Investigation into the role of extracellular histones in the process of cardiomyocyte death during ischaemia-reperfusion injury of the heart

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Acknowledgements

I begin in the name of Allah, the Most Gracious, the Most Merciful.

I would like to thank my supervisors, Professor Derek Yellon and Professor Sean Davidson, for their guidance, advice and patience throughout my PhD research.

I would also like to thank my mother and father for their countless sacrifices, and my family and friends for their support. I would especially like to thank my beloved sister Ettrut. You have always been my biggest fan and a constant source of love and encouragement throughout my life.

Finally, I dedicate this thesis and my career to the memory of my beloved late grandfather:

Syed Mastan Shah.

Thank you for your vision, strength of character, and moral compass. If I have achieved anything, it is because you are the giant upon whose shoulders I stood.

Mohammed Karar-Haider Hussain Shah

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Signed Declaration

I, Mohammed Shah confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.' All technical assistance relevant to the results presented herein is duly acknowledged.

Signed:

11/05/22

Mohammed Karar-Haider Hussain Shah

Impact Statement

This work has shown that histones, which are part of intracellular DNA, are released from rat cardiomyocytes during ischemia-reperfusion (IR) injury of the myocardium. A significant amount of histone H4 is released (10ug/ml – 50ug/ml) from the rat myocardium during IR injury. We also demonstrated a positive correlation between the amount of histone released and the actual size of the infarct on histological analysis.

When we exposed rat cardiomyocytes to histones, the cells died very quickly. We can protect these rat cardiomyocytes from the deadly actions of histones by using both the generic histone neutralising drug called mCBS and the histone H4 specific neutralising drug called HIPe. The degree of protection of the cell is the same when we target either all histones or just histone H4 suggesting that histone H4 is the major culprit of histone induced cardiomyocyte cytotoxicity.

Using a Langendorff ex-vivo Rat coronary artery occlusion model we were able to demonstrate that mCBS not only neutralised circulation extracellular histone during ischemia reperfusion injury of the heart but also reduced the size of the infarct.

It was previously thought that histones cause their cytotoxic effects by activating the innate immune system through Toll Like Receptors. By activating Toll Like Receptors it was thought that intracellular formation of the Caspase dependent inflammasome was a crucial step in its cytotoxic effect. Our mechanistic experiments demonstrate that the cytotoxicity occurs in a Toll Like Receptor independent process, this was supported by our collaborators Prof Solnheims team in a Nature publication.

We were also able to demonstrate HIPe a novel protein specifically developed to target histone H4 is cardioprotective to isolated cardiomyocytes exposed to toxic concentrations of histone.

Finally the results of our discovery and mechanistic understanding of histones allowed the team at the Hatter Cardiovascular to demonstrate in a live rat cardiac ischemia-reperfusion model that HIPe is cardioprotective and reduces infarction size after Myocardial Infarction. All our findings have been published in peer reviewed publications.

Myocardial infarction is a major cause of death and morbidity worldwide.
We have identified a novel target and successfully used a novel pharmacological agent in a rat which reduced the size of the myocardial infarct after myocardial infarction. The size of the infarction directly correlates with the degree of mortality and morbidity after a Heart Attack. Identification of therapies that protect cardiomyocytes from the cytotoxic effects of myocardial infarction, may potentially significantly change the treatments for myocardial infarctions and help us treat this devastating illness better in the future. The next stage of this experimental work will be to try and replicate these findings in a human model.

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Abstract

Acute myocardial infarction causes lethal cardiomyocyte injury during ischaemia and reperfusion (I/R). Histones have been described as important Danger Associated Molecular Proteins (DAMPs) in sepsis. The objective of this research was to establish whether extracellular histone release contributes to myocardial infarction. Isolated, perfused rat hearts were subject to I/R. Nucleosomes and histone-H4 release was detected early during reperfusion. Sodium-b-O-Methyl cellobioside sulfate (mCBS), a newly developed histone-neutralizing compound, significantly reduced infarct size whilst also reducing the detectable levels of histones. Histones were directly toxic to primary adult rat cardiomyocytes in vitro. This was prevented by mCBS or HIPe, a recently described, histone-H4 neutralizing peptide, but not by an inhibitor of TLR4, a receptor previously reported to be involved in DAMP-mediated cytotoxicity. In an isolated rat cardiomyocyte model, HIPe significantly protected cardiomyocytes from the cytoxic effects of Histones. Histones released from the myocardium are cytotoxic to cardiomyocytes, via a TLR4-independent mechanism. The targeting of extracellular histones provides a novel opportunity to limit cardiomyocyte death during I/R injury of the myocardium.

Investigation into the role of extracellular histones in the process of cardiomyocyte death during ischaemia - reperfusion injury of the heart

Despite our increasing understanding of the pathogenesis of myocardial infarction, it remains a leading cause of premature death in the Western world (1, 2). The widespread adoption of percutaneous coronary intervention has resulted in a significant reduction in the duration of coronary ischaemia (2) once the clinical diagnosis of coronary artery occlusion and ST elevation myocardial infarction is made. Early restoration of blood flow to the myocardium can prevent excessive myocyte death. Conversely, delaying treatment is associated with worse outcomes and death (3). As a consequence of this success in reducing death early following STEMI, there has been a corresponding increase in the incidence of heart failure (4, 5). Paradoxically, both ischaemia and the subsequent reperfusion cause cardiomyocyte death, which results in profound “remodelling” of the heart, altering the cardiac geometry of the ventricle as well as the cellular and molecular biology of the myocardium (6)

In order to help preserve cardiomyocytes after ischaemia- reperfusion injury, attention has turned to understanding the process of myocyte death and inflammation after myocardial reperfusion (7) There is increasing evidence that ischaemia-reperfusion-induced inflammation may actually be contributing to myocyte death, excessive scar formation, and poor ventricular remodelling. Unfortunately, the results of the majority of clinical trials into the use of anti-inflammatory therapies

have been disappointing, illustrating our lack of understanding of the molecular events that occur as a consequence of ischaemia-reperfusion-induced inflammation in the myocardium. One neglected target is the process by which cardiomyocytes, which are viable at the point of reperfusion, die during reperfusion. This type of cell death is unlike phagocytosis or apoptosis, but is more chaotic and results in the ruptured cell spewing its intracellular contents into the extracellular matrix (8-10). In so doing, the dying cells propagate the inflammatory response through the reperfusion zone, as the intracellular contents act as danger-associated molecular patterns (DAMPs), which are ligands for activation of the innate immune system (11). This type of cell death has been labelled pyroptosis, and attempts to identify and target these DAMPs to save myocytes from the deleterious effects of reperfusion have been mixed(11). This is often because many identified DAMPs, such as HMGB1, have complex roles in both inflammation and the healing of the damaged myocardium (12). Extracellular macromolecular DNA and extracellular histones have recently been identified as DAMPs, which have the benefit of not possessing any complex or pleiotropic effects. There are a number of pharmacological agents that can degrade or neutralise extracellular DNA and histones, which provide a potential route to target this DAMP and attenuate the deleterious effects of ischaemia/reperfusion injury. However, there is a lack of consensus on whether this strategy has the potential to be translated into a viable treatment in the clinical setting.

The role of inflammation during MI

Once a coronary artery is occluded, if reoxygenation is not restored promptly, the resulting ischaemic conditions lead to subsequent necrotic (oncotic) and dying tissue, generating DAMPs or alarmins. Some examples of DAMPs include high mobility group box-1 (HMGB1), heat shock proteins, adenosine, extracellular RNA, matrix fragments, and interleukin (IL)-1α, all of which can stimulate the innate immune response, initiating a post-infarction inflammatory process. The subsequent inflammatory response removes dead or dying tissue and initiates the process of tissue repair involving a complex interplay of leukocytes, interleukins, and extracellular signalling cascades. A crucial component of the innate immune response is leukocyte infiltration into the necrotic myocardium. Leukocytes, the vast majority of which originate from within the blood or lymphatic tissue, play an important role during the process of myocardial infarction and repair (13-15). Neutrophils, the most abundant leukocyte, infiltrate the injured myocardium early during ischaemia, appearing within 12-24 hours (16, 17). After infiltrating the infarcted myocardium, they cause (18, 19) proteolysis, generation of oxygen-free radicals(20)the release of pro-inflammatory cytokines and stimulation of the complement cascade(21). The resulting immune cascade is beyond the scope of this review; however, its complexity and the presence of multifunctioning mediators is undoubtedly why an effective anti-inflammatory therapy has not yet been developed.

Clinical trials to target inflammation during MI

There have been many attempts to target the immune response during and after an MI; however, the results of the majority of clinical trials have been disappointing. Earlier attempts were focused on using corticosteroids, an idea born out of animal studies showing that they conferred protection upon cardiomyocytes during ischaemia(22). However, despite this promising theory and clinical trials into the use of corticosteroids, a meta-analysis of randomised control trials demonstrated there is no significant benefit or any detrimental effect with the use of corticosteroids after a myocardial infarct(23) A huge body of animal studies has given further tantalising hope of identifying new treatment targets in preventing the inflammatory damage in ischaemia-reperfusion injury. Targeting integrin signalling with the use of antibodies to CD 18 has consistently, in a multitude of animal models, demonstrated a reduction of infarct size after ischaemia -reperfusion. Chemokines such as CCR5(24) and SDF(25) , inflammasomes and TGF-β(26) are some of the other targets that have demonstrated some potential in animal models of ischaemia-reperfusion injury.

In humans, clinical trials have been conducted in myocardial ischaemia in the absence of a defined reperfusion injury. This classically manifests as a non-ST elevation myocardial infarction. Although there is a spectrum of pathophysiological overlap between STEMIs and NSTEMIs, the former is often managed with prompt mechanical revascularisation, which creates a definitive timepoint where the injured myocardium transitions from ischaemia to reperfusion. Unfortunately, there is little clinical data on the use of immunomodulatory therapy in the context of STEMIs. Inclacumab, a P-selectin inhibitor that prevents the binding of leukocyte surface

adhesion molecules such as P-selectin (27-29), was used in a randomised trial of 544 NSTEMI patients; however, the inconclusive results only showed a trend towards a reduction in troponin in the treatment arm (30). The HALT-MI study investigated the inhibition of the CD11/CD18 integrin receptor on leukocytes. 420 patients with an acute MI were enrolled; however, treatment with the antibody did not reduce infarct size (31). Tocilizumab, the anti-IL6 receptor antibody, reduced levels of high sensitivity troponin during in-hospital stay in 117 NSTEMI patients (32). Over a decade ago, the APEX-AMI trial examined Pexelizumab, a humanised monoclonal antibody that binds to the C5 component of the complement cascade during PCI after myocardial infarction. A total of 5,745 patients were recruited; however, there was no perceived benefit in the treatment arm(33).

The COLCOT Trial (Colchicine cardiovascular outcomes trial) was randomized placebo-controlled trial were 4661 patients who suffered a myocardial infarction within 30 days were randomly assigned to treatment with a placebo or colchicine (34). Colchicine is a potent anti-inflammatory medication, its mechanism of action is through the inhibition of tubulin polymerization leading to effects on cellular adhesion molecules, inflammatory chemokines, and generation of the inflammasome. The study demonstrated that the use of low-dose colchicine within the first 3 days after MI is associated with a reduction of 48% in the risk cardiovascular death, cardiac arrest, Myocardial Infarction, stroke, or urgent hospitalization for angina requiring coronary revascularization, in comparison with placebo. This study has on heightened the interesting in targeting the inflammasome and its synthesis in the pathogenesis of myocardial infarction.

Despite these mixed findings, the search for an effective anti-inflammatory therapy

for myocardial ischaemia continues. One possible future avenue should be the development of immunomodulatory therapies designed to target ischaemic-reperfusion injuries to negate the effects of pyroptosis and necrosis. Reperfusion also plays an important role in post-ischaemia leucocytosis, as the primary source of neutrophils in the infarct zone is recruitment from the coronary circulation. Reperfusion permits neutrophils from the circulation to enter the ischaemia zone more quickly and in greater numbers, resulting in morphological changes within the infarct zone, including oedema, interstitial haemorrhage and cardiomyocyte swelling. Targeting the inflammatory cascade at the point of reperfusion may allow us to modulate the healing process within the ischaemic penumbra and prevent parts of the reperfusion injury itself.

DAMPs as a potential target

A key part of immune activation is immunostimulation via DAMPs(35). Identification of specific DAMPs released during ischaemia-reperfusion injury may provide valuable drug targets. The appearance of excessive amounts of intact, high-molecular-weight, extracellular DNA is one of many differences between controlled cell death pathways such as apoptosis and uncontrolled necrotic cell death(36, 37). Intracellular DNA is systematically degraded when cells undergo apoptosis(37); however, during ischaemia cells die via a process of necrosis/oncosis during which nuclear and mitochondrial DNA is released into the blood. Mitochondrial DNA is a well-recognised DAMP that has been shown to stimulate innate immunity via Toll-like receptor 9a as well as stimulate the release of pro-inflammatory cytokines. MtDNA

has also been implicated in the process of neutrophil extracellular trap formation (NETosis) in renal inflammation and trauma.

Given that each eukaryotic cell carries approximately 6pg of DNA and the human myocardium contains approximately five billion cells, a significant myocardial infarct consisting of ~one billion dead cardiomyocytes has the potential to release ~one mg nuclear DNA into the extracellular space(38, 39). This large quantity of DNA is then free to diffuse within the necrotic milieu of the infarct and peri-infarct zone. Elevated levels of cell-free DNA are associated with a variety of conditions, including trauma, tumour malignancy and sepsis, each of which are themselves associated with a degree of immune activation or inflammation.

Within the nuclease of the cell, each DNA nucleosome core consists of superhelical DNA wound around an octamer of histones, consisting of two copies of each of the core histones H2A, H2B, H3 and H4. The linker histone H1 binds to the complete nucleosome core particle and forms higher-order structures. After a necrotic event, nuclear DNA may circulate freely as DNA fragments or isolated histones, both of which are well-recognised DAMPs(40). The extent to which extracellular mammalian DNA is cytotoxic was first demonstrated by Xu et al., who showed that intravenous injection of isolated histones into mice was lethal within minutes(41). Curiously, the administration of intact nucleosomes did not have this effect. It has now been shown that this effect is mediated through TLR2 and TLR4, both crucial receptors involved in the activation of the innate immune system(42).

Another large source of extracellular DNA comes from infiltrating neutrophils. In

response to pro-inflammatory stimuli, neutrophils can discharge their nuclear DNA, forming an extracellular net of chromatin that comprises DNA and histones(43). This process is termed NETosis, which ultimately kills the neutrophil but creates a histone-rich mechanical mesh surrounding the neutrophil. The mechanical net is thought to entrap DAMPs and cellular debris, providing stimuli for the activation of infiltrating leukocytes. These neutrophil extracellular traps (NETs) can subsequently break down and release histones, causing further damage to tissue remote from the initial necrotic site(44). NETs also play a crucial role in thrombosis, platelet aggregation, and occlusion of blood vessels, further exaggerating coronary ischaemia(45). Thrombi aspirated from the coronary arteries of patients who suffered ST elevation myocardial infarction demonstrate that the burden of NETosis positively correlates with infarct size and negatively correlates with ST segment resolution(46).

The occurrence of circulating extracellular DNA is understood to occur during the normal physiological state in both humans and rodents. Its concentration is tightly controlled by extracellular DNAases. The crucial role of removing DNA from the extracellular space in normal physiology is highlighted by the fact that the DNAse II-knockout mouse model is lethal shortly after birth(47). Furthermore, deficiencies in the normal biological processing of extracellular DNA digestion are linked to autoimmune diseases such as SLE(48-50). It is now believed that during cellular processes that involve the destruction of a large number of cells, such as erythropoiesis, the amount of available extracellular DNA remains within a manageable quantity through continuous degradation to maintain a level below the immunostimulatory threshold If, however, the threshold is breached in conditions such as necrotic cell death, the DNA may act on the same pathogenic receptor

pathways, stimulating an innate immune response(51-53). A well-documented finding in autoinflammatory conditions is the presence of circulating cell-free DNA incorporated into immune complexes(54), confirming the ability of self-DNA to act as a self-antigen. Previously, it was believed that the presence of unmethylated CG dinucleotides in microbial DNA conferred foreign DNA with the ability to interact with TLR9 and stimulate the innate immune response. Mammalian purified DNA dinucleotides are mostly methylated, so in theory, they should not exhibit an immunostimulatory response, but mammalian DNA complexed with either histone or certain DNA-binding proteins has been demonstrated to induce TLR9-mediated signaling(55, 56). Extracellular mammalian DNA has been shown to have this effect both by interacting with TLR9(57) to activate the innate immune system, but also via a Toll-like receptor (TLR)-independent mechanism that increases the transcription of type 1 Interferon, a potent pro-inflammatory cytokine (58-60). Unlike naked DNA, cell-free chromatin contains abundant proteins that may contain epitopes that helper T cells identify as foreign. Antibodies to chromatin precede the occurrence of antibodies to anti-DNA antibodies, suggesting chromatin plays a crucial role in developing an autoinflammatory response to self-DNA(61).

Do histones act as DAMPs or not?

Necrosis is induced by ischaemia and releases histones(62) either as part of nucleosome fragments or on their own. These extracellular histones have also been shown to trigger inflammation and cell death, either by stimulating pro-inflammatory cytokines resulting in the activation of cell death pathways or through the process of

NETs. In human observational studies, raised histone serum levels have been demonstrated in multiple trauma patients and correlate with the severity of coagulopathy, endothelial damage, and inflammation(63). A large body of evidence demonstrates that histone-induced cell toxicity plays a crucial role in cell death during ischaemia/reperfusion injury of the myocardium. There is some debate on whether histones exert their cytoxic action via a TLR-dependent or independent process.

Isolated histones are known to activate TLR2 and TLR4; furthermore, TLR knockout mice are protected from the lethal effects of histones administered intraenously(42). In an ischaemic stroke model, histone infusion increased the infarct size and, conversely, histone neutralisation via an antibody infusion resulted in a reduction in infarct size(64). Similar cytoxic effects of histones have been demonstrated in kidney injury(65), sepsis(41), and even hair follicle death(66). There is also increasing evidence that histones can cause cytotoxicity independent of immunostimulation, damaging lung endothelial cells and stimulating an influx of intracellular calcium and subsequent necrosis(67).

However, more recently, it has been proposed that histones may cause cell cytotoxicity via a TLR-independent mechanism. A recent study demonstrated that histone endothelial-mediated cytotoxicity played a crucial role in the development of atherosclerosis(68). The authors demonstrated that histone H4 was the most cytotoxic histone in a rat model of endothelial cells. They were able to demonstrate that histone H4 formed a pore within the cell wall of smooth muscle cells, puncturing the intracellular space and resulting in rapid extravasation of intracellular contents.

Antibody blockade of histone H4 in hypercholesterolemic mice with established vascular lesions leads to the generation of plaques with reduced vulnerability and increased lesion smooth muscle cell content. Furthermore, this cytotoxicity of histone H4 was also inhibited by a novel peptide termed histone inhibitory peptide, which they postulated would bind to extracellular isolated histone H4, changing the shape of the histone and preventing it from forming a pore in the cell wall. This seminal work has led to a debate on how histones may exhibit their cytotoxicity and how we could potentially neutralise this effect in pathology. However, until we can prove otherwise, the overwhelming literature would suggest histones function as DAMPs and activate TLR stimulating intracellular cell death pathways.

DNA and cell death (inflammasome activation and pyroptosis)

It has been shown that extracellular DNA and histones can function as an alarmin or DAMP by activating TLRs. In addition to playing a crucial role in the innate immune system, TLR can activate intracellular cell death pathways or pyroptosis. During the process of ischaemia/reperfusion, evidence suggests that cell death pathways such as pyroptosis may contribute to infarct development and subsequent poor remodelling of the myocardium(69-71). This has culminated in an interest in targeting pyroptosis to limit the degree of cell death during myocardial infarction (72-74). The difficulty presented by this is the understanding that IR injury is multi-factorial and that pyroptosis inhibition may represent one aspect of a multi-targeted approach to reducing infarct size.(74)

The role of histones in sepsis, endothelial dysfunction and hyperinflammatory states have previously been investigated. It has been demonstrated that by targeting extracellular histone in animal models of sepsis, ameloriation of sepsis and the lethal complication of sepsis is possible. Xu et al (41)used both antibodies to histone to save mice from the lethality of Histone infusion. It is important to note that the use of antihistone antibodies is limited as there is the risk of developing autoimmune proinflammatory conditions when used in humans(75). Furthermore, it was demonstrated that Activated Protein C cleaves extracellular histone and reduces histone H3 and H4 induced cytoxicity and endothelial dysfunction. Furthermore Baboons challenged with lethal dose of E.Coli induced sepsis can be saved with treatment of activated protein C (76).

During ischaemia, necrotic cell debris, including DNA fragments, is released into the extracellular space, creating a pro-inflammatory milieu. Stimulation of TLRs on surviving cardiac cells in the border zone of the infarct area leads to the activation of downstream intracellular signalling pathways, which convene to result in NF-κB-mediated expression of the protein components that make up the NLRP3 inflammasome (77, 78). Following a secondary trigger, the individual protein components, now present in the cytoplasm of the cell, conglomerate to form a multiprotein oligomer, also called the inflammasome complex (79, 80). This complex is now able to interact with procaspase one and leads to the conversion of procaspase into the active caspase-1 form (81). Caspase-1 begins the subsequent activation of the pro-inflammatory cytokines IL-1B and IL-18 (82). An additional substrate of caspase-1 is the cytosolic protein gasdermin D (GSDMD) (83-85). The cleavage of GSDMD by caspase-1 releases N-terminal fragments, which oligomerise

within the cell membrane to form pores (84, 86). These pores cause the loss of cell membrane integrity, leading to pyroptotic cell death (87). The pores also increase membrane permeability to IL-1Beta and IL-18, leading to their extracellular release, where they amplify the inflammatory response and mediate further injury (24, 69, 70, 88-95).

Evidence for a role of pyroptosis in myocardial infarction.

