saline. Panel C: Group IV, pre-treatment with I.V. P8 peptide. Panel D: Group V, pre-treatment with I.V. random peptide. P8 treated animals showed little or no C3 deposition compared to Groups II, III, or V............................................... 150
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AICD</td>
<td>Activated induced cell death</td>
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</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
<td></td>
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<tr>
<td>AP-1</td>
<td>Activator protein</td>
<td></td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ATP</td>
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<tr>
<td>B cell</td>
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<td>CD</td>
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<td>Cytotoxic T lymphocyte-associated antigen 4</td>
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<td>Fragment, crystallizable</td>
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<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>HIF</td>
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<td>IL</td>
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<td>Interferon</td>
<td></td>
</tr>
<tr>
<td>IPC</td>
<td>Ischaemic preconditioning</td>
<td></td>
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<tr>
<td>IR</td>
<td>Ischaemia reperfusion injury</td>
<td></td>
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<tr>
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<td>Intravenous</td>
<td></td>
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<tr>
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<td>LOP</td>
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<td>MAC</td>
<td>Membrane attack complex</td>
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<td>MBL</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complexes</td>
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<tr>
<td>MI</td>
<td>Myocardial infarction</td>
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</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide Coenzyme</td>
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<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
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</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
<td></td>
</tr>
<tr>
<td>NMDS</td>
<td>N-methyl-D-aspartic acid</td>
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<tr>
<td>NMHC-II</td>
<td>Non-muscle heavy chain type 2</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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NOS = Nitric oxide synthase  ml = millilitre
PAF = Platelet aggregating factor  µl = microlitre
PBMC = Peripheral blood mononuclear cell  mg = milligram
PDC = Pyruvate Dehydrogenase Complex  µg = microgram
PGE₂ = Prostaglandin E2  pg = picogram
PKA = Protein kinase A  mm = millimetre
PKC = Protein kinase C  kDa = Kilodalton
PMN = Polymorphonuclear cells  RI = Reperfusion injury
RI = Reperfusion injury  ROS = Reactive oxygen species
SEM = Standard error of the mean  SCID = Severe combined immunodeficiency
SOD = super oxide dismutase  Tc = T cell (cytotoxic)
T cell = T lymphocyte  Tcm = T cell (central memory)
TCR = T cell receptor  Tem = T cell (effector memory)
TGF = Transforming growth factor  Th = T cell (helper)
Th = T cell (helper)  Tim = T cell (intermediate memory)
TLR = Toll-like receptors  Tr = T cell (regulatory)
VCAM = Vascular cell adhesion molecule  VEGF = Vascular endothelial growth factor
VEGF = Vascular endothelial growth factor  WT = Wild type
XO = Xanthine oxidase
Chapter One: Introduction
1.1 ISCHAEMIA INTRODUCTION

Ischaemia is the term applied to a deficiency of blood supply to any part of the body relative to its local requirements. The inevitable result of ischaemia is hypoxia, or a deficiency of oxygen to that tissue. Oxygen is but one of many substances transported around the body by the circulatory system but its lack is the one felt first by most tissues. An inability to provide sufficient arterial perfusion pressure to a tissue, or the inability to return blood from a tissue via the capillary-venous systems, results in ischaemia.

Tissue ischaemia is one of the most common pathologies encountered in clinical practice. The systems affected by this ischaemia and the aetiologies are widely disparate and the specialties, both medical and surgical, that diagnose, treat and, in certain cases, cause this ischemia are many. Within the context of either the clinic or the laboratory, awareness of maximal ischaemia time or time to irreversible damage of a specific tissue must be known to safely treat effected patients and design realistic clinical scenarios in the laboratory situation.

Many tissues in the body are extremely vulnerable to ischaemic injury including the myocardium and the central nervous system, where a mere matter of minutes is sufficient to induce irreversible cellular injury (Reimer et al., 1977). Constituents within the limbs including muscle, bone, skin and nerves all tolerate ischaemia relatively well, with a maximum tourniquet time of approximately two hours considered acceptable, however, there is little evidence to support this arbitrary period (Miller et al., 1979) (Sjostrom et al., 1982). Much of the literature focuses on reperfusion injuries sustained in the myocardium; the vasculature of the limbs however differs greatly from both the heart and the brain where tissues are markedly more ischaemia intolerant.
The ischaemic cascade is a series of biochemical reactions that are initiated in aerobic tissues after seconds to minutes of ischemia.

1. Lack of oxygen causes the cell’s normal process for making ATP for energy to fail.
2. The cell switches to anaerobic metabolism, producing lactic acid.
3. ATP-reliant ion transport pumps fail, causing the cell to become depolarized, allowing ions, including calcium (Ca++), to flow into the cell.
4. The ion pumps can no longer transport calcium out of the cell, and intracellular calcium levels get too high.
5. The presence of calcium triggers the release of the excitatory amino acid neurotransmitter glutamate.
6. Glutamate stimulates AMPA receptors and Ca++-permeable NMDA receptors, which open to allow more calcium into cells.
7. Excess calcium entry overexcites cells and causes the generation of harmful chemicals like free radicals, reactive oxygen species and calcium-dependent enzymes such as calpain, endonucleases, ATPases, and phospholipases in a process called excitotoxicity. Calcium can also cause the release of more glutamate.
8. As the cell's membrane is broken down by phospholipases, it becomes more permeable, and more ions and harmful chemicals flow into the cell.
9. Mitochondria break down, releasing toxins and apoptotic factors into the cell.
10. The caspase-dependent apoptosis cascade is initiated, causing cellular apoptotic death.
11. If the cell dies through necrosis, it releases glutamate and toxic chemicals into the environment around it. Toxins poison nearby cells, and glutamate can overexcite them.
1.2 CELLULAR RESPIRATION

Cellular respiration is the set of the metabolic reactions and processes that take place in the cells of organisms to convert biochemical energy from nutrients into adenosine triphosphate (ATP), and then release waste products. The reactions involved in respiration are catabolic reactions that involve the redox reaction (oxidation of one molecule and the reduction of another). Respiration is one of the key ways a cell gains useful energy to fuel cellular reformations. In mammals metabolism occurs predominantly under aerobic conditions but the body and cells themselves are capable of anaerobic activity albeit for a limited time.

1.2.1 MITOCHONDRIA AND AEROBIC METABOLISM

Aerobic respiration requires oxygen in order to generate energies (ATP). Although carbohydrates, fats, and proteins can all be processed and consumed as reactant, it is the preferred method of pyruvate breakdown in glycolysis and requires that pyruvate enter the mitochondrion in order to be fully oxidized by the Krebs cycle. The product of this process is energy in the form of ATP by substrate-level phosphorylation, NADH and FADH$_2$. The reducing potential of NADH and FADH2 is converted to more ATP through an electron transport chain with oxygen as the "terminal electron acceptor". Most of the ATP produced by aerobic cellular respiration is made by oxidative phosphorylation. This works by the energy released in the consumption of pyruvate being used to create a chemiosmotic potential by pumping protons across a membrane. This potential is then used to drive ATP synthase and produce ATP from ADP and a phosphate group. Aerobic metabolism is 19 times more efficient than anaerobic metabolism (which yields 2 mol ATP per 1 mol glucose). They share the initial pathway of glycolysis but aerobic metabolism continues with the Krebs cycle and oxidative phosphorylation. The post glycolytic reactions take place in the mitochondria in eukaryotic cells.
Inhibition of the electron transport chain and oxidative phosphorylation, ordinarily maintained by the mitochondria, results in ATP and phosphocreatine levels decreasing after 3 hrs. of ischemia and ultimately cell death (Reimer et al., 1983, Hartung et al., 1985). In vivo investigations and several in vitro models have demonstrated that inability to restore mitochondrial function after reperfusion was correlated with a similar incapacity to reverse cellular damage in general; indeed tissue recovery after ischemic injury may depend on the maintenance of mitochondrial integrity (Vogt and Farber, 1968, Jennings, 1976, Trump et al., 1976, Mittnacht et al., 1979).

Glycolysis is a metabolic pathway that is found in the cytosol of cells in all living organisms. The process converts one molecule of glucose into two molecules of pyruvate (pyruvic acid), it makes energy in the form of two net molecules of ATP. Four molecules of ATP per glucose are actually produced; however, two are consumed for the preparatory phase. The initial phosphorylation of glucose is required to destabilize the molecule for cleavage into two pyruvate. During the pay-off phase of glycolysis, four phosphate groups are transferred to ADP by substrate-level phosphorylation to make four ATP, and two NADH are produced when the pyruvate are oxidized. The overall reaction can be expressed this way:

\[ \text{Glucose} + 2 \text{NAD}^+ + 2 \text{P}_i + 2 \text{ADP} \rightarrow \text{pyruvate} + 2 \text{NADH} + 2 \text{ATP} + 2 \text{H}^+ + 2 \text{H}_2\text{O} \]

Pyruvate is oxidized to acetyl-CoA and CO\(_2\) by the Pyruvate dehydrogenase complex (PDC). The PDC contains multiple copies of three enzymes and is located in the mitochondria of eukaryotic cells and in the cytosol of prokaryotes. In the conversion of pyruvate to acetyl-CoA one molecule of NADH and one molecule of CO\(_2\) is formed. This step is also known as the link reaction, as it links glycolysis and the Krebs cycle.

The Krebs cycle is also known as the citric acid cycle or the tricarboxylic acid cycle. When oxygen is present, acetyl-CoA is produced from the pyruvate molecules created
from glycolysis. Once acetyl-CoA is formed, two processes can occur, aerobic or anaerobic respiration. When oxygen is present, the mitochondria will undergo aerobic respiration, which leads to the Krebs cycle. However, if oxygen is not present, fermentation of the pyruvate molecule will occur. In the presence of oxygen, when acetyl-CoA is produced, the molecule then enters the citric acid cycle inside the mitochondrial matrix, and gets oxidized to CO₂ while at the same time reducing NAD to NADH. NADH can be used by the electron transport chain to create further ATP as part of oxidative phosphorylation. To fully oxidize the equivalent of one glucose molecule, the Krebs cycle must metabolize two acetyl-CoA. Two waste products, H₂O and CO₂, are created during this cycle.

The citric acid cycle is an 8-step process involving 18 different enzymes. Throughout the entire cycle, acetyl-CoA changes into citrate, cis-aconitate, isocitrate, α-ketoglutarate, succinyl-CoA, succinate, fumarate, malate, and finally, oxaloacetate. The net energy gain from one cycle is 3 NADH, 1 FADH₂, and 1 GTP; the GTP may subsequently be used to produce ATP. Thus, the total energy yield from one whole glucose molecule (2 pyruvate molecules) is 6 NADH, 2 FADH₂, and 2 ATP.
Figure 1. Metabolism.
1.2.2 Anaerobic Respiration

Without oxygen, pyruvate is not metabolized by cellular respiration but undergoes a process of fermentation. The pyruvate is not transported into the mitochondrion, but remains in the cytoplasm, where it is converted to waste products that may be removed from the cell. This serves the purpose of oxidizing the electron carriers so that they can perform glycolysis again and removing the excess pyruvate. This waste product varies depending on the organism. In skeletal muscles, the waste product is lactic acid. This type of fermentation is called lactic acid fermentation. The ATP generated in this process is made by substrate-level phosphorylation, which does not require oxygen.

Fermentation is less efficient at using the energy from glucose since 2 ATP are produced per glucose, compared to the 38 ATP per glucose produced by aerobic respiration. This is because the waste products of fermentation still contain plenty of energy. Glycolytic ATP, however, is created more quickly. For multicellular organisms, during short bursts of strenuous activity, muscle cells use fermentation to supplement the ATP production from the slower aerobic respiration, so fermentation may be used by a cell even before the oxygen levels are depleted, as is the case in sports that do not require athletes to pace themselves, such as sprinting.

1.2.3 Oxidative Stress

Oxygen, although a slow reactant, has a tendency to form incompletely reduced reactive oxygen species (ROS): free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them. Examples are hydroxyl radical, superoxide, hydrogen peroxide, and peroxynitrite, but to name a few. Within the cell, ROS can cause genetic degeneration and physiological dysfunction, eventually leading to cell death and
progressive ageing of the organism (Abele, 2002). Oxidative stress occurs within cells as a result of increased oxidant generation, decreased anti-oxidant protection and/or a failure to repair oxidative damage.

The creation of ROS can indeed result in cellular damage but ROS are also part of normal bodily function and in fact the main source of these in vivo is simple aerobic respiration. ROS are also produced by peroxisomal oxidation of fatty acids, microsomal cytochrome P450 metabolism of xenobiotic compounds, arginine metabolism, and tissue specific enzymes and are broken down by others (Hayes and McLellan, 1999). Additionally, and importantly, in cases of ischaemia and reperfusion, cellular components of the immune system release ROS when activated and during phagocytosis (Tate and Repine, 1983). The principle cellular damage results from the ROS-induced alteration of polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA. Mechanisms of mitochondria-derived muscle damage around ischaemia include production of ROS, onset of mitochondrial permeability and apoptosis triggered by mitochondrial loss of cytochrome c (Borutaite et al., 2003, Lesnefsky et al., 2001, Weiss et al., 2003).

1.3 ISCHEMIA REPERFUSION INJURY

Ischemia causes tissue damage; and, somewhat surprisingly, restoration of blood flow can cause even more damage. This is counterintuitive because it might be expected that the return of blood flow would be of immediate benefit to ischemic tissues. The first clue to the existence of reperfusion injury came in the 1960s, when it was recognized that systemic shock and acidosis followed the restoration of blood flow after the prolonged clamping of major arteries (Baue and McClerkin, 1965, Vetto and Brant, 1968). This phenomenon was referred to as “declamping shock.” It is now appreciated that declamping shock is more than just the flushing out of the “toxic metabolites” that accumulate in ischemic tissues.
Cerra et al. first described reperfusion injury in 1975 (Cerra et al., 1975). They initially studied myocardial pedicles in dogs and, in an experiment that was designed to detect the extent of reperfusion injury, found that restoration of blood flow was associated with sub-endothelial haemorrhagic necrosis. Following this initial finding they reviewed the clinical notes and autopsy finding of 25 patients who died after aortic valve replacement. They discovered that five of these patients probably died as a result of a reperfusion injury— all of these patients had sub-endothelial haemorrhagic necrosis at autopsy and had been subjected to more than 70 minutes of cardiopulmonary bypass. It was concluded that reperfusion was the commonest cause of myocardial infarction after surgery for the replacement of an aortic valve.

The phenomenon of “no-reflow” is well documented in the plastic and reconstructive surgery literature. It consists of progressive vascular damage during reperfusion and an expanding zone of decreased blood flow (Kloner, 1993). In 1978, May et al. described the phenomenon in a free flap experiment and hypothesized that the no reflow phenomenon is caused by cellular swelling, intravascular aggregation, and the leakage of intravascular fluid into the interstitial space (May et al., 1978a). More recent animal studies have shown that the no-reflow phenomenon provokes an inflammatory response in post-capillary venules, which can be minimized by inhibiting the trans-endothelial migration of inflammatory cells (Scott Isenberg, 2003).

Reperfusion injury has a wide clinical relevance. It influences the outcomes of patients after cardiovascular and transplant surgery, as well as influencing the clinical recovery of patients after stroke and myocardial infarction.

Lengthy periods of ischaemia will eventually result in irreversible cell death but the resolution of ischaemia and reperfusion initiates a cascade of events in the already damaged tissues to which they now may be more vulnerable (Grace, 1994). Clinically, the advent of early reperfusion strategies within the context of myocardial infarction limits the injury sustained by the myocardium with infarct size reduction and improves overall survival (Kim and Braunwald, 1993, Boyle and Weisman, 1993). For these
patients the ill effects of reperfusion are countered by the gravity of prolonged ischaemia, which the myocardium tolerates poorly. Application of these principles has revolutionised the management of patients and is now the acceptable standard of care in acute myocardial infarction.

1.3.1 Systemic Reperfusion Injury

Not alone does reperfusion injury cause damage at the tissues directly affected by a period of hypoxia/ischaemia and reperfusion, significant systemic effects may also be encountered. High circulating levels of proinflammatory cytokines responsible for leukocyte activation including TNF-α, IL-6, IL-8 and IL-10 are released systemically following reperfusion such as that encountered after abdominal aortic aneurism repair (Barry et al., 1997, Yang et al., 2000, Neumann et al., 1995, Oz et al., 1995). Aside from this cytokine release, leukocytes activated by local factors at the time of reperfusion but remaining within the systemic circulation travel to distant vascular beds. The circulating pro-inflammatory cytokines then activate the vascular endothelium around the body including the extensive pulmonary microcirculation (the first vascular bed encountered by venous blood returned to the heart) where the stiffened, activated cells may become trapped resulting in pulmonary sequestration which can result in an acute pulmonary microvascular injury, non-cardiogenic pulmonary oedema and eventual adult respiratory distress syndrome which can be fatal (Paterson et al., 1989b, Anner et al., 1988). By means of neutrophil manipulation and depletion and other strategies aimed against cell adhesions on involved endothelium, these effects have been attenuated with preservation of the pulmonary microvascular barrier (Klausner et al., 1988, Welbourn et al., 1991).
1.4 THERAPIES FOR ISCHEMIA REPERFUSION INJURY

Numerous substances and therapeutic strategies have been employed in an attempt to treat IR in muscle and skin. A number of these studies have shown positive results but many have not been substantiated by large-scale follow-up or cross over to larger animal/human trials. A number of the more thoroughly investigated and reported interventions are outlined below.

1.4.1 PRE-CONDITIONING IN ISCHEMIA REPERFUSION INJURY

Preconditioning is the term applied to the phenomenon observed when tissues are exposed to a short period of a stressor followed by a period free from this stressor, which renders the tissue more resistant to subsequent injury. First described in the context of ischaemia and ischaemic preconditioning, other modalities of preconditioning have been investigated including chemical and thermal preconditioning (Murty, 1986). Despite numerous in vivo and in vitro investigations and the identification of numerous putative mediators and pathways the exact mechanisms of preconditioning at a cellular level have yet to be fully elucidated.

In 1986, Reimer et al., in an attempt to examine the contributions of high energy phosphate depletion and catabolite accumulation in the development of ischemic injury, conducted a series of experiments in the dog heart where ATP and catabolite concentrations were measured after a series of four 10 min coronary artery occlusions. These were then compared with ATP concentrations measured after a single 40 min occlusion. After 40 minutes, ATP levels in dogs subjected to serial ischemia were higher
than in those exposed to a single ischemic event. Further, they noted that, of the seven dog hearts exposed to serial ischemic events, six did not develop myocardial necrosis (Reimer et al., 1986). This paradox, that intermittent ischemia may not have an additive but rather a protective effect against subsequent ischemia, was tested in 1986 by Murray et al. They subjected dog hearts to four cycles of alternating five-minute periods of ischemia and reperfusion. This was followed by a prolonged ischemic episode. With this protocol, infarct size was limited to 25% of that in the control group (which had no preceding cycles of ischemia and reperfusion). This form of myocardial protection was termed ischemic preconditioning (Murry, 1986).

Repetitive short periods of ischemia and reperfusion before prolonged ischemia reduce the extent of reperfusion injury (Mounsey et al., 1992b). The protective effect of such ischemic preconditioning is biphasic. The early phase (first 24 hours) has several triggers, but the most important appears to be adenosine, with the subsequent activation of protein kinase C. The late phase (up to 72 hours) has a wide variety of triggers including endotoxins, nitric oxide, adenosine agonist, opioid agonist, and tumour necrosis factor-α (Brown et al., 1992). Inducible nitric oxide synthetase (Takano et al., 1998) and cycloxygenase-2 (Shinmura et al., 2000) appear to be the main mediators of delayed preconditioning.