A number of animal studies have provided evidence that self-DNA may be an effective target for inhibiting inflammation and myocyte death during ischaemia-reperfusion injury. In a murine Langendorff model, it has been demonstrated that in vitro histones caused cardiomyocyte toxicity (96). In an in vivo heart ischaemia - reperfusion model, DNAase one treatment disrupted extracellular cytotoxic chromatin, resulting in a reduction in myocardial histone concentration(97). This correlated with a significant improvement in left ventricular remodelling and cardiomyocyte survival Ge et al supported this finding in a murine model of ischaemia -reperfusion in which DNAase plus recombinant tissue-type plasminogen activator resulted in a reduction of infarct size as well as reducing the density of neutrophil-associated NETs(98). Although this also leads to an improvement in left ventricular remodelling, curiously this effect was not observed when DNAse or rt-PA was administered on its own(98). Savchenko et al. administered DNAse to PAD-4 -/- mice that do not produce NETS, as well as to wild types(99). The study demonstrated that MI/R injury caused an increase in nucleosome concentration, neutrophil infiltration, and histone H3 at the site of injury. Treatment with DNAse

resulted in an improvement in cardiac contractile function by a similar degree in both wild type and PAD-4 -/- deficient mice. This suggests that DNA fragments contribute to cardiomyocyte dysfunction during reperfusion irrespective of NET activity, possibly by acting as a DAMP. Using an in vivo rat model, Downey's group made similar findings, with DNAse administered after 30 min of coronary artery occlusion resulting in a significant reduction in infarct size(100). Interestingly, the addition of a mitochondrial DNA inhibitor Endonuclease III with DNAse resulted in a greater reduction in infarct size than that seen with DNAse alone. This would suggest that nuclear DNA could act through a different pathway than the well-recognised DAMP, mitochondrial DNA(100).

Endothelial dysfunction plays a crucial role in ischaemia -reperfusion injury, contributing to myocardial stunning, microvascular obstruction and exposing the myocytes to toxic stimuli, which contribute to lethal myocardial injury (101-104). Interestingly, heparin consists of a high concentration of negatively charged sulphated proteoglycans, which bind to histones and inactivate them through high-affinity electrostatic interactions(105). It has also long been shown that heparin protects the coronary endothelium and myocardium from I/R injury (106-109). Both heparin and chondratin sulphate have been shown to protect vascular endothelial cells from histone-induced cytotoxicity in vitro(110, 111). Furthermore, heparin derivatives reduce infarct size in a rat model of ischaemia -reperfusion by inhibiting caspase-dependent cell death pathways(112). Agents that possess negatively charged proteoglycans but lack any anticoagulative effect may provide a model for the development of anti-histone therapy. Sodium-β-O-Methyl cellobioside sulphate (mCBS) is one such compound currently in development with SIRTEX Laboratories.

Extracellular histone contains a large number of basic residues on its surface, which gives histones a high positive charge. Pharmacologists have long hypothesised that since histones are highly cationic, and polyanions may interact electrostatically with histones and neutralise their cytopathic and prothrombotic properties. Heparin is the most commonly recognised polyanion and has demonstrated a strong affinity for extracellular histones. Animal studies have demonstrated that heparin protects against the cytotoxic effect of histones on endothelial cells; however, the use of heparin as an anti-histone therapy is limited by its anticoagulant properties. However, these studies established the preclinical proof-of-concept that polyanions could neutralise the negative effects of unbound histones (105, 113). Researchers at the University of Australia investigated the smallest polyanions that neutralised the cytotoxic activity of extracellular histones and found them to be sulphated disaccharides.

It is important to note that there are other methods of neutralising histones RNA-aptamers recombinant thrombomodulin and heparin. One exciting development is the use of RNA-aptamers, they are chemically stabilized nucleic acid bio-drugs, they are synthetic structure RNA or DNA oligonucleotide ligands that bind with high affinity and specificity to their targets. Aptamers specific to Histone have been shown to prevent pathological evidence of histone toxicity in a murine model (114)

mCBS (originally developed at the University of Australia and Sirtex pharmaceuticals) is a sulphated disaccharide of glucose with a methylated group in the β anomeric configuration at the terminal reducing end. In the salt form, it consists

of seven sodium counter ions, which rapidly dissociate when introduced to an aqueous environment to form the free base, a highly anionic product that is the active agent.

Its small molecular polyanionic structure has been shown in pharmacodynamics (PD) studies in murine and rabbit models to mediate a reduction in histone-induced systemic toxicity (confidential data from Sirtex Laboratories) and can be given in much higher doses without any anticoagulant effect. As a novel compound, mCBS has not yet been investigated for its ability to protect the heart from ischaemia-reperfusion injury. We propose that mCBS will act as a cation potent scavenger of the free histones released during ischaemia-reperfusion of the heart. This will subsequently attenuate the early release of pro-inflammatory interleukins and inhibit excessive tissue necrosis, scar formation, and poor ventricle remodelling. Second, we propose that mCBS may also disrupt the process of NETosis, further improving the patency of the coronary vasculature during ischaemia-reperfusion.

HIPe or mCBS

The best method to neutralise extracellular histones is still not known. Historically, the most well-studied histone neutralisers have been heparin and polyanions; however, there now exist newer therapies that are designed to inhibit the cytotoxic actions of histones via alternative mechanisms. One such compound developed specifically targets the structure of the histones and prevents it from exerting its action on the cell membrane. The seminal article recently published in Nature by Silvestre-Roig et al(68) demonstrated that histones may exert their cytotoxic actions

via a TLR-independent process. They demonstrated that histone H4 forms a pore within the cell wall of the smooth muscle cells, causing cell lysis. Atomic force microscopy of artificially reconstituted membrane bilayers incubated with histone H4 showed the appearance of pores. To further validate this theory, the investigators performed small-angle X-ray scattering on small unilamellar vesicles incubated with the histone H4 N terminus, which resulted in a dose-dependent membrane deformation rich in negative Gaussian curvature. These Gaussian curvature values were comparable to those obtained from other membrane-remodelling proteins. The investigators then took this theory one step further and developed a peptide that interacted with the histone H4 cell membrane interactions by binding to the N terminus of the Histone H4 molecule. This peptide was named histone inhibitory peptide (HIPe). The researchers then administered the peptide to mice with pre-exisiting atherosclerotic lesions and demonstrated that treatment with HIPe resulted in atherosclerotic lesions with a larger number of smooth muscle cells and a reduction in the vulnerability of the plaque. The authors concluded that chronic inflammation in the wall of arteries, perpetuated by extracellular histone release either via the process of NETosis or direct release from cell lysis, leads to smooth muscle cell cytotoxicity, contributing to vascular damage and the perpetuation of atherosclerosis. The rapid cell lysis of smooth muscle cells and endothelial cells ultimately creates a vulnerable architecture that may predispose the atherosclerotic plaque to rupture and occlude the vessel.

HIPe has only been used in the context of atherosclerotic mice; however, we postulate that a similar effect may also occur during the process of ischaemia-reperfusion injury of the myocardium. The cytotoxicity of histones to smooth muscle

cells could also be prevalent within cardiac myocytes during ischaemia and may be perpetuating the ongoing inflammation within the myocardium, further increasing the amount of tissue death and the subsequent size of the infarct of the heart.

Both mCBS and HIPe have unique advantages and disadvantages. mCBS is a polyanion that interacts with histone via an electrostatic connection and thus makes no preference for which histone it can bind to. It is reasonable to assume that the compound has equal affinity for histones 1–5 and that it does not exert a preferential effect on one histone over the other. Histones H2A, H2B, H3, H4, and H1 are frequently detected on the cell surface or the cytoplasm of immune cells in response to stress(115-117). mCBS non-specificity for histones gives it a distinct advantage over HIPe, which is a peptide that was tailor-made to interact with only histone H4. However, this non-specificity may also be a disadvantage, because a polyanion will not differentiate between extracellular histones and other cations that are released during a necrotic episode. It may be reasonable to assume that even during normal pathological conditions, the blood is a rich medium for cations, which may also bind to a non-specific polyanion such as mCBS. Theoretically, this may reduce its ability to bind to extracellular histones once the drug is in the bloodstream or in a pro-inflammatory environment where there will be a high concentration of free radicals and cations.

Neither drug has been tested in the environment of ischaemia -reperfusion injury of the myocardium, so at this stage, these are just hypothetical arguments into the pharmacodynamics of each individual therapy. The biggest disadvantage of HIPe lies in its cost of manufacturing. Professor Oliver Sonelheim, from Germany who

pioneered the therapy, has been kind enough to gift us with a small quantity for our research. With this small amount, we had enough to conduct in vitro cell-based experiments as well as in vivo rat coronary artery occlusion experiments (see details in the results section of this thesis). However, the amount we would need to conduct ex vivo Langendorff experiments would cost us an estimated £40,000. At this stage, we felt it would be more sensible not to use HIPe in a Langendorff model, and in the future, if the compound is deemed promising enough, we could consider this experiment.

Chapter 2 – Hypothesis and Aims

Original hypotheses

1. Extracellular histones are released from the myocardium during the process of ischaemia- reperfusion injury.

2. Ischaemia -reperfusion injury increases the amount of extracellular histone present in myocardial tissue.

3. Extracellular histones are cytotoxic to healthy cardiomyocytes in vitro.

4. mCBS protects cardiomyocytes from the cytotoxic effects of histones in vitro.

5. HIPe protects cardiomyocytes from the cytotoxic effects of histones in vitro.

6. mCBS reduces the detectable amount of histone released during ischaemia-reperfusion injury.

7. Extracellular histones are cytotoxic to cells during ex vivo ischaemia-reperfusion injury of the myocardium.

8. Endogenous histones administered to the ex vivo heart using an isolated rat heart Langendorff apparatus are associated with a larger myocardial infarct.

9. mCBS is cardioprotective during ischaemia- reperfusion injury of the

myocardium ex vivo by neutralising the cytotoxic actions of extracellular histones.

10. mCBS is cardioprotective during ischaemia -reperfusion injury of the myocardium in an in vivo rat coronary artery occlusion model.

11. Extracellular histones do not function as a traditional danger-associated molecular pattern.

12. The cytotoxic actions of histones are independent of TLR and TLR-dependent intracellular cell signalling pathways.

13. Histone H4 is present during ischaemia -reperfusion injury of the myocardium.

14. Targeting histone H4 by using HIPe in an in vivo rat coronary artery occlusion model is cardioprotective.

15. Targeting total extracellular histone by using mCBS in an in vivo rat coronary artery occlusion model is cardioprotective.

Aims

The aim of this PhD research was to investigate the role extracellular free histones play in excessive cardiomyocyte death during ischaemia- reperfusion injury. Extracellular histones have been demonstrated to be cytotoxic in many pathological processes, and they have been detected in the aspirated clot of patients suffering ST elevation myocardial infarctions. More recently, it has been shown that histone H4 is implicated in the cytotoxicity of smooth muscle cells of coronary arteries of mice during the process of atherosclerosis(68).

The first step was to confirm the presence of a cytotoxic concentration within the myocardium of extracellular histones. To investigate this, I used ELISA-based protein assays coupled with the ex vivo Langendorff isolated rat heart model.

The next step was to demonstrate the cytotoxic effects of histone. In an ideal situation, I would have liked to demonstrate the cytotoxicity of histone H4 on its own, but it is very difficult to purify histone into histone H4 for commercial or research purposes. As such, we opted to use histone derived from calf thymus, which is a mixture of a multitude of histones, including histone H4. To see whether extracellular histone is cytotoxic, we tried to incubate isolated rat cardiomyocytes in the presence of histones. To assess the degree of death, we stained the cells with propidium iodide and then analysed the cells under the microscope and counted the percentage of dead cells. We also ascertained the level of death by using an LDH assay kit to calculate the amount of LDH released by the cells.

Next, we tested the effect of retrogradely perfusing hearts undergoing ischaemia-reperfusion on a Langendorff apparatus with exogenous histones to demonstrate that, in an ex vivo model, extracellular histones are also cytotoxic.

The next step was an investigation into the mechanics of how extracellular histones and histone H4 interact with the cell membrane. Immunohistochemistry staining of histone H4 in hearts subjected to ischaemia - reperfusion injury demonstrated how the extracellular movement of histone H4 alters when the organ is subjected to ischaemia- reperfusion. We hypothesised that cell death in the form of pyroptosis or other forms of uncontrolled cell death would result in the release of fractured parts of DNA, including histones and specifically histone H4. The experiments demonstrated that during ischaemia- reperfusion histone H4 congregates around the cardiomyocyte membrane, mimicking the environment of our in vitro cell-based experiments. Histone H4 is cytotoxic to smooth muscle cells in the coronary arteries, which suggests similar cytotoxicity may occur during ischaemia reperfusion injury.

Furthermore, we looked more deeply into the mechanism of how histone achieves this cytotoxicity. Studies within the literature are divided; several studies demonstrate this cytotoxicity is via activation of a Toll-like receptor(40, 118). This would suggest that extracellular histones are DAMPs that activate intracellular cell death signalling pathways such as pyroptosis. The problem with this theory is that inflammasome-dependent intracellular cell death signalling pathways require a second priming signalling to allow the formation of the intracellular inflammasome. TLR activation on its own should not activate an intracellular cell death pathway. Silvestre-Roig et al(68) demonstrated a more complete theory as to how histone H4 delivered its

lethal effect to smooth muscle cells in the coronary artery. They proposed that the protein forms a pore within the myocardium, a process independent of TLR. We challenged the notion that histone toxicity is a TLR-dependent process and followed the Silvestre-Roig et al. theory.

The techniques I utilised to delve into the mechanism of histone-induced cytotoxicity were based around isolated rat cardiomyocytes, LDH ELISA assays as well as cell staining and microscopy. Through help from our international collaborators, we also had access to TLR transfected human embryonic kidney (HEK) reporter cells. We were able to block TLR with TLR antagonists, which we co-cultured with histones and isolated cardiomyocytes to demonstrate any differences.

The human embryonic kidney (HEK) reporter cells are very useful because they can demonstrate the TLR-dependent and NF-κβ dependent processes. TLR receptor activation triggers cytosolic adaptor proteins that induce downstream signalling cascades, which in turn promote the transcription of inflammatory mediators including IL-1β and the release of NF-κβ HEK reporter cells. HEK-Blue™-hTLR4 cells are created by cotransfection of the human TLR4 and SEAP (secreted embryonic alkaline phosphatase) genes into HEK293 cells. The SEAP reporter gene is placed under the control of NF-κβ activation. As well as this, the CD14 co-receptor gene is transfected into these cells to enhance the TLR response.
Activation of the TLR with a ligand activates NF-κβ, which induces the production of SEAP. Levels of SEAP can be determined to quantify the degree of TLR4 activity. NF-κβ is also a crucial intracellular downstream enzyme for the activation of inflammasome-dependent intracellular cell death pathways, such as pyroptosis. If

histone cytotoxicity is TLR-dependent, this may suggest that histones function as stimulators of intracellular cell death pathways. Unfortunately, I did not have access to these cell lines, nor do I have the expertise to use them, but our collaborators at the University of Oslo under the supervision of Professor Stensløkken (undertook studies as part of a collaboration and I was able to use the data obtained within this thesis.

Another way of inhibiting inflammasome-dependent intracellular cell death pathways is by the use of a Caspase-1 inhibitor such as emiricasan. As such, to help delineate the role of inflammasome-dependent cell death pathways, we also assessed the effect of compounds such as emiricasan on the effects of histones on cardiomyocytes. Should histone cytotoxicity require activation of intracellular cell signalling pathways, then caspase inhibitors should prevent the formation of the inflammasome and subsequent activation of pyroptotic-like cell death. Thus, the use of caspase inhibitors to reverse the effects of histone provided us with further mechanistic insight into how extracellular histones exert their actions.

After gaining a greater mechanistic understanding, I then determined whether neutralising the amount of circulating extracellular histones and neutralising histone H4 released during ischaemia -reperfusion injury protects cardiomyocytes from the deleterious effects of extracellular histones. In order to test this, I tested two histone neutralising agents. First, mCBS is a non-specific histone neutralising agent, and second, HIPe is a peptide that specifically targets histone H4. These compounds were tested in a multitude of experimental models. We then tested these compounds in an in vitro isolated rat cardiomyocyte model. This technique involves isolating

cardiomyocytes via collagenase perfusion of a rat heart and collecting the resulting cells to incubate in the medium. The cells can then be subjected to histones at increasing concentrations to determine whether there is a dose-dependent cytotoxic response. The amount of death can be assessed by propidium iodide stating or calculating the amount of LDH released using an ELISA assay. Within this model, I also tested whether blocking TLR activation with a TLR antagonist reversed any cytotoxic action of histones. The histone-exposed cells were also incubated in the presence of the compounds mCBS and HIPe to assess their ability to negate the actions of extracellular histones.

To further clarify whether mCBS is cardioprotective, the next step was to examine the compound in the Langendorff isolated retrogradely perfused heart in the model of ischaemia reperfusion injury ex vivo. There are two techniques in the Langendorff model: regional ischaemia or global ischaemia. I initially established the regional ischaemia technique, which uses a carefully placed suture in the superior part of the midline groove in order to cause a regional area of ischaemia in the left anterior descending artery territory. The global ischaemia technique involves interrupting the flow of the perfusate to the whole of the myocardium to generate global ischaemia within the entirety of the myocardium. The hearts were subjected to a period of ischaemia followed by two hours of reperfusion in both techniques, after which the hearts were removed and stained as detailed below to calculate the size of the infarct. If the treatment with mCBS is cardioprotective, then hearts that are perfused with mCBS would show a reduction in the total infarct size.

Ideally, I would have liked to test both mCBS and HIPe in this model; however, as it

is a continuous flow model, it requires larger amounts of the drug. This is not an issue with mCBS; however, HIPe is considerably more expensive to manufacture, and we would have needed over £30,000 of HIPe to conduct the full set of experiments. As such, we felt at this stage we should only trial mCBS.

We translated ourhypothesis into an in vivo rat model. For this, we had the help of Dr David He an expert in conducting the non-recovery rat coronary artery occlusion model. We tested both HIPe and mCBS, administered to rats before the onset of ischaemia -reperfusion. This is a non-recovery model, and the rat is sacrificed immediately after reperfusion of the myocardium. It is then isolated from the heart and stained with Evans blue to demarcate the area not at risk, after which triphenyl tetrazolium chloride (TTC) is used to determine the infarcted area. Crucially, we were able to use both mCBS and HIPe within this model, as we were fortunate enough to have been gifted with a small amount of HIPe by Professor Oliver Soehnlein at the University of Munich, Germany.

A possible improvement on the in vivo rat coronary artery occlusion model is the recovery model, where the rat is awoken after ischaemia reperfusion injury of the myocardium and then the effect of the infarct is assessed at a later date using an echocardiogram.

Establish experimental models

- Establish the ex vivo retrogradely perfusated isolated rat heart with regional ischaemia -reperfusion injury Langendorff model.
- Establish the same isolated perfused rat Langendorff model but with global ischaemia.
- Clarify which model would better suit my experiments.
- Establish a protein assay to quantify the concentration-free histones and free histone H4 that is released from the myocardium of rat hearts in the Langendorff model.
- Establish a method to accurately calculate the exact amount of histone H4 released during ischaemia- reperfusion injury.
- Establish a cell culture model to incubate isolated cardiomyocytes.
- Establish the best way to assess cell death in cultured cardiomyocytes.

Experimental aims

To determine the role of extracellular histones in ischaemia/reperfusion injury

- Quantify the amount of histone released during ischaemia reperfusion injury of the myocardium.
- Identify the concentration of histone H4 released from the rat myocardium during ischaemia reperfusion.
- Does the amount of histone H4 released from the myocardium correlate with the size of the infarction?
- Investigate the movement of extracellular histone H4 in the myocardium after ischaemia reperfusion.

To investigate the effect of exogenous histone on cardiomyocytes

- Investigate the effect of exposing isolated rat cardiomyocytes to increasing concentrations of exogenous histone.
- Investigate the effect of exposing the isolated ex vivo rat heart to ischaemia-reperfusion injury to increasing doses of histone.

To investigate the mechanism of histone and cardiomyocyte interaction

- Investigate whether histone effect on cardiomyocytes is mediated via Toll-like receptors.
- Does extracellular histone activate an intracellular NF-κB mediated cell

signalling pathway in cardiomyocyte?

- Does extracellular histone activate a caspase-1 dependent inflammasome mediated cell death pathway?

Does mCBS inhibit the effects of extracellular histones?

- Study the effects of mCBS on histone-exposed isolated rat cardiomyocytes.
- Study the effects of mCBS on histone release during ischaemia/reperfusion injury in an ex vivo isolated rat heart.
- Study the effects of mCBS on infarct size of ex vivo isolated rat hearts undergoing ischaemia reperfusion injury, is mCBS cardioprotective.

Does HIPe, which selectively targets histone H4, inhibit the effects of extracellular histones?

- Study the effects of HIPe on histone-exposed isolated cardiomyocytes.
- Study the effects on histone H4 release during ischaemia reperfusion injury in an ex vivo isolated rat heart.
- Ideally, we would like to trial HIPe on the Langendorf ex vivo model; however, the quantity of HIPe required would be extremely expensive to buy, so this experiment is not feasible

Can we induce cardioprotection in an in vivo rat coronary artery occlusion model?

- Study the effects of treating rats with mCBS and the effect on the infarct size

of the myocardium after ischaemia reperfusion injury.

- Study the effects of treating rats with HIPe and the effect on the infarct size of the myocardium after ischaemia reperfusion injury.

Quantify which extracellular histone is cytotoxic.

- Compare different classes of the histone linker protein in an in vitro isolated. rat cardiomyocyte model to compare the cytotoxicity of each protein.
- Compare different classes of the histone linker protein to compare the cytotoxicity of each protein in the Langendorff isolated retrogradely perfused heart model of ischaemia reperfusion injury ex vivo.

Chapter 3 – Research Methodology

Ex vivo Langendorff perfused heart model of ischaemia- reperfusion injury.

Retrograde mammalian heart perfusion was pioneered in the latter part of the 19th century by, amongst others, Oscar Langendorff, whose name has now become synonymous with the technique, as well as Carl Ludwig and Elias Cyon (119). It has proved to be an extremely useful and insightful technique that has allowed scientists over the centuries to gain a considerable understanding of cardiac physiology and pathology. There are a large number of variations of this technique that have allowed researchers to explore arrhythmias, coronary function, cardiomyocyte biology, and the pathology of diabetes and hypertension, to name a few. Its usefulness has not diminished over the years, and with advancements in the technology of monitoring, its role at the forefront of present and future research endeavours is assured.

In line with Langendorff's own observation, the principles behind the research method remain the same today. Following anaesthesia, the heart is isolated from the animal and cannulated through the aorta so that a flow perfusion buffer can pass through the aorta retrogradely, forcing the aorta valves to shut and allowing the buffer to pass through the coronary ostia into the coronary vasculature. Once established, the heart can then have physiological parameters assessed, the heart can be exposed to compounds through the perfusion buffer, and ischaemia-reperfusion injury can be administered to the heart either locally by attaching a suture to the anterior artery or globally by interrupting the flow of the perfusate buffer. An important limitation of the technique is that the heart is isolated from blood circulation. The heart has few resident immune cells; the most prominent cells to

enter the ischaemic zone after infarction are neutrophils recruited from the peripheral circulation. As such, one must be aware that the Langendorff model is an immune naïve model, which in turn is also an advantage as it allows us to study the effect of cell-cell inflammation in the absence of the circulating immune system.

The flow of perfusate into the myocardium can be achieved via two mechanisms: constant pressure or constant flow. Constant pressure is simpler to achieve and requires a column of fluid to be placed at a height above the cannula through which the aorta is attached. Varying the height of the fluid helps maintain a continuous, constant hydrostatic perfusion pressure by the force of gravity. Using this method, the pressure of the flow through the heart can be increased by raising the height of the buffer fluid level in the column, and vice versa. A constant flow requires a peristaltic roller pump through which the perfusion buffer passes. They require a commercially available pump and thus may be more expensive, but offer the distinct advantage of being able to maintain a constant perfusion pressure into the heart irrespective of the state of the heart's vascular bed. This is particularly useful in the setting of ischaemia -reperfusion injury, as the ischaemic insult can impair the vascular bed via microvascular obstruction to such a degree that the ischaemic zone may not be perfused as effectively by a constant pressure system.

During my experiments, I used both techniques, the constant pressure system for the setting of ischaemia- reperfusion injury, but when I was isolating cardiomyocytes, I required the more robust buffer delivery system offered by a constant flow system. See figure 3.1

Coronary flow

Coronary flow can be an important indicator of vascular tone and the state of the vascular bed. It is also important to ensure the heart does have some coronary flow through the reperfusion period of ischaemia- reperfusion injury because the method of identifying the size of the infarction of the heart is based on being able to stain enzymatic byproducts of cellular death such as LDH. If there is an inefficient flow of perfusate of the heart, excessive LDH could be held up around the heart even in the infarcted site, potentially causing a false positive effect.

In a peristaltic pump providing constant flow Langendorff, the signal voltage that alters the pump speed can provide a real-time indicator of the coronary flow. In a constant pressure system, the amount of effluent collected over a certain time period can be used to estimate the flow of perfusate through the vascular bed. The latter has a greater deal of inaccuracy, given that there are other factors in the heart that may interfere with the flow of perfusate out of the myocardium.

Left ventricular systolic and diastolic functions

Crucial to the measurement of intraventricular pressure in the Langendorff model is the placement of a balloon within the left ventricle. The balloon must be made of semi-compliant material and be attached to a tube made out of non-compliant material. The balloon is placed through the mitral valve into the left ventricle and then inflated. The semi-complaint material of the balloon ensures minimal trauma to the

left ventricle while still providing a conduit to transfer pressure changes from the intraventricular space into the balloon and along the tube. The material of the balloon must be able to generate a frequency response curve that neither dampens nor amplifies the recorded signal of the rat heart; stretched cellophane is a successful material for this task. The non-compliant tube must be able to transfer any pressure changes to a calibrated pressure transducer. The entire system from the pressure transducer, the tube and balloon must be filled with fluid without air bubbles, as air is semi-compliant and may dampen any pressure transfer.

As the ventricle contracts during systole, the pressure transducer can estimate this force into a pressure given in mmHg and termed developed pressure. The transducer is also able to estimate the pressure in the ventricle at the end of diastole and records this as end-diastolic pressure (EDP). The importance of the balloon lies also in the positive physiological effect it has: once inflated, it stretches out the ventricular wall and as a result of the Frank-Starling effect, improves the efficiency and strength of the systolic contraction.

Temperature

Maintaining strict temperature regulation is a crucial part of any biological experiment, especially within an ex vivo organ. There are two ways the temperature is controlled. In one, the column of perfusate buffer is wrapped within a water jacket from the reservoir at the top, through the length of the column and even into the bubble trap on top of the aortic cannula. The water within the jacket is heated via a

heat pump and maintained at 36–38°C, which ensures that the perfusate buffer reaches the heart at the correct temperature. Another way is to place the heart in a water bath below the cannula. This is also water jacketed like the rest of the column, ensuring the heart is always surrounded by a temperature-controlled environment of perfusate buffer.

Typically, the pulmonary artery is incised and a temperature probe is placed within it. The probe is placed within the pulmonary artery as it is in a convenient location just next to the aortic cannula, but this also ensures that the pulmonary artery remains open. As the aortic root and subsequent pulmonary trunk are sutured onto the cannula, pulmonary hypertension can rapidly transfer to the right ventricle, impairing the systolic function of the heart. An incision within the pulmonary artery prevents this problematic complication. Through the temperature probe, the heart can be maintained at a strict temperature range to ensure efficient cardiomyocyte contractility and function.

Variations in the temperature of the heart can have profound effects on the heart rate and myocardial contractility; this is compounded in a rat heart as it has a relatively high surface area to volume ratio(120). Indeed, the importance of temperature maintenance is demonstrated by the fact that hypothermia and hyperthermia have both been associated with an effect on the Langendroff heart similar to that seen with preconditioning(121, 122). This only further highlights the importance of continuous temperature monitoring and adjustment to maintain temperature parameters as close to physiologically normal as possible.

Krebs–Henseleit Buffer

The most commonly used perfusate buffer is a modified version of the Krebs–Henseleit buffer (KHB), composed of NaCl 118.5 mM, $NaHCO_3$ 25.0 mM, KCl 4.7 mM, MgSO4 1.2 mM, KH2PO4 1.2 mM, glucose 11 mM, and CaCl2 of 1.3 mM, which will have a pH of 7.4 at 37°C when bubbled with 5% CO_2. This is modified to reflect the later discoveries that the calcium content in a rat heart was significantly lower than the 2.5 mM of CaCl2 that was originally proposed back in 1932, and this now better reflects physiologic calcium, with a range between 1.2 and 1.8 mM.
The two main limitations of the buffer are the glucose concentration and the myocardium's preference for using fatty acids as a substrate over glucose in normoxic conditions. However, the use of fatty acids within the perfusate provides a difficult challenge, as the resultant solution would froth up and clog the flow through the apparatus as a result of the 5% CO_2 that is bubbled through it. This makes the use of fatty acids within a perfusate unfeasible. Glucose is the second-best option as it easily dissolves into the perfusate and can be utilised by the cardiomyocytes for ATP production. Unfortunately, the second issue is that the concentration of glucose required within the perfusate is at supra-physiological levels. With the increasing awareness of the effect of poor glucose control on mortality in patients who suffer ST elevation infarction, there is a greater understanding to maintain as close to physiological levels of glucose. However, the high glucose concentration is needed to help overcome the lack of free fatty acids within the perfusate and is, unfortunately, a "necessary evil" in order to allow the heart to function as close to normal as possible.

Regular maintenance

A common reason for experimental failure is not taking the importance of regular cleaning of the apparatus seriously. It is easy to overlook clean apparatus; however, the presence of endotoxins within the tubing and glass of the apparatus can significantly impair the function of the myocardium. The perfusate buffer is rich in glucose and, coupled with the bacteria-philic temperature of the water jacket, the Langendorff apparatus is an ideal site for bacterial growth. To minimise the effects of endotoxins on the experiment, it is vitally important to practise regular cleaning. The entire column and tubing are washed in boiling (at least 80°C) water every morning before commencement of the experiment and at the end of the day after the experiment has concluded. Every two to three weeks, the entire column is washed in a 10% hydrochloric acid wash, which also helps clear calcium deposits. The strict maintenance of this cleaning regimen is of paramount importance, and without endotoxin lead, myocardial damage can interfere with heart performance and function.

Ventricular pacing

The denervated ex vivo rat heart lacks the neuro-hormonal control of normal heart rate, and this method of isolating the rat heart also severs the blood supply to the SA node. As a result, the Langendorff heart can be very bradycardic (HR 200–300 in comparison to a normal rat heart (500–600). The difficulty with a bradycardic heart that lacks neuro-hormonal control is the erratic nature of the heartbeat, which in turn

causes erratic temperature control, flow rate, and develops pressures of the left ventricle. There is also the risk of bradycardia-related arrhythmia, which would impair perfusion of the heart and potentially exaggerate infarct size. It is possible to conduct successful Langendorff in this state, but one method to avoid these possible complications is the use of an external ventricular pacing device. This can be achieved relatively easily by purchasing a commercially available pacing box (or building one oneself). Once the heart is mounted on the cannula, a stainless steel needle can be placed through the right atrium into or close to the septal wall, with a crocodile clip acting as the ground attached to any metallic part of the Langendorff rig. In our experiments, we opted to use this technique to pace the rat heart at 400 BPM, with an RR interval of 10m/s and a voltage output between 1–5 mV. Just as with the Langendorff apparatus, it is vitally important to clean the pacing wires and box twice a day with alcohol. The perfusate buffer that is in frequent contact with the pacing wires and the electrical charge running through the system means the pacing wires rapidly oxidise and rust, which results in increased impedance in the system and ultimately, failure to pace the ventricle. The pacing wires were thus cleaned and stored in ethanol at the end of the experiment and washed in distilled water before the commencement of each experiment.

Exclusion criteria

Reproducibility of infarct size is a crucial part of establishing a functioning and reliable experimental model. Through careful adherence to the techniques and repeated practice, an operator can establish a reproducible infarct size that can be

used to compare a treatment to establish whether a treatment is cytotoxic or cardioprotective. An important step in maintaining this reproducibility is establishing a set of exclusion criteria for the heart during the stabilisation period. These criteria ensure that the quality of the ex vivo perfused heart is stable enough to cope with the stresses of the ischaemia reperfusion injury. In our lab, we have a well-established set of exclusion criteria to minimise the time the heart spends out of the rat's body and to identify whether the process of cannulation has unduly damaged the heart. An example of this is an excessively high coronary flow rate, which may be a reflection of rupture of the ventricular wall, or a low coronary flow rate, which may be a result of the cannula causing trauma to the ostium of the aorta. Both these complications would result in inaccuracies throughout the experiment and ultimately compound the final result. Thus, the use of exclusion criteria is also a quality control check that ensures the heart is as healthy as possible prior to the commencement of an intervention. (You may be asked to add both the inclusion & exclusion info)

Positive control

An important part of any experimental model is the existence of a therapy that can be used as a positive control. The positive control can be used as an assessment tool to ensure that the experimental model is functioning well, and it can also be used to compare it to the effectiveness of any potential treatment. The most well-known positive control in the field of ischaemia -reperfusion injury of the myocardium

is ischaemic preconditioning. It has long been established in both in vivo and ex vivo settings that exposing the myocardium to brief periods of ischaemia and reperfusion prior to the onset of prolonged ischaemia and reperfusion results in a significantly smaller infarction size(123). This positive effect of ischaemic preconditioning is termed cardioprotection and is a widely used tool or benchmark with which to compare cardioprotective strategies. Murray et al. first identified that four 5 min cycles of alternating occlusion and reflow of the left anterior descending (LAD) coronary artery of a canine heart prior to the onset of 90 min of occlusion and three days of reperfusion resulted in a 75% reduction in the size of the infarct(124, 125). There remains no single unifying theory as to how IPC protects cardiomyocytes from cell death, but it is thought to be related to the activation of a number of cell signalling pathways such as the reperfusion injury salavage kinase (RISK) pathway. Add a general review ref

This technique has been successfully utilised in the Langendorff model to provide a method to corroborate cardioprotective strategies. Once the heart has been cannulated to the perfusion cannula and is stabilised, one can interrupt the flow of the perfusate to the heart by turning off the cannula for periods of five min, followed by five min of reperfusion. This cycle is repeated three times, after which the heart is immediately subjected to a prolonged period (35 mins) of ischaemia followed by two hours of reperfusion. An important part of being able to learn and establish the Langendorff model is mastering the use of IPC to reduce the size of the infarct after 35 minutes ischaemia and 2 hours of reperfusion.

Infarct size analysis

A crucial part of the experiment is being able to identify the necrotic area within the heart after ischaemia- reperfusion injury. Figure This is achieved by staining the infarcted heart with a TTC stain; the method of doing this is outlined clearly in the methodology section. Triphenyl tetrazolium chloride (TTC) is used to differentiate between metabolically active and inactive tissue. In living tissue, the white compound is degraded into a red staining compound called 1,3,5-triphenylformazan. The degradation is a redox reaction and occurs when TTC comes into contact with lactate dehydrogenase and other enzymes that play an important role in cellular oxidisation. Thus, when TTC comes into contact with living cells, it is converted to 1,3,5-triphenyl formazan and stains the tissue red; however, where there is cell necrosis and an absence of dehydrogenase enzymes, the tissue is stained white. This technique is widely used in biology to characterise living tissue.
Cardiomyocyte isolation. See figure 3.2

Another experimental model I used widely in my PhD research is the rat cardiomyocyte isolation and culture. The methods behind isolating the cardiomyocytes are discussed in detail in the method section. However, the technique behind the method is heavily reliant on retrograde ex vivo organ perfusion. An ex vivo rat heart needs to be digested in a safe and controlled manner to separate the myocardium into its cells, cardiomyocytes, without killing or damaging the cardiomyocytes themselves. To achieve this, we have to simultaneously perfuse the heart with nutrients and oxygen, but at the same time ensure a suitable

collagenase is rapidly and efficiently distributed throughout the heart to dissolve the extracellular matrix that binds cardiomyocytes together. Collagenase is an enzyme that breaks the peptide bonds in collagen, a crucial component of connective tissue, and in doing so, degrades the rat heart into individual cells that can be collected. The most efficient way to achieve this is via the use of the Langendorff perfusion technique with a constant flow mode. Digestion with collagenase will invariably impair the function of the myocardium, reducing the flow of the collagenase and perfusate buffer to the cardiomyocyte. As such, a constant flow device is needed to help maintain the flow of the perfusate despite the deteriorating heart.

Another important consideration is the contents of the buffer that is used to perfuse the myocardium. The heart will need to be perfused with three or four different buffers with varying levels of calcium to ensure the effective function of the collagenase. Initially, the heart is perfused with a calcium-containing buffer to stabilise the myocardium, after which a calcium-free buffer is utilised to arrest the normal contractions within the myocardium. It is only after this that the heart is perfused with a buffer containing collagenase in order to digest the extracellular matrix. A detailed description of this can be found in the Methods section.

Once isolated and collected, the isolated cardiomyocytes can be plated in an appropriate medium for further study.

Figure 3.1. A diagram of the Langendorff apparatus (adapted with permission from Bell et al)

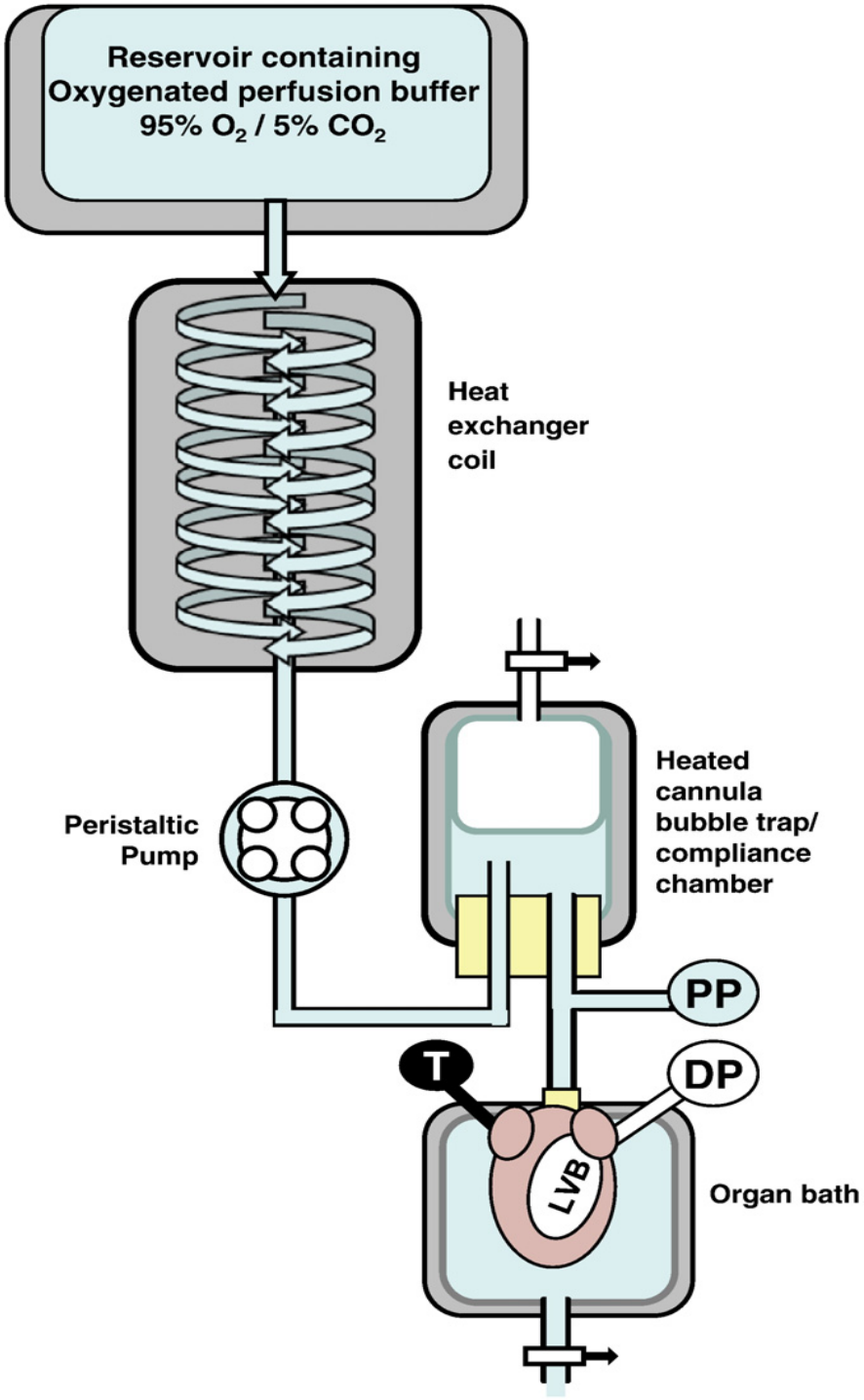
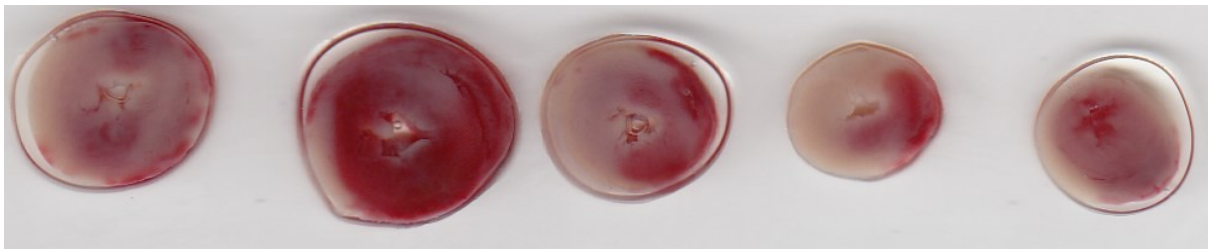


Figure 3.2 – Demonstrates histological analysis of Rat Hearts subjected to 30mins of global ischemia followed by TTC staining.

The stained segments demonstrate viable myocardial cells. The unstained segments demonstrate necrotic tissue of the infarct size.



Chapter 4 – Materials and Methods

General animal usage

All the animals used were treated in accordance with the United Kingdom (Scientific Procedures) Act of 1986. All the animals used throughout were male Sprague-Dawley (SD) rats weighing 300-400g, obtained from a central animal unit within UCL.

mCBS

Methyl 2,2',3,3',4',6,6’-hepta-O-sulfonato-β-cellobioside heptasodium salt was acquired from Sirtex Medical Ltd and stored as a salt at -20°C.

What about HIPe?

Rat handling and care

All the rats were housed in an environment where they were fed, well looked after and familiarised with handling. They were taken from the animal-handling unit on the day of the experiment. The rats were humanely restrained in a plastic cone and subjected to an intraperitoneal injection of 90mg/kg of 20% pentobarbitone. The rats were left with water in a small cage while the drug took effect. The operator made regular checks on their respiratory rate and general alertness. The animals were deemed dead once we were unable to elicit hindlimb and corneal reflexes. The chest cavity was opened via a clamshell thoracotomy, and the heart was harvested via an incision to the aorta. The heart was immediately placed in ice-cold Krebs–Henseleit buffer (KHB).

Krebs–Henseleit buffer (KHB)

KHB was prepared fresh every day, mixing a solution with 118 mM NaCl, 25 mM $NaHCO_3$, 11 mM d-glucose, 4.7 mM KCl, 1.22 mM MgSO4.7H2O, 1.21 mM KH2PO4, and 1.84 mM CaCl2.2H2O.

Ex vivo Langendorff retrograde perfusion of the heart

Once harvested, the isolated rat hearts are transferred to the Langendorff retrograde perfusion apparatus. The method of this experiment is based on the Langendorff methodology previously described by Bell et al(123).

The principles of the technique are based on retrograde perfusion of the coronary arteries. The heart is attached to a cannula via the aorta, and a blood substitute is passed through the cannula into the aorta. The perfusate flows at a constant pressure and is forced into the coronary arteries of the heart, allowing the perfusate to flow into the tissue of the myocardium. This provides a medium for the cardiomyocytes to survive and contract in an environment that simulates normal physiology. The Langendorff apparatus holds the perfusate above the cannulae and flows into the aorta via gravity. By maintaining a constant supply of perfusate into the Langendorff apparatus, gravity ensures that the perfusate flows at a constant pressure of 60–80mmhg in order to replicate the pressure that would be experienced by the aorta in vivo.

The rat heart and the aorta are identified, and the aorta is then placed onto a cannula and held in place using two 3–0 calibre cotton sutures. The cannula is attached to the Langendorff apparatus, which contains KHB solution. Once securely

in place, the tap is switched and a KHB solution aerated with oxygen is passed through the Langendorff apparatus into the heart. This step would be timed to ensure the heart is attached to the rig less than two minutes after severing the aorta.