The protective effect of ischemic preconditioning is related to the duration of each cycle of ischemia and reperfusion (ischemia-reperfusion cycle) and the number of cycles. Zahir et al. have reported that ischemic preconditioning with 10-minute cycles is superior to 5-minute cycles and that three cycles are superior to one or two cycles (Zahir et al., 1998d). Ischemic preconditioning improves the ischemic tolerance of various tissues including skeletal muscle (Mattei et al., 2000, Gurke et al., 2000, Gurke et al., 1996b, Gurke et al., 1996a). In skeletal muscle IP resulted in reduced infarct size in pigs (Mounsey et al., 1992a) and attenuation of capillary no-reflow in dogs (Jerome et al., 1995). The effectiveness of ischemic preconditioning of flaps has been proven in many animal models (Carroll et al., 1997, Zahir et al., 1998c).
Surgical delay—to promote angiogenesis and thus improve flap survival—has been a standard clinical practice in flap surgery, especially in high-risk patients. Zahir et al. have found that ischemic preconditioning and surgical delay had similar benefits when evaluating the survival of a musculocutaneous flap model (Zahir et al., 1998a). Surgical delay has the major disadvantage of requiring an extra surgical procedure, whereas ischemic preconditioning can be performed during the flap elevation.

Originally ischaemic preconditioning described an immediate adaptation to brief vascular occlusion however it is now apparent that two types of protection occur, which differ in respect of both their onset and cellular mechanisms dubbed “early” and “delayed” preconditioning. The effects of early preconditioning develop within minutes and have benefits that work best when followed by “early” ischaemia that is to say less than 1 hour. Delayed preconditioning on the other hand becomes apparent 12–24hrs after reperfusion and its effects can last for 2 to 3 days. Given its rapid onset it is of little surprise that early preconditioning is independent of protein synthesis, while delayed preconditioning is dependent on altered gene expression and synthesis of new proteins (Ishida et al., 1997). Several proteins have been proposed as possible effectors, including nitric oxide synthase (NOS), cyclooxygenase-2, aldose reductase, antioxidant enzymes (particularly Manganese-Super Oxide Dismutase), and heat shock proteins (Bolli, 2000, Carroll and Yellen, 1999).

The preconditioning stimulus was originally reported as a brief insult to a tissue and that this particular tissue was then protected from subsequent and more severe injury. This scenario is now termed “local” preconditioning as a second form has been discovered termed “remote”. Remote preconditioning is a similar brief noxious stimulus to a tissue but the subsequent injury does not necessarily occur in the preconditioned tissue but elsewhere in the body; nonetheless preconditioning still affords it protection. The type and magnitude of benefit derived appear similar but it may now be possible to avoid any unnecessary injury to the target tissue by carrying out a remote preconditioning protocol in an area that may tolerate it better.
In response to ischemic preconditioning, a signal is generated that is transduced into an intracellular message to influence the protective effector mechanisms. It is likely that substances released from the ischaemic tissues act in a paracrine fashion to do this. Cardiac models have suggested a number of molecules including NO, ROS, adenosine, bradykinin, catecholamines, opioids and angiotensin II (Williams et al., 1999). Adenosine seems likely to be the most important of these and as a breakdown product of ATP it is released in volume into the extracellular space at the onset of ischaemia (Cohen et al., 2000). The action of adenosine (via it type I and II receptors) has been shown to inhibit neutrophil oxidative metabolism and adhesion to endothelial cells, to increase membrane stability and energy production by promoting glucose transport, and to reduce Ca\(^{++}\) influx through the activation of ATP-dependent K\(^{+}\) channels (Cronstein et al., 1986, Grisham et al., 1989, Wyatt et al., 1989, Howell et al., 2000). Adenosine receptors (I and II) coupled to G proteins are thus the likely initiators of the preconditioning response. The G protein dissociates and activates a membrane-bound phospholipase that divides phosphatidylinositol biphosphate into two intracellular second messengers; inositol triphosphate, which releases Ca\(^{++}\) from non mitochondrial intracellular stores, and diacylglycerol (DAG), which activates specific isoforms of protein kinase C (PKC) (Speechly-Dick et al., 1994, Ishida et al., 1997). PKC is known to regulate a number of biological processes such as metabolism, ion transport, and gene expression and allows phosphorylation of effector molecules, most probably ATP sensitive (K\(^{+}\)) channel, 5'-nucleotidase, and cAMP-protein kinase A activation (PKA) (Cutrin et al., 2002).

It has been proposed that in the mitochondria, K\(^{+}\) channel openers target an ATP-sensitive K\(^{+}\) channel implicated in the regulation of mitochondrial ion and volume homeostasis. At baseline physiological conditions K\(^{+}\) channel opening is regulated by the amount of ATP available within the cell. Given that the action PKC is likely to occur via K\(^{+}\) ATP channels it is unsurprising that both are found to be activated in preconditioning (Speechly-Dick et al., 1995). However, at the low levels of ATP experienced during preconditioning, the K\(^{+}\) channel is likely to be inhibited (Light et al., 1995). Opening of these channels causes preservation of mitochondrial calcium
homeostasis and ATP levels, thus improving cell viability in the face of otherwise harmful ischaemia. Downstream effects include stimulated production of adenosine from AMP and this is thought to preserve the endothelial barrier function, block leukocyte adhesion (reduced expression of adhesion molecules), superoxide radical production, and phagocytic activity of neutrophils (Ishida et al., 1997, Simkhovich et al., 1998, Tong et al., 2000, Ricciardi et al., 2001). The increased levels of AMP during ischaemia in turn triggers catabolic pathways that generate ATP, while switching off anabolic pathways that consume ATP (Peralta et al., 2001). This has been translated from the laboratory into clinical practice through reduced prescription of glibenclamide based diabetes mediations as these function through inhibition of the K+ ATP channels thereby abolishing the potential positive effect of preconditioning in a high risk patient cohort.

In cardiac ischaemic, preconditioning induced by five minutes of ischaemia reduces myocyte high energy phosphate compounds by a mere 29% but nonetheless reduced ATP depletion, lactate production and glycogenolysis/anaerobic glycolysis during subsequent prolonged ischaemia (Murry et al., 1990). This means that absolute ATP levels are unlikely to be the direct trigger for preconditioning but the reduction in cellular acidity secondary to reduced ATP catabolism may have a more important role as a stimulus (Granger, 1988).

Quite apart from the effects of preconditioning on myocytes, it also protects endothelial cells of the vasculature. Multiple studies in the arterial vessels of the heart and the limbs of both animal and human models have shown that after preconditioning the endothelium preserves its ability to dilate to flow and to vasodilatory compounds (Richard et al., 1994, Kharbanda et al., 2001). Recently it has been demonstrated that NO may have direct effects on the K+ ATP channels and therefore be a means of action of early preconditioning, additionally it has been shown to have effects in late preconditioning (Sasaki et al., 2000). After reperfusion NO levels are reduced possibly through reduced availability of precursors or inactivation by the ROS that have been generated and so its protective effects on the vascular endothelium are lost (Vinten-Johansen et al., 1999). In
this manner, NO may cause preconditioning and the preconditioning may itself then be a means of preserving levels of NO post reperfusion.

Few clinical studies have been carried out to investigate the utility of pre-conditioning due, for the most part, to practical concerns. In 1998, Restifo and Thomson reported good results after using acute ischemic preconditioning on pedicled transverse rectus abdominis musculocutaneous flaps in six patients (Restifo and Thomson, 1998). In 1999, Cheng et al. studied the effectiveness of ischemic preconditioning on pedicled groin flaps in 12 patients with severe hand injuries; early pedicle division, after assessments using Doppler measurements, was possible after a mean period of 8.4 days, with a success rate of 90 percent (Cheng et al., 1999). Kharbanda and colleagues carried out the first clinical trial of remote ischaemic preconditioning in children undergoing cardiac surgery in 2006. Four cycles of ischaemic preconditioning (5 minute duration) reduced the inotrope requirements, airway resistance and troponin levels intra-operatively and post-operatively (Cheung et al., 2006). A further study in patients undergoing abdominal aortic aneurism repair also demonstrated reduced incidence of post-operative myocardial injury/infarction and renal impairment after two 10-minute cycles of preconditioning through cross-clamping of a common iliac vessel prior to aortic surgery (Ali et al., 2007).

1.4.2 POST-CONDITIONING

Zhao et al. were the first to report in 2003 the application of post conditioning to limit lethal reperfusion injury in experimental myocardial ischemia reperfusion. Post conditioning differed from its preconditioning counterpart in that the mechanical intervention focused exclusively on events occurring during reperfusion (Figure 2) and identified the early moments of reperfusion as a key therapeutic window (Zhao et al., 2003a). Later studies by Halkos et al. revealed that post conditioning also reduced cardiomyocyte apoptosis and contracture, coronary endothelial dysfunction,
microvascular injury, tissue oedema, and organelle dysfunction (Halkos et al., 2004). There is a very narrow window for the post conditioning to be effective. The delay in applying the first re-occlusion can only be short, but the available data are surprisingly sparse. In rats *in vivo*, IR injury reduction by post conditioning was lost when the first re-occlusion was shifted from 10 to 60 seconds reperfusion (Kin et al., 2004). In rabbits *in vivo*, protection was achieved when the post-conditioning manoeuvre was initiated at 30 seconds reperfusion but was lost at 60 seconds or 10 minutes reperfusion (Yang et al., 2004). However, in several studies in mice, rabbits, dogs, and also in studies in patients, ischaemic post conditioning still reduced IR injury when the delay to the first re-occlusion ranged from 60 to 180 seconds (Skyschally et al., 2009).
Figure 2. Infarct size reduction by pre- and post conditioning. Coronary occlusion, followed by reperfusion is associated with infarct development in the ischaemic risk zone (Control). Brief periods of ischemia and reperfusion performed either before index coronary occlusion or immediately after index coronary occlusion can significantly reduce infarct size. From Zhao et al., Am J Physiol Heart Circ Physiol, 2003.
Whether or not post-conditioning provides as powerful protection as preconditioning is unclear at present, and efficacy might be dependent on the post-conditioning protocol and the duration of the preceding ischemia (Skyschally et al., 2009). A technical problem, which may result from repeated occlusion of the target vessel, specifically in small animal models, is the failure to achieve complete reperfusion, and such failure of reperfusion may confound any potential protection.

Remote ischaemic post-conditioning, in which applying a conditioning stimulus of one or more cycles of brief ischemia and reperfusion to a remote organ or tissue protects the ischemic tissue, may be hugely beneficial in the clinical setting. Kerendi et al. first reported that one 5 min cycle of renal artery occlusion/reperfusion applied immediately before myocardial reperfusion could reduce myocardial IR injury by 50% in an in vivo rat model (Kerendi et al., 2005). Myocardial IR limitation, attenuated apoptotic cell death, and less oxidative stress have been observed with remote ischaemic post conditioning using in vivo rabbit models in which the protective stimulus has been applied to either the renal artery (three cycles of 30 s reperfusion/occlusions at the end of the index ischemia) (Liu et al., 2007), the femoral artery (Li et al., 2006), or the carotid artery (four cycles of 60 s reperfusion/occlusions just prior to myocardial reperfusion) (Gritsopoulou et al., 2009). Andreka et al. applied the remote ischaemic post conditioning stimulus (four 5 min cycles of hind-limb ischemia/reperfusion) at the immediate onset of myocardial reperfusion and observed myocardial injury reduction at 72 h using an in vivo pig model (Andreka et al., 2007).

All of the above interventions have a very narrow therapeutic window. No late or salvage therapies have been described which influence outcome in IR injury more than a few minutes after the initiation of reperfusion.
1.4.3 Statins

Statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) have shown therapeutic effect on IR in many different organs. In skeletal muscle, Dillon et al. reported that pretreatment with pravastatin reduces the tissue oxidative damage and edema associated with reperfusion injury in rat skeletal muscle (Dillon et al., 2006). Similarly, Cowled et al. found that simvastatin inhibits ischemia-reperfusion–induced neutrophil infiltration in rat skeletal muscle (Cowled et al., 2007).

1.4.4 Hyperbaric Oxygen

Hyperbaric oxygen has been shown to improve outcomes of IR in various organs and tissues, including skeletal muscle. By observation with confocal microscopy, Khiabani et al. found that hyperbaric oxygen inhibits IR–induced neutrophil adhesion by blocking CD18 surface polarization in rat muscle (Khiabani et al., 2008). Jones et al. reported that hyperbaric oxygen reduction of IR–induced neutrophil polarization of CD18 and adherence to intercellular adhesion molecule-1 is mediated through a nitric oxide mechanism that requires nitric oxide synthase (Jones et al., 2010).

1.4.5 Nitric Oxide

Nitric oxide is important in IR. As to whether nitric oxide exacerbates or protects from IR remains an issue of some disagreement. In homeostasis, nitric oxide is produced by endothelial nitric oxide synthase at a level far in excess of the superoxide anion, which allows nitric oxide to scavenge reactive oxygen species, regulate vascular tone, and
prevent platelet aggregation and thrombus formation. During ischemia, however, the ability of endothelial nitric oxide synthase to generate nitric oxide is compromised because of the requirement for oxygen as a substrate. As a result, nitric oxide is deficient in the ischemic tissue. However, in the early phase of reperfusion, superoxide is produced at a level far beyond the level of nitric oxide, which causes superoxide to quench nitric oxide and results in subsequent production of the highly toxic peroxynitrite anion.

Supplementation of nitric oxide donors during ischemia and reperfusion has been investigated in many IR injury models and has shown conflicting results. For example, Ward et al. showed that nitric oxide synthase inhibition significantly increases IR–induced microvascular permeability in ischemic skeletal muscle. Conversely, IR–induced microvascular permeability was not increased in the animal that received nitric oxide synthase inducer in ischemic skeletal muscle. They surmise that nitric oxide plays a role in preventing IR in skeletal muscle (Ward et al., 2009). However, Zimiani et al. reported conflicting results. They reported that nitric oxide plays a detrimental role in ischemia-reperfusion injury of skeletal muscle (Zimiani et al., 2005).

1.4.6 ANTIOXIDANTS

The reactive oxygen species burst during early reperfusion is thought to be one of the earlier triggers that initiate IR. To eliminate reactive oxygen species, numerous antioxidants have been investigated. Most of these antioxidants have shown positive results by reducing infarct size, microcirculatory alterations, superoxide production, mitochondrial dysfunction, metabolic dysfunction, and histologic damage, in the skeletal muscle or skin (Erkanli et al., 2005, Ozyurt et al., 2007, Neumayer et al., 2006, Bolcal et al., 2007). However, no single antioxidant or combination of antioxidants that has been shown to reduce IR in randomized control trials.
Chapter Two: POLOXAMER 188 DECREASES MUSCLE INJURY AND MORTALITY FROM ISCHEMIA-REPERFUSION INJURY IN A MURINE HIND LIMB MODEL OF INJURY
2.1 INTRODUCTION

Managing oxygen metabolism is essential for tissue health and successful recovery from disease. Under normal physiological conditions, many cellular mechanisms exist to regulate oxygen chemistry. However, in disease or following transient disruption of oxygen delivery, oxygen regulatory control is lost resulting in reperfusion injury. Reperfusion triggers a cascade of acute inflammatory events leading to cellular death resulting in tissue dysfunction and necrosis (Carroll and Esclamado, 2000). Complex inflammatory and immunological signalling pathways that have not been fully elucidated modulate IR injury. Clinically, IR injury is an important factor limiting what can be accomplished in the fields of trauma (Tadros et al., 2000), vascular (Norwood et al., 2004, Mallick et al., 2004), transplantation (Kosieradzki and Rowinski, 2008, Desai et al., 2008), and plastic surgery (Chait et al., 1978, May et al., 1978b).

There are many pathways activated in IR injury. A well-described example, and one of the earliest events in the process, is related to the binding of circulating natural antibodies and the activation of complement (Austen et al., 2003). First described in myocardial reperfusion by Hill and Ward (Hill and Ward, 1971), complement activation was shown to involve the release of C3a and C5a anaphylatoxins that induce degranulation of mast cells with release of histamine and other chemical mediators. The final common pathway of IR injury is oedema, attraction of activated leukocytes, and the formation of membrane-attack complexes.
(Austen et al., 1999) all of which lead to disruption of cell membranes and resulting cell death.

The structural integrity of the cell membrane lipid bilayer is critical for cellular viability. To maintain cell membrane integrity, cells possess membrane repair mechanisms that allow recovery from routine day-to-day trauma (Marks et al., 2001). Preconditioning up regulates these paths to allow greater injury tolerance. However when normal membrane repair mechanisms are exhausted, usage of membrane sealing polymers can be effective in preserving cell viability if administered before extensive molecular degradation has occurred (Hannig et al., 2000). One class of membrane sealing polymers are poloxamers [pol(y)ox(y)mers] which are biocompatible multi-block surfactant copolymers that have been used as pharmaceutical excipients in blood banking for decades (Toth et al., 2000). Poloxamer surfactants have important ‘surface-active’ properties that adsorb to damaged cell membranes resulting in a shift of water interfacial tension in the direction that favours membrane sealing.

One member of this chemical class is Poloxamer 188 (P188), which is a tri-block copolymer of poly(oxyethylene) and poly(oxypropylene) and abbreviated POE-POP-POE. P188 has an average molecular weight of approximately 8400 kilo Daltons. The POE chains are hydrophilic due to their short carbon unit between oxygen bridges, whereas the POP centre is hydrophobic due to the larger propylene unit. In addition to their membrane-sealing properties, poloxamers are oxygen free radical scavengers (Marks et al., 2001), which add to their value as a trauma therapeutic. Dextran (10 kDa), a purely hydrophilic polymer, was
chosen as a control treatment because its molecular weight is similar to P188, it tends to adhere to cell surfaces, and has historically been used as a control for P188 effects (Lee et al., 1992, Greenebaum et al., 2004, Collins et al., 2007)

P188 has been shown to be effective in preserving cell membrane structure and viability following electroporation, high-dose ionizing irradiation and superoxide injuries (Hannig et al., 2000, Lee et al., 1999, Lee et al., 1992). This protection is thought to be due to P188 inserting into cell membrane defects and “sealing” the cell, thereby preventing loss of transmembrane ion gradients and intracellular proteins. The physical chemistry of the membrane sealing has been well demonstrated using in vitro lipid monolayer models. P188 was shown to insert into the lipid monolayer when surface tension decreased after membrane poration, and was squeezed out when the membrane integrity was restored (Maskarinec et al., 2002).

In this experiment we investigate if a single dose of the non-ionic synthetic surfactant Poloxamer 188 administered to achieve a maximal sub-critical micelle concentration in the extracellular fluid compartment can mitigate skeletal muscle injury caused by 2 hours of ischemia and twenty-four hours reperfusion. Parameters of muscle injury include histologic evidence of myocyte fibre damage and depletion of ATP (an index of metabolic dysfunction). The extent of ischemic skeletal muscle necrosis is known to correlate with the extent of ATP depletion (Petrasek et al., 1994). In addition, a series of experiments were extended out to 7 days reperfusion to determine whether administration of P188 influenced mortality following hind limb ischemia reperfusion.
2.1.1 Poloxamer 188 Structure

Poloxamer 188 is a tri-block co-polymer surfactant composed of two hydrophilic, polyoxyethylene (EO) segments and one central, hydrophobic, polyoxypropylene (PO) segment joined through ether oxygen linkages in an a:b:a construct (Figure 3). It has a molecular weight of 8400 kDa. It has a melt point of 52°C and a >10% solubility in water at 25°C (BASF Corporation and Jersey, 1999). Unlike polyethylene glycol (PEG), which is a highly polar, purely hydrophilic, molecule, P188 is an amphiphilic surfactant copolymer with a low detergency.