Once in place, a temperature probe was inserted into the pulmonary artery to ensure the temperature was maintained between 36.5–37.5°C. Second, an incision was made to remove the left atrial appendage. Through the incision, a balloon was passed into the left ventricle and inflated until LVEDP 5–20 mmHg. From this balloon, we can calculate LV developed pressure. Third, a small incision was made in the right atrium and a pacing probe was inserted into the right atrium. The heart was paced at 360–380 BPM throughout stabilisation but not ischaemia. To calculate the flow rate of perfusate through the myocardium, a small boat was placed under the heart for 30 seconds. The resultant collection was measured and then doubled to calculate the coronary flow rate (CFR) in ml/minute. To ascertain histone release, perfusate from the beating heart was collected prior to ischaemia and then at the point of reperfusion. They were immediately frozen for assessment at a later date.

Following the successful placement of the heart and the placement of the monitoring equipment, there is a ten-minute pre-stabilisation phase in which nothing is done to the heart in order to allow the heart to recover from the insult of the procedure.

The next stage is 30 minutes of stabilisation, where the heart is monitored for 30 minutes, with observations recorded every ten minutes.

This is followed by a period of ischaemia, which, depending on the protocol, may be 35 or 45 minutes.

Finally, the heart undergoes reperfusion after the period ischaemia for two hours.

Regional ischaemia

To stimulate regional ischaemia and reperfusion, part of the myocardium needs exclusion from perfusion for a short period. A 3–0 suture was placed through the thickness of the heart around the origin of the left anterior descending coronary artery. To stimulate ischaemia, this suture was tightened to occlude the flow of perfusate to the anterior wall of the LV. To stimulate reperfusion, the suture was then realised to allow perfusate to travel through the ischaemic section. The heart was not paced during ischaemia but was paced at 360–380BPM during the reperfusion stage.

Global ischaemia

To stimulate global ischaemia and reperfusion, the entire myocardium needs to have a controlled period where myocardial perfusion is halted. To achieve this, the flow of perfusate into the myocardium is halted. This stimulates ischaemia in the entirety of the myocardium. To maintain an adequate temperature in the heart, the heart needs to be placed within a water bath where the temperature can be controlled to keep it constant 36.5–37.5°C. During reperfusion, the myocardium is extremely pro-arrhythmogenic, and as such, the heart is not paced. The size of the infarct also means the heart may not return to sinus rhythm, so arrhythmias greater than three minutes are accepted and do not form part of the exclusion criteria.

Assessment of infarct size

At the end of the period of reperfusion, the heart was analysed using two separate methods depending on whether it was subjected to global ischaemia or regional ischaemia. Hearts that underwent regional ischaemia were immediately injected with 1–2 ml Evans blue dye 0.25% in aqueous solution after tying off the suture around the left coronary artery, staining the myocardium in the areas not supplied by the occluded LCA. Excess dye was wiped and gently washed off, and the heart was removed from the cannula and weighed. It was then wrapped to stop dehydration of the external surface and frozen for 15 minutes at -80°C. However, in the global ischaemia model, hearts were not injected with Evans blue and instead immediately frozen.

Once the heart was frozen, it was dissected into five slices approximately 2 mm in width from the basal part up to the apex. The resultant slices of the heart were incubated in 0.1% 2,3,5-triphenyl tetrazolium chloride (TTC) in an aqueous solution for 15 min at 38°C. TTC is a redox indicator, and in solution reacts with dehydrogenases present only in viable cells. The resultant reaction stains the viable myocardium red, leaving the infarcted or necrotic area a contrasting white. In the regional ischaemia model, the heart also has the third blue colour to define the area of the heart that was perfused throughout ischaemia and represents the healthy tissue outside the infarct zone.

The three colours were then intensified by incubation for a minimum of one hour at room temperature in 10% neutral buffered formalin.

Following this, slices were compressed gently en face between two glass plates, and

their basal surfaces were scanned into a digital bitmap file at 600 dots per inch using a flatbed scanner.

Once scanned, the images were analysed using Image J software, the different regions were measured, and the total size of the infarct was calculated and represented as a percentage. In regional ischaemia, this was represented as a percentage of the infarct size to ischaemic zone, while in the global ischaemia heart, this was calculated as a percentage and labelled as the infarct size (because the whole heart is the ischaemic zone).

Using mCBS with ex vivo Langendorff retrograde perfusion of the heart

Isolated rat hearts were perfused on a Langendorff apparatus, as previously described. Hearts were randomly allocated to receive either KHB as a control or mCBS dissolved in KHB at a concentration of 100μg/ml as the treatment arm. Hearts in the treatment arm were initially perfused with standard KHB, but five minutes before the onset of ischaemia, the hearts were switched to mCBS and then perfused with mCBS throughout ischaemia and reperfusion. The hearts were then removed and analysed, as previously described.

Calculating dose of mCBS

Using data from the Sirtex laboratory, we were able to calculate an appropriate dose of mCBS to perfuse the Langendorff model. In their 14-day IV infusion rat study, over the dose range 300 to 3000 mg/kg/day, the mCBS plasma steady-state concentrations were reached within five hours of infusion, and the mean ranged from

20.9 to 242 μg/mL. After the end of infusion, the mean mCBS plasma concentrations declined rapidly at a mean estimated t1⁄2 value ranging from 0.653 to 0.742 hr. Furthermore, using a rabbit lung injury model, Sirtex was able to show that 99mTc-radiolabelled nanoparticles were coated with histone proteins, which rapidly accumulated in the lungs of the rabbit after IV injection. The dose of 100 mg/kg mCBS.Na reduced accumulation in the lung by 66%, and 50 mg/kg reduced levels by 44% relative to the negative controls. Thus, we estimated that achieving a dose of 100ug/ml in a rat in vivo was easily possible given the pharmacology data coupled with the demonstration in a lung model that such a dose was effective in inhibiting the accumulation of histone. We decided that a continuous perfusion of 100ug/ml through the Langendorff apparatus was an effective dose to use.

Collection of perfusate

Through the Langendorff experiment, coronary effluent is continuously discharged from the rat heart. Samples of this effluent were collected during stabilisation, immediately at the point of reperfusion and after 1 minute, 5 minutes, 10 minutes, and 30 minutes of reperfusion. The samples were immediately frozen at -80°C for analysis at a later date.

Detection of histones

Perfusate from the Langendorff experiment was analysed for the presence of DNA material, including histones, with a commercially available ELIZA assay (Sigma-Aldrich). The perfusate was allowed to thaw back to liquid at room temperature. The

ELIZA plate, which was pre-seeded with anti-histone antibody, was aliquoted with 20μL of perfusate, and a secondary antibody to DNA was added to the sample as well a streptavidin-based fluorescent marker. After two hours of incubation followed by multiple washes, the level of fluorescence was analysed with a plate reader at 450 nM wavelength.

DELFIA technique to quantify the concentration of histone H4

DELFIA® (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay) time-resolved fluorescence technology (TRF) is a wash-based assay similar to ELISA, but with a wider dynamic range that makes it ideal to capture smaller and more obscure molecules such as histone H4.

In a DELFIA immunoassay, the primary antibody is added to a microplate, giving you much more flexibility. To start with, 5–10 µl of each perfusate sample was diluted to 100 µl in PBS, added to high-binding ELISA plates, and then incubated overnight at 4°C. The plates were washed three times with DELFIA wash buffer (PerkinElmer, Cambridge, UK). The wells were blocked with 100 µl 1% BSA in PBS for 1 h at room temperature and then washed three times. Primary antibodies (CD81 clone JS-81; HSP70 clone N27F3–4) were added at 1 µg / ml and plates incubated for 2 h at room temperature. After washing three times, goat anti-rabbit IgG or goat anti-mouse IgG1 was added (1:2000 in blocking buffer) and incubated for 1 h at room temperature. Plates were washed three times, and 1:1000 streptavidin–europium conjugate in DELFIA Assay Buffer (PerkinElmer) was added and incubated for 1 h. The plate was washed six times and 100 µl of the DELFIA Enhancement Solution

was added to the plate, which releases the europium from its chelate, allowing the formation of a new, highly fluorescent chelate in solution. Finally, the plate was shaken for 2 × 5 min on the plate reader. Time-resolved fluorimetry was performed using a Pherastar plate reader (BMG Labtech), with excitation of 337 nm, detection at 620 nm, integration time set at 200 μs, and lag time of 60 μs.

A plate reader illuminates the sample using a specific wavelength as a result of the illumination, the sample fluoresces, and a second optical system collects the emitted light, separates it from the excitation light (using a filter or monochromator system), and measures the signal using a photomultiplier tube.

The amount of analyte is proportional to the emission signal, which can be quantified by interpolation from a standard curve. This allows us to quantify the amount of histone H4 released within the perfusate of the myocardium.

Isolation of primary adult rat ventricular cardiomyocytes

A buffer was prepared by creating a solution containing 130mM NaCl, 5.4mM KCl, 1.4mM $MgCl_2$, 0.4mM Na_2HPO_4, 4.2mM HEPES, 10mM glucose, 20mM taurine and 10 mM creatine. The pH was stabilised at 7.4 and the buffer was stored at 37°C. Rats were obtained, anesthetised, and the heart removed using the technique previously described. The aorta of the heart was attached via a cannula to a Langendorff rig, and the heart was first perfused with buffer containing 750μM $CaCl_2$. This step allows the blood and debris to wash out. The heart was then digested by perfusing the heart through the cannula with a buffer containing 0.06% collagenase and 0.01% protease with 100μM $CaCl_2$. The ventricle of the heart was then severed and mechanically broken down using scissors into smaller segments. The resultant

solution was incubated in an agitator for 15 minutes to allow further digestion of the tissue. The solution was then sieved through a mesh to remove excess debris, and the cardiomyocytes were collected with low-speed centrifugation. The resultant cardiomyocyte solution was then gradually reintroduced with calcium through buffer washes with 500μM $CaCl_2$ and then 1mM $CaCl_2$ before being resuspended in Medium 199 (ThermoFisher) supplemented with 5 mM creatine, 2 mM carnitine, 5 mM taurine (Acros), 50 units/ml penicillin and 50 μg/ml streptomycin. ARVCs were seeded in 96 well plates on areas preincubated for at least one hour with 20-40 μg/ml laminin to facilitate cell adherence. Cardiomyocytes were stabilised overnight in a conventional tissue culture incubator at 37°C and 5% CO_2 before use for experiments.

Rat adult cardiomyocytes and histone co-cultures

Isolated rat cardiomyocytes were prepared using the technique described above and seeded onto a 24-well microplate. Calf thymus-derived histone was purchased (Sigma-Aldrich) and dissolved in phosphate buffer solution. The microplates were treated with a control and increasing doses of histones and left to incubate for one hour at 37°C and 5% CO_2. After the period of incubation, the wells were treated with propidium iodide (PI). PI is a fluorescent intercalating agent that binds to intact DNA and fluoresces red; it is not membrane-permeable and so will bind to necrotic or apoptotic cells, providing a marker of cell death.

Assessing cell death using propidium iodide

2μL/ml of PI (1mg/ml) solution was added to each well at the end of the incubation time, and each laminin-coated field was examined under a fluorescent microscope. The cells were left to incubate in PI for a further 15 minutes, after which they were visualised under a microscope. Two images in each group were acquired on a Leica TCS SP5 confocal microscope using 40x (MCECs) and 63x (cardiomyocytes) magnification objectives and 543 nm (20%) and 405 nm (9%) lasers.
To estimate cell death, each photograph was anonymised by assigning a number according to a randomised list generated for each experiment. The anonymised photographs were analysed using ImageJ software. Dead cells were defined as those that showed evidence of uptake of PI, which was measured as the membrane fluorescence intensity for cardiomyocytes.

Assessing cell death using an LDH assay

The Thermo Scientific Pierce™ LDH cytotoxicity assay kit was used to quantify cellular cytotoxicity. Cell death releases LDH into the cell culture medium, and extracellular LDH in the media can be quantified by a coupled enzymatic reaction in which LDH catalyses the conversion of lactate to pyruvate via NAD + reduction to NADH. Diaphorase then uses NADH to reduce a tetrazolium salt (INT) to a red formazan product that can be measured at 490 nm.
Cardiomyocyte cells were cultured as described above. After incubating with the desired therapies, the culture wells had 20 ul of medium extracted from the well. The medium was transferred to a new plate and mixed with reaction mixture. After a 30-minute room temperature incubation, reactions were stopped by adding stop solution. Absorbance at 490 nm and 680 nm was measured using a plate-reading

spectrophotometer to determine LDH activity as described above.

Treatment with mCBS

Isolated rat cardiomyocytes were exposed to histones as well as either control, 25 μg/ml or 100 μg/ml concentration of mCBS. Confidential data from the manufacturers of mCBS (Figure 4.1, Sirtex) showed in an in vivo cell culture model doses of 100 μg/ml of mCBS was sufficient to protect against the cytotoxic effects of histones, which is why we used an equivalent dose. When cultured HMECs are exposed to histones (400 μg/mL), 37% remain viable, whereas 56% take up PI. When mCBS.Na, 100 ug/ml was added prior to histone exposure, the toxic effects of histones on the viability of HMECs were reversed, with 74% remaining viable and 20% dead based on PI uptake. This protective effect is concentration dependent, with a lower amount of mCBS (25 μg/mL) providing a reduced level of protection. This data from Sirtex Laboratory was used to help estimate the doses required for the cell culture studies.

Figure 4.1 – Cultured HMECs in the presence of extracellular histones.

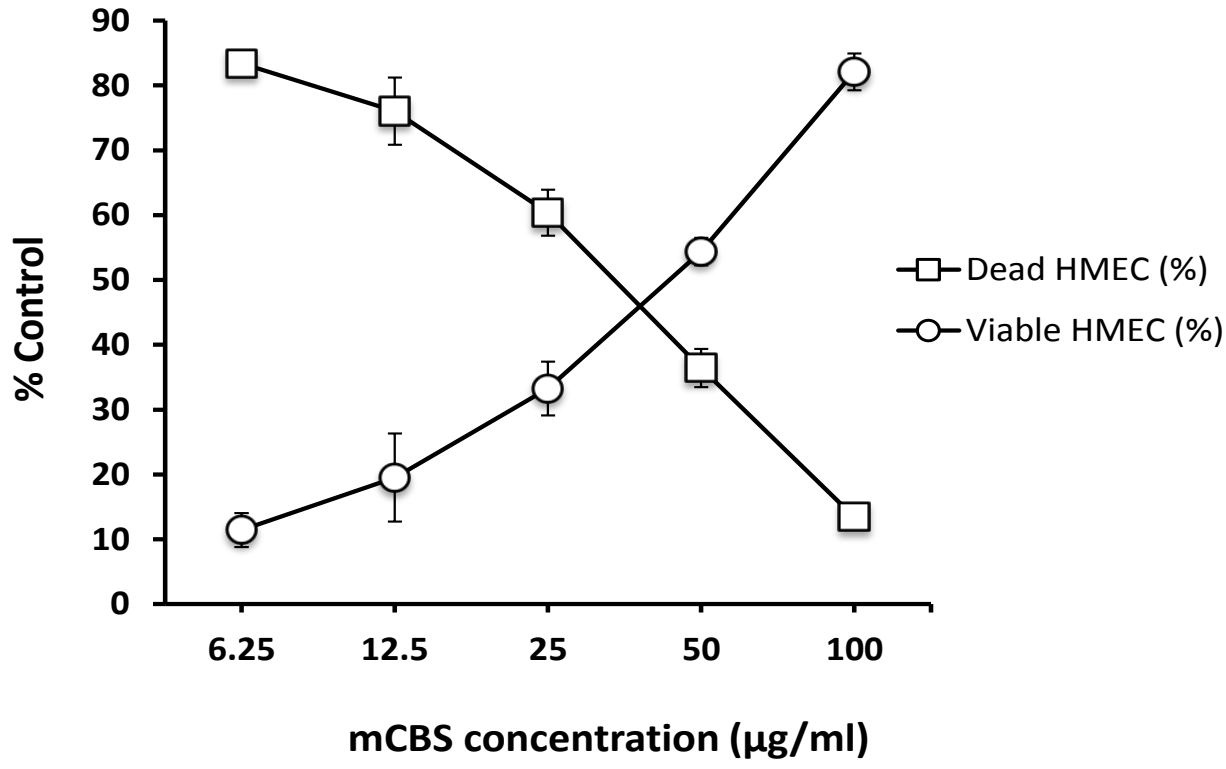


Figure 4.1. Cultured HMECs were exposed to 400 µg/mL of histones for 60 min, followed by treatment with mCBS.Na (100 µg/mL) for ten min. Calcein-AM and PI were added for the last five min, and the extent of uptake was analysed using flow cytometry. Approximately 30% of HMECs reverted from PI uptake to PI exclusion and calcein-AM uptake following treatment with mCBS.

Treatment with HIPe

Silvestre-Roig et al. was the first publication to demonstrate the theory that histone H4 interacts with the cell membrane to induce a permeable pore in the wall of the cell, causing cell death. Using molecular dynamic studies, they demonstrated that this effect was caused by the interaction of the N terminus of the histone H4 molecule. They then screened for peptides that interacted with the N terminus of the histone molecule. The cyclical peptide HIPe (histone inhibitory peptide) showed a potential ability to efficiently disturb histone H4 membrane interactions; they further proved HIPe's ability to achieve this effect using atomic force microscopy and conventional confocal microscopy demonstrating the ability of the peptide to disrupt the histone H4 membrane interaction. Furthermore, they demonstrated that inhibition of histone H4 cytotoxicity in vascular smooth muscle cells was achieved with recombinant histone H4 when it was incubated with 100 μg/ml of peptide HIPe for one hour before addition to murine SMCs. We used the same dose for our experiments using isolated rat cardiomyocytes.

Treatment with TLR antagonists

TAK-242 (Resatorvid), a small-molecule-specific inhibitor of Toll-like receptor 4 (TLR4) signalling, inhibits the production of LPS-induced inflammatory cytokine release by binding to the intracellular domain of TLR4 (126). It has been shown that TAK-242 inhibits the Toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP) or Toll/interleukin-1 receptor domain-containing adaptor protein-inducing interferon-β-related adaptor molecule (TRAM) in human embryonic kidney (HEK)

293 cells(127). It is an effective treatment for inhibiting TLR4-mediated inflammation in a host of different cell lines and animal models, as well as attenuating the post-inflammatory response of ischaemia reperfusion injury of the myocardium(126, 128-130).

Statistical analysis

The sample size is stated in the figure legends, and data are plotted as single values or means ± SEM. Statistical analyses were performed using Student's t-test, 1-way or 2-way ANOVA with posthoc tests. Pearson's or Spearman's correlation tests were performed, where indicated after a Kolmogorov-Smirnov test for normality. GraphPad Prism 5.0 was used for statistical analyses and graph production (GraphPad Software). A p-value of <0.05 was considered significant.

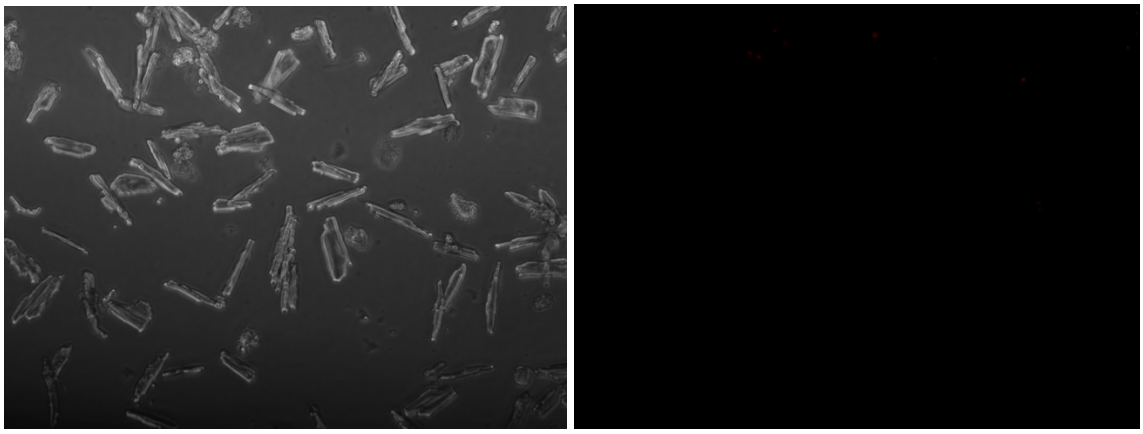
Chapter 5 – Results of the Investigation into the effects of histones on isolated rat cardiomyocytes

Extracellular histones have been detected in several different pathological processes ranging from sepsis to atherosclerosis. Histones in conjugation with NETs have also been detected in the thrombus burden of patients suffering from ST elevation MI. During the process of necrosis or pyroptosis, DNA fragmentation will occur, and theoretically, DNA fragments, including histones, will be released into the extracellular matrix. It has been shown that histones are cytotoxic to human endothelial cells(110, 131), kidney cells and pulmonary cells. However, histones have not been shown to be cytotoxic to rat cardiomyocytes. The first step in our hypothesis was to ascertain the effect of histones on isolated rat cardiomyocytes.

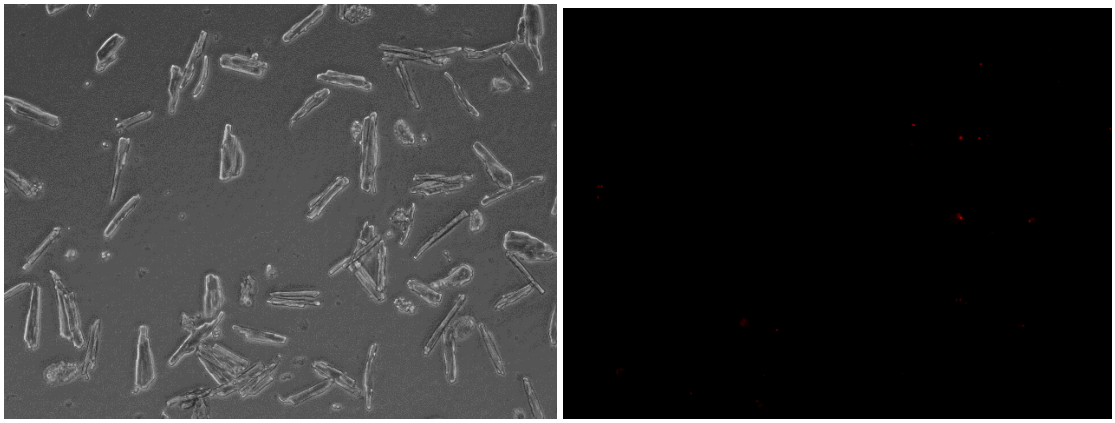
Isolated rat cardiomyocytes were incubated in the presence of increasing doses of histones. The greater the concentration of histones in the medium, the greater the degree of cytotoxicity of cardiomyocytes. At concentrations greater than 50 μg/ml histones caused 100% cell death. Death was calculated by PI staining as well as counting the number of viable, rod-shaped cells

Histone is provided in a lyophilised form, and putting it into a solution required a discussion with Fisher Pharmaceuticals, who advised us to dissolve the protein in phosphate buffer saline at a concentration of 1 ml per 1 mg and use aggressive agitation to dissolve the compound. This method was successful, with aggressive agitation and the use of a sonification device.