Figure 3. Chemical structure of a poloxamer. Poloxamers differ through varying numbers and rations for a and b.
2.1.2 POLOXAMER 188 AS AN EMULSIFICATION AGENT

The water soluble tri-block copolymers, which includes P188, have had, and continue to have, many uses in the medical and pharmaceutical industries including their uses as emulsifying, wetting, thickening, coating, solubilizing, stabilizing, dispersing, lubricating and foaming agents (Chu B, 1996). P188 is FDA approved for use as an emulsifying agent in perfluorochemical solutions. These are chemically inert organic products of fluorination synthesis that dissolve large amounts of oxygen and have been used as blood substitutes.

A number of studies have shown P188 to inhibit platelet aggregation and fibrin clot formation. Carr et al. showed in 1991 that addition of P188 to plasma caused an increase in fibrin fibre size and a significant decrease in time to fibrin clot lysis when r-tPA was added (Carr et al., 1991). A number of studies have shown that the addition of P188 to whole blood inhibits platelet aggregation induced by red blood cells (Armstrong et al., 1995), and by various chemical agonists (Edwards et al., 1998).

Initially it was theorized that P188 improves microvascular blood flow by reducing blood viscosity, especially under low shear (low flow) conditions and by reducing adhesive frictional forces. (Carr et al., 1991, Smith et al., 1987). Subsequent work by Lechmann & Reinhart has cast doubt on this theory. Their study found that lower concentrations of P188 had no effect on blood or plasma viscosity, and surprisingly found that high concentrations of P188 in fact increased blood and plasma viscosity (Lechmann and Reinhart, 1998). These data suggest that P188 may exert its anti-thrombotic and anti-platelet aggregation properties through protection of red blood cells from membrane damage and subsequent ADP leakage and platelet activation.
2.1.3 Poloxamer 188 and Cell Salvage In Vitro

In 1992 Lee et al. demonstrated that P188 reduced leakage of a fluorescent dye after electroporation. They hypothesized that because membranes form spontaneously when surfactants are mixed in an aqueous solvent at a sufficient concentration, that it may be possible to seal damaged cell membranes by exposing them to a non-cytotoxic non-ionic surfactant, possibly by incorporating the surfactant into the membrane defects.

Rat skeletal muscle cells were grown in vitro and incubated in a carboxyflourescin solution to produce a cytoplasm loaded with a membrane-impermeable fluorescent dye. Cells were then subjected to a 4-millisecond electrical field pulse and fluorescent dye leakage from the cell measured. Cells were bathed in PBS supplemented with P188 or dextran. As is demonstrated in Figure 4, P188 significantly retarded the rate of dye loss following injury, more so than the dextran polysaccharide and control (Lee et al., 1992).
Figure 4. Fluorescent dye leakage following electroporation of rat muscle cells *in vitro*. 

In a later paper from this same group they showed that super-physiologic heating to temperatures of between 45-65°C caused significant fluorescent dye leakage. This was significantly reduced by the addition of P188 (Padanilam et al., 1994).

Further to these initial results P188 has been shown to have a protective affect on a number of cell types subjected to a number of forms of injury *in vitro*. Greenebaum *et al.* carried on their work with adult rat skeletal muscle cells and in 2004 showed that P188 protected these cells from the effects of high-dose ionizing radiation injury. Cells were exposed to 10, 40 or 80 Gray of gamma radiation and were treated post-exposure with P188 or dextran. Cell viability was assessed by means of a fluorescent dye viability assay. Dextran had no effect compared to Control, whereas P188 at 1 and 2 mM concentration increased the number of viable cells (Figure 5) (Greenebaum et al., 2004).
Merchant et al. later looked at the effect of P188 on lethally heat-shocked human fibroblast cultured in vitro. They subjected human foreskin derived fibroblasts to super-physiologic temperatures of 43-48°C for 20, 40, or 60 minutes. They discovered that addition of P188 post-injury improved the functional contractile capacity of the fibroblasts. These data are presented in Figure 6 below.
Figure 6. Effect of P188 on fibroblasts subjected to heat shock. From Merchant et al., JSR, Feb. 1998.
In 2001 Marks et al. demonstrated that P188 reduced injury in rat embryonic hippocampal cells cultured in vitro. Cells were exposed to a number of injury-inducing stimuli including N-methyl-D-aspartate and menadione, all potent necrosis-inducing agents. As well as increasing the number of viable cells following injury, P188 was seen to also protect the functionality of these cells. The authors proposed that this protection was due to P188’s ability to restore membrane integrity after disruption as well as preventing plasma membrane peroxidation. Their schematic for this action is represented below in Figure 7 (Marks et al., 2001).
Figure 7. Schematic diagram of membrane-targeted mechanisms causing NMDA-induced neuronal death and the actions of P188 on the plasma membrane. From Marks et al., FASEB J, Feb. 2001.
Serbest *et al.* further expanded the role of P188 in neuroprotection following injury in their 2006 paper. They demonstrated that P188 protected rat PC2 cells (a sympathetic neuron cell line) from mechanical shear injury *in vitro*. Their standard injury caused >40% cell loss of viability at 24h post-injury. Treatment with P188 however, reduced this to approx. 7% cell loss. They found that both acute necrosis and delayed apoptosis was significantly reduced by addition of P188. This suggests that membrane sealing post-acute injury may not only rescue cells from acute cellular death but also staves off the secondary cascades leading to delayed apoptotic cell death (Serbest *et al.*, 2006).

Further *in vitro* work by Kilinc *et al.* examined a model of diffuse axonal injury on cultured primary chick forebrain neurons and characterized the resulting structural and morphological changes. They found that fluid shear stress injury induces axonal beading, the "hallmark" morphology of diffuse axonal injury. Furthermore, beads co-localized with microtubule disruption, also characteristic of diffuse axonal injury. P188 reduced axonal beading to control levels indicating that axolemma integrity was maintained (Kilinc *et al.*, 2007). These data were backed up by a very similar study by Serbest *et al.* albeit with a less subtle measurement of outcome (Serbest *et al.*, 2005).

The group of Haut in search of treatments for post-traumatic cartilage damage initially examined effects of P188 on cartilage chondrocytes. In their first report (Phillips and Haut, 2004), they showed that addition of P188 added directly after induction of chondrocyte trauma, by applying high unconfined static compression to bovine cartilage explants, saved chondrocytes from early death. The number of live cells was significantly higher in P188-treated cartilage (approximately 75%) than in the controls (approximately 50%) after trauma. In 2005, the group reported similar results on rabbit cartilage tissue in vivo (Rundell *et al.*, 2005). Half of the dead chondrocytes could be saved by intra-articular injection of P188 just before blunt trauma. In both studies, they used histochemistry by use of a Live/Dead Cytotoxicity Kit as the only outcome parameter. In 2006 the same group reported that P188 was also effective in reducing the percentage of cells with DNA fragmentation as shown by TUNEL staining in impacted explants (Baars *et al.*, 2006). These data suggest that early P188 intervention was effective in preventing
DNA fragmentation of injured chondrocytes. A subsequent paper from Guo et al supported these data. They found that P188 could help restore the integrity of cell membranes in cartilage damaged by blunt mechanical trauma (Guo et al., 2008).

A subsequent paper by Jansen et al. examined the effects of P188 on preventing or reducing blood induced chondrocyte necrosis on human cartilage tissue explants. They found that after 48 hours of exposure to 10% volume/volume human blood that P188 had no effect on proteoclycan release, synthesis rate, or content (Jansen et al., 2008).

2.1.4 POLOXAMER 188 AS AN IN VIVO THERAPEUTIC IN ANIMAL MODELS

As well as demonstrating its ability to reduce cell injury in vitro, Lee et al. also demonstrated in their 1992 paper that P188 had the capacity to prevent in vivo injury. They isolated a biceps femoris muscle flap and exposed it to a 150 V/cm electrical field. The electrical impedance of the muscle was measured and used as a measure of cell membrane integrity. Following exposure to the electrical injury the animals given an intravenous treatment of P188 (460mg/Kg) or a dextran control solution. They found that post-shock resistivity, or membrane integrity, was significantly improved by pre-treatment and post-treatment with P188 (Lee et al., 1992). These date are represented graphically in Figure 8.
Figure 8. Normalized resistivity for flaps immediately post electroporation, 1h post IV saline, 1h post IV dextran, 1h post IV P188, and immediately after injury in animals pretreated with P188. From Lee et al., PNAS, May 1992.
Further studies on rat skeletal muscle *in vivo* showed that after electroporation injury P188 was capable of decreasing oedema, and improving compound muscle action potential, an indication that muscle function was protected. This was in addition to histological and MRI evidence of cytoprotection (Collins et al., 2007). CMAP results are shown in Figure 9.
Figure 9. CMAP amplitudes in control, shocked lactated Ringers (LR) treated, and P188 treated rats. From Collins et al., Biochem & Biophysics Acta, Jan. 2007.
Much interest has been focused on the therapeutic potential of P188 in the treatment of Duchenne muscular dystrophy. Duchenne Muscular Dystrophy is a genetic disease caused by the lack of the protein dystrophin. Dystrophic muscles are highly susceptible to contraction-induced injury, and following contractile activity, have disrupted plasma membranes that allow leakage of calcium ions into muscle fibres. Therefore if P188 can seal these membrane defects muscle injury may be ameliorated. Initial studies by Quinlan et al., using a C57 BL/10 MDX mouse model which is genetically deficient in dystrophin, showed P188 to be ineffective in preventing muscle breakdown as assessed by fluorescent microscopy (Quinlan et al., 2006).

Further work by Ng et al. using isolated lumbrical muscles from dystrophic (mdx) mice, demonstrated for the first time that P188 is effective in reducing the force deficit in a whole mdx skeletal muscle. A reduction in force deficit was also observed in mdx muscles that were exposed to a calcium-free environment. These results, coupled with previous observations of calcium entry into mdx muscle fibres during a similar contraction protocol, support the interpretation that extracellular calcium enters through sarcolemmal lesions and contributes to the force deficit observed in mdx muscles. The results provide a basis for potential therapeutic strategies directed at membrane stabilization of dystrophin-deficient skeletal muscle fibres (Ng et al., 2008).

Similarly, in a 2005 paper in Nature, Yasuda et al. show that intact, isolated dystrophin-deficient cardiac myocytes have reduced compliance and increased susceptibility to stretch-mediated calcium overload, leading to cell contracture and death, and that application of the membrane sealant poloxamer 188 corrects these defects in vitro. In vivo administration of poloxamer 188 to dystrophic mice instantly improved ventricular geometry and blocked the development of acute cardiac failure during a dobutamine-mediated stress protocol (Yasuda et al., 2005).

Another area of investigation of the beneficial effects of P188 is in relation to myocardial injury secondary to myocardial infarction and post-reperfusion injury. Justicz et al. showed that single does administration of P188 in association with mannitol reduced
infarct size in a canine model of myocardial infarction as assessed by tetrazolium blue staining (Justicz et al., 1991). These animals underwent 90 minutes of LAD occlusion and 24 hours of reperfusion. This reduction in infarct size and area at risk is clearly visible in Figure 10 below.

A further study by Schaer et al. examined the role of 4 hours and 48 hours of P188 infusion following 90 minutes of LAD occlusion on infarct size as assessed after 72 hours of reperfusion. They found that an intravenous bolus of 75mg/Kg P188 bolus followed by 150 mg/Kg/h infusion for 4 hours did not significantly reduce infarct size as assessed by triphenyl tetrazolium chloride staining. Left ventricle ejection fraction was not reduced compared to control either. Plasma creatine phosphokinase levels were, however, significantly reduced. Interestingly 48 hours of P188 infusion at the same rate resulted in significant improvements in infarct size, left ventricle ejection fraction and plasma creatine phosphokinase levels. This study also suggested a relationship between P188 levels and neutrophil chemotaxis suppression (Schaer et al., 1994).

Another study by the same group examined the effects of P188 on canine myocardial injury following a lengthier three hours of LAD occlusion. Regional myocardial blood flow was measured using coloured microspheres. Myocardial infarct size and area at risk were determined by post-mortem histochemical staining. Compared with controls, poloxamer 188-treated dogs showed no significant increase in collateral blood flow during the final 2 hours of a 3-hour coronary artery occlusion. In addition, P188 treatment had no beneficial effect on infarct size or left ventricular function in this model. Increased collateral blood flow is unlikely to be a beneficial mechanism of poloxamer 188 in myocardial infarction. These data also question the benefit P188 to reduce reperfusion injury in the setting of more prolonged (3-hour) coronary occlusion (Kelly et al., 1998).

Using an explanted rat heart in a Langendorff perfusion model Watanabe & Okada showed that pre-treatment with P188 completely protected the heart from the potent cardio-toxic effects of lysophosphatidylcholine. Heart rate, left ventricular developed pressure, and left ventricular end-diastolic pressure were all significantly adversely
affected by lysophosphatidylcholine administration. P188 had the effect of normalizing these values to control levels. The authors hypothesized that this effect was through sarcolemma membrane sealing by P188 and prevention of Ca^{2+} influx (Watanabe and Okada, 2003).
Skeletal and myocardial muscle are not the only tissues to have been shown to be amenable to protection or treatment with P188. Neurologic tissues as demonstrated in vitro can be protected from various injuries by P188. These results have been replicated in in vivo injury models. Colbassani et al. showed in 1989 that P188 increased cerebral blood flow in areas of severe or moderate ischemia following occlusion of the middle cerebral artery of a rabbit. The authors hypothesized that this effect may be due to P188 inhibiting adhesive interactions among cells and proteins in the microcirculation (Colbassani et al., 1989). Of note this paper was published before the vast body of evidence showing P188’s membrane sealing functions.

A further paper by Mezrow et al. showed that P188 significantly improved neurological outcome and survival in dogs following prolonged hypothermic circulatory arrest. The animals were subjected to cooling to 10°C and the heart was arrested for 150 minutes before rewarming and weaning from bypass. There were no deaths in seven P188 treated dogs versus three deaths in the six control dogs. P188 treated dogs also had less neurological dysfunction (Mezrow et al., 1992).

Similar survival results were seen when Mayer et al. subjected rabbits to haemorrhagic shock followed by resuscitation with volume replacement, whole blood transfusion, or whole blood with P188. There were significantly more animals surviving at the end of the monitoring period in the two groups that received poloxamer 188 (numbers of animals alive after 3 hours = 7 of 8 in the High group and 6 of 8 in the Low group) compared to the Transfusion (4 of 8) and Volume (2 of 8) groups (Mayer et al., 1994). This study did not examine any parameters other than survival rates.

Zhang et al., however, looked at myocardial injury, pulmonary inflammation, small bowel damage, renal tubular necrosis, hepatic central lobular necrosis and apoptosis of splenic germinal centres as well as survival in a model of prolonged hypotensive resuscitation in the rat. They found that P188 improved auto resuscitation, reduced fluid requirements and increased the survivable duration of hypotensive resuscitation by over 3 hours as well as reducing the degree of tissue injury seen in all the measured parameters
(Zhang et al., 2009). These results suggest that P188 may play a role as an adjunct to hypotensive resuscitation.

In 2004 Borgens et al. showed that single dose administration of P188 after an acute crush injury to the guinea pig spinal cord preserved anatomical integrity of the cord and produced a rapid recovery of nerve impulse conduction through the lesion compared to control. 3D reconstruction of all serial histologic sections comprising the injured segments of spinal cord showed that P188 treatment reduced the amount of pathologic cavitation of the cords and was associated with an increased volume of intact parenchyma (Figure 11). A statistically significant improvement in recovery of reflex action below the level of the injury was also seen (Borgens et al., 2004).
Figure 11. Comparison of cyst formation between P188 and vehicle-treated spinal injuries. 3D reconstructions of injured spinal cord segments. The dark red regions are cysts (labelled c). From Borgens et al. J Neurosci Res. April, 2004.
Further in vivo neurological work with P188 was carried out by Curry et al. Sprague-Dawley rats underwent striatal infusion of quinolinic acid to produce a spherical excitotoxic lesion. Each animal then received either vehicle or P188 at 10 minutes, 4 hours, or both time points after surgery by direct cisterna magna injection. Animals were killed at 1 week, and brains were stained immunohistochemically for the neuronal marker Neu-N. Volumes of neuronal loss were calculated and compared between groups. They found that the animals that received intrathecal poloxamer at the early injection time had statistically smaller lesions than controls. Those animals that received poloxamer at both injection times also had statistically smaller lesions (Figure 12). The group that received poloxamer at the late injection time only did not have significantly decreased lesion size (Curry et al., 2004).

A further study by the same group showed that a single dose of P188 protects against early neuronal loss after intra-cranial haemorrhage. Their study used Sprague-Dawley rats that received an intracisternal injection of either P188 or control 10 minutes after striatal infusion of 50 µL of autologous blood (Cadichon et al., 2007).

Quinn et al carried out another important study of the effects of P188 on neuronal tissue. In their study foetal rat dopaminergic tissue was dissociated in media with or without P188 and then cultured for 1 week or transplanted into the striatum of rats with unilateral 6-hydroxydopamine lesions of the nigrostriatal dopaminergic pathway. Foetal dopaminergic cell survival and reinnervation of the host brain were examined using tyrosine hydroxylase immunohistochemistry and stereological quantification. The number of surviving tyrosine hydroxylase-immunoreactive cells in vitro and in vivo was significantly increased by 2.2-fold by incubating foetal dopaminergic cells with P188 during tissue dissociation. Furthermore, the striatal reinnervation in Parkinsonian rats that received intrastriatal transplants of P188-exposed dopaminergic cells was significantly enhanced compared with rats that received non-P188-treated cells (Quinn et al., 2008).
Figure 12. Photomicrographs of coronal sections of rat brain stereotactically injected with quinolinic acid and stained with immunohistochemical marker Neu-N. A; lesion after intrathecal injection of control. B; lesion after intrathecal P188 injection. From Curry et al. Neurosurgery, Oct. 2004.
A further area of potential therapeutic use for P188 is in the area of burn injury. In 2001 Baskaran et al. demonstrated that P188 reduced the size of the zone of stasis and the zone of coagulation in a controlled mouse dermal burn model. Mice were given P188 intravenously at a dose of 200mg/Kg 20 minutes after being subjected to a standard burn injury. It was found that control animals had a 1mm zone of coagulation and 3mm zone of stasis following the injury. Treatment with P188, however, totally eliminated this zone of stasis. At 24 hours post-injury it was seen that P188 reduced the size of the zone of coagulation or necrotic area by 40% (Figure 13) (Baskaran et al., 2001).
Figure 13. Effect of P188 on coagulation area 24h after the burn. Fluorescein/dextran was injected 24h after the burn, and fluorescent images were obtained. From Baskaran et al. JSR, Nov. 2001.
Similar results were found in a different model of burn injury. Birchenough et al. applied P188 topically to rat mesenteric microvessels that had been thermally injured. They characterized blood flow in microvessels as normal or abnormal (i.e., sludging and stasis). Topical treatment with P188 reduced the percentage of capillaries with abnormal blood flow from 62% to 23%. In venules, this treatment resulted in a decrease from 54% to 34% (Birchenough et al., 2008).