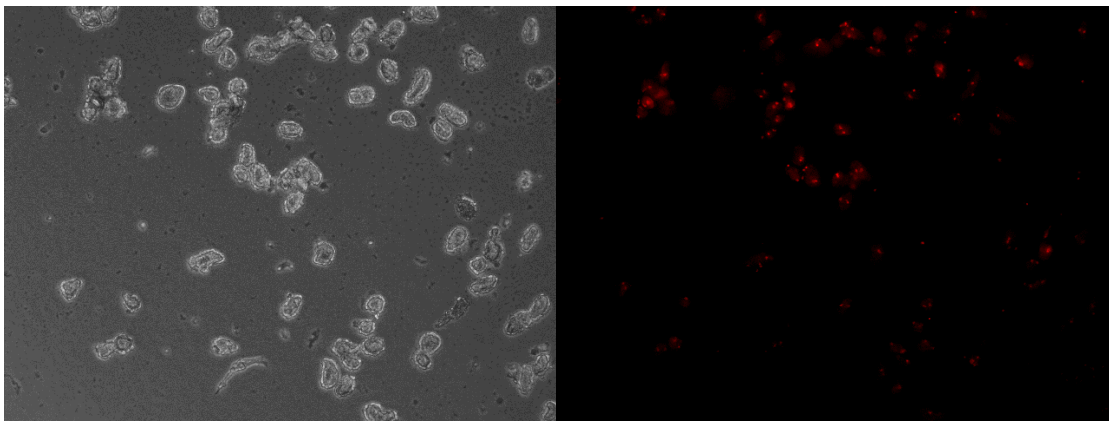
Figure 5.1 – Isolated Rat Cardiomyocytes cultured in the presence of extracellular histones



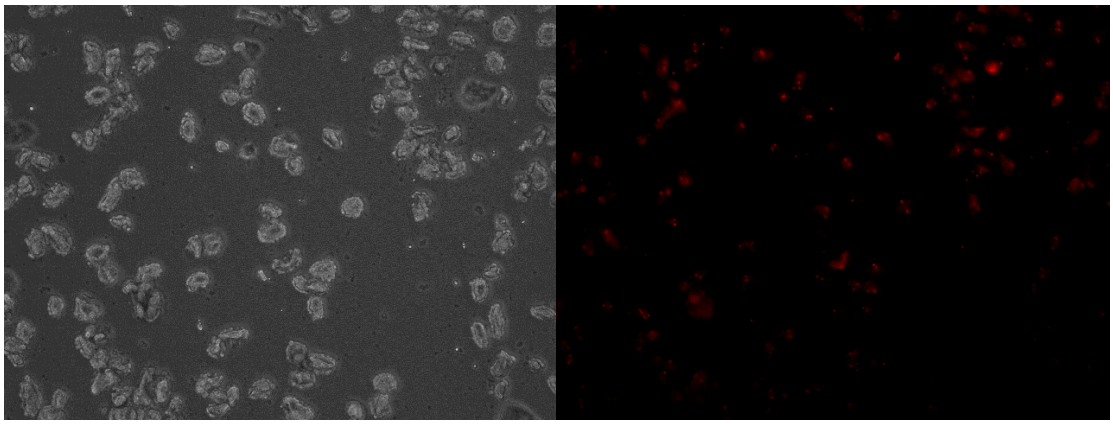
Control—Rat cardiomyocytes incubated in a buffer.



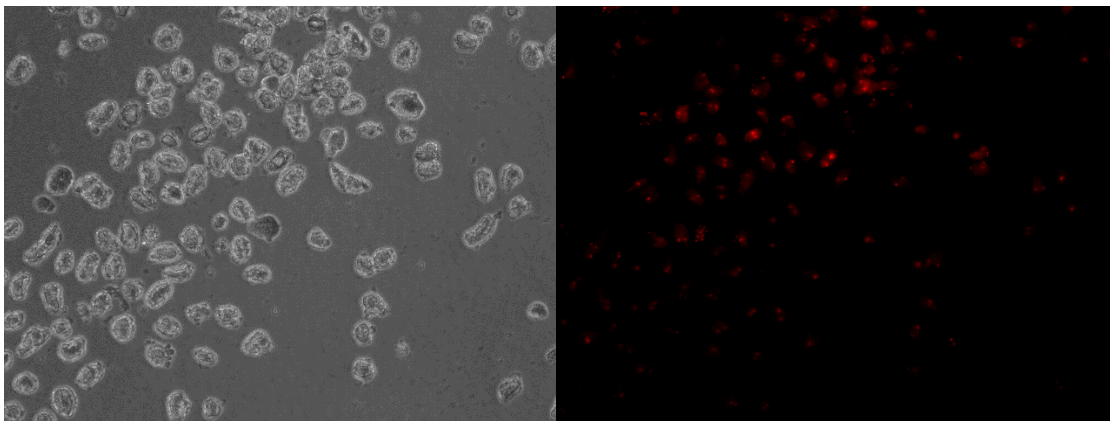
—isolated rat cardiomyocytes incubated with 1 μg/ml concentration of histones.



50μg/ml of histones – isolated rat cardiomyocytes incubated with 50μg/ml of Histones



100μg/ml of histones – isolated rat cardiomyocytes incubated with 100μg/ml of Histones



200μg/ml of histones – isolated rat cardiomyocytes incubated with 200μg/ml of Histones

Figure 5.1 Histones incubated in the presence of isolated rat cardiomyocytes. Shown side by side is the Standard Microscopic image of the cardiomyocytes followed by Flurosence Microscopy after the same cells after being stained with Propidium Iodide. In the control group where no histones were added, there was little (18%±3.7%) detectable cell death. At 1μg/ml there was some detectable (31%±11%), however at 50μg/ml and greater doses, there was extensive cell death at (>95%).

Figure 5.2. - Isolated Rat Cardiomyocytes are exposed to increasing concentrations fo histones.

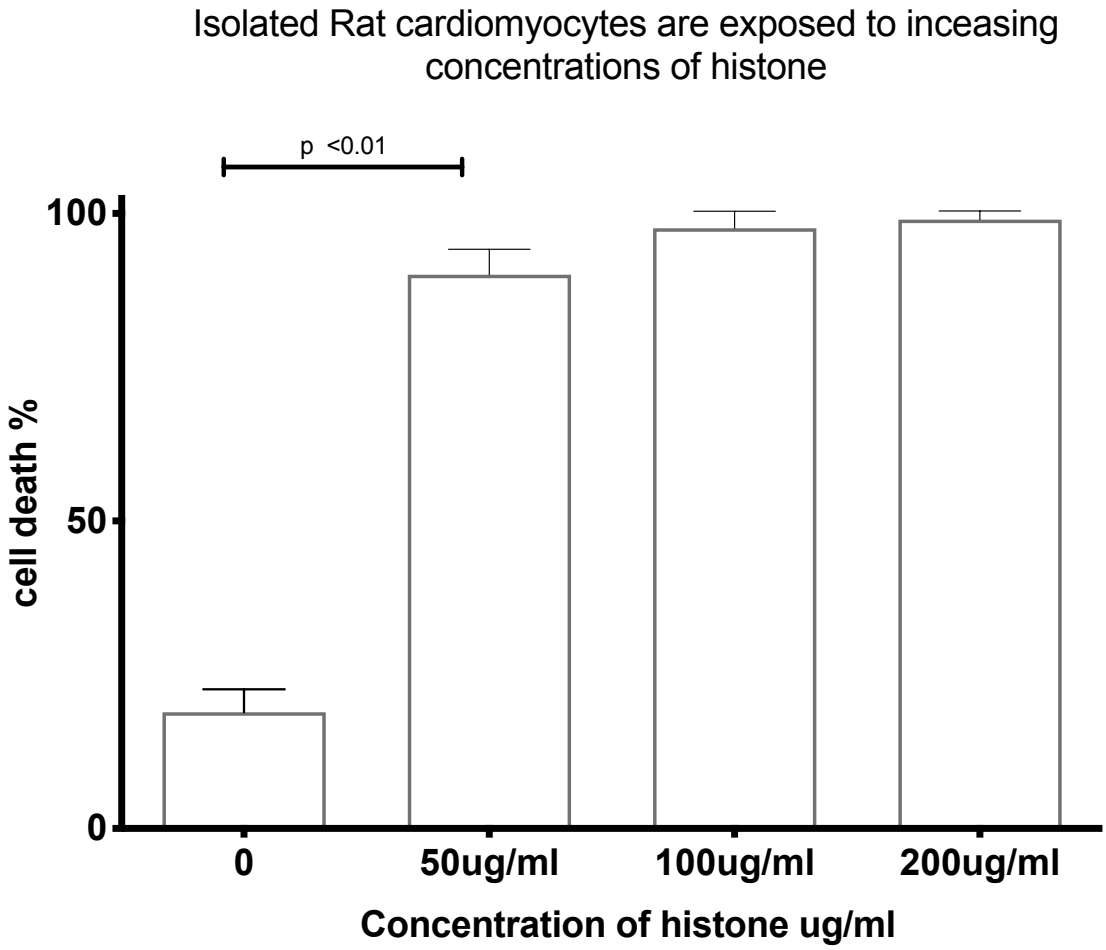


Figure 5.2 Isolated rat cardiomyocytes were incubated in the presence of increasing concentrations of extracellular histones. Cells were then labelled with PI, and using a cell counter, the incubation wells were visualised through a microscope. The percentage of cell death was estimated. There was a significant increase in the number of dead or dying cardiomyocytes when the cells were exposed to 50 ug/ml histone in comparison to the control. At 100 ug/ml and 200 ug/ml there was near to 100% cell death ($p<0.01$ when compared to control)

Discussion

Extracellular histones are cytotoxic to hepatic cells(118, 132), alveolar cells(67), kidney cells, and smooth muscle cells(68) in both in vitro and in vivo animal and cellular models. The levels of circulating histones in human patients with sepsis positively correlate with the degree of illness and are associated with the onset of toxic cardiomyopathy. Our experiments are the first to demonstrate in an in vitro cellular model that histones are directly cytotoxic to cardiomyocytes. Indeed, patients with severe sepsis have been shown to circulate levels of extracellular histones over 50 ug/ml(133) and in a mouse heart, a concentration of 5 ug/ml of histones is enough to induce cardiac arrhythmias and depressed LV function(96). This is an important first step in our hypothesis, as cellular or necrotic death as seen in ischaemia -reperfusion injury is associated with increased levels of extracellular histones. Furthermore, clots aspirated from patients who have suffered a STEMI have demonstrated high concentrations of extracellular histones in the form of NETS. This demonstrates the potential of identifying a new cytotoxic protein that is theoretically released in abundance during the process of ischaemia- reperfusion injury of the myocardium. The identification of such an element may pave the way for a potential target in limiting the excessive cardiomyocyte death that occurs in the myocardium after an infarction.

The current model has taught us that any concentration of histone above 50 ug/ml results in close to 100% cellular death. The next investigation determined the lowest feasible in vitro dose of histone that is still cytotoxic. Given that human patients with florid sepsis have circulating extracellular histone concentrations of between 20-70

ug/ml (133) we trialled the effects of lower doses of extracellular histones on cardiomyocytes to establish a dose response and also show whether pathological levels of extracellular histones are cytotoxic. Alternatively, it is important to state that during the pathological event of ischaemia -reperfusion injury of the myocardium, disorganised cell death processes like pyroptosis and necroptosis would theoretically be a major source of extracellular histones. As such, the interaction of extracellular histones with cardiomyocytes would be at a theoretical concentration that is far greater than the doses we have used. However, we aimed to establish a model of histone cytotoxicity that we can then experiment with using histone neutralising agents, so we decided to see if we could establish a dose-related response.

Chapter 6 – Results, does histone-induced cardiotoxicity occur at pathological doses, and can mCBS in vitro inhibit the cytotoxic effects of histones on isolated rat cardiomyocytes?

Isolated rat cardiomyocytes were incubated with lower doses of histones, 10μg/ml, 20μg/ml and 40μg/ml concentrations. The rationale for using lower doses is discussed in the previous chapter. The cells were also incubated with a vehicle (cell culture medium) with a low dose of mCBS (25 μg/ml) and a high dose of mCBS (100 μg/ml). mCBS protected rat cardiomyocytes from the cytotoxic effects of Histones in a dose-dependent manner. These doses were chosen based on confidential data released from Sirtex Laboratory that demonstrated the mCBS at 100 ug/ml was effective at protecting human endothelial cells from the cytotoxic effects of histone in vitro studies, as discussed above. mCBS is provided as a salt so it dissolved easily into the culture medium, which was prepared using the techniques discussed in the methods section.

We were able to discuss our experimental protocol with scientists from Sirtex Laboratory, who advised us on how best to handle and store the mCBS. As it is an experimental drug, we were one of the first users of the compound outside of the Sirtex Laboratory. However, it must be stressed as at no point was Sirtex involved in collecting or analysing the data and neither I nor anybody else who worked in the laboratory have any undisclosed connection with or interest in Sirtex.

Figure 6.1. – Isolated Rat Cardiomyocytes incubated in the presence of histones and mCBS

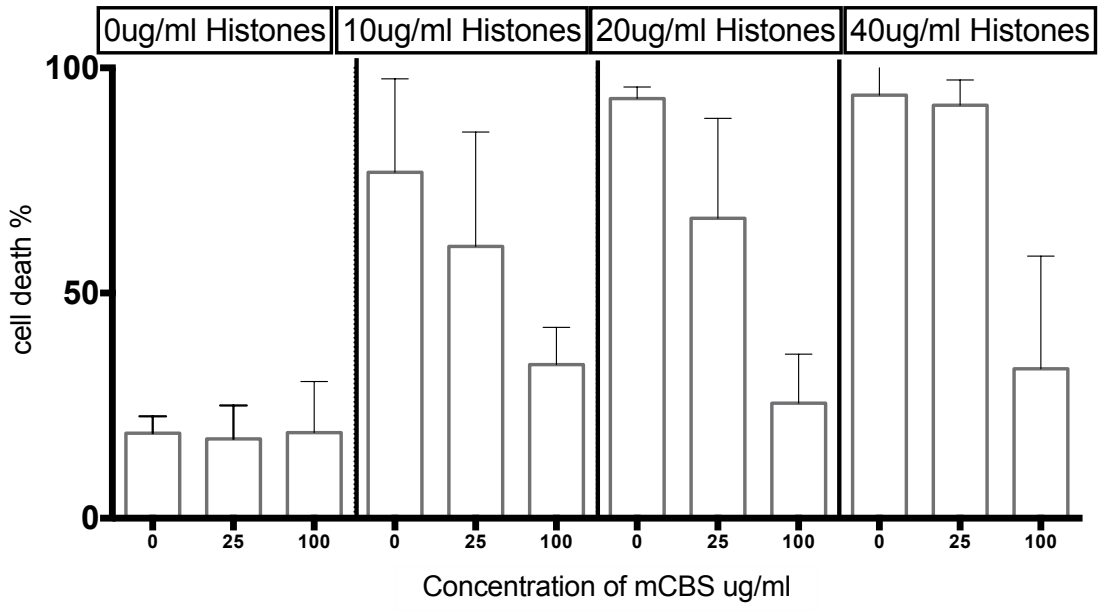


Figure 6.1 Isolated rat cardiomyocytes incubated with increasing doses of pure histones. They were also treated with a low and high dose of mCBS (n = 4). The percentage of cell death was calculated using PI staining and expressed as a percentage of dead cells in comparison to the total number of cells. Histones were shown to be cytotoxic to cells in a dose-dependent fashion, and mCBS protected isolated rat cardiomyocytes from the cytotoxic effects of extracellular histones. When histones were incubated at a concentration of 40 ug/ml there was a significant reduction of cell death from 97.5±4% in the vehicle group to 33.7±10% in the treatment (100 ug/ml of mCBS) group (p = 0.02).

Discussion

This experiment was able to demonstrate that at lower doses of 10 ug/ml of histones, there was still significantly more cardiomyocyte death in comparison to the placebo. 10 ug/ml was the lowest dose tested, which is still greater than the 5 ug/ml which has previously been perfused into an isolated mouse heart to demonstrate cardiac toxicity in the form of cardiac arrythmias(96). The in vitro exposure of extracellular histone to isolated cardiomyocytes has not been conducted before, and there is no equivalent experiment in the literature. The second reason we chose the lowest dose of 10 ug/ml was that this was the lowest dose we could accurately pipette the dissolved histone. This was because the histone was too difficult to dissolve in smaller doses of a solution, as discussed in the previous chapter. Finally, there is also, in the literature, a study examining sepsis-induced cardiomyopathy in patients being treated in an intensive care unit. This study showed that in 65 patients, circulating histone concentrations greater than 75 ug/ml were associated with much higher cardiac troponin levels (mean 147.8 pg/mL) compared with those with circulating histones less than 75 μg/mL ((mean 28.8 pg/mL) $p<0.01$) (133). Increased circulating cardiac troponin levels are a well-recognised marker of cardiac necrosis.

Extracellular histone concentration in these patients is a reflection of the peripheral circulation, i.e., the amount of histone within the bloodstream. The source of this histone is cellular; therefore, it is feasible to suggest that the concentration of extracellular histone in the extracellular matrix is far greater than 75 ug/ml. This is an important factor in understanding the importance of our results because, in ischaemia- reperfusion injury, we hypothesise that cellular necrosis exposes neighbouring healthy cardiomyocytes to concentrations of extracellular histones in

concentrations far greater to the peripheral circulation. If a concentration of 75ug/ml in the peripheral circulation is enough to induce evidence of myocardial cell necrosis, then it is reasonable to suggest that our concentration of 40ug/ml to have a similar effect.

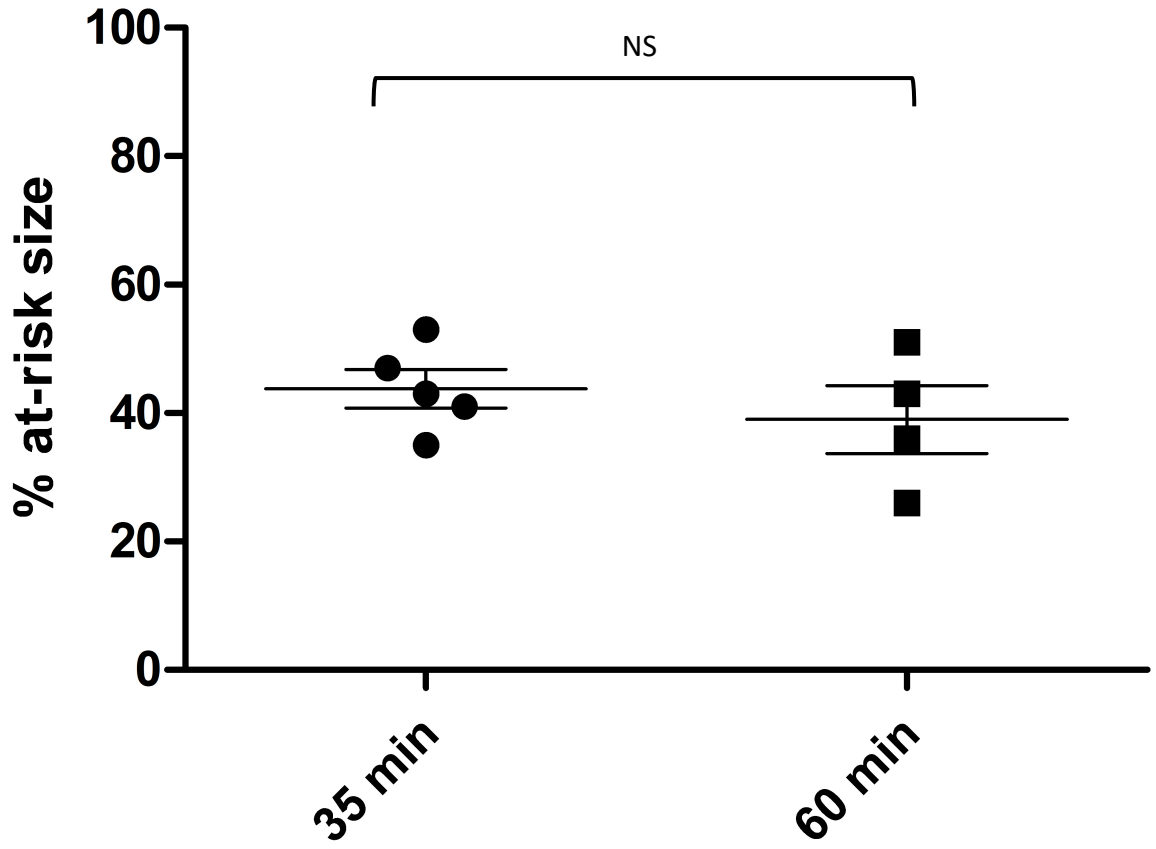
Chapter 7 - Results, establishing the Langendorff ex vivo retrograde heart perfusion model with regional ischaemia.

The next step in testing my hypothesis is establishing a reproducible ex vivo model of ischaemia- reperfusion injury. I chose the Langendorff isolated perfused rat model; however, this technique requires an element of technical skill that needs to be learned and then practised until the model produces reproducible data. Previous operators in the laboratory have demonstrated a consistent control infarct size of approximately 40–50% in regional ischaemia (when the LAD is ligated in the same area) and 70–80% in global ischaemia (when the whole heart is devoid of oxygenated buffer)(123). We opted to aim for n = 4 in both groups, which is similar to published literature and the numbers previous operators had used. This number was also estimated based on a power calculation.

Figure 7.1 shows the result of establishing the Langendorff ex vivo retrograde heart perfusion model; however, despite the relative reproducibility of the infarct size, the overall infarct size was relatively small. The difficulty with having such a small infarct size is the inability to demonstrate any reduction in infarct size in a treatment arm because the level of cell death is already limited.

Figure 7.1 and 7.2 Demonstration of infarct size after 35mins of ischemia vs 60mins ischemia in a Langendorf ex-vivo model of rat coronary artery occlusion.

Figure 7.1



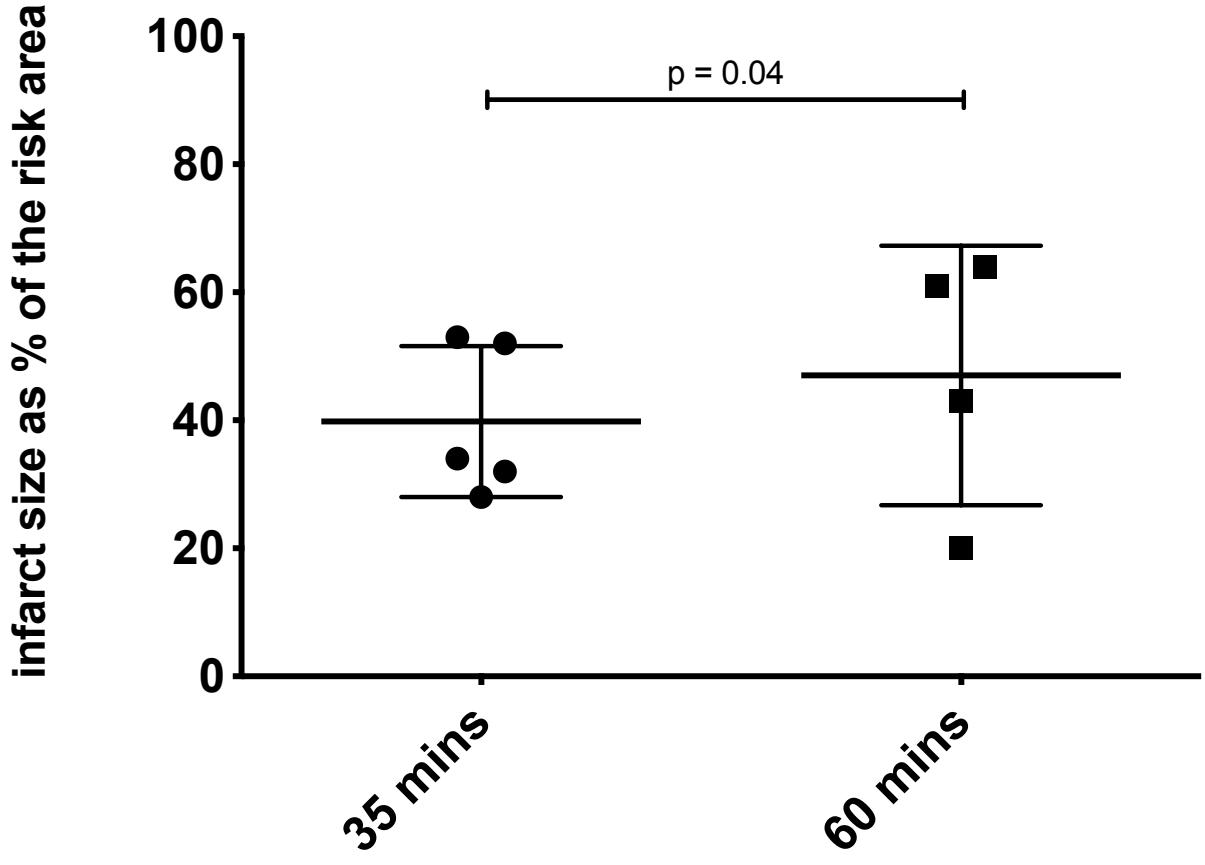


Figure 7.1 and 7.2 Demonstrates regional ischaemia in a Langendorff model of 35 minutes of ischaemia versus 60 minutes of ischaemia. There is no statistically significant difference in % at-risk zone, but 60 minutes does result in a statistically significant change in infarct size (p = 0.04).

After discussing the matter with my supervisors and re-evaluating my technique, I identified several methods to improve reproducibility and increase the infarct size and cell death.

First, I took my Langendorff apparatus apart and rebuilt it in accordance with a stricter set of guidelines based on Bell et al(123). I initiated regular acid washing to ensure that the apparatus was free from excessive debris. I redesigned my LV balloon, which measures intraventricular pressure, so that it had a lower profile and was made of a less rigid material, which reduced trauma to the ventricle.

I introduced a new exclusion criterion, which included coronary flow rate after reperfusion >8ml/minute. This is because an adequate flow rate is required to wash away cellular debris after a period of ischaemia. This cellular debris may contain enzymes such as LDH, which will also stain red in the presence TTC, exaggerating the actual amount of surviving tissue.

Finally, I also decided to introduce pacing at a rate of 360–380 BPM into my model, which helped with keeping a constant flow rate and temperature.

Figure 7.3 and 7.4. - A comparison of area at risk and infarct size between the new protocol and the old protocol of Langendorf ex-vivo rat coronary artery occlusion model.

Figure 7.3

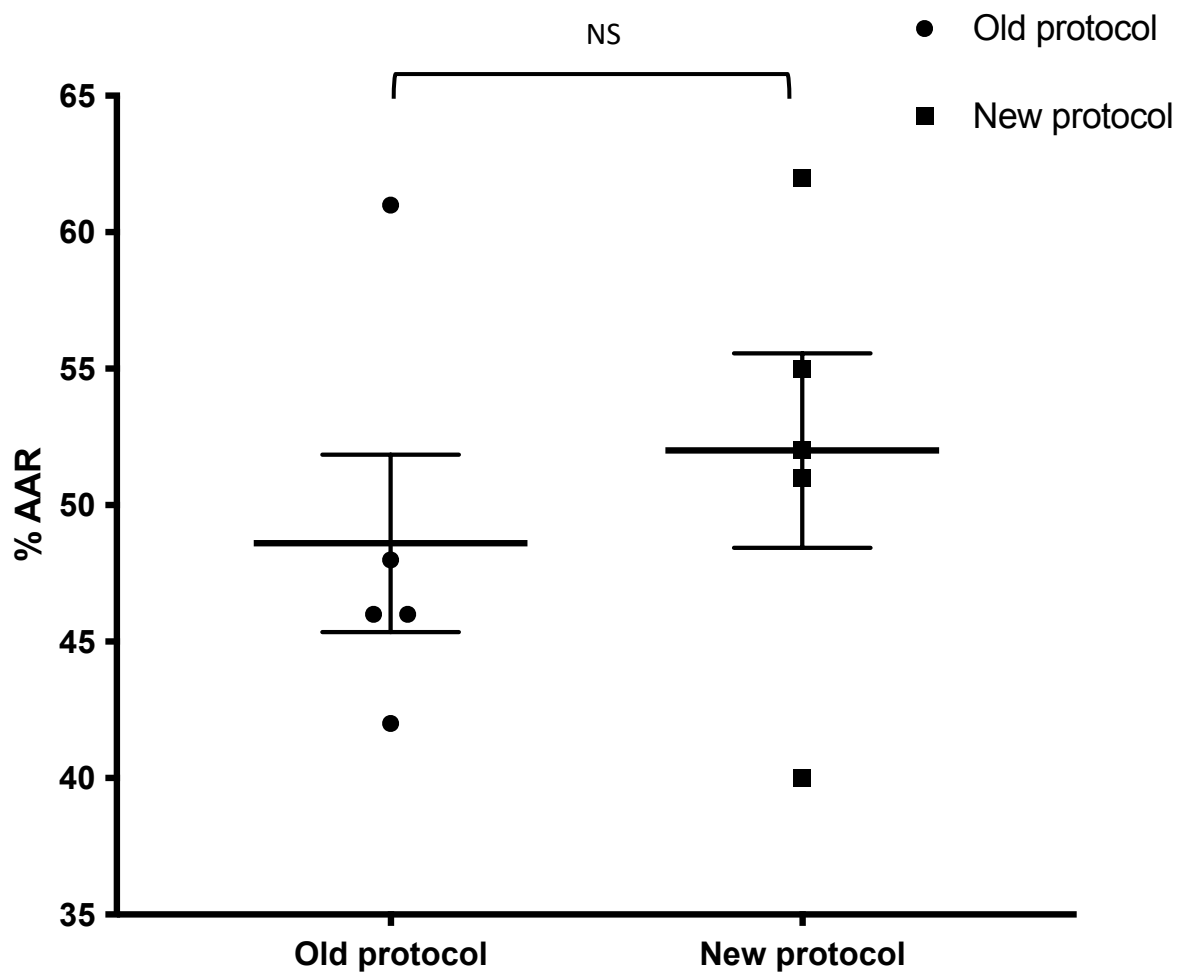


Figure 7.4

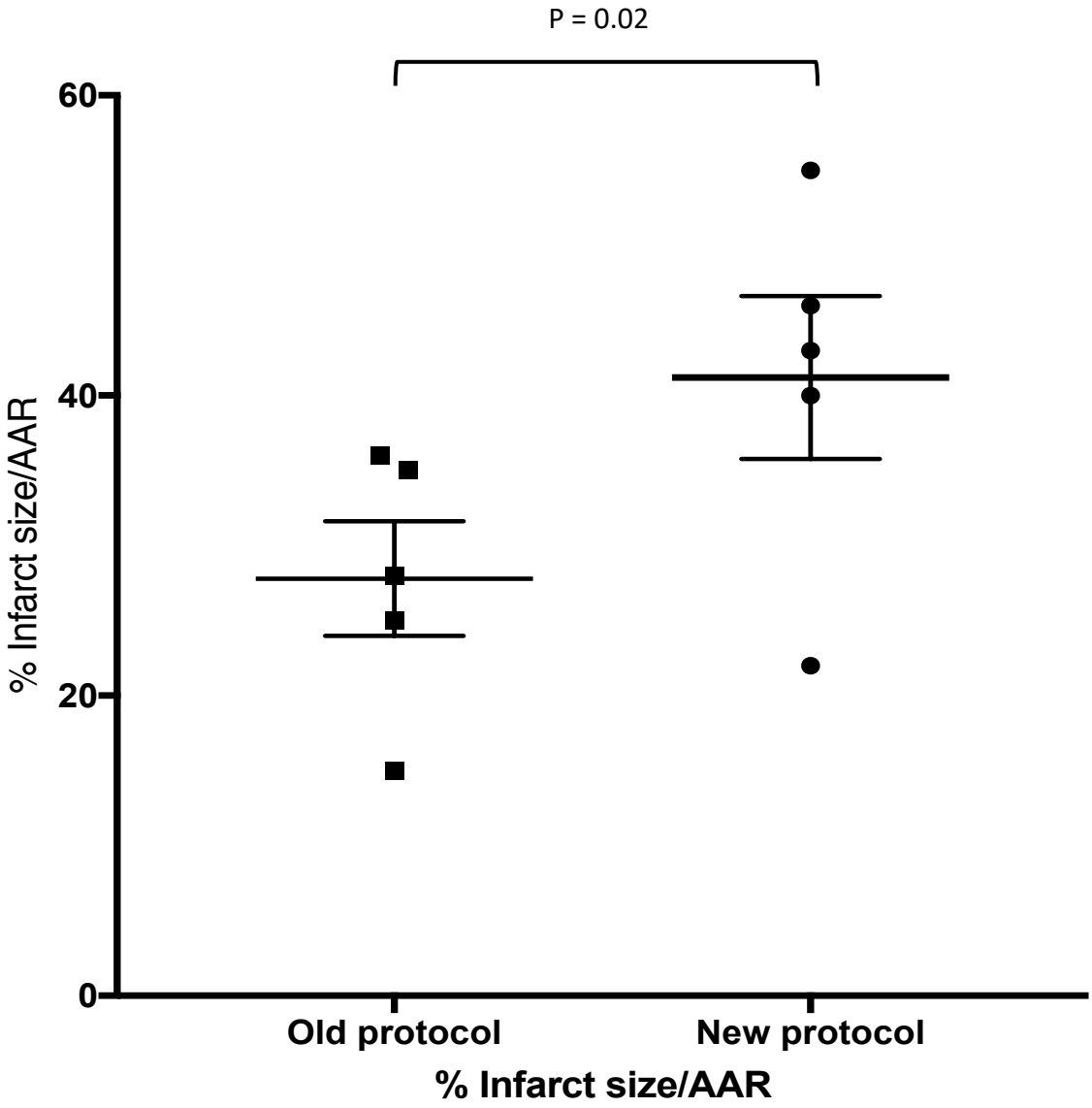


Figure 7.3 and 7.4. A comparison of area at risk and infarct size between the new protocol and the old protocol. There is a significantly larger infarct size with the new protocol in comparison to the older protocol, which demonstrates an improvement in my technique.

Establishing a positive control for the Langendorff protocol

We decided to use ischaemic preconditioning (IPC) as a positive control for our model of ischaemia -reperfusion injury. As discussed in the introduction, ischaemic preconditioning is the process of exposing the myocardium to short cycles of ischaemia followed by reperfusion. It has been well established that exposing the myocardium to IPC prior to ischaemia-reperfusion injury both in vivo and ex vivo leads to a significant reduction in infarct size(134). Within the Langendorff model of regional and global ischaemia, the method of IPC is the same. The stabilisation period of 30 minutes is replaced with three cycles of five minutes of ischaemia followed by five minutes of reperfusion. The ischaemia is brought on by switching off the flow of oxygenated buffer to the heart for five minutes.

To prove that my model of IPC results in a smaller infarct size, the Langendorff experiment was repeated with n = 4 in both groups. Hearts were attached to the Langendorff apparatus as previously described and subjected to 35 minutes of regional ischaemia followed by two hours of reperfusion. Hearts that were subjected to three cycles of five minutes of IPC demonstrated a significant reduction in the total infarct size, but no change in the area at risk. This correlates with previously observed data and allowed us to have a functioning positive control in future experiments.

Figure 7.5. and 7.6 – The Effect of IPC on the at risk zone and infarct size in a Langendorf ex-vivo rat coronary artery occlusion

Figure 7.5

At risk zone 35mins regional ischemia versus IPC and 35 mins regional ischemia

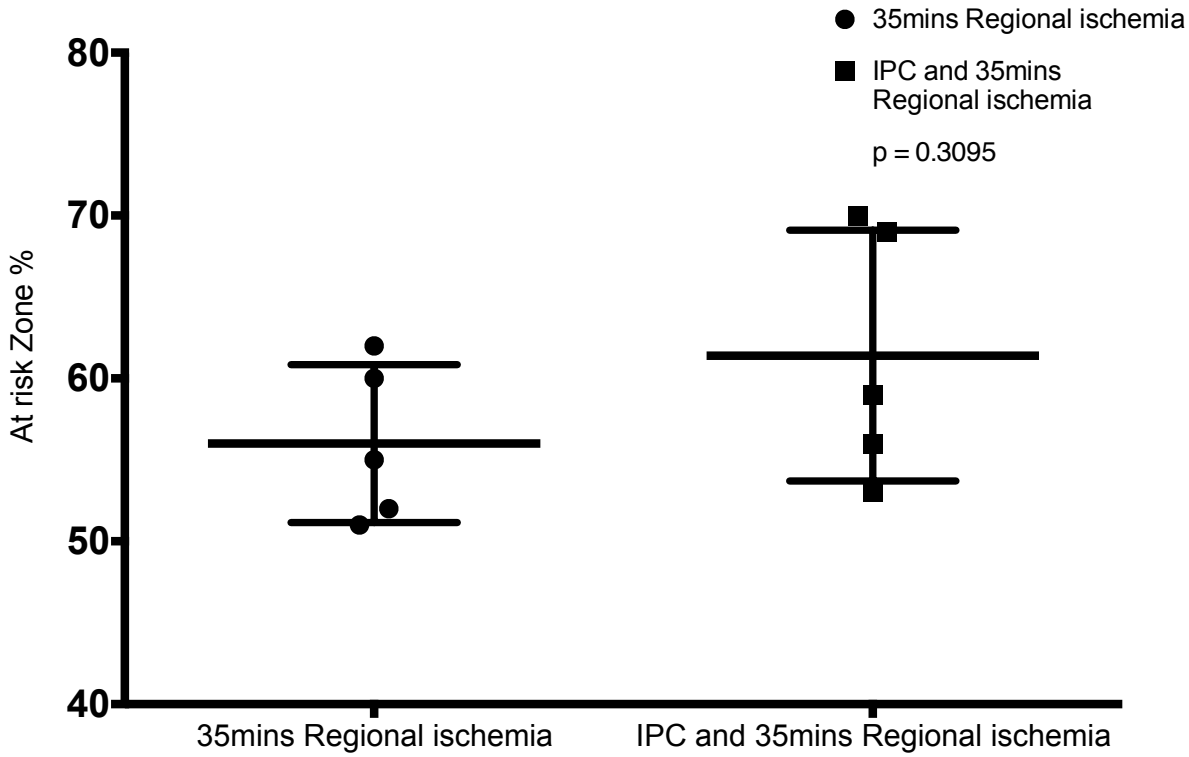


Figure 7.6

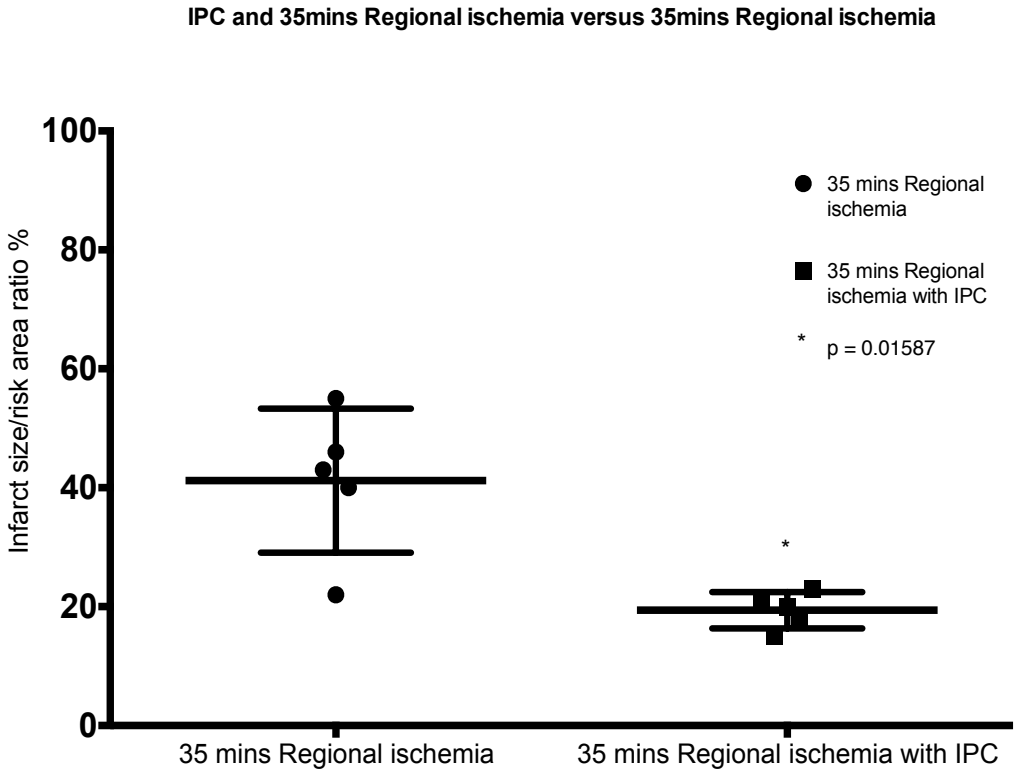


Figure 7.5 and 7.6. Langendorff ex vivo isolated rat heart perfusion. When hearts were subjected to IPC prior to 35 mins of global ischaemia, there was a significant reduction (p = 0.01) in the total infarct size/at-risk area ratio. Crucially, there was no difference in the at-risk area between the two groups (p = 0.39).

Is mCBS able to cardioprotect in a Langendorff ex vivo retrograde heart perfusion model with regional ischaemia?

Hearts were subjected to 35 mins regional ischaemia using the methods described above. There was no statistically significant difference in the size of the infarct between mCBS and the control. However, there was a statistically significant improvement in the coronary flow rate during reperfusion in the treatment group in comparison to the control group ($p < 0.05$).

Figure 7.7. – The effect of mCBS and IPC on infarct size after 35mins ischemia in a Langendorff ex-vivo rat coronary artery occlusion model.

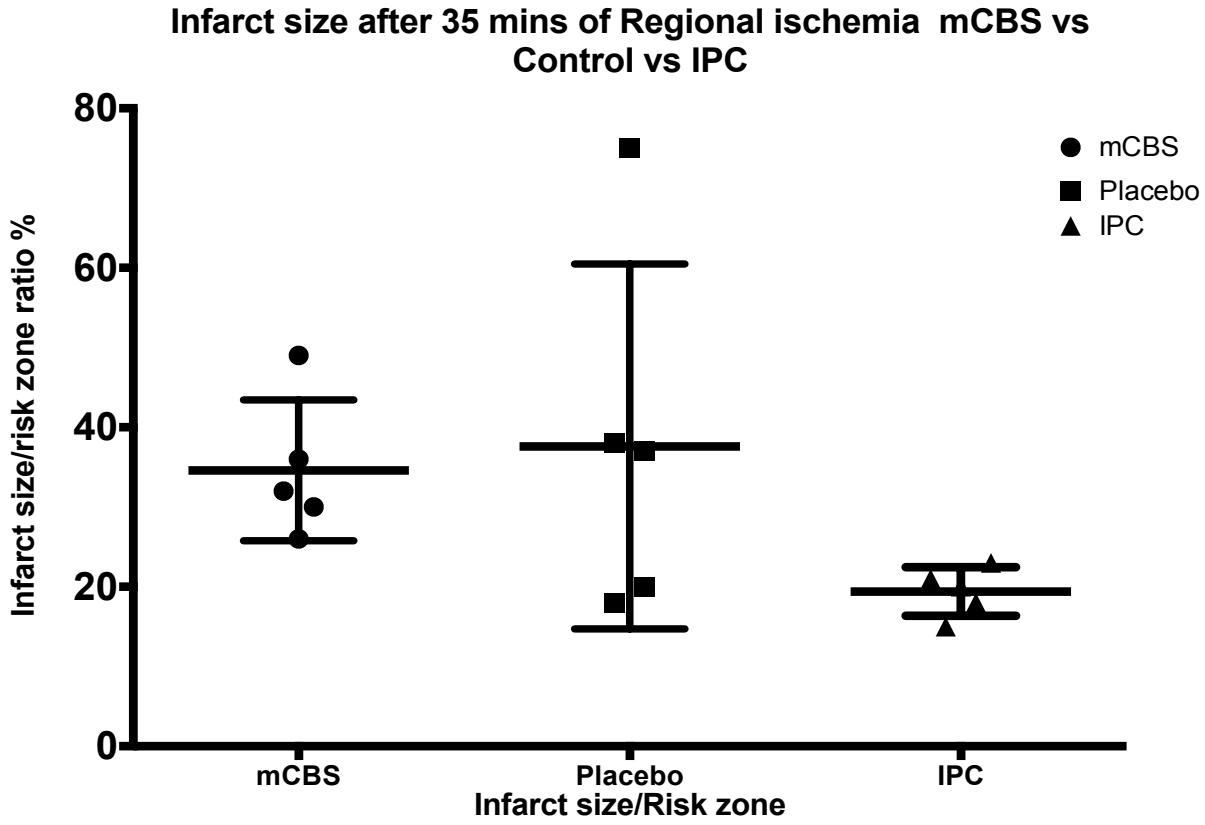


Figure 7.7 Hearts were subjected to 35 mins of regional ischaemia in the presence of control or mCBS. After TTC staining and analysis, there was no statistically significant difference between the treatment group and the control ($p = 0.91$). Interestingly, there was no statistical difference between the control arm and ischaemic preconditioning, although there was a definite trend towards significance ($p = 0.10$)

Figure 7.8 – Coronary flow rate in the presence of mCBS compared to placebo.