2.1.5 PoloXamer 188 in Human Trials

A number of human trials evaluating the clinical utility of P188 have been carried out. These have focused on two specific areas; treatment of acute vaso-occlusive sickle cell crisis, and acute myocardial infarction. An initial pilot study by Adams-Graves et al. in 1997 investigated the effects of P188 when given during the acute painful episode of sickle cell disease. They conducted a randomized, double-blinded, placebo-controlled trial incorporating 50 patients with sickle cell crisis. Endpoints included: (1) painful episode duration, (2) days of hospitalization, (3) quantity of analgesics used, and (4) pain intensity scores. Compared with placebo P188-treated patients showed a 16% to 45% decrease in duration of painful episodes, a 1- to 2-day reduction in hospital stay, a threefold to fivefold reduction in analgesic requirements, and a 1-point reduction (using a 5-point scale) in average pain intensity scores at 72 hours. P188 was well tolerated; no clinically significant differences were observed between treatments with respect to adverse experiences or other safety measures (Adams-Graves et al., 1997).

A subsequent larger, multi-centre, randomized, double blind, placebo-controlled trial conducted between March 1998 and October 1999 involved 250 patients with sickle cell crisis. Patients were randomly assigned to receive an intravenous infusion of purified P188, 100 mg/kg for 1 hour followed by 30 mg/kg per hour for 47 hours (n = 127), or a
matching volume of saline placebo (n = 128). Similar outcome measures as the previous study were employed. This time however there was only a very small, albeit significant, difference in duration of painful episode seen (141 hours vs. 133 hours) (p=0.04) (Figure 14). There were small, but non-significant, reductions in time to discharge, visual analogue pain scores, and total analgesic use associated with P188 treatment (Orringer et al., 2001).
Figure 14. Kaplan-Meier analysis of proportion of patients remaining in crisis over time.

A 2004 study by Ballas et al. looked at the effect of P188 on acute chest syndrome. Acute chest syndrome is the most common cause of death in patients with sickle cell anaemia. Its management is primarily palliative. Forty-three patients with sickle cell disease and acute chest syndrome were treated with doses of P188 as high as 2960 mg/day by continuous intravenous infusion. No evidence of renal toxicity or other limiting adverse events were found. The ANOVA ($p=0.627$) and regression analyses ($p=0.185$) indicated that there was no relationship between increasing dose and the duration of the acute chest syndrome episode. Moreover, ANOVA ($p=0.295$) analysis indicated that there was no dose relationship between increasing dose and the duration of hospitalization. At the two highest dose levels there was a trend for a decrease in hospitalization time but the differences were not statistically significant (Ballas et al., 2004).

Acute myocardial infarction is the other area which investigation of poloxamer 188 therapy has focused on. As outlined previously there are a number of studies that showed P188 to reduce infarct size and reduce morbidity following myocardial infarction in canine models (Justicz et al., 1991, Schaeer et al., 1994). The first human trial was reported in Circulation in 1996. Schaer et al. randomized 114 patients to a 48-hour infusion of poloxamer 188 or vehicle placebo beginning immediately after the initiation of thrombolytic therapy for acute myocardial infarction. P188-treated patients demonstrated a 38% reduction in median myocardial infarct size compared with placebo (16% vs. 26%; $p=0.031$), greater median myocardial salvage (13% vs. 4%; $p=0.033$), and a 13% relative improvement in median ejection fraction (52% vs. 46%; $p=0.020$). Poloxamer 188 treatment also resulted in a reduced incidence of reinfarction (1% vs. 13%; $p=0.016$).

In a companion study O’Keefe et al. investigated using P188 as an adjunct to primary percutaneous transluminal coronary angioplasty for acute myocardial infarction. Patients were randomized in a 2:1 manner to receive a P188 infusion for 48 hours post-angioplasty or placebo control. Measured outcome endpoints included reinfarction, cardiogenic shock, death, and infarct size. There was no significant difference in any outcome measure between P188 and control. Of note there was a noted elevation in
serum creatinine level in 12% of patients receiving P188 (p=0.048) (O'Keefe et al., 1996).

The much larger CORE (Collaborative Organization for RheothRx Evaluation) group reported their results in Circulation in 1997. They randomized almost 3000 patients presenting with acute myocardial infarction. Patients receiving P188 were randomized to receive a 1-hour bolus only (regimen A, n = 844), an additional 11-hour infusion at a low dose (target serum concentration of 0.5 mg/mL) (regimen Y, n = 490), or an additional 23-hour infusion at a low dose (regimen B, n = 483). Three higher doses were discontinued because of high rates of renal dysfunction (8.8%). Renal dysfunction was also observed at lower doses (regimen A, 3.1%; Y, 2.7%; and B, 4.1%) compared with the control patients (1.0%). In this study of 2948 patients, P188 had no effect on mortality, reinfarction, or cardiogenic shock and an adverse effect on renal function, left ventricle ejection fraction, and various clinical manifestations of left ventricle dysfunction and/or heart failure (CORE, 1997).

2.1.6 POLOXAMER 188 AND LIPID MONOLAYERS

The most widely espoused mechanism of action of P188 is that of cell membrane sealing, whereby P188 inserts into cell membrane defects by means of interaction with the hydrophobic portion of the lipid membrane. Despite numerous studies demonstrating P188’s ability to reduce or prevent injury in various models none has been able to directly prescribe the mechanism through which this occurs.

Although cell membranes are composed of lipid bilayers, the Langmuir lipid monolayer system serves as a model for the outer leaflet of the cell membrane. These lipid monolayers are two-dimensional surface films which are well established as model
biological membranes (Mohwald, 1990). In their 1996 paper Sharma et al. used an artificial lipid monolayer system that they subjected to electroporation. For both charge pulse and voltage clamp experiments, P188-treated membranes exhibited a statistically higher threshold voltage, and longer latency time. Also, P188-treated membranes were found to have a relatively lower conductance, longer time required for the porated membrane to reach a certain conductance value, and longer postelectroporation time constant. Furthermore, addition of poloxamer 188 was found to reduce the membrane capacitance by approximately 4-8% in 5 minutes. These findings suggest that poloxamer 188 adsorbs into the lipid bilayers, thereby decreasing their susceptibility to electroporation (Sharma et al., 1996).

A further study by Maskarinec et al. looked directly at P188 interaction with a lipid monolayer by means of concurrent Langmuir isotherm and fluorescence microscopy. They found that P188 inserts into monolayers at surface pressures equal to and lower than approximately 22 mN/m at 30 degrees C; this pressure corresponds to the maximal surface pressure attained by P188 on a pure water subphase. Because the equivalent surface pressure of a normal bilayer is on the order of 30 mN/m, the lack of P188 insertion above 22 mN/m further suggests the poloxamer selectively adsorbs into damaged portions of electroporated membranes, thereby localizing its effect. P188 was also found to be "squeezed out" of the monolayers at high surface pressures, suggesting a mechanism for the cell to be rid of the poloxamer when the membrane is restored (Maskarinec et al., 2002)
2.2 MATERIALS & METHODS
2.2.1 PoLOXAMER 188 TREATMENT

Control-treated mice received 150 µL NS+dextran (6.0 mM, MW=8 kDa, Sigma Aldrich Inc.). P188-treated mice received 150 µL NS+P188 (6.0 mM Pluronic F68, Sigma Aldrich Inc.). Both treatments were administered intravenously via tail vein injection. Based on extracellular fluid volume estimated at 24% total body weight, the P188-treated mice received a dose such that the final concentration of P188 in the blood was 0.15mM, which is below critical micelle concentration. This is equivalent to a dose of 300mg/kg.

For the dose-response experiment, animals received either 150 µL NS+P188 6.0 mM, 150 µL NS+P188 12.0 mM, 150 µL NS+P188 3.0 mM, or 150 µL NS+P188 0.6 mM to give final blood concentrations of 0.15mM, 0.3mM, 0.075 mM, and 0.015mM respectively. Treatment was administered at one of four different time points; 10 minutes prior to ischemia, 10 minutes prior to reperfusion, two hours after reperfusion beginning, or four hours after reperfusion beginning (Figure 15).
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Figure 15. Time points for different P188 administration

### 2.2.2 Animal Care Protocol

Animal care and experimental procedures complied with “Principles of Laboratory Animal Care” (Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication No. 86-23, Revised 1996) and were approved by the Massachusetts General Hospital’s Institutional Review Board. C57BL6 mice (22–28 g) (Jackson, Bar Harbor, ME) were initially anesthetized by intra-peritoneal administration of sodium pentobarbital (60 to 90 mg/kg in a bolus of 0.4 ml normal saline). During the pre-ischemic, ischemic, and initial 3h of reperfusion intervals, animals were placed on a heating pad to maintain the body temperature at 37°C. Following reperfusion
experiments, mice were returned to their cages in the vivarium and were allowed access to water and chow *ad libitum*. Mice were kept in a 12h:12h light-dark cycle, and the room temperature was kept constant between 24 –26°C.

Animals were not administered post-procedural analgesia as previous studies have suggested that one of the most important protective factors in preconditioning is opioids. They are believed to play a triggering role in the reduction of ischemia reperfusion injury (Dickson et al., 2001, Tubbs et al., 2002, Schultz et al., 1997).

Our model is an inflammatory model where we are looking for an inflammatory response that we believe is responsible for a protective effect against I/R injury. Salicylates are anti-inflammatory drugs and all anti-inflammatory drugs have been documented in the literate to significantly reduce ischemia reperfusion injury (I/R) (Bolli et al., 2002). Salicylates are also blood-thinning agents that decrease viscosity and the risk of clotting while increases blood flow, all of which will effect the I/R injury to the animal. Similarly paracetamol has been documented in the literature to significantly protect against I/R injury in many animal models (Merrill et al., 2004, Golfetti et al., 2003, Leshnower et al., 2006).

### 2.2.3 *In Vivo* Hindlimb Ischaemia

The McGivney hemorrhoidal ligator band (MHL) is an established model of tourniquet-induced hind limb IR injury in mice (Kyriakides et al., 2000, Wakai et al., 2001, Woodcock et al., 2000). Tourniquet models are frequently used for murine studies of IR models because experimental models of angiogenesis in mice have demonstrated the need to simultaneously occlude several branches of the femoral and iliac arteries to achieve reliable levels of limb ischemia. The rich collateral blood supply surrounding the murine pelvic girdle provides substantial collateral flow usually from iliac and tail
branches, which perfuse the posterior thigh. While popular and widely used, the MHL model has been criticized for its inability to control for nonspecific neuromuscular damage due to the crushing force of the rubber band on the underlying tissue. Crawford et al. overcame this problem by demonstrating that orthodontic rubber bands provided complete hind limb ischemia with much less crushing force (Figure 16).
Figure 16. Murine hind limb with orthodontic rubber band in situ and corresponding laser Doppler image confirming complete ischemia of the limb.
Thirty minutes after the induction of anaesthesia, the MHL was used to apply an orthodontic rubber band for 2h periods of hind limb ischemia followed by reperfusion. The rubber band was placed above the greater trochanter bilaterally. Mice remained anesthetized throughout the duration of ischemia with supplemental anaesthesia (sodium pentobarbital) as needed. For tissue viability, animals were allowed to recover from anaesthesia after the ischemic period, and after 24h of reperfusion, the animals were euthanized and the tissue was harvested.

### 2.2.4 Muscle Histology

Limbs from mice that underwent hind limb ischemia and reperfusion were fixed in 4% paraformaldehyde for 8 hours. The gastrocnemius muscle from each limb was dissected out, rinsed in Dulbecco Phosphate Buffered Saline (PBS) for 1 hour, and serially dehydrated in graded acetone. Each sample was embedded using JB-4 Embedding Kit (Polysciences Inc, Warrington, Pa) under vacuum conditions, cut in cross-section at 2-µm thickness using a motorized microtome (Leica Microsystems Inc, Bannockburn, Ill), and stained with Masson trichrome.

JB-4 is a water-soluble, GMA based, plastic resin kit intended for use in the preparation of embedded samples for high-resolution light microscopy. Widely used for research and clinical diagnosis. JB-4 yields semi-thin sections (0.5µm-2µm) with excellent morphological preservation. Clear casts are obtained in 90 minutes or less at room temperature.

Masson's trichrome is a three-colour staining protocol. It produces red keratin and muscle fibres, blue or green collagen and bone, light red or pink cytoplasm, and dark brown to black cell nuclei.
The trichrome is applied by immersion of the fixated sample into Weigert's iron haematoxylin, and then three different solutions, labelled A, B, and C:

Weigert's haematoxylin is a sequence of three solutions: ferric chloride in diluted hydrochloric acid, haematoxylin in 95% ethanol, and potassium ferricyanide solution alkalized by sodium borate. It is used to stain the nuclei.

Solution A, also called plasma stain, contains acid fuchsin, Xylidine Ponceau, glacial acetic acid, and distilled water.

Solution B contains phosphomolybdic acid in distilled water.

Solution C, also called fibre stain, contains aniline blue.
Figure 17. Mason trichrome stained section of murine gastrocnemius showing a mix of injured and uninjured muscle fibres as well as a neurovascular bundle.
2.2.5 **Muscle Injury Scoring**

Stained slides were examined under light microscopy at ×200 magnification (Nikon E600 Upright Microscope, Tokyo, Japan). Multiple images were acquired from the entire muscle section, and each image was assigned a serial number, using SPOT Insight Digital Camera (Diagnostic Instruments, Sterling Heights, MI). The blinded observer then examined the images of each muscle in random order using a random number generator ([www.random.org](http://www.random.org)) until a minimum of 600 muscle fibres per section had been scored.

Muscle fibres were scored as uninjured or injured based on the morphology of the individual fibres. Scoring was carried out in an all-or-nothing fashion where fibres were deemed to either be injured or uninjured. Uninjured fibres were characterized as having well-defined borders, uniform texture and colours, and easily identifiable satellite cells and pericellular nuclei. Injured fibres, on the other hand, had interrupted or ragged borders, inconsistent texture and colour, breaks in the cytoplasm, and nuclei detachment (Figure 18).
Figure 18. Sample of Mason trichrome stained murine gastrocnemius muscle following IR injury. Each muscle fibre is deemed injured (I) or uninjured (U). A percentage injury score is generated once 600 individual fibres are scored for each muscle.
2.2.6 Tissue Adenosine Triphosphate Levels

Samples of frozen muscle (200 mg) were homogenized with a polytron homogenizer in a test tube containing 10% trichloroacetic acid. Samples were centrifuged for 10 minutes at 10,000g, and supernatants were diluted in PBS. The ATP levels were measured using ATPlite Luminescence Assay according to the manufacturer's protocol (PerkinElmer Life, Boston, MA). ATPLite is an Adenosine TriPhosphate (ATP) monitoring system based on firefly (Photinus pyralis) luciferase. ATP is a marker for cell viability because it is present in all metabolically active cells and the concentration declines very rapidly when the cells undergo necrosis or apoptosis. The ATPLite assay system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. This is illustrated in the following reaction scheme:

\[
\text{ATP} + \text{D-Luciferin} + \text{O}_2 \xrightarrow{\text{LUCIFERASE}} \text{Oxyluciferin} + \\
\text{Mg}^{2+} + \text{AMP} + \text{PP}_i + \text{CO}_2 + \text{Light}
\]

The emitted light is proportional to the ATP concentration within certain limits.

Top counts were read using 1450 MicroBeta plate reader (PerkinElmer Life). Concentrations of the unknowns were extrapolated off the standard curve and expressed as nanomole per milligram tissue weight.
2.2.6.1 ATP EXTRACTION

Once the gastrocnemius muscle is harvested an approximately 200mg piece of muscle is snap frozen in liquid nitrogen and placed at –80°C until extraction.

1- Remove tissue to be extracted from –80°C freezer into liquid nitrogen container.
2- Prepare 10% TCA (Trichloroacetic acid 6.1N, Sigma #T0699-100ML) in ultra pure H2O and 1X DPBS with Ca/Mg (1X Dulbecco’s Phosphate-Buffered Saline, Invitrogen #14040-133). Place both solutions on ice.
3- Label and pre-cool 3 sets of 1.5 micro centrifuge tubes for each sample to be extracted by placing them on ice before use.
4- Add 1ml of ice cold 10% TCA to each 200mg of tissue sample and homogenize in 15ml round bottom centrifuge tube and place on ice until all the samples has been extracted. Transfer the homogenate into a labelled pre-cooled 1.5 centrifuge tube (homogenize one sample at time) and place back on ice.
5- Clear the homogenate by centrifuging @ 10,000xg, 4°C for 10 minutes.
6- Dilute an aliquot of each sample 50X with 1X DPBS by adding 20ul of the supernatant into 980ul DPBS on ice and vortex (acid concentration will be 0.2%).
7- Further dilute the samples to 500X or 1000X by adding 50ul of the 50X-diluted sample into 450 or 950ul cold DPBS. Vortex and place on ice.
2.2.6.2 Standards Preparation

Standard ATP is provided as 10mM (10^{-3} M) frozen aliquots in the –20°C freezer in 50ul volume each. Dilute standard 10 times with 450ul cold ultra-pure H_{2}O to make a 100uM solution. Make a serial dilution in pre-cooled micro centrifuge tubes to generate the following concentrations: 50, 25, 12.5, 6.25 µM ATP standards in water. Place tubes on ice until are ready to do the assay. Dilute the 10^{-3} M solution 100 times with 495µL cold ultra-pure H2O. That will make it 10^{-5} or 10µM. Further serial dilute this to give 5, 2.5, 1.25, and 0.625 µM ATP standards.

2.2.6.3 Assay Procedure

Using a black flat bottom 96 well plate Greiner bio-one (part #655076) (E&K scientific EK-255076). Reconstitute one vial of the lyophilized substrate solution with 5ml of substrate buffer just before use.

1- Add 50µl of the mammalian cell lysis solution to each well using the motorized pipette.
2- To the standard wells add 90µl of 1X DPBS.
3- Add 10µl of the standard samples into a designated well in duplicate.
4- Add 100µl of 50X diluted sample into a designated well.
5- Shake the plate on orbital shaker for five minutes at room temperature at 700rpm.
6- Add 50µl of the substrate solution per well and shake on the orbital shaker at 700 rpm for 5 minutes in the dark.
7- Keep the plate protected from light. Measure the luminescence by counting for 30 seconds each using PerkinElmer 1450 Micro Beta Jet micro plate luminescence counter.

8- Calculate the standard curve and extrapolate the concentration of the unknowns (Figure 19).
Figure 19. Example of an ATP Standard curve used to extrapolate the concentration of unknown values.
2.2.7 **Hindlimb Mortality**

Animals were allowed to awaken following bilateral in vivo limb ischemia applied as previously described. They were allowed free access to chow and water and were followed for seven days or until death. Kaplan-Meier survival curves were plotted and curves were compared.

2.2.8 **Statistical Analysis**

Statistical analysis was performed with Instat (GraphPad, San Diego, CA). Data were expressed as means +/- SEM. Comparisons were made using ANOVA and Student’s t-test. A P value <0.05 was considered significant. *Post-hoc* comparison was made using Tukey Kramer parametric analysis. Kaplan-Meier survival curves were compared using the Log-Rank test.
2.3 RESULTS
2.3.1 Dose Response to P188 Administration

NS+P188 given prior to the initiation of reperfusion demonstrated a classical sigmoidal dose-response curve with the optimal minimum dose seen to be 300mg/kg (Figure 20) (n=8 for each group).

Figure 20. Dose response curve when P188 given 10 minutes prior to reperfusion.
2.3.2 GASTROCNEMIUS MUSCLE FIBRE INJURY

2.3.2.1 PRE-ISCHAEMIA TREATMENT

After 24 hours of reperfusion, there was a statistically significant, six-fold decrease in the amount of cell injury in animals treated 10 minutes prior to ischemia with NS+P188 (8%) (n=15) compared with control animals (50%) (n=8) (P<0.001) (Figure 22). There was no difference in the degree of injury seen in those treated with Dextran and those given Normal Saline. A representative photomicrograph of injured and uninjured myocytes is presented in Figure 21.

Figure 21. Microscopic Skeletal Muscle Morphology: A. Mix of injured and uninjured muscle fibres in a control animal (60% injury). B: Uninjured Fibres in a P188 treated animal.
Figure 22. P188 protects against IR injury in muscle fibres when given prior to onset of ischemia (p<0.001).
2.3.2.2 Pre-Reperfusion Treatment

When administered 10 minutes prior to the release of the bilateral hind limb tourniquets and the commencement of the 24 hour reperfusion period NS+P188 (n=10) (12%) provided a significant, more than two-fold difference in percentage myocyte injury as compared to NS+dextran control (n=9) (29%) (p<0.001) (Figure 23).
Figure 23. P188 protects against IR injury in muscle fibres when given prior to onset of reperfusion (p<0.001).
2.3.2.3 Post-Reperfusion Treatment

When given two hours after the release of the bilateral hind limb tourniquets NS+P188, the histological injury score was 15% in the NS+P188 (n=14) treated group compared to 40% in the NS+dextran group (n=10) (p<0.05) (Figure 24). In contrast, when P188 was administered 4 hours post reperfusion (n=6), myocyte injury at 24 hours reperfusion was identical to dextran controls (n=7) (Figure 25).
Figure 24. P188 protects against IR injury in muscle fibres when given 2 hours following onset of reperfusion (p<0.05).
Figure 25. No difference is seen in muscle injury scores when P188 is given 4 hours after the onset of reperfusion.
2.3.3 Effect of P188 on Muscle ATP Levels

When administered 10 minutes prior to reperfusion, both NS+P188 (n=8) and NS+dextran (n=9) treated animals showed a significant decrease in cellular ATP levels compared to sham animals (n=10) (p<0.001). After 3, 6, and 24 hours of reperfusion animals treated with NS+P188 had significantly greater levels of ATP than those treated with NS+dextran (6.23 ng/mg vs. 2.53 ng/mg at 3h) (Figure 26) (p<0.05), (6.15 ng/mg vs. 2.83 ng/mg at 6h) (Figure 27) (p<0.05), and 5.55 ng/mg vs. 2.41 ng/mg at 24h) (p<0.05) (Figure 28).
Figure 26. Cellular ATP levels after 3 hours of reperfusion. Treatment administered 10 minutes prior to reperfusion beginning. Animals treated with P188 had 6.23 ng/mg ATP vs. 2.53 ng/mg in those treated with Dextran (p<0.05).
Figure 27. Cellular ATP levels after 6 hours of reperfusion. Treatment administered 10 minutes prior to reperfusion beginning. Animals treated with P188 had 6.15 ng/mg ATP vs. 2.83 ng/mg in those treated with Dextran (p<0.05).
Figure 28. Cellular ATP levels after 24 hours of reperfusion. Treatment administered 10 minutes prior to reperfusion beginning. Animals treated with P188 had 5.55 ng/mg vs. 2.41 ng/mg in those treated with Dextran (p<0.05).
When given 2 hours after reperfusion beginning animals treated with NS+P188 (n=8) had significantly greater levels of ATP than NS + dextran control animals (n=6) after 24 hours of reperfusion (2.2. ng/mg vs. 0.49 ng/mg) (Figure 29, Figure 30).

Figure 29. Cellular ATP levels after 24 hours of reperfusion. Treatment administered 2 hours after initiation of reperfusion. Animals treated with P188 had 2.2. ng/mg vs. 0.49 ng/mg in those treated with Dextran (p<0.05).
Figure 30. Individual animal ATP levels. Concentration in µM is extrapolated from a standard curve based on luminescence readings.
2.3.4 Effect of P188 on Mortality

When P188 was administered 10 minutes prior to reperfusion, 75% of the mice survived to 7 days (n=12) as compared to only 25% of the dextran control mice (n=12) (p=0.007) (Figure 31).

P188 treatment (n=12) two hours after reperfusion continued to provide a significant survival advantage over dextran control (n=12) (P188- 50% vs. Control-8%) (p=0.032) (Figure 32).
Figure 31. Kaplan-Meier survival curve when treatment given 10 minutes prior to the start of reperfusion. 75% of P188 animals survived for 7 days compared to 25% of control animals (p=0.0077).
Figure 32. Kaplan-Meier survival curve when treatment given 2 hours after the start of reperfusion. 50% of P188 animals survived for 7 days compared to 8.3% of control animals (p=0.032).
2.4 DISCUSSION

Previous studies have established P188 as a membrane-sealing polymer that interacts directly with monolayers (Wu et al., 2005) and damaged membranes (Maskarinec et al., 2002). P188 is effective in stabilizing membranes and improves survival and recovery of a variety of cell types from an array of injuries including radiation injury, electroporation and mechanical trauma (Borgens et al., 2004, Merchant et al., 1998, Ng et al., 2008, Phillips and Haut, 2004). P188 has not, however, been previously used in skeletal muscle IR injury. Various reports (Greenebaum et al., 2004, Lee et al., 1992, Maskarinec et al., 2002, Wu et al., 2005) have described the essential conditions for P188 mediated membrane sealing in various types of cells. It has been shown that the surfactant monomer is the active agent, not the surfactant micelle. For membrane-sealing purposes, P188 is typically administrated at concentrations well below the critical micelle concentration of 0.1–1.0 mM at physiological temperatures. These experiments demonstrate that a single dose of the non-ionic surfactant P188 given prior to or after the onset of ischemia or reperfusion modulates murine skeletal muscle injury and survival following bilateral lower extremity ischemia reperfusion.

Poloxamer 188 has been shown to remain primarily in extra-cellular fluid in rodents, canines and humans after IV administration with little or no intracellular uptake (Grindel et al., 2002). Renal clearance via glomerular filtration accounts for over 90% of P188 clearance (Jewell et al., 1997). Mean renal clearance of purified poloxamer 188 is estimated at 5.21 L/h, and elimination half-life is estimated to be 7.65 hours (Jewell et al., 1997). As myocyte injury begins almost immediately following initiation of reperfusion and is irreversible within four hours of reperfusion, administration of a single dose of P188 either prior to or during the ischemic period or in the early reperfusion period
should ensure an adequate therapeutic plasma level during the period when the myocytes are still salvageable.

The potential therapeutic benefits of P188 for treatment of IR injury has been previously recognized and investigated (Grindel et al., 2002, Phillips and Haut, 2004). However, in previous studies the therapeutic rationale for P188 therapy was mostly directed toward the well-established effect of P188 on blood rheology. P188 has the effect of reducing the effective viscosity of blood, making it easier to perfuse through stenotic arteries. In previous human trials investigating P188 as an adjunct to coronary artery transluminal angioplasty, treatment was administered by continuous intravenous infusion for several days. However, this therapeutic approach was found to be associated with renal dysfunction. Increases in serum creatinine levels were seen in between 2.3% to 8.8% of patients treated with P188. Greater creatinine rises were seen with increased P188 doses. P188 in these studies was administered at doses of 300mg/Kg/h and upwards for 48h (CORE, 1997, O'Keefe et al., 1996). These investigators later demonstrated reduced renal injury by reducing the polydispersity of commercially available Poloxamer 188.

In this study, a different approach was taken. The therapeutic strategy was to augment the natural membrane repair process to render the ischemic tissue to be more injury tolerant. Co-polymer surfactant sealing of disrupted cell membranes occurs within the time frame of seconds after surfactant membrane contact. Thus, we hypothesized that a substantial therapeutic benefit to IR injured tissue could be derived from a single bolus P188 administration that would avoid prolonged renal exposure to high concentrations. This report is particularly significant for showing therapeutic benefit from a single-dose administration of P188 for IR injury.

In an experimental protocol where P188 was administered 10 minutes prior to the onset of ischemia, there was a marked decrease in skeletal muscle injury. This pre-ischemic treatment protocol mimics clinical conditions where arterial occlusion occurs in a planned fashion, such as aortic cross clamp during arterial reconstructions or skeletal
muscle tissue free flaps (Figure 22). Systemic and local complications associated with planned vascular occlusions are usually manageable with fluid resuscitation and close management of patients’ hemodynamic profile in an intensive care setting. In contrast, treatment of patients with acute arterial occlusions (i.e. popliteal embolus or a thrombosed free flap), therapeutic interventions are more difficult due to the development of the no-reflow phenomenon. In these clinical scenarios, therapeutic intervention can only effectively begin immediately before reperfusion. To mimic this clinical situation, P188 was administered immediately prior to reperfusion. Even when P188 was administered during ischemia, prior to reperfusion, it effectively decreased skeletal muscle injury to an equivalent level observed when this single dose was administered prior to ischemia (Figure 23).

The restoration of blood flow, i.e. reperfusion of an acutely ischemic limb or flap, triggers a complex cascade of biochemical, immunological, and cellular events which results in muscle oedema, myocyte necrosis, apoptosis, and impaired muscle function (Wang et al., 2008, Austen et al., 2004). One of the earliest events involved in the process of reperfusion injury is the binding of circulating natural antibody (IgM), and the subsequent activation of complement. Previous findings in hind limb, intestinal, myocardial, and burn models have found that injury is mediated by a local effect of specific natural IgM with subsequent complement activation (Boes et al., 2000, Zhang et al., 2004). Many inflammatory pathways, including the generation of reactive metabolites of molecular oxygen, are activated during IR injury (Lindsay et al., 1989). The formation of reactive oxygen metabolites contributes to the formation of chemotactic stimuli, the expression of, and/or activation of, adhesion molecules, and reduces the concentration of, the anti-adhesive agent, nitric oxide. These events lead to neutrophil infiltration and adhesion to post capillary venules. Plugging of capillary venules during reperfusion is the basis of the no-reflow phenomenon, and plays a crucial role in the genesis of IR injury.

Therapeutic interventions that begin a few hours after the onset of reperfusion have to overcome a well-established pathologic process. These experiments demonstrate that
after two, but not four hours reperfusion, P188 significantly reduces skeletal muscle injury (Figure 24, Figure 25). The observed temporal effect of P188 on limiting skeletal muscle injury even when administered during reperfusion lends credence to the concept that P188 reduces injury by membrane sealing.

IgM binding to affected tissues begins during the ischemic injury and continues for up to 6 hours post-initiation of reperfusion. Complement activation, as indicated by C3 deposition on endothelial and muscle cells, is seen within minutes of reperfusion (Chan et al., 2004b). Similarly mast cell activation and degranulation is seen within one hour of the initiation of reperfusion (Abonia et al., 2005). Neutrophil depletion has been shown to protect against IR injury, but only when carried out immediately after initiation of reperfusion (Iwahori et al., 1998). As these pathways have already been activated prior to our administration of P188 it is unlikely to exert its major effect through direct interaction or interference with these pathways. Thus as a therapeutic agent, P188 appears to be effective in the most relevant emergent clinical conditions, i.e. after the onset of established ischemia.

In addition to experiments that confirmed a temporal relationship between P188 administration and effective protection against IR induced skeletal muscle necrosis, a dose-response relationship was demonstrated between calculated P188 concentration and the extent of muscle necrosis. Maximal protection against skeletal muscle injury was observed at a dosage of 300 and 600 mg/kg (Figure 20). The direct relationship between the concentration of P188 and limiting muscle injury suggests a specific mechanism whereby this drug has beneficial effects.

Based on the observations obtained from the dose-response curve, experiments were designed to determine whether P188 therapy resulted in preservation of skeletal muscle ATP levels. P188 administered 10 minutes prior to reperfusion and two hours after reperfusion effectively preserved levels of skeletal muscle ATP levels (Figure 28). While ATP levels did not return to pre-ischemic baseline after treatment with P188, these results
are consistent with previous studies with canine gracilis muscle where recovery of energy stores was incomplete even after two days of reperfusion (Rubin et al., 1992).

In a series of mice where P188 was administered 10 minutes prior to reperfusion, overall survival at 7 days was 80%, significantly better than mice treated with normal saline and dextran (Figure 31). This protective effect on mortality was also observed when P188 was administered 2 hours after beginning reperfusion (Figure 32). While treatment with P188 is clearly effective from a histologic and biochemical perspective when given up to two hours reperfusion, the most important factor in recovery from limb ischemia, i.e. survival appears to be related to early treatment, if possible prior to reperfusion.

Previous work on skeletal muscle IR injury has focused on the effects of pre-treatment or pre-conditioning with a variety of agents (Chan et al., 2006b, Kyriakides et al., 2001, Schroeder et al., 1996). In the clinical setting pre-treatment is often not a viable option as the onset of ischemia can be a sudden unpredictable event. Limited protection has been demonstrated with therapies given at the initiation of reperfusion. Ischemic post-conditioning (Zhao et al., 2003b), levosimendan (du Toit et al., 2008) and acetylcholine (Lu et al., 2006) have shown reduced infarct size in myocardial ischemia reperfusion models when given at the time of reperfusion. However, no effective late therapies have yet been developed. Our results suggest that P188, which has been FDA approved for other uses, could become a beneficial clinical option as it provides a late, salvage therapy as well as effective pre-reperfusion protection.
2.5 CONCLUSION

In conclusion, our study demonstrates that administration of P188 attenuates skeletal muscle ischemia reperfusion injury and reduces mortality in a murine hind limb model. We feel that this drug, given as a single dose, may be a potential therapeutic intervention for skeletal muscle IR injury in the clinical setting.
Chapter Three: A NOVEL, NATURAL IgM-BINDING, PEPTIDE DECREASES MURINE SKIN FLAP INJURY FROM ISCHEMIA REPERFUSION INJURY
3.1 INTRODUCTION

Ischemia-reperfusion injury is modulated by complex inflammatory and immunological signalling pathways that have to date not been fully elucidated. Clinically, IR injury is responsible for significant morbidity in the fields of trauma, vascular, transplant, and plastic surgery.

The binding of circulating natural antibodies and the activation of complement (Carroll and Esclamado, 2000). First described in myocardial reperfusion by Hill and Ward (Hill and Ward, 1971), complement activation was shown also to involve the release of C3a and C5a anaphylatoxins that induce degranulation of mast cells with release of histamine and other chemical mediators. The final common pathway is oedema, attraction of activated leukocytes, and the formation of membrane-attack complexes that damage membranes, in net leading to tissue necrosis (Khalil et al., 2006).

Attempts to reduce IR injury by blocking the complement cascade at various levels have shown some success. Pre-treatment of rodents with recombinant soluble complement receptor type 1, a potent global inhibitor of complement, showed profound protection in different organ models (Hill et al., 1992, Huang et al., 1999, Kyriakides et al., 2001, Lehmann et al., 2001, Stammberger et al., 2002, Weisman et al., 1990a, Zacharowski et al., 1999). Both C4 deficient knockout mice and C3 deficient knockout mice were protected from local injury in the hind limb and intestinal ischemia reperfusion models (Weiser et al., 1996, Williams et al., 1999). In a myocardial IR study complement inhibition with C1 esterase inhibitor therapy suppressed endothelial adhesion molecule expression and reduced infarct size (Buerke et al., 1995, Buerke et al., 1998). In rat intestinal models (Arumugam et al., 2002, Wada et al., 2001) and myocardial models (Vakeva et al., 1998) blockade of C5/C5a with antibodies or inhibitors resulted in protection from IR injury.
Zhang et al. isolated an IR-specific natural immunoglobulin M (IgM) producing cells using B-1 lymphocyte hybridomas constructed from peritoneal cells. A single IgM producing clone, CM22, was identified that restored IR injury in immunodeficient mice [RAG-1 -/-] in an intestinal model of IR injury with evidence of IgM, C3 and C4 complement deposition in damaged tissue (Zhang et al., 2004). In addition, Austen et al. demonstrated restoration of reperfusion injury in antibody repertoire-altered Cr2/-/- mice in a hind limb IR model when reconstituted with this specific IgM (Austen et al., 2004).

The identification of CM22 as a natural self-reactive antibody led to the search for injury neoantigens. Zhang et al. hypothesized that circulating IgM would bind self-antigens exposed during ischemia and that these complexes could be isolated and the antigens identified by proteomic techniques. When the immune complexes in injured tissues of RAG-1/-/- mice reconstituted with CM-22 antibody were investigated, non-muscle myosin heavy chain (NMHC-II) A and C were determined to be the involved antigen (Zhang et al., 2006a). It has been shown in intestinal tissue and hind limb that a synthetic peptide (P8) representing a conserved region of NMHC-II binds IgM CM-22 in vitro and blocks reperfusion injury in wild type mice when given I.V. as a competitive inhibitor (Chan et al., 2004a, Zhang et al., 2006a).

In the past, there have been many attempts to mitigate ischemia-reperfusion injury through various experimental means. One of the more effective methods of protection has been the process of ischemic preconditioning, whereby tissue is exposed to short cycles of ischemia and reperfusion prior to critical ischemia. This phenomenon was first described in 1986, when Murry et al. discovered that brief cycles of ischemia and reperfusion within the canine heart exert a myocardial protective effect from a longer ischemic insult (Murry, 1986). This has been subsequently investigated in large animal models and within various tissue types, including cardiac (Cleveland et al., 1996, Yellon et al., 1993), hepatic (Yoshizumi et al., 1998), skeletal muscle ( Schroeder et al., 1996), musculocutaneous flaps (Carroll et al., 1997, Zahir et al., 1998b), and skin flap models (Coskunfirat et al., 2006), although the main
investigational focus has historically been on cardiac tissue. In this study we used ischemic preconditioning of the skin flap as a positive control.

In plastic surgery, composite tissues composed of muscle as well as skin and subcuticular tissues are often utilized. In addition, as composite tissue transplantation becomes more commonplace, protection of the tissue from IR injury will assume greater importance. The aim of our study was to investigate the potential protective effects of the synthetic peptide P8 in an axial island skin flap model and to establish the possibility that tissue loss in this model is attributable to reperfusion injury.

3.1.1 The Complement System

The complement system was discovered many years ago as a heat labile component of normal plasma comprised of a set of proteins that work to eliminate microorganisms and other antigens from the body (Kinoshita, 1991). More than half a century after its discovery, it became clear that complement played a major role in the initiation and control of inflammation in general (Frank and Fries, 1991). It has been shown that the complement system consists of three activation pathways: a single terminal pathway, regulatory proteins, and complement receptors (Kinoshita, 1991).

The complement system can be activated through different mechanisms: 1) the classical pathway, 2) the lectin pathway, and 3) the alternative pathway (Figure 33). Each pathway has a different mechanism for cascade initiation (Mollnes et al., 2002). The classical pathway is activated by antigen-antibody interaction, which then leads to activation of C1q, followed by C2- and C4-dependent cleavage of C3 (by C3 convertase C4b2a) and, ultimately, cleavage of C5 by formation of the C5 convertase (C4b2a3b).