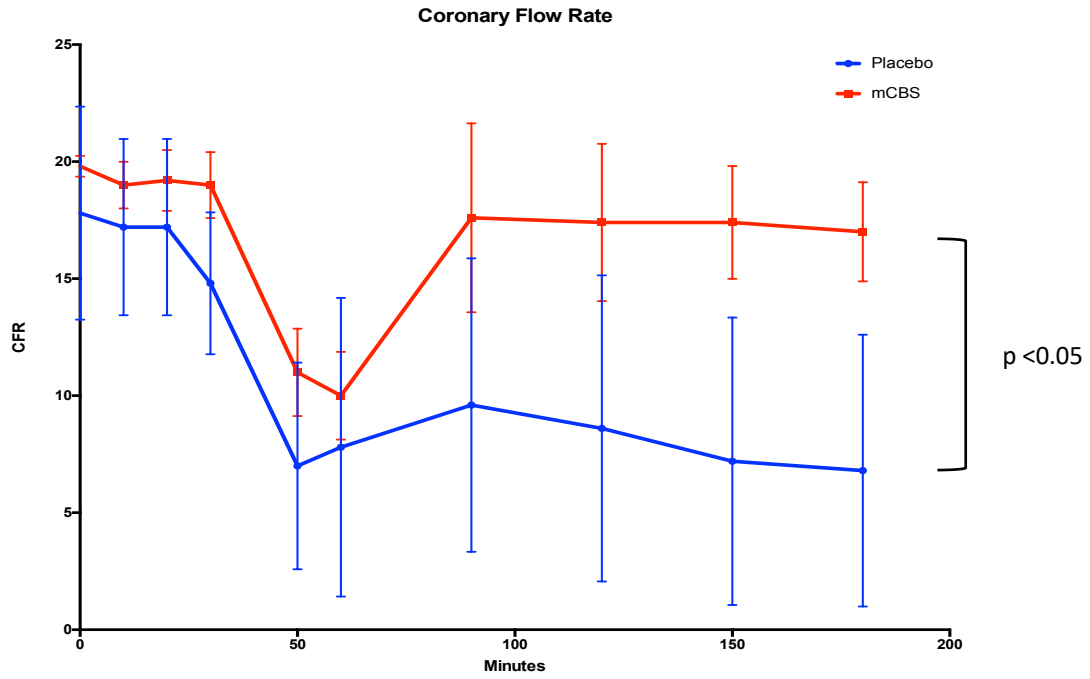


Figure 7.8. The coronary flow rate through the myocardium was significantly improved during reperfusion in the treatment arm in comparison to the placebo arm ($p < 0.05$).

Chapter 8 - Results, are histones and nucleosomes released from the heart during regional and global ischaemia- reperfusion injury?

The experiments designed in this chapter were aimed at ascertaining evidence for the existence of free histones in the heart during ischaemia -reperfusion injury. Perfusates from the heart were collected from the Langendorff experiments described above prior to the onset of ischaemia and at the point reperfusion, 1 minute after reperfusion, 5 minutes, 10 minutes, and 30 minutes after reperfusion. The perfusates were analysed using the methods described above, using an ELISA assay.

Rationale for the establishment of a global ischaemia model

The regional ischaemia model is associated with some difficulties, which may explain some of the negative findings. First, reproducibility is challenging, as demonstrated by the stark differences in infarct size between repeated experiments. Infarcts range from 20% to 70%; this level of difference changes the power calculations. This would mean that to demonstrate a statistically significant change in infarction size of 50% we would need n = 35 in each group. These numbers are too large and would take months to complete. Second, the concentration of histones in the perfusate in the regional ischaemia model is below the detection limit of our ELISA assay. If we are unable to quantify the concentration of free histones during ischaemia-reperfusion then we will be unable to prove that the treatment arm reduces the concentration of circulating free histones.

To confront these problems with our experimental model, we decided to switch to a

model of global ischaemia in a Langendorff ex vivo retrograde heart perfusion protocol. The global ischaemia model is associated with a much larger infarct, typically over 60%, which will allow us to power the study with smaller n numbers. Second, the larger infarct will mean a greater level of cell necrosis and theoretically a larger amount of circulating intracellular contents, including histones. The method of collecting perfusate from a heart after global ischaemia is most likely to yield a greater degree of cell debris. This is because there remains a coronary flow rate throughout the period of ischaemia in a regional ischaemia model, as well as a constant perfusion pressure of the perfusate into the tissue of the myocardium. However, in the global ischaemia model, there is no perfusate flowing through the entire myocardium. This may allow a greater accumulation of intracellular debris from ischaemia to enter the perfusate at the point of reperfusion, which is when we collect the perfusate for analysis.

Establishing the Langendorff ex vivo retrograde heart perfusion model with global ischaemia without pacing

Hearts were attached to the Langendorff apparatus as previously described and subjected to 35 mins global ischaemia or 35 mins global ischaemia with preceeding IPC.

There was a significant reduction in the size of the infarct ($p = 0.09$) with IPC. Using this model with a mean infarct size of 50% ($n = 6$) would be adequate to show a statistically significant 50% reduction in infarct size.

Figure 8.1. – Infarct size comparison between control and IPC in a global ischemia model of Langendorf ex-vivo rat coronary artery occlusion model.

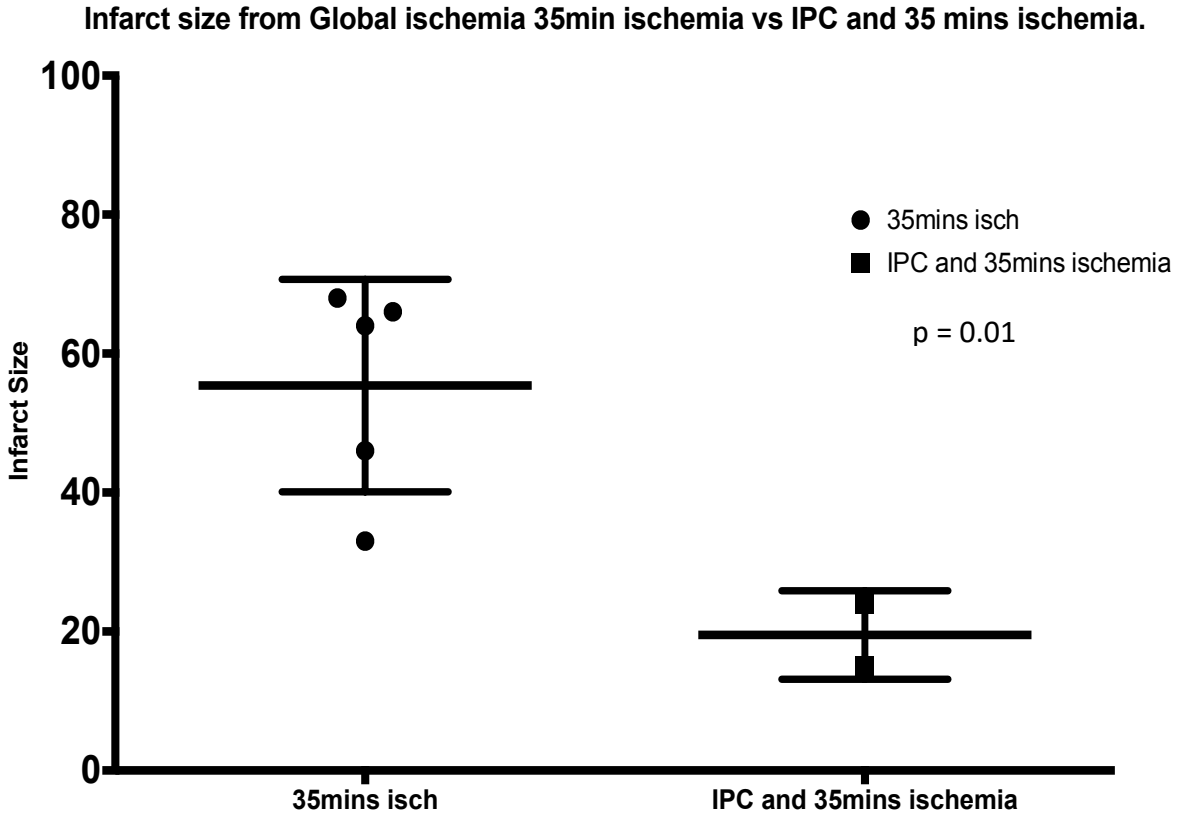


Figure 8.1 Hearts subjected to 35 mins global ischaemia demonstrated a statistically significant (p = 0.01) reduction in infarct size when they were subjected to ischaemic preconditioning prior to the onset of ischaemia.

Are histones and nucleosomes released from the heart during global ischaemia - reperfusion injury?

Perfusates from the heart were collected from the Langendorff experiments described above prior to the onset of ischaemia and at the point of reperfusion, 1 minute after reperfusion, 5 minutes, 10 minutes, and 30 minutes after reperfusion. The perfusates were analysed using the methods described above, using an ELISA assay.

As can be seen in figure 8 below, histones and nucleosomes were undetectable prior to the onset of ischaemia. During reperfusion, there was a detectable level of histones bound to nucleosomes; after ten minutes, these nucleosomes were undetectable. IPC resulted in a non-significant reduction ($p = 0.07$) in the detectable concentration of histones and nucleosomes present in the perfusate.

Figure 8.2 Histone release during reperfusion in a Langendorff model.

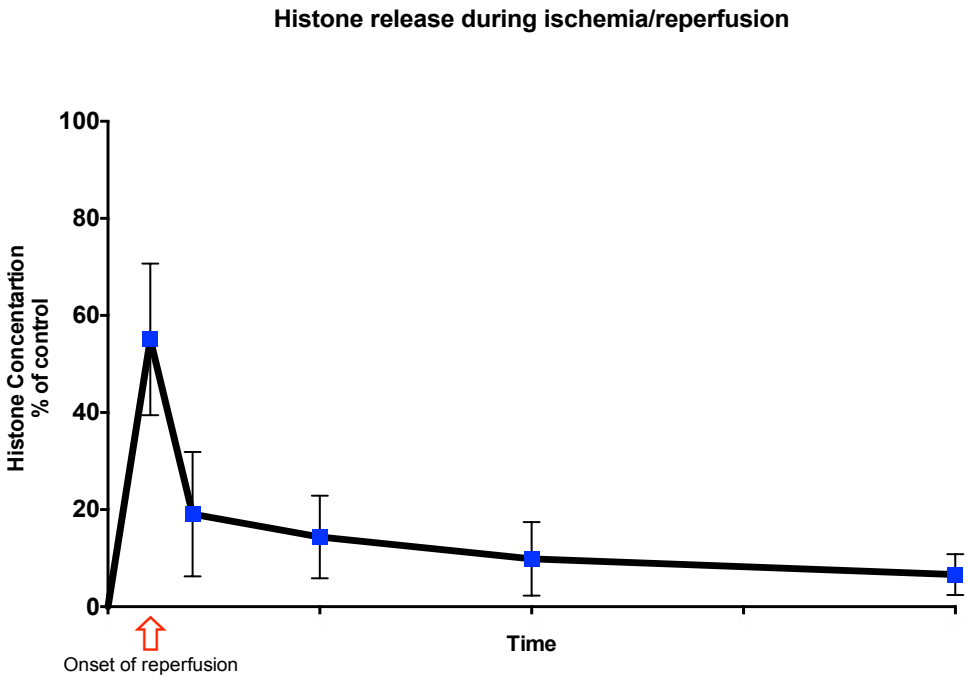
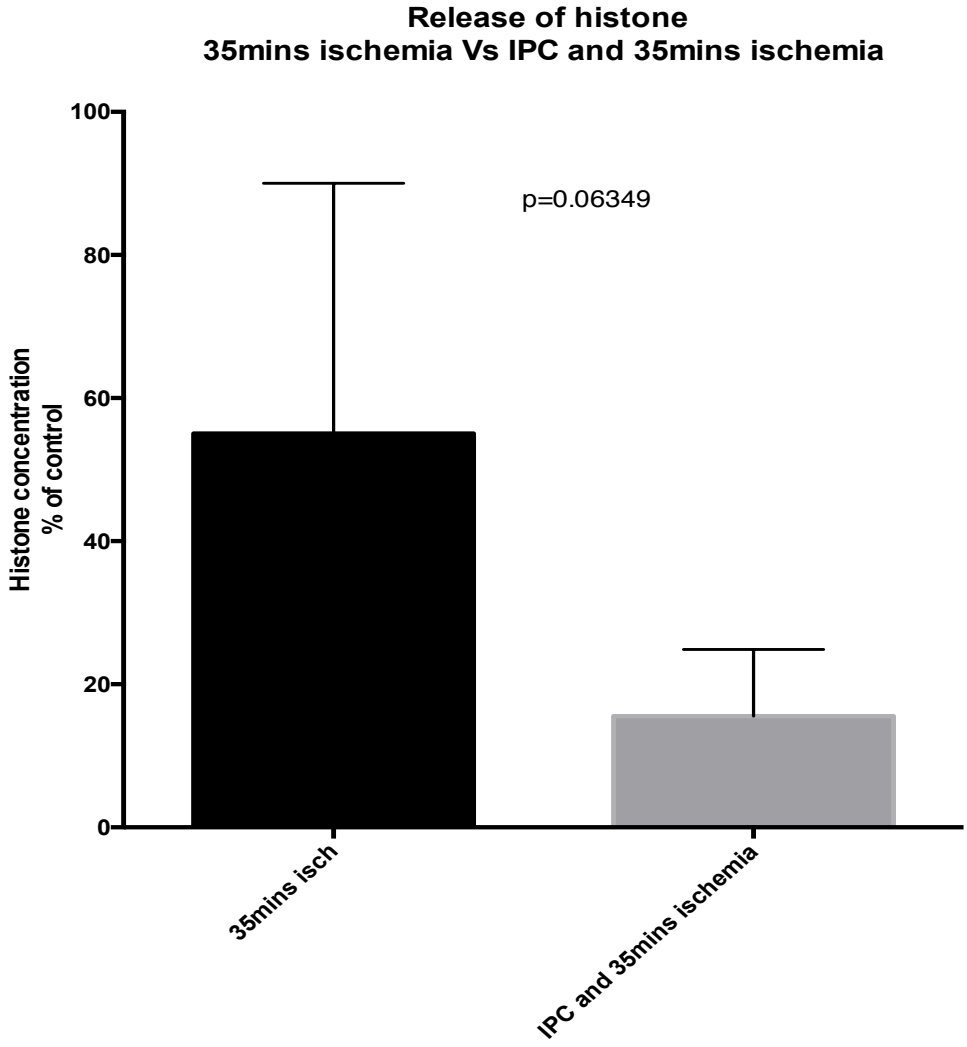


Figure 8.3. – Histone release during reperfusion in a Langendorff model



Figures 8.2 and 8.3. Perfusate collected from hearts undergoing ischaemia-reperfusion injury demonstrated a significant increase in the concentration of histones and nucleosome released at the point of reperfusion. The concentration of histones decreased after one minute of reperfusion and were undetectable after five minutes of reperfusion. IPC non-significantly (p = 0.06) reduced the concentration of circulating histones and nucleosomes in the perfusate were collected immediately at the point of reperfusion.

Chapter 9 – Results , is mCBS able to cardioprotect in a Langendorff ex vivo retrograde heart perfusion model with global ischaemia?

The Langendorff experiment with global ischaemia was repeated using the techniques described above. It was decided to increase the duration of ischaemia to 45 minutes in order to maximise the amount of cell death and subsequent concentration of histone that would be released. Hearts were subjected to 45 min ischaemia and two hours of reperfusion with the presence of mCBS or KHB. mCBS treatment resulted in a significant reduction in the total infarct size ($p = 0.02$), with $n = 6$ in both groups. IPC also resulted in a non-significant reduction in infarct size, $n = 2$. There was no detectable difference in coronary flow rate between the two groups.

Figure 9.1 Infarct size from a Langendorff ex-vivo rat coronary artery occlusion model, Control vs mCBS vs IPC.

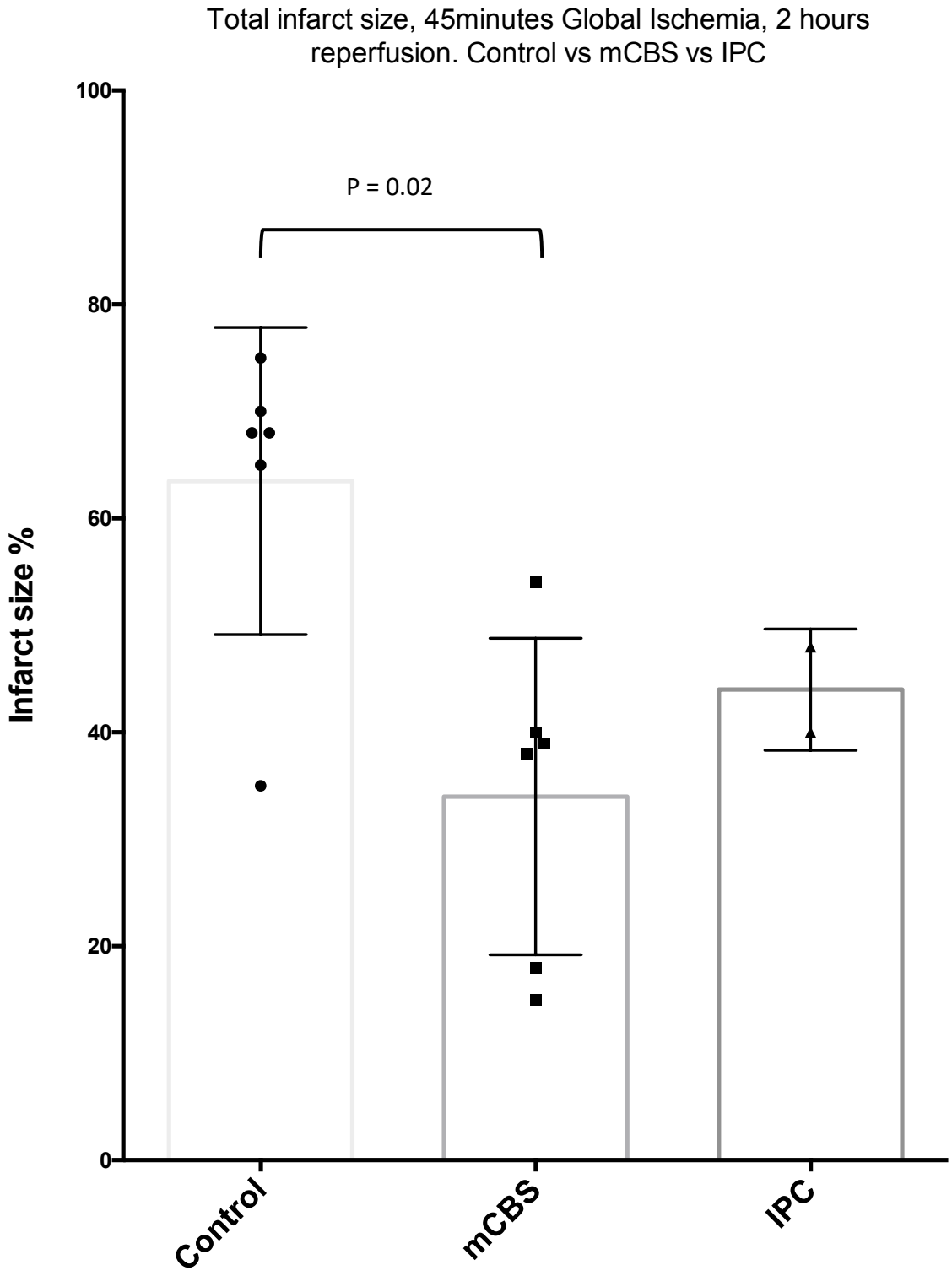


Figure 9.1 Isolated perfused Langendorff rat hearts subjected to 45 minutes of global ischaemia and two hours of reperfusion treated with a vehicle (Krebs buffer) or mCBS. Treatment with mCBS showed a significant reduction ($p = 0.02$) in infarct size. IPC n = 2 was used as a positive control. mCBS n = 6 and Control n = 6.

Does mCBS affect the amount of histone and nucleosome released after global ischaemia?

There was no statistical difference between the amount of histone as a nucleosome that was released between hearts treated with mCBS and placebo ($n=5$, $p = 0.6$). However, the ELISA assay detects histones as part of a nucleosome, and the amount of free histone was not measured. This is because ELISA assays specific to individual histones are more expensive and much harder to find. As a result of budget constraints, we opted to use a cheaper alternative.

Figure 9.2. – Amount of histone released from the perfusate of hearts undergoing global ischemia in a Langendorff model.