In the lectin pathway, serum mannose binding lectin (MBL), which is homologous to C1q, recognizes microbial surface mannose and triggers activation of MBL-associated
proteases (MASP1–3). This interaction leads to the same formation of C3 and C5 convertases.

The alternative pathway is activated by presence of lipopolysaccharide (LPS), and, to a certain extent, spontaneously generated C3b. In this pathway, C3 binds to factor B and forms a complex, which is cleaved by factor D to form the alternative C3 convertase, C3 (H₂O) Bb. Properdin acts as an amplifying activator and stabilizes this complex, enabling the cleavage product C3b to bind to it, thus forming the alternative C5 convertase (C3b3bBb).

All pathways, therefore, use C3 and cleave C5, which results in the powerful pro-inflammatory cleavage products C5a and C5b-9. These two products of complement activation are believed to be mainly responsible for IR injury. C5a has been shown to exert numerous pro-inflammatory effects such as a chemotactic effect on neutrophils (Shin et al., 1968), release from phagocytic cells of granular enzymes (Goldstein and Weissmann, 1974), vasodilatation, and increased vascular permeability (Schumacher et al., 1991). C5b-9 has been demonstrated to have major contribution to complement-mediated tissue injury after IR (Zhou et al., 2000, Ito et al., 1996), and causes relaxation of coronary arteries (Stahl et al., 1995).
Figure 33. Schematic of the complement system.
3.1.2 Complement in Ischaemia Reperfusion Injury

Ischemia-reperfusion injury is now recognized as a highly complex cascade of events that includes interactions between vascular endothelium, interstitial compartments, circulating cells, and numerous biochemical entities. Inflammation is known to be a key mediator of IR injury and considerable data exist demonstrating the significance of the innate immunity (Boros and Bromberg, 2006, Huang et al., 2007, Rabb, 2002).

Parenchymal damage occurs from both direct microvascular dysfunction from hypoxia and the subsequent inflammatory response. Acute ischemia leads to oxygen deprivation and adenosine triphosphate depletion resulting in direct parenchymal damage through tissue necrosis. Upon restoration of blood flow to the ischemic tissue, a “no-reflow” phenomenon occurs. Capillaries and microcapillaries are not perfused, leading to further tissue damage. The mechanisms of failed capillary perfusion include endothelial cell swelling, capillary narrowing due to interstitial oedema, and intravascular sludging from haemoconcentration and capillary vasoconstriction that is mediated by many chemokines and cytokines. These events lead to direct microvascular dysfunction and parenchymal damage (Eltzschig and Collard, 2004, Menger and Vollmar, 2007, Molitoris et al., 2002).

In IR injury, complement activation was described during myocardial infarction over 30 years ago and has led to numerous investigations on the contribution of the complement system to IR tissue injury (Hill and Ward, 1971).

Accumulating evidence supports a major role for the serum innate immune response in the propagation of IR injury, namely natural IgM and the complement system. Early observations that transient depletion of complement C3 reduced inflammation in a rat model of myocardial infarction suggested a role for innate immunity in IR (Hill and Ward, 1971). Subsequently, Weisman et al. demonstrated that pre-treatment of rats with a specific soluble inhibitor of complement C3 (sCR1) dramatically reduced injury in a similar model of myocardial infarction and further established the importance of
complement as a mediator of injury (Weisman et al., 1990b). Subsequent studies using the sCR1 inhibitor in various animal models, including porcine (Chai et al., 2000, Huang et al., 1999) and mouse (Hill et al., 1992), and in various tissues as diverse as the central nervous system, intestine, or skeletal muscle confirmed and generalized the concept that the complement system was an important mediator of IR.

Although the complete mechanism of injury is not clear, a role for the complement membrane attack complex (C5–C9) is supported by observations of deposition of complement C9 within reperfused heart tissues (Schafer et al., 1986, Robert-Offerman et al., 2000), reduction in injury in mice deficient in C5 or treated with C5-specific antibody (Fleming et al., 2003, Homeister et al., 1992, Zhao et al., 2002, Austen et al., 1999), and C6-deficient rabbits (Ito et al., 1996, Kilgore et al., 1998). Importantly, IR injury in the skeletal muscle model is not only complement dependent but requires intact mast cells, as mice deficient in mast cell protease 5, one of the chymotryptic proteases found in granules, are protected from full injury (Abonia et al., 2005).

The first indication that natural antibody was involved in initiation of IR derived from studies in mouse models bearing complete deficiencies in innate immune proteins such as complement components C3 and C4 and natural IgM. Results from these studies suggested that brief periods of ischemia led to an alteration in surface epitopes, and this change resulted in binding by natural IgM and activation of the complement system (Williams et al., 1999, Weiser et al., 1996). Further support that specific natural IgM was involved followed from two separate reports that mice deficient in complement receptors CD21 and CD35 were protected in an intestinal model of RI despite a normal level of circulating IgM (Fleming et al., 2002, Reid et al., 2002).

Reconstitution of the animals with pooled IgM (isolated from WT mice) or engraftment with WT peritoneal B-1 cells restored pathogenic IgM and IR. Notably, reconstitution of Cr2−/− mice with pooled murine IgG alone did not restore histological injury but enhanced neutrophil infiltration when combined with IgM (Fleming et al., 2002). More recently, identification of a single monoclonal IgM, which alone could restore injury in
both intestine and hind limb models of IR in antibody-deficient (RAG-1\(^{-/-}\)) mice, provided further support for the hypothesis that innate recognition of stress-induced self-antigens was involved in initiation of IR. To identify IR-specific natural IgM-producing cells, a panel of hybridomas was generated from peritoneal cells enriched in B-1 cells, and their IgM product was screened in vivo for reconstitution of injury in RAG-1\(^{-/-}\) mice. One clone, CM22, was identified in which its IgM restored significant IR injury. Deposition of IgM, C4, and C3 within reperfused tissues correlated with pathogenesis after treatment of mice reconstituted with IgM isolated from CM22 but not from other hybridomas. Sequence analysis of pathogenic IgM identified an apparent usage of germ-line V\(_H\) and V\(_K\) genes in CM22 Ig heavy and light chains, respectively. Therefore, a single pathogenic IgM clone was identified, confirming a crucial link in the mechanism of classical pathway complement-mediated induction of IR injury (Austen et al., 2004, Zhang et al., 2004).

In 2006, Zhang et al. identified a highly conserved region within nonmuscle myosin heavy chain (Hc) type II (NMHC-II) A and C as the target for natural IgM and initiation of injury in murine models of skeletal and intestinal IR (Zhang et al., 2006a). They provide five lines of evidence to support a role for NMHC-II as the critical self-ligand in IR: (a) protein sequence analysis of natural IgM-bound NMHC-II isolated from lysates of IR tissue; (b) binding of NMHC-II by pathogenic IgM (IgM\(^{\text{CM-22}}\)) in an ELISA assay; (c) initiation of intestinal IR in RAG-1\(^{-/-}\) mice with an anti-pan-myosin antibody; (d) sequence homology with a synthetic peptide isolated from phage display library that binds pathogenic IgM\(^{\text{CM-22}}\) and blocks intestinal IR in WT mice; and (e) a synthetic peptide representing a conserved region of NMHC-II binds IgM\(^{\text{CM-22}}\) in vitro and blocks IR in WT mice in two distinct tissues. Collectively, these results support the model that natural IgM recognition of NMHC-II expressed on the surface of ischemic tissue results in activation of the complement system and acute inflammation. These findings suggest a general mechanism by which stress, such as hypoxia, leads to expression of a highly conserved self-antigen on the cell surface and recognition by the innate immune system.
resulting in acute inflammation as well as providing a therapeutic target for blocking the IgM $^{CM-22}$.

It has been shown in intestinal tissue and hind limb that a synthetic peptide (P8) representing a conserved region of NMHC-II binds IgM CM-22 in vitro and blocks reperfusion injury in wild type mice when given IV as a competitive inhibitor (Chan et al., 2006b, Zhang et al., 2006a). Chan et al. showed intravenous administration of P8 led to significant attenuation of muscle injury (13 +/- 1.8 injured fibres/50 counted) after reperfusion injury compared to animals receiving saline (26 +/- 2.3) or the same mass of a random peptide (22 +/- 2.3). This level of protection from injury was comparable to that seen in the absence of antibody altogether. Also, P8-treated animals exhibited a marked decrease in deposition of IgM (as well as C3) in comparison to saline treated controls.
Figure 34. Deposition of CM22 IgM, C4, and C3 on injured intestinal tissues.

Representative cryosections of intestinal tissues were harvested after intestinal IR from RAG-1/−/− mice pre-treated with either IgM from CM31 (a, d, g, j, m, and p) or CM22 (b,
c, e, f, h, i, k, l, n, o, q, and r). All sections were stained with anti-IgM-biotin followed by streptavidin-Alexa 568 (red) and counterstained with 4', 6-diamidino-2-phenylindole (violet). Cryosections were costained with anti-C4-FITC (green in a–i) and anti-C3-FITC (green in j–r). The co-localization of IgM and C4 (red _ green _ yellow) is represented in g–i. Co-localization of IgM and C3 is represented in p–r (red _ green _ yellow). High-magnification (X400) images of CM22 treatment (c, f, i, l, o, and r) were taken from the same region as X100 magnification marked by white boxes. From Zhang et al., PNAS, March 2006.
3.1.3 Toll-like receptors in ischaemia reperfusion injury

Toll-like receptors (TLRs) represent a family of transmembrane proteins that have a major role in the pathogen-induced inflammation. They detect specific pathogen-associated molecular patterns such as peptidoglycan (TLR-2) or lipopolysaccharide (TLR-4) (West et al., 2006) deriving from various microorganisms including bacteria, fungi and viruses (Kawai and Akira, 2007a, Kawai and Akira, 2007b). TLR activation leads to the initiation of a pro-inflammatory response through the release of cytokines, chemokines, interferons and the activation of inflammatory cells (Schnare et al., 2006, Zhong et al., 2006). In addition, TLRs recognize distinct endogenous danger molecules released from damaged tissues in non-physiological sites or amounts, such as heat-shock proteins, hyaluronan, high-mobility group box 1 protein (HMGB1) and fibrinogen (Johnson et al., 2003). Accumulating evidence highlights the important role of TLRs in innate host defence in the healthy intestinal mucosa, maintaining mucosal homeostasis. Recent observations imply that the continuous recognition of selective commensals by TLR2 and TLR4 under steady-state conditions is essential in mucosal protection against exogenous injury (Arumugam et al., 2009).

After IR injury TLRs can respond to various endogenous cellular and extracellular components whose structure is altered as a result of released cellular enzymes. This includes low-molecular-weight hyalouronic acid, fibrinogen, fibronectin, β-defensins, heparin sulfate proteoglycans and heat shock proteins HSP60 and HSP70. In response to ligand binding to TLR on immune cells, a number of innate immune responses occur, including NF-κB activation, cell activation, and the production of proinflammatory cytokines (Arumugam et al., 2009).

TLRs have been recently suggested to play a key role in IR in several territories. Experimental data provide strong evidence that the pathogenesis of IR injury following
kidney transplantation in humans involves signalling through TLR4 expressed in donor kidney cells (Kruger et al., 2009). More specifically, it has been demonstrated that IR up regulates HMGB1, HSP70 and biglycan, which in turn up regulate pro-inflammatory genes via TLR4 signalling in kidney tissue. Additional data suggest a potential TLR involvement in myocardial IR injury; IRAK-1, HSP60 and HSP70 become quickly activated following myocardial ischemia and act subsequently as TLR ligands promoting TLR pro-inflammatory actions (Chao, 2009). Supporting these findings, Feng et al. recently found that inhibition of TLR signalling in mice results in reduced neutrophil recruitment and attenuated cytokine/adhesion molecule production, smaller infarct sizes, and better preserved ventricular function following myocardial IR injury (Feng et al., 2008). TLRs also play a role in neuronal IR injury. They have been shown to induce cytokine production by intrinsic glial cells and to up regulate the expression of adhesion molecules that facilitate the infiltration of lymphocytes into the ischemic brain region, which probably contributes to neuronal damage after cerebral ischemia (Kilic et al., 2008).

3.1.4 CYTOKINES IN ISCHAEMIA REPERFUSION

Cytokines are low-molecular-weight (approximately 25 kDa) regulatory proteins or glycoproteins released from various cells, mainly from leukocytes, usually by various activating stimuli, and regulate the development and effector functions of immune cells. Most cytokines show autocrine and/or paracrine action and a few of them exhibit endocrine action. Ischemia reperfusion-induced acute injury causes the synthesis of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α (Takada et al., 1997, Donnahoo et al., 1999). Cytokine production following ischemia reperfusion occurs through the interaction between cytokines and the transcriptional response directly induced by hypoxia itself. Ischemia activates transcription factors such as NF-κB, heat shock factor-
1, and hypoxia-inducible factor-1 (HIF-1) (Cao et al., 2003, Eickelberg et al., 2002). Direct blockade of a number of cytokines, including IL-1, IL-6, and IL-8 has been shown to attenuate renal injury during ischemic acute kidney injury, while IL-4 and IL-10 modulation can worsen disease (Furuichi et al., 2006, Haq et al., 1998).

Chemokines are a subgroup of cytokines, which are released by tissues and composed of 90 to 130 amino acid residues. Their basic functions are chemotaxis and activation of leukocytes. Chemokines have three subtypes according to the number of amino acids between the first two cysteines; CC, CXC, and CX3C families. Chemokine induction during the inflammatory response after ischemia reperfusion injury has been reported in several organs such as brain, heart, liver, and kidney (Furuichi et al., 2006). In post-ischemic tissues, chemokines are induced by ROS, cytokines, complement activation, TLR-mediated pathways, and the NF-κB system. ROS has been reported to induce chemokine production in brief myocardial ischemia in a murine model (Nossuli et al., 2001) and a canine model (Lakshminarayanan et al., 2001). ROS is known to trigger cytokine and chemokine cascades through NF-κB activation. Pro-inflammatory cytokines, such as tumour-necrosis factor (TNF)-α and interleukin (IL)-1β, stimulate chemokine production in post-ischemic tissues of myocardial ischemia reperfusion injury models (Chandrasekar et al., 2000) and hepatic ischemia reperfusion injury models (Colletti et al., 1995). Complement activation can also stimulate chemokine induction.
3.1.5 Cellular Mediators of Innate Immunity in Ischaemia Reperfusion

3.1.5.1 Macrophages

Macrophages play roles in both innate and adaptive immunity. Activated macrophages exhibit potent phagocytic activity and secret several important cytokines such as IL-1, IL-6, IL-8 (CXCL8), IL-12, and TNF-α. Monocyte adherence is observed after 2 hours following reperfusion, and inhibition by anti-B7-1 antibody attenuates renal IR injury (De Greef et al., 2001). Macrophages infiltrate into the outer medulla of post-ischemic kidneys and remain into the recovery phase (Ysebaert et al., 2000). Early monocyte/macrophage influx could be mediated by microvascular basement membrane heparin sulfate proteoglycans binding to L-selectin and monocyte chemoattractant protein-1 (MCP-1) (Celie et al., 2007). Although macrophages are suspected to play a role in repair after acute injury, their precise role is yet to be elucidated. One study demonstrated that osteopontin knockout mice revealed fewer infiltrating macrophages and less fibrosis compared to wild-type mice (Persy et al., 2003). Macrophages play a role in the early injury phase of ischemia reperfusion-induced acute injury (Day et al., 2005, Jo et al., 2006).

3.1.5.2 Neutrophils

Neutrophils play key roles in innate immune response by phagocytosis, producing reactive oxygen, nitrogen species and antimicrobial peptides. Reports show that renal injury is reduced after ischemia reperfusion when treatments inhibiting neutrophil infiltration or activity are used (Kelly et al., 1996), however, other studies failed to find a
protective effect of neutrophil blockade or depletion (Thornton et al., 1989, Rabb et al., 1994).

Neutrophils accumulate in reperfused tissues including skeletal muscle and are a cause of injury and not present merely as a consequence of the injury itself (Menger et al., 1992) (Jerome et al., 1994) (Korthuis et al., 1988). Two requirements exist for neutrophil mediated damage. Firstly these early inflammatory cells must reach the site of injury and secondly there must be cell-to-cell interactions. Both of these requirements are aided by the expression and presence of cell adhesion molecules (CAMs) on the surface of the neutrophils; CAMs can then bind to their corresponding ligand on the endothelial walls of lymph nodes and vessels in the various target tissues (Fischer et al., 1988). Neutrophils aggregating in and around the walls of reperfused vessels exit the vasculature and enter the surrounding tissues by a process of margination and extravasation (Kloner et al., 1991, Kurtel et al., 1992, Paterson et al., 1989a). Surface marker activated neutrophils have a more potent release of their cytotoxic oxidants when compared to their inactivated and circulating fellow cells with deposition of their contents directly into the cytoplasm of the adherent target cell (Shappell et al., 1990) (Suzuki et al., 1989). Also this combination of this cell-to-cell and local platelet-to-cell aggregation contributes to microvascular plugging and may prevent reflow within affected vessels. Although neutrophils are less likely to cause direct renal injury compared to their effect during cardiac or skeletal muscle ischemia reperfusion injury, they likely have a contributory role by plugging renal microvasculature and releasing oxygen free radicals and proteases (Friedewald and Rabb, 2004). A phase I human trial blocking ICAM-1 showed lower rates of transplant ischemic injury (delayed graft function) in treated group (Haug et al., 1993). Blockade of platelet-activating factor (PAF), known to play a role in neutrophil adherence to endothelium, also had a protective effect in a rat cold ischemia reperfusion injury model (Riera et al., 1997).
3.1.5.3 DENDRITIC CELLS

Dendritic cells have been shown to participate in ischemia reperfusion injury in a number of studies. In a rat transplant ischemia reperfusion model, recruitment of recipient MHC class II-positive leukocytes into the kidney was demonstrated despite no sign of acute rejection, and some of them were identified as dendritic cells (Penfield et al., 1999). Both total number and MHC class II expression of renal dendritic cells are increased after ischemia reperfusion injury (Kim et al., 2005). Dendritic cell-endothelial cell binding and migration seem to be facilitated during the initial inflammatory response (Schlichting et al., 2006). Resident dendritic cells are the predominant TNF-secreting cell in early ischemia reperfusion-induced acute kidney injury (Dong et al., 2007) and ischemia reperfusion causes abnormal dendritic cell trafficking into the transplanted kidney (Loverre et al., 2007).

3.1.5.4 T-CELLS IN ISCHAEMIA REPERFUSION

Many studies involving a number of organ systems have shown T-cell trafficking into organs following IR injury. Previously believed to be “passive observers” in the inflammatory response, there is now a significant body of evidence in different organ systems showing the role of T cells as direct mediators of IR injury. T cells have been shown by immunohistochemistry in the post ischemic brain within 24 hours of reperfusion and localize to the stroke boundary zone near blood vessels (Schroeter et al., 1994, Jander et al., 1995). Yilmaz et al. demonstrated that Rag1−/− mice, deficient in both T and B cells, subjected to middle cerebral artery occlusion had significantly reduced cerebral infarct size and neurologic damage compared to WT control mice (Yilmaz et al., 2006). Rag1−/− mice reconstituted with splenocytes from WT mice were no longer protected from injury. In the same study, the authors showed that mice deficient of
CD8\(^+\) T cells, CD4\(^+\) T cells, and interferon (IFN)-\(\gamma\) had reduced cerebral infarct compared to WT mice. Hurn et al. examined mice with severe combined immunodeficiency (SCID), lacking both B and T cells, 22 hours after middle cerebral artery occlusion. They found that compared to WT mice, there was a greatly reduced infarction size in the SCID mice. This also demonstrated that T cells and possibly B cells were mediators of brain IR injury (Hurn et al., 2007).

T cells have been demonstrated to mediate IR injury in the lung. Using a syngeneic lung transplant model in Lewis rats, it was shown that recipient CD4\(^+\) T cells infiltrated lung grafts within 1 hour of reperfusion and up-regulated the expression of CD25 over the ensuing 12 hours. When compared to T cell–deficient nude rats (rnu/rnu), WT Lewis rats had decreased oxygenation and increased peak airway pressures indicating more severe injury in the mice with functional T cells. There were significantly higher levels of IFN-\(\gamma\) in transplanted lung tissue of recipient WT rats compared to nude rats. To confirm that T cells were key mediators of injury, nude rats that were reconstituted with T cells from heterozygous rats. These reconstituted nude rats developed the injury pattern seen in WT rats after 12 h of reperfusion (de Perrot et al., 2003). Geudens et al. also showed a pathogenic role of T cells in a lung IR injury model in SCID mice. SCID and control mice underwent 90 minutes of ischemia. After 4 hours of reperfusion, there was a significant decrease in neutrophils and interleukin (IL)-1\(\beta\) in SCID mice compared to controls (Geudens et al., 2007).

T lymphocytes have also been shown to be pathogenic during myocardial IR injury. Yang et al. investigated myocardial infarct size in RAG1\(^{-/-}\) mice and controls following 45 minutes of left anterior descending coronary artery occlusion. RAG1\(^{-/-}\) mice had significantly smaller infarct size compared to that of control mice. After reconstitution of RAG1\(^{-/-}\) mice by adoptive transfer with CD4\(^+\) T cells, the infarct size of the reconstituted RAG1\(^{-/-}\) mice was significantly greater than that of the RAG 1\(^{-/-}\). RAG1\(^{-/-}\) mice reconstituted with CD4\(^+\) T cells from IFN-\(\gamma\)^{−/−} showed no increased myocardial infarct size indicating that the cytokine IFN-\(\gamma\) may be an important mediator of IR. They also examined T-cell depletion and its effect on myocardial infarct size. CD4\(^+\) depleted mice,
but not CD8$^+$ depleted mice had a significantly decreased infarct size compared to control mice again implicating CD4$^+$ T cells’ deleterious role in IR injury (Yang et al., 2006).

Intestinal IR injury has been shown to be mediated by T lymphocytes. Shigematsu et al. occluded the superior mesenteric artery of both WT and SCID mice for 45 minutes, followed by 30 or 360 minutes of reperfusion. They examined both extravasation of albumin and T-cell adhesion following reperfusion. In the SCID mice, there was a significant decrease in intestinal leakage of albumin compared to the WT at 30 minutes. Restoration of the WT levels of albumin extravasation was seen in SCID mice reconstituted with T cells from WT mice. T-cell adhesion was significantly increased after 6 hours of reperfusion but was not significantly increased 1 hour after reperfusion. This suggests that T cells can have a pathogenic effect during IR injury even in the absence of adhering to vascular endothelium. Tissue myeloperoxidase activity was also significantly decreased in the SCID mice when compared to WT, suggesting a role for T cell recruitment of neutrophils (Shigematsu et al., 2002). Recruitment of neutrophils early in the reperfusion injury could account for endothelium damage rather than direct cytotoxic effects of the T cells themselves.

Experimental data also have implicated T cells as mediators of hepatic IR. Zwacka et al. BALB/c (WT) demonstrated the pathogenesis of CD4 cells in liver IR injury and athymic (nu/nu) mice were subject to lobar hepatic IR injury. Although there was no significant difference between the 2 groups the acute injury phase (3–6 hours), the nu/nu mice had significantly reduced injury 16–20 hours post ischemia, both serologically and histologically, when compared to the WT mice. In vivo depletion of CD4$^+$ T cells in WT mice also resulted in decreased serologic and histopathologic injury, whereas adoptive transfer of CD4$^+$ T cells into the nu/nu mice restored the injury. In vivo depletion of CD8$^+$ T cells had no effect on the injury pattern seen (Zwacka et al., 1997).

Many studies have been performed demonstrating the pathogenesis of T cells in renal IR injury. Rabb et al. studied renal IR injury in mice deficient of CD4 and CD8 T cells (CD4/CD8$^{-/-}$). At 2 days post IR injury, CD4/CD8$^{-/-}$ mice had significantly improved
renal function, significantly less neutrophil infiltration and markedly decreased tubular atrophy scores compared to WT controls. This suggests a protective effect of T cell depletion on renal IR (Rabb et al., 2000). Burne et al. examined the effects of renal IR injury on T cell–deficient mice (nu/nu), CD4-deficient mice (CD4−/−), and CD8-deficient mice (CD8−/−). These mice were subject to 30 minutes of warm ischemia. Both nu/nu mice and CD4−/− mice had significantly less histopathologic and serologic injury compared to WT mice. CD8−/− mice had similar functional and structural injury patterns as seen in WT mice. Reconstitution of nu/nu mice by adoptive transfer with T cells from WT mice restored the injury phenotype, as did adoptive transfer of isolated CD4+ T cells into the CD4−/− mice. Transferring CD4+ T cells from CD28-deficient mice was not sufficient to restore the injury phenotype in nu/nu mice, nor was transferring CD4+ T cells from IFN-γ–deficient mice into nu/nu mice. This suggests that both costimulation with CD28 and a TH1 milieu of CD4+ T cells are important contributors to renal IR injury (Burne et al., 2001).

Savransky et al. examined the effect of T cell receptor (TCR) depletion on renal IR injury. Knockout αβ-TCR and γδ-TCR mice were subjected to warm IR injury. The αβ-TCR–deficient mice were protected from serologic kidney injury 24 hours after ischemia-reperfusion, as measured by serum creatinine, compared to WT mice. Both αβ-TCR and γδ-TCR–deficient mice had significantly decreased histopathologic renal injury compared to WT mice (Savransky et al., 2006). Similar protective effects of TCR deficiency were found by another group who found both αβ-TCR and γδ-TCR–deficient mice had significantly decreased serologic and histopathologic renal injury compared to WT mice 72 hours following ischemic reperfusion. There was also a significant decrease in infiltrating CD4+ T cells in the TCR-deficient mice compared to WT mice (Hochegger et al., 2007).
3.1.5.5 B-Cells in Ischaemia Reperfusion

B cells play an important role in the adaptive immune response. Once activated, each cell produces and releases thousands of a single specific and unique antibody or B-cell receptor before undergoing cell death. This antibody aids in immune response by identifying foreign objects and facilitating their removal, destruction, or neutralization. Specific epitopes on foreign antigen are bound by antibody. This binding action alone can prevent damaging effects of the foreign particle; alternatively, bound antibodies leads to pathogen removal by macrophages and granulocytes. The antibody antigen complex also triggers direct pathogen destruction by complement pathway activation.

While there has been much effort in discerning the role of the T-cell population in IR injury, currently, there has been little work describing the role of the B cell in IR. While the role of the T cell can be either pathogenic or protective depending on timing of the injury and type of T cell, the role of the B cell has been found to be predominantly pathogenic in all organs systems ranging from the intestine, heart, kidney, and skeletal muscle (Austen et al., 2004, Burne-Taney et al., 2003, Burne-Taney et al., 2006, Busche et al., 2008, Chan et al., 2006b, Weiser et al., 1996, Zhang et al., 2006a, Zhang et al., 2004). Various models have been employed to determine the effects of B cells on IR injury using mice that are deficient in either the B cells or complement components which interact with B-cell receptor. Austen et al. used complement receptor–2 knockout mice (CR2<sup>−/−</sup>) in their IR injury model. CR2<sup>−/−</sup> mice have defective B-1 cells resulting in a deficiency of natural immunoglobulin M (IgM). This deficiency confers protection from IR injury. They found that that muscle injury in the CR2<sup>−/−</sup> mice reconstituted with CM22, a self-reactive IgM secreting B-1 cell clone, had similar injury compared to WT mice reconstituted with saline and compared to CR2<sup>−/−</sup> mice reconstituted with WT serum. The injury to the CR2<sup>−/−</sup> mice reconstituted with CM22 was significantly greater than in CR2<sup>−/−</sup> mice reconstituted with saline and CR2<sup>−/−</sup> mice reconstituted with a
different IgM clone, CM31. The results suggest that CM22 is a mediator of IR and suggests that B cells are pathogenic in IR injury (Austen et al., 2004).

It was subsequently discovered that B-cell isotype IgM mediates IR injury. The IgM isotype is secreted as a pentamer or hexamer. It’s known functions include detection of the A and B antigen on red blood cells and complement activation. The B cell clone, CM22, was found to be the only clone to completely restore intestinal IR injury in RAG 1−/− mice (Zhang et al., 2004). Subsequent work showed CR2-deficient mice had reduced IR injury in the intestine that was easily reversed with prepared IgM (Fleming et al., 2002, Reid et al., 2002). Zhang et al. have shown that natural IgM is implicated in myocardial IR injury also (Zhang et al., 2006b).

Using mice deficient in C3, C4, or IgG, Williams et al. were able to show that the mechanism of action involves the activation of the complement cascade by the classical pathway in the intestine (Williams et al., 1999). Austen et al. showed similar results in the hind limb (Austen et al., 2004). In addition, at least in cardiac tissue and skeletal muscle, the lectin pathway is also implicated in the pathogenesis of IR injury (Busche et al., 2008, Chan et al., 2006a).

In skeletal muscle, the two pathways appear to cause differing pathology. Chan et al. have found that isolated blockade of the classical pathway alone is protective against vascular permeability and remote pulmonary injury but not histologic muscle injury. However, blocking the lectin pathway alone protects against histological injury but not vascular permeability or remote lung injury. Activation of both the classical complement pathway and the MBL pathway are required for the full spectrum of disease associated with IR injury (Chan et al., 2006b).
A free radical is any species capable of independent existence that contains one or more unpaired electrons occupying an atomic orbital by itself. Radicals are therefore formed by the loss or gain of an electron from a non-radical. The presence of an unpaired electron usually makes the species highly reactive, although different free radical species differ greatly in their reactivity. “Redox” or oxidation-reduction reactions are those reactions that involve exchange of electrons between molecular species.

The diatomic “ground state” oxygen molecule has two unpaired electrons, each in a different orbital, and fulfills the definition for a free radical. If this molecule accepts an electron the product is superoxide radical (O$_2^-$). Many of the important free radicals are derived from oxygen. Another important radical is nitric oxide (NO$^\cdot$). Many of these have important intermediates, such as hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^-$), which are not free radicals but which are highly reactive and may be responsible for some of the biologic effects attributed to free radicals. Therefore, the term reactive oxygen species (ROS) is used to encompass these species. Non-radical ROS also include ozone and singlet oxygen.

Sources of free radicals are the mitochondrial electron transport chain, the enzymes xanthine oxidase, NADPH oxidase, lipoxygenase/cyclooxygenase and nitric oxide synthase (NOS), and auto-oxidation of various substances, particularly catecholamines. The tetravalent reduction of molecular O$_2$ by the mitochondrial electron transport chain is necessary for generating biologic energy. To accomplish this there are four mitochondrial complexes (I–IV) involved in energy conservation. The reduction is not 100% efficient, however, and 1%–4% of available oxygen is normally incompletely reduced and leaks from the electron transport chain in the form of O$_2^-$. O$_2^-$ is produced at complex I and complex III. This process becomes greatly accelerated at supranormal O$_2$ tensions or after mitochondrial injury and is believed to be a primary source of ROS during ischemia and reperfusion. Cellular hypoxia decreases the activity of complex IV (cytochrome oxidase);
when O₂ is reintroduced, leakage of free radicals from more proximal complexes is greatly accelerated (Kevin et al., 2005).

Xanthine oxidase is a major source of ROS after reperfusion of ischemic tissue in several organs (Terada et al., 1992, Brown et al., 1988). During ischemia, purine precursors are degraded to the nucleotide derivatives hypoxanthine and xanthine and the enzyme xanthine dehydrogenase is converted to xanthine oxidase. Xanthine oxidase catalyses oxidation of hypoxanthine and xanthine to uric acid while reducing O₂ to O₂⁻ and H₂O₂.

Phagocytic cells generate O₂⁻ from O₂ when these cells are activated. This is the so-called “respiratory burst,” which is catalysed by the enzyme NADPH oxidase. ROS generated from this source play a central role in host defence. Neutrophils also provide a major source of free radicals during and after IR injury (Kawahito et al., 2000).

There are endogenous antioxidant systems that counteract the potential for injury to cellular structures by regulating the balance of individual ROS and their reactants. These endogenous antioxidants are up regulated when exposure of the cell to ROS is increased; thus the term “redox homeostasis” has been coined. However, under pathologic conditions such as ischemia-reperfusion, ROS formation can rapidly overcome antioxidant defences and cellular injury ensues.

Major endogenous antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione, Coenzyme Q10 (ubiquinone), and vitamins C and E (Figure 35). SOD exists in 3 isoforms: MnSOD is within mitochondria, CuZnSOD is in the cytoplasm, and extracellular SOD is located extracellularly associated with glycosaminoglycans. During IR endogenous antioxidant systems become activated to high levels in response to increased free radicals; such is the ROS production, however, that these rapidly become depleted so that IR leads to oxidant damage (McColl et al., 1998).

Free radicals exert their biologic effects by obtaining an electron from any molecule that may yield it, including lipids, proteins, and DNA. When the generation of ROS is
excessive, like in IR, the cell cannot inactivate the free radicals produced, and cell injury occurs. This injury ranges from minimal to cell death. Three important pathological molecular responses develop as a consequence of the overwhelming and unopposed effect of ROS: 1. Lipid peroxidation of membranes, 2. Nuclear and mitochondrial DNA fragmentation, and 3. Sulfhydryl-mediated protein cross-linkage. All these events directly contribute to cell injury, the degree of which will depend on the time and intensity of the IR insult (Jassem and Heaton, 2004). Lipid peroxidation causes particularly destructive effects on cell membranes. Modification of protein structure on attack by free radicals or their reactive products can alter function or enhance susceptibility to proteolysis. Proteins that are misfolded or partially unfolded are most sensitive to oxidation; thus, oxidative denaturation of dysfunctional proteins is believed to serve a physiologic housekeeping function. At the same time, consumption of ROS in this process aids in cellular antioxidation (i.e., scavenging). ROS are strongly implicated in the pathogenesis of post ischemic myocardial stunning, necrosis, apoptosis, and vascular dysfunction (Kloner et al., 1994). Cell death after IR results from necrosis and apoptosis. ROS have been shown to directly induce apoptosis after IR injury (Maulik et al., 1998).
Figure 35. The major sources of superoxide radicals (xanthine oxidase system and nicotinamide adenine dinucleotide phosphate system) and the fate of superoxide.
Administration of exogenous antioxidants has been extensively investigated as a means to attenuate IR injury. Investigations in a variety of animal models have shown beneficial effects of several drugs. However, clinical trials have furnished inconsistent results. SOD decreases infarct size in animal models when given during ischemia and reperfusion (Kilgore et al., 1994), or in animals given a gene transfer for SOD (Li et al., 2001). Human trials have been disappointing. One small clinical trial involving 23 patients reported protective effects of SOD against premature ventricular contractions after thrombolysis (Murohara et al., 1991), but a larger trial involving 120 patients undergoing angioplasty in the setting of acute infarction found no such change, nor was there any improvement in global function, i.e., SOD did not attenuate stunning (Flaherty et al., 1994).

Paracetamol is a phenol and, like many other phenols, it has antioxidant properties. The studies of Merrill et al. demonstrated protective effects of paracetamol in animal models (Merrill et al., 2001). Paracetamol attenuated production of OH radical in the post ischemic myocardium and improved post ischemic recovery in guinea pig hearts.
3.2 MATERIAL AND METHODS
3.2.1 Animal Care Protocol

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Massachusetts General Hospital and followed all the policies outlined in the NIH Guide for the Care and Use of Laboratory Animals. Following surgery, as described below, mice were returned to their cages, allowed water and chow ad libitum, kept on a 12 hour light/dark cycle, and room temperature was between 20-22°C. On the seventh postoperative day, the mice were given an overdose of pentobarbital (200 mg/kg IP).

3.2.2 Dorsal Skin Flap

Mice were anesthetized by intraperitoneal administration of sodium pentobarbital 60 to 90 mg/kg. Additional doses were given as necessary during surgery. During the surgical procedure animals were placed on a heating pad to maintain the body temperature at 37°C. The dorsum of each animal was shaved with an electric animal shaver and residual hair was removed thoroughly with depilatory cream. The surgical area was cleansed and treated with povidone-iodine for disinfection.

Dorsal lateral thoracic artery pedicled island skin flaps measuring 3.5 by 1.5 centimetres were raised on the right dorsolateral area of the mice. The medial border of the flap was on the midline of dorsum, the lateral border was on the right axillary line, and the cranial and caudal borders were on the infrascapular line and the iliac crest, respectively (Figure 36). The flap contains skin, subcutaneous tissue, panniculus carnosus and superficial fascia. After flap elevation, a medical grade silicon sheet (Bioplexus, Ventura, CA) was placed on the muscle bed as a barrier to vascular invasion and sutured in place with 7-0 polypropylene. Placing a non-crushing 7mm microvascular clamp on the vascular pedicle
produced flap ischemia (Figure 37). Interrupted sutures were used at the cranial borders of the flaps in order to provide easy clamp removal. Mice were permitted to recover from anaesthesia during the ischemia time and were kept in cages with free access to food and water. At the end of 10 hours of ischemia, clamps were taken out by removing the interrupted sutures on cranial border of the flap and then re-sutured. On the seventh postoperative day, the mice were given an overdose of pentobarbital (200 mg/kg IP), following flap viability assessments. Flourescin perfusion studies were used to validate the ischemia produced by this method as well as allowing visualization of flap reperfusion following release of the clamp.
Figure 36. The dorsal skin flap marked out after depilation. The medial border of the flap is midline of dorsum, the lateral border is right axillary line, and the cranial and caudal borders are on the infrascapular line and the iliac crest, respectively.
Figure 37. The flap is raised on the dorsal lateral thoracic vascular pedicle. Flap ischemia is achieved by placing a non-crushing 7mm microvascular clamp across both artery and vein.
Figure 38. Intravenous flourescin is seen throughout the mouse skin except for the skin flap with vascular clamp in situ, confirming flap ischemia.
Figure 39. Within minutes of clamp release flourescin is seen filling the vascular tree of the flap, confirming reperfusion.
Figure 40. Within 30 minutes of clamp release the entire flap is filled with flourescin, confirming complete flap reperfusion.
3.2.3 Skin Flap Viability

Flap viability was assessed by drawing the viable and nonviable areas of each flap on transparent paper. The border between viable and non-viable flap areas was determined by visual inspection based on dark colour, eschar formation, and lack of capillary refill. The areas were calculated using the ImageJ program and the necrotic area was expressed as a percentage of total flap area (Figure 41). One evaluator who was blinded as to the treatment groups carried out all evaluations.
Figure 41. Flap viability assessment is carried out at Day 7 following IR injury. The non-viable area is marked within the heavy black line. This area is expressed as a percentage of the total flap area.
3.2.4 Flap Pre-Conditioning

Twenty-seven (22-25g) C57BL/6 mice were used in this experiment. Mice were separated into 3 groups:

- **Group I**: Sham Group (Without Ischemia) (n=7)
- **Group II**: Saline Control Group (Saline inj.+10 hrs. Ischemia) (n=9)
- **Group III**: Preconditioning Group (2 x cycles of 20 min Ischemia followed by 20 min Reperfusion prior to 10 hrs. Ischemia) (n=11)

In Group I, flaps were repositioned and sutured with a continuous 6-0 polypropylene suture and no other procedure was performed. Group II had a flap elevated as before, but were subjected to ten hours of clamp ischemia. Thirty minutes prior to flap elevation and clamp ischemia, 0.25 cc saline was administered via tail vein injection. Group III underwent two cycles of twenty minutes of ischemia produced by pedicle clamping followed by twenty minutes of reperfusion prior to the clamp being placed for the 10-hour ischemia time.

3.2.5 P8 Peptide Administration

Forty-one (22-25g) C57BL/6 mice were used in this experiment. Mice were separated into four groups.

- **Group I**: Sham Group (Without Ischemia) (n=7)
- **Group II**: Saline Control Group (Saline inj.+10 hrs. Ischemia) (n=9)
- **Group III**: CM22 Binding Peptide Group (P8 inj.+10 hrs. Ischemia) (n=18)
- **Group IV**: Control Peptide Group (Control Peptide inj.+10 hrs. Ischemia) (n=7)
As before Group I, flaps were repositioned and sutured with a continuous 6-0 polypropylene suture and no other procedure was performed. Group II had a flap elevated and were subjected to ten hours of clamp ischemia. Thirty minutes prior to flap elevation and clamp ischemia, 0.25 cc saline was administered via tail vein injection. Group III were given 25µg/0.25 cc peptide P8 (NGNNVNGNRNNN) (Biopeptide Co., San Diego, CA) prior to clamp removal, and Group IV were given 25µg/0.25 cc random 12-mer peptide (LDKNKDPLNETV).

3.2.6 SKIN FLAP IMMUNOHISTOCHEMISTRY

16 C57BL/6 mice were subjected to injury with immunohistochemical analysis of the flaps after 3 hours of reperfusion. They were divided in to the four groups:

Group I: Control Group (Just 10h Ischemia) (n=4)
Group II: Saline Control Group (Saline inj.+10 hrs. Ischemia) (n=4)
Group III: CM22 Binding Peptide Group (P8 inj.+10 hrs. Ischemia) (n=4)
Group IV: Control Peptide Group (Control Peptide inj.+10 hrs. Ischemia) (n=4)

Treatments and surgical procedures were applied as described above. After 10 hours ischemia and 3 hours reperfusion, flaps were harvested. Flaps were embedded in paraffin and cut in cross-section followed by deparaffinization. Samples were stained with either anti-IgM or anti-C3 antibodies.

To assess IgM deposition, sections were blocked for twenty minutes and then incubated in a 1:100 dilution of biotinylated goat anti-mouse IgM (Jackson Labs, Bar Harbor, ME) for 30 minutes at 25°C. Sections were developed with Vectorstain ABC (Vector labs, Burlingame, CA).
To assess C3 deposition, we used a 1:100 dilution of goat anti-mouse C3 (MP Biomedicals, Solon, Ohio) for 30 minutes at 25°C. Biotinylated rabbit anti-goat IgG (Sternberger Monoclonals, Lutherville, MD) was used at a 1:100 dilution and sections were incubated for 30 minutes.

After washing sections were reacted with avidin/streptavidin conjugated to horseradish peroxidase for 30 minutes and then developed with diaminobenzidine.

### 3.2.7 Statistical Analysis

Statistical analysis was performed with Instat (GraphPad, San Diego, CA). Data were expressed as means +/- SEM. Comparisons were made using ANOVA and Student’s t-test. A P value <0.05 was considered significant. Post-hoc comparison was made using Tukey Kramer parametric analysis.
3.3 RESULTS
3.3.1 **Statistics**

Data in these experiments were deemed “normal” following Kruskal-Wallis testing and therefore parametric tests were used throughout. All groups were compared using analysis of variance (ANOVA) and Tukey’s multiple comparison tests. No corrections were made for multiple testing. All data is represented as mean value and standard error of the mean. Significance was taken at P < 0.05.

3.3.2 **Effect of Pre-Conditioning on Flap Necrosis**

The mean percentage of necrosis and the standard error of the means for each group are shown in Figure 42 & Figure 43. The percentage of flap necrosis was 1.11 ± 1.11% (SEM) in Group I, the sham surgery group where flaps that were not rendered ischemic. Group II, animals with flaps subjected to 10 hours of ischemia, developed 33.36 ± 4.04% necrosis by the end of the 7 days of observation. Group III animals which underwent two cycles of ischemic preconditioning, had 18.82% ± 1.86% necrosis.

ANOVA showed statistically significant differences among all groups (p<0.001). With pair wise comparisons there were significant differences between the animals treated with preconditioning and the other groups.
Figure 42. Bar-graph demonstrating % necrosis of murine skin flaps after 7 days. Group III subjected to 10 hours of ischemia following preconditioning with two twenty-minute cycles of intermittent ischemia and reperfusion. Group I flaps were not clamped. Group II treated with IV saline. Results expressed as mean +/- SEM. P<0.001 for comparison of Group III to all controls.
Figure 43. Statistical analysis of results of flap ischemia experiments with Tukey’s Multiple Comparison Test.
3.3.3 Effect of P8 IgM Blocking Peptide on Flap Necrosis

The mean percentage of necrosis and the standard error of the means for each group are shown in Figure 44 & Figure 45. The percentage of flap necrosis was $1.11 \pm 1.11\%$ (SEM) in Group I, animals with flaps that were not rendered ischemic. Group II, animals with flaps subjected to 10 hours of ischemia, developed $25.78 \pm 4.26\%$ necrosis by the end of the 7 days of observation. Group IV, animals with ischemic flaps that were treated with P8 peptide had $14.61 \pm 2.77\%$ necrosis. This represents a 43% reduction in necrosis compared to animals treated with saline. Animals treated with random peptide had $42.05 \pm 4.51\%$ flap necrosis.

ANOVA showed statistically significant differences among all groups ($p\leq 0.001$). With pair wise comparisons, there was no significant difference between the P8 group and the sham group, which did not undergo ischemia. There were significant differences between the animals treated with P8 and the other ischemia groups (ischemia + saline & ischemia + random peptide). There were no significant differences with two by two comparisons between the ischemia + saline, and the random peptide groups.
Figure 44. Bar-graph demonstrating % necrosis of murine skin flaps after 7 days. Group I flaps were not clamped. Group II treated with IV saline. Group III treated with a random peptide and Group IV administered the CM22 binding P8 peptide. Results expressed as mean +/- SEM. P<0.001 for comparison of Group IV to all controls.
Figure 45. Statistical analysis of results of flap ischemia experiments with Tukey’s Multiple Comparison Test.
3.3.4 Immunohistochemistry

In the 10 hours ischemia and 3 hours reperfusion experiment, there was IgM and C3 deposition on the endothelium of medium-sized vessels of the pedicled flaps. Untreated, saline treated and random peptide treated groups had qualitatively similar amounts of deposition. P8 treated animals exhibit a significant qualitative decrease in IgM and C3 deposition (Figure 46 & Figure 47).
Figure 46. 200X photomicrographs of IgM deposition on medium vessels of murine skin flaps after 10 hours ischemia and 3 hours of reperfusion, as assessed by anti-IgM immunoperoxidase reaction. Panel A: Group II, no pre-treatment. Panel B: Group III, pre-treatment with IV saline. Panel C: Group IV, pre-treatment with IV P8 peptide. Panel D: Group V, pre-treatment with IV random peptide. P8 treated animals showed little or no IgM deposition compared to Groups II, III, or V.
Figure 47. 200X photomicrographs of C3 deposition on medium vessels of murine skin flaps after 10 hours ischemia and 3 hours of reperfusion, as assessed by anti-C3 immunoperoxidase reaction. Panel A: Group II, no pre-treatment. Panel B: Group III, pre-treatment with I.V. saline. Panel C: Group IV, pre-treatment with I.V. P8 peptide. Panel D: Group V, pre-treatment with I.V. random peptide. P8 treated animals showed little or no C3 deposition compared to Groups II, III, or V.
3.4 DISCUSSION

We developed a model of a composite pedicle flap on the dorsum of the common inbred C57BL/6 mouse (Tatlidide et al., 2009). When the pedicle is clamped with a microclip for 10 hours and then observed for a week, 30% of the flap is lost to necrosis (Figure 41). This situation simulates loss of free flap tissue perfusion due to microvascular thrombosis, followed by restoration of flow from revision of the anastomosis. Treatment of animals with IV saline or with a random 12 amino acid peptide did not improve the tissue loss (Figure 42). However, treatment of animals with the 12 amino acid P8 peptide significantly reduced the tissue loss (Figure 42). As this peptide was designed to compete with natural injury antigens for the binding site on pathogenic antibodies that cause diverse reperfusion injuries, these data suggests that the loss of tissue in this situation is due to reperfusion injury and is potentially reversible with appropriate treatment. The hallmark of reperfusion injury is the significant amplification of injury by deposition of IgM, with resultant complement co-deposition and damage. Examination of tissue sections reveal deposition of IgM (Figure 46) and complement C3 (Figure 47) in the same flap vessels, a process that is reversed by P8 peptide. These data confirm that P8 is acting through its anticipated mechanism and, therefore, that the prevention of tissue loss is a result of preventing reperfusion injury in the flap.

Tissue injury during reperfusion after prolonged periods of ischemia is the result of multiple factors. One of the earliest events involved in the process of IR injury is the binding of circulating natural antibody (IgM), and the subsequent activation of complement. Previous findings in hind limb, intestinal, myocardial, and burn models have found that injury is mediated by a local effect of specific natural IgM with subsequent complement activation and that this effect can be mitigated by intravenous pre-treatment with a 12-mer peptide (P8) (Boes et al., 2000, Zhang et al., 2006a, Suber et al., 2007). The results of our study demonstrate that blocking the IgM and complement cascade in pedicled skin flaps in mice improves tissue survival.
Attempts to inhibit complement activation during IR injury have targeted the classical pathway (C1 esterase inhibitor and C1 antagonists), alternative pathway (C3 and Factor B), the lectin pathway (MBL antibodies), and all three pathways (sCR1 and anti-C5/C5a) (Siemionow and Arslan, 2004). It has been suggested that classical pathway activation is an antibody-independent process and that intracellular moieties exposed by ischemia may bind C1q directly (Kagiyama et al., 1989, Rossen et al., 1988). Natural IgM has however been shown to be an essential factor in complement activation; mice deficient in IgM were protected from intestinal IR injury, the same animals reconstituted with pooled wild-type IgM had restoration of injury (Williams et al., 1999).

This discovery has lead to the hypothesis that IR can be activated by recognition and binding of pre-existing natural IgM to neoantigen expressed by hypoxic cells (Weiser et al., 1996, Williams et al., 1999). Natural IgM is a first line of defence against foreign pathogens and is a component of the innate immune system (Boes et al., 2000). B-1 cells are the major source of this pre-existing or natural IgM. These cells are found mainly within the peritoneal and pleural cavities of humans, mice, and other animals (Kantor and Herzenberg, 1993). Recent studies using Cr2-/- mice that are deficient in both complement receptors 1 and 2, which are important for B1 cell development, have shown these mice to have a markedly decreased IR injury. These mice have normal serum concentrations of IgM, however they have markedly reduced levels of natural IgM specificities hence the decreased injury seen in intestinal IR models. This protection is eliminated by intravenous reconstitution of IgM from normal mice or by engraftment of knockouts with peritoneal cells from isogenic normal mice (Fleming et al., 2002, Reid et al., 2002).

A similar mechanism of IR injury has been demonstrated in the rat (Padilla et al., 2007), supporting the hypothesis that the role of natural IgM in IR injury is highly conserved and is similar across mammalian species. There is limited, but increasing, evidence to suggest that a similar innate immunological response is responsible for IR injury in humans. Recently Zhang et al. have shown that human natural IgM can induce IR injury in a murine intestinal model (Zhang et al., 2008). It has also been shown that IgM along
with complement and C-reactive protein is deposited in infacted human myocardium following acute myocardial infarction (Krijnen et al., 2005).

In 2004 Zhang et al. identified a specific natural antibody (IgM CM-22) that initiates intestinal and skeletal muscle reperfusion injury in antibody-deficient mice. This discovery made it possible to examine the initiation of acute IR injury and to isolate and characterize the self-antigen involved (Zhang et al., 2006a). It is likely that the pathogenic property of this IgM is inherent to its binding site; therefore, it is likely that there is a specific antigen(s) on the cells expressing the injury phenotype to which the IgM binds (Chan et al., 2006b). This led to the identification of a highly conserved region within nonmuscle myosin heavy chain type II (NMHC-II) A and C as the target for natural IgM and initiator of reperfusion injury in murine skeletal and intestinal models (Bresnick, 1999).

These findings by Bresnick suggest that IR injury is initiated at the endothelium surface, as is found in the intestinal model, and that NMHC-II is the major self-ligand in ischemic skeletal muscle and that the NMHC-II epitope is mobilized to the cell surface by hypoxia-related events. Although these experiments suggest that NMHC-II is the major antigen exposed during reversible reperfusion injury, other epitopes are likely exposed on the surface of injured endothelium, but in the absence of specific natural IgM are insufficient to mediate inflammation (Zhang et al., 2006b).

Zhang et al. isolated synthetic peptides to bind pathogenic IgM CM-22 and block reperfusion injury in wild type mice. They accomplished this using a M13 phage display library consisting of random 12-mer amino acid sequences, which were screened with the specific IgM. 10 phage clones were isolated. One of these clones, P8 bound with the highest affinity. All three isoforms of NMHC-II include a motif that shows similarity with the P8 sequence. These results suggest that IgM CM-22 binding to phage P8 is specific for the peptide region and that the synthetic peptide could be used as a mimetope for the actual antigen (Zhang et al., 2006a).
Interestingly, Zhang et al. found no exact matches when the amino acid sequence of the
12-mer peptide from the P8 clone was compared to that of all known mammalian protein
sequences. P8 is however homologous to a number of protozoan antigens. The
specificity of the interaction of P8 with IgM in the prevention of reperfusion injury was
reinforced when random peptides of similar size did not exhibit this protection (Chan et
al., 2006b).

Although P8 was shown to be effective at lower concentrations in intestine (Zhang et al.,
2006a), Chan et al. used twenty micrograms of peptide in order to block skeletal muscle
ischemia reperfusion (Chan et al., 2006b). Because blood supply to the skin is not as
abundant as to skeletal muscle and the flap which we raised would contain a significantly
lower proportion of circulating blood than hind limb, we chose to use 25 micrograms of
these 12-mer peptides in our study.

It has been shown that ischemia alone is not sufficient to produce visible histological
muscle injury. Structural evidence of muscle fibre damage can be seen however after
only a short period of reperfusion. IgM deposition has been demonstrated as early as five
to ten minutes after ischemia and continued throughout reperfusion. Complement C3
deposition is not seen during the ischemic period but increases dramatically once
reperfusion begins (Zhang et al., 2004).

Because IgM deposition starts during ischemia and prior to reperfusion, the timing of
peptide administration is imperative. For attenuation of reperfusion injury to skeletal
muscle, Chan et al. used P8 intravenously 30 minutes prior to ischemia (Chan et al.,
2006b). Similarly in an intestinal model Zhang et al. pre-treated wild type mice with P8
five minutes before reperfusion to test whether this peptide represented a mimetope for a
major self-antigen. This pre-treatment before reperfusion blocked any apparent injury
(Zhang et al., 2006a). We chose pre-treatment 30 minutes before ischemia similar to
Chan et al. As this peptide is being administered in a preventative capacity prior to any
injury occurring it mirrors the elective surgical experience rather than that of traumatic IR injury.

In this study, skin flaps were analysed by immunohistochemistry with antibodies to IgM and C3. P8 treated animals exhibit a significant qualitative decrease in IgM and C3 deposition compared to all other groups. These results are in accordance with the findings of Chan et al. who showed P8 treated skeletal muscle did not show any IgM or C3 deposition after reperfusion (Chan et al., 2006b) and with those of Zhang et al. who determined that P8 treated animals did not show IgM, C3 or C4 deposition in the microvilli in an intestinal IR model (Zhang et al., 2006a). As demonstrated in other investigations (Chan et al., 2006b, Weiser et al., 1996), our study showed that IgM and C3 deposition is seen mainly on the endothelium. This was shown in all groups except the flaps treated with P8.

Although it is reported that during ischemia, exposure of other forms of myosin heavy chain (such as smooth muscle) is possible (Zhang et al., 2006a), we could not detect any IgM or C3 deposition on the panniculus carnosus in this study. Despite the absence of the panniculus carnosus layer in human skin the lack of IgM or C3 deposition seen in these experiments lead us to feel that this model closely correlates with IR injury as seen in human skin flaps.

Chan et al. reported a 41% reduction in injury with P8 treatment compared to the random peptide in a skeletal muscle model of IR injury (Chan et al., 2006b). In our study, there was a 65 % reduction in necrosis compared to the control peptide group and a 56% reduction in necrosis compared to the saline control group. In comparison with previously published hind limb and intestinal models, our skin flap model showed a higher degree of protection with the P8 peptide (Chan et al., 2006b, Zhang et al., 2006a). This apparent increased efficacy of P8 in preventing IR injury in skin compared to hind limb and intestine may reflect an increased susceptibility to IgM mediated IR injury in skin flaps.
Ischemic preconditioning is a simple, protective phenomenon that mitigates IR injury in experimental models. Brief periods of ischemia followed by brief periods of reperfusion have been shown to condition tissues to prolonged periods of ischemic insult and result in smaller areas of necrosis. The gold standard for assessing the effect of preconditioning in the heart model is the measurement of infarct size. Preconditioning does not entirely prevent the IR injury, but it does cause a significant reduction in the size of the infarct (Tsai et al., 2004). The results of our study, similar to the heart studies, showed that preconditioning the flaps reduced the level of ischemia-related injury by 43%, but did not eliminate the infarct altogether.

Different numbers of IR cycles and different duration of the ischemia and reperfusion periods have been used for effective preconditioning in various tissues. Murry et al. found that four sequential 5-min ischemia and reperfusion cycles reduced the infarct size in canine heart by 25% caused by 40 min of coronary artery occlusion compared with nonconditioned ischemic controls (Murry, 1986). Using a rat musculocutaneous flap model, Zahir et al. have shown that three 10-min cycles of ischemic preconditioning are superior to 5-min cycles, but they also suggested that longer than 10-min cycles may also be effective and should be studied (Zahir et al., 1998a). More recently, Coskunfirat et al. showed efficacy for reducing injury in rat epigastric skin flap with two 15-min preconditioning cycles (Coskunfirat et al., 2006). In this experiment, we used the regimen of two 20-min cycles of preconditioning that we have shown to be an effective regimen in mice hind limb models (Eberlin et al., 2009).

3.5 CONCLUSION

These results lead us to conclude that blockage of a specific IgM can protect against IR injury in an axial skin flap. This type of protection could be useful in improving results in free flap transfer and composite tissue transplantation.
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