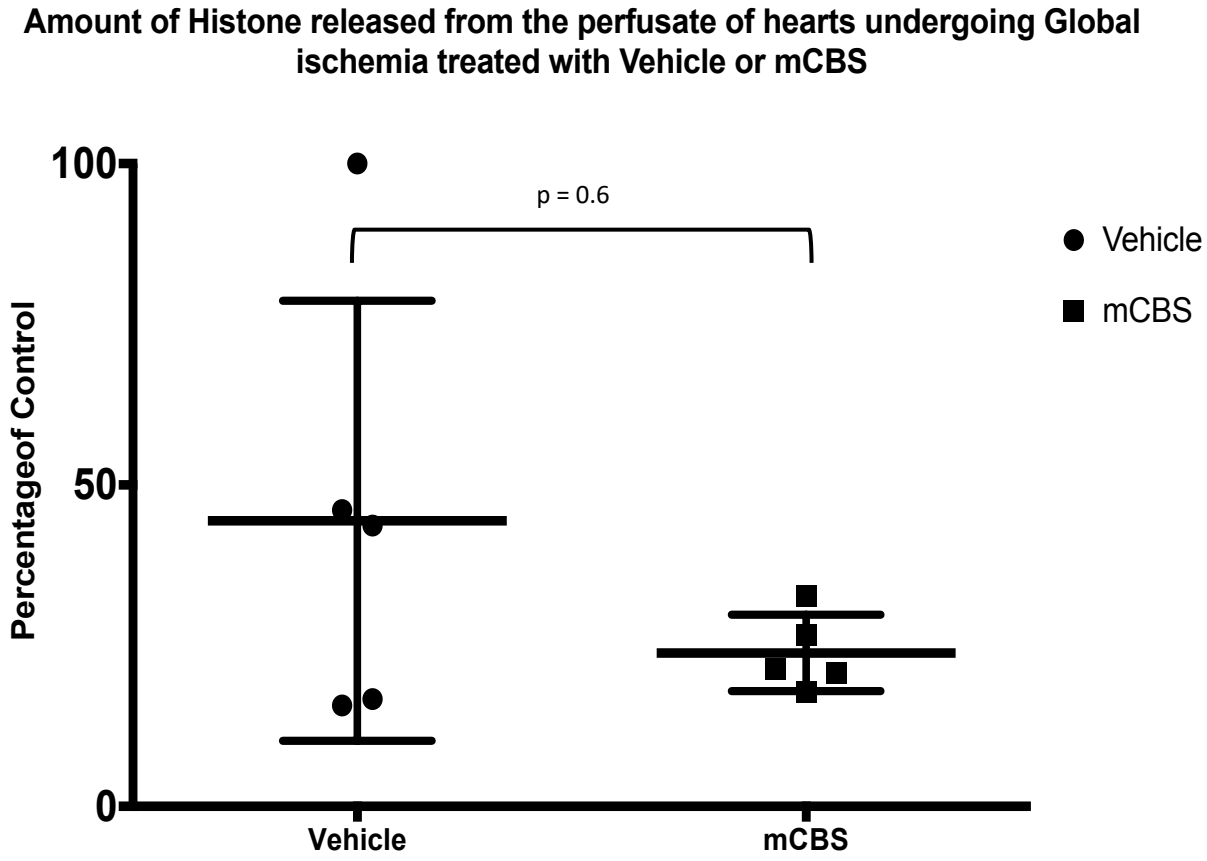


Figure 9.2. The perfusate of hearts undergoing global ischaemia demonstrated no difference in the amount of detectable histone present within the sample. P = 0.6

Discussion

In our Langendorff model, mCBS was cardioprotective during global ischaemia-reperfusion injury. This would replicate our findings in the cell isolation model, which also demonstrated that mCBS was cardioprotective when isolated cardiomyocytes were exposed to histones. However, in this model, we were unable to show that there was a reduction in the amount of circulating histone in the perfusate of the heart during the Langendorff experiment. TWe believe the reason for this finding is that our ELISA assay was able to detect nucleosomes but not free histones. Nucleosomes have been demonstrated multiple times not to be cytotoxic; however, free histones have demonstrated cytotoxicity in several cell lines. The most accurate way to have assessed the perfusate would have been with an ELISA against H1-H6 histone, but the cost of this experiment would have been over our budget. However, the crucial finding in this experiment was the cardioprotective mechanism of mCBS.

Chapter 10 – Results, Quantifying the amount of histone H4 released from the perfusate of hearts undergoing global ischaemia reperfusion injury in a Langendorff apparatus.

The crucial role of histone H4 as a potential lead cytotoxic agent was identified in the seminal work by Professor Soehnleins' team in his recent Nature paper(68) who identified H4 as the agent that orchestrated chronic inflammation in a rat endothelium model and achieved this by directly interacting with the cell membrane of the endothelial cells independent of any activation of innate immunity. This important discovery midway through my research allowed us to focus our attention on H4 and set about to design an experiment where we could identify whether histone H4 was present in high concentrations during ischaemia -reperfusion injury of the myocardium. The use of an ELISA assay was deemed too expensive, so we used another immunohistochemical analysis technique called dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA).

A suitable plate is first coated with a capture antibody. After overnight incubation, the plate was washed three times. The perfusate from the heart was then added and incubated for 1.5 hours before a further three washing steps. A detection antibody was incubated for 1.5 hours before three more washing steps. At this point, the histone H4 was sandwiched between the two antibodies. The final addition of an enhancement solution increased the sensitivity of the assay. The lanthanide chelate was dissociated, and a new highly fluorescent chelate was formed within a protective micellar solution. The fluorescence emitted was measured with a plate reader capable of TRF detection.

Perfusate from hearts undergoing ischaemia-reperfusion injury were collected using the methods described above. The perfusate was then analysed for the presence of histone H4 using the DELFIA technique as described above.

Using DELFIA to detect histone H4, we found a significant increase of free unbound histone H4 at reperfusion ($n = 8$, $p < 0.01$). The concentration of histone H4 in the perfusate of hearts peaked immediately after the point of reperfusion and then stayed elevated throughout the reperfusion stage.

Figure 10.1. Concentration of histone H4 in the perfusate of hearts attached to a Langendorff apparatus undergoing ischemia/reperfusion.

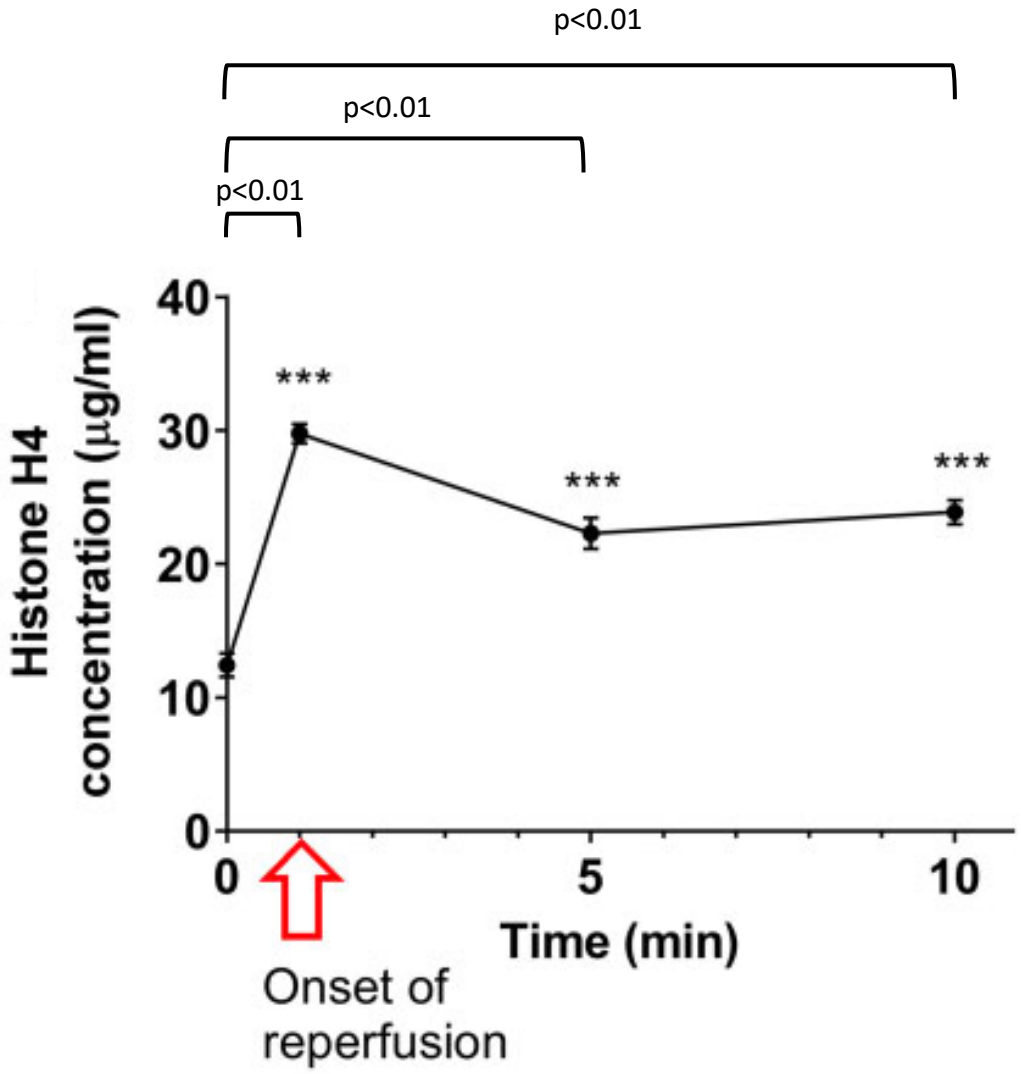


Figure 10.1 The concentration of histone H4 in the perfusate of hearts following global ischaemia reperfusion injury in hearts attached to the Langendorff apparatus reached a maximum of 29.8 ± 0.7 mg/mL.

We could further ascertain a positive correlation between the total infarct size in rat hearts undergoing global ischaemia in a Langendorff apparatus and the amount of histone H4 released from the rat heart at the point of reperfusion. The greater the degree of infarction, the greater the amount of histone H4 released from the myocardium.

Figure 10.2. Correlation between infarct size and concentration of extracellular histone release, in a Langendorff ex-vivo rat coronary artery occlusion model.

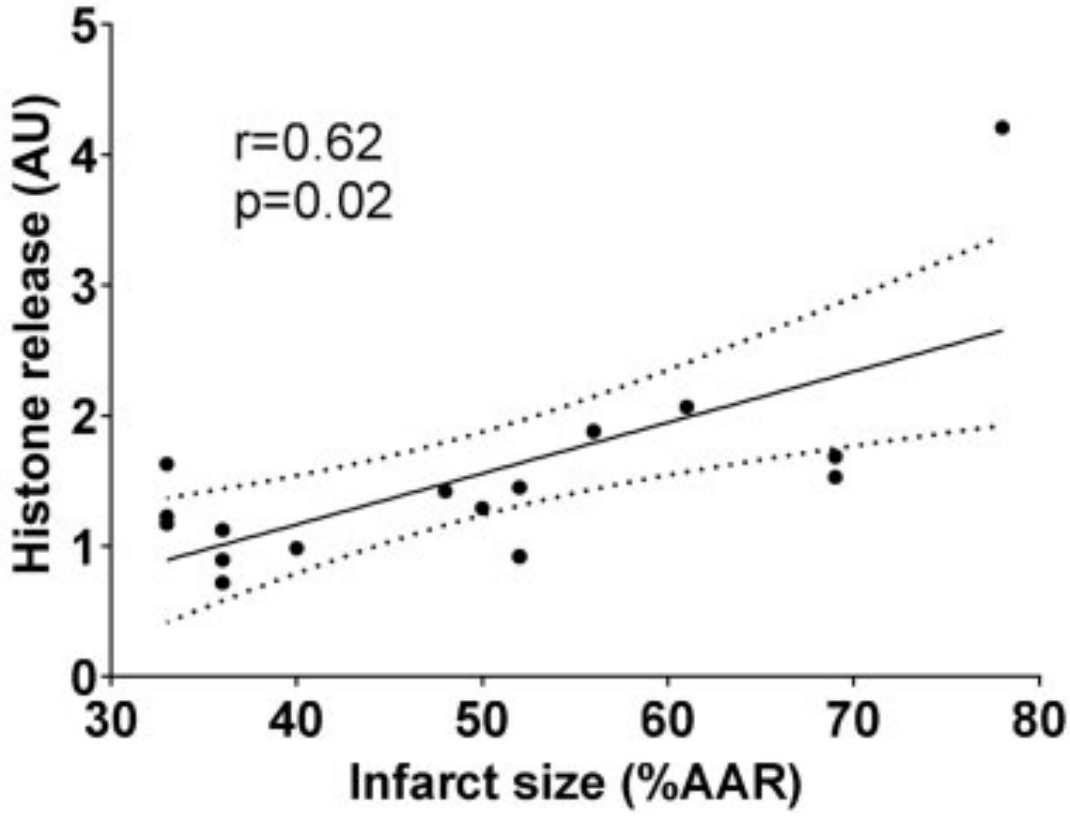


Figure 10.2 There was a positive correlation between infarct size and the concentration of free unbound histone H4 in the perfusate released immediately at the onset of reperfusion.

Discussion

These experiments demonstrated that histone H4 is present in large quantities during the process of ischaemia -reperfusion injury, and there is a positive correlation between the amount of Histone H4 released and the size of the area of total infarction. This means larger areas of cell death are associated with larger amounts of histone H4 release. The identification of histone H4 in the perfusate is an important step in providing evidence for the mechanistic effect that free histone H4 plays in the process of ischaemia -reperfusion injury.

Chapter 11 – Results, HIPe reduces infarct size in vitro via a mechanism independent of TLR4

The next stage of the research was to ascertain whether specifically targeting histone H4 was a viable method to negate its cytotoxic effects, as demonstrated in previous studies. Based on the seminal work of Silvestre-Roig et al(68)., there already exists an inhibitory peptide specifically synthesised as a research compound that is shown to significantly attenuate the cytotoxic effects of histone H4. As a result of our collaboration with the group that conducted that work, we were fortunate enough to receive from Professor Oliver Soehnleins' team in Munich, Germany, a small amount of the histone inhibitory peptide (HIPe.) The small quantities of the compound and the extraordinary cost of synthesising a new peptide meant that we were only able to use HIPe in selected experiments where smaller quantities of the drug could be used. The in vitro isolated rat cardiomyocyte model was an ideal model for testing the compound.

The primary rat cardiomyocytes were seeded on a 24-well microplate as described previously. They were further treated with vehicle or calf thymus-derived histones (Sigma-Aldrich) dissolved in a phosphate buffer solution and then incubated for 1 h at 37ºC and 5% CO_2. The cyclic peptide HIPe was then incubated with the histone-exposed cardiomyocytes at the indicated concentrations as previously mentioned according to the details outlined by Silvestre-Roig et al. Cell death was ascertained by using propidium iodide and lactate dehydrogenase techniques as described earlier. The cells were treated with propidium iodide (PI) for 15 min, after which they were visualised under a fluorescence microscope. PI is a fluorescent DNA

intercalating agent that can enter cells via damaged membranes and bind to DNA in the nucleus of cells and fluoresces red, thus providing a marker of cell death. After blinding to treatment, photographs of the incubated cells were analysed using ImageJ software. Dead cells were defined as those that showed evidence of uptake of PI, which was measured as the membrane fluorescence intensity for cardiomyocytes.

As the second marker of cell death, the concentration of lactate dehydrogenase (LDH) released from dead cells was estimated using a commercially available LDH assay kit (Thermo Scientific).

When cardiomyocytes were incubated in the presence of 40 ug/ml of histones, there was a significant increase in the amount of cell death in comparison to the placebo. The co-incubation of HIPe with cardiomyocytes was able to significantly prevent the amount of cell death, as calculated by quantifying the amount of LDH released as described earlier. 100 ug/mL but not 25 lg/mL HIPe was also able to prevent the cytotoxic effects of 40 ug/mL histones in the LDH assay (36.6% ± 3.8% vs. 58.7% ± 8.7%, $N = 5$, $P < 0.01$).

In order to further clarify whether this cytotoxic effect of histone H4 occurs via a TLR mechanism, we also incubated these cells with the well-recognised TLR4 inhibitor, TAK-242. As demonstrated by Silvestre-Roig et al(68) H4 histone has a direct cytotoxic effect on the endothelium of rat coronary arterial tissue by punching a pore within the membrane of the cell. This direct cytotoxic effect is unlike what was previously thought regarding the mechanism through which histone kills cells. Some evidence suggests that it may be by activating TLR4 receptors as a conventional DAMP. However, researchers including Silvestre-Roig et al. have provided evidence

of the direct cytotoxicity of Histone H4. The dose for the use of TAK-242 was based on Yang et al (135) who conducted a similar study with LPS in isolated rat cardiomyocytes, the team found the effects of LPS were inhibited at 1ug/ml. However the incubation wells we used were 5mls in size and the stock solution of our TAK-242 was too dilute for us to accurately pipette 1ug/ml into the well, we choose 5ug/ml in order to maintain accuracy and reproducibility.

In our experiments, co-incubation of isolated rat cardiomyocytes exposed to 40ug/ml of histones caused a significant increase in the amount of cell death that was significantly reversed with the addition of mCBS and HIPe. However, the addition of the TLR4 inhibitor compound TAK-242 did not affect the degree of cell death caused by free histones suggesting that that the cytotoxic mechanism is TLR4 idependent.

Figure 11.1. LDH release in isolated rat cardimyocyte model after incubation with histone,mCBS, HIPe and TAK-242.

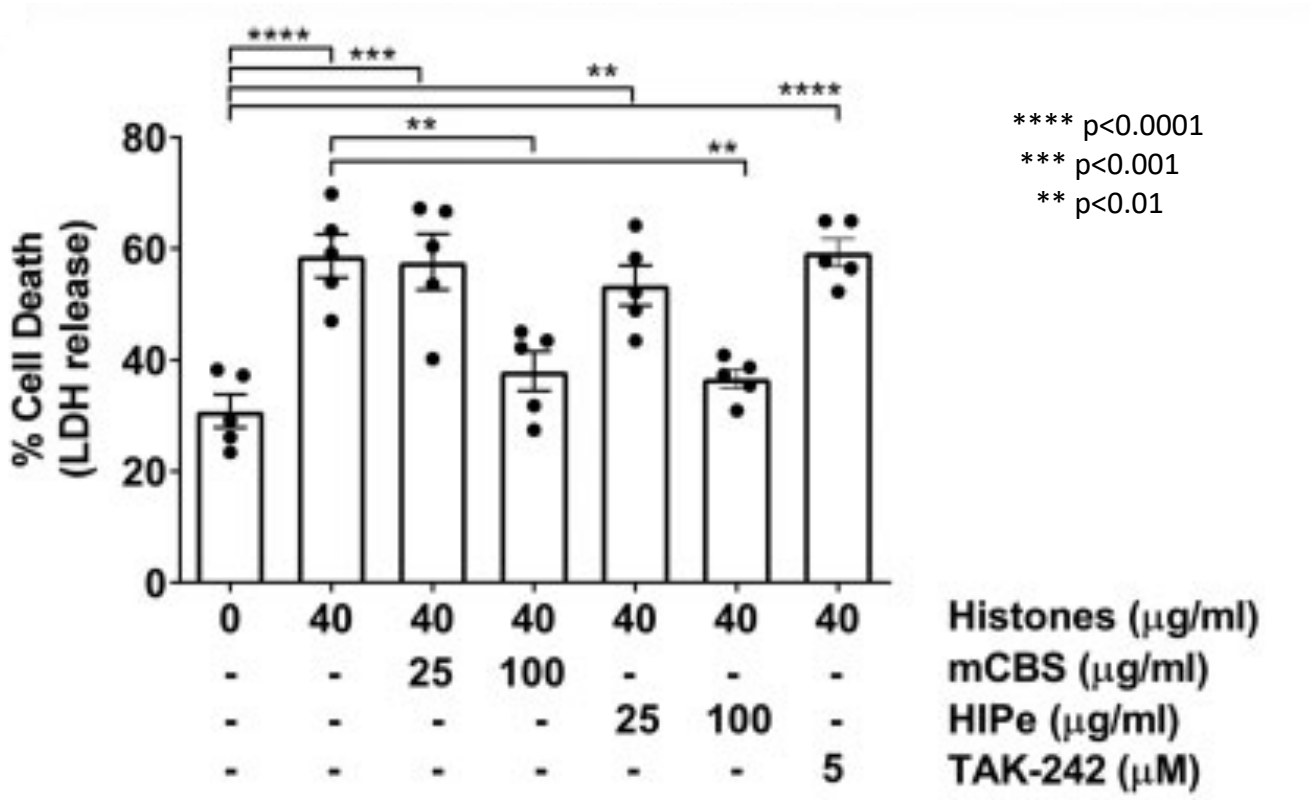


Figure 11.1 LDH release was used to assay cell death in primary cardiomyocytes treated with the drugs at the concentrations indicated. N = 5 independent biological experiments. Analysis by one-way ANOVA and Tukey post-test. (** P < 0.01, *** P < 0.001, **** P < 0.0001).

Chapter 12 – Histone cytotoxicity occurs via a TLR independent mechanism.

Further experiments to demonstrate histone cytoxicity was independent of TLR activation and an in vivo rat model of coronary artery occlusion were conducted by collabaroters and colleagues. I have included these experiments in the thesis in order to help explain the interesting findings of my work and provide further evidence to support my hypotheisis. Although the experiments were not conducted by me, I was deeply involved in designing the experiments and analysing the data produced.

The commercially available HEK-Blue mTLR4 cell line stably transfected with murine TLR4, MD-2, and CD14 co-receptor genes and an NF-κB-inducible Secreted Embryonic Alkaline Phosphatase reporter gene was used to assess TLR4-dependent NF-κB activity. This was purchased from Invivogen, San Diego, CA, USA. The parental cell line HEK-Blue Null1 served as a negative control, this was also purchased from Invivogen, San Diego, CA, USA. Both cell types were cultured according to the manufacturer's instructions

Cytotoxicity was measured from the accumulation of formazan, metabolized from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), by mitochondrial dehydrogenase which is only activated in viable cells.

HEK-Blue mTLR4 and HEK-Blue Null1 cells were seeded in a 96-well plate and treated overnight with mCBS. MTT was dissolved in PBS (0.5 mg/mL) and filter sterilized (0.22 mm) was added to the wells in serum-free cell medium in a 1:10 dilution and incubated for 30 min, at 37°C, in 2% CO2. Intracellular formazan was dissolved in dimethyl sulfoxide (DMSO) before absorbance was measured at 550 nm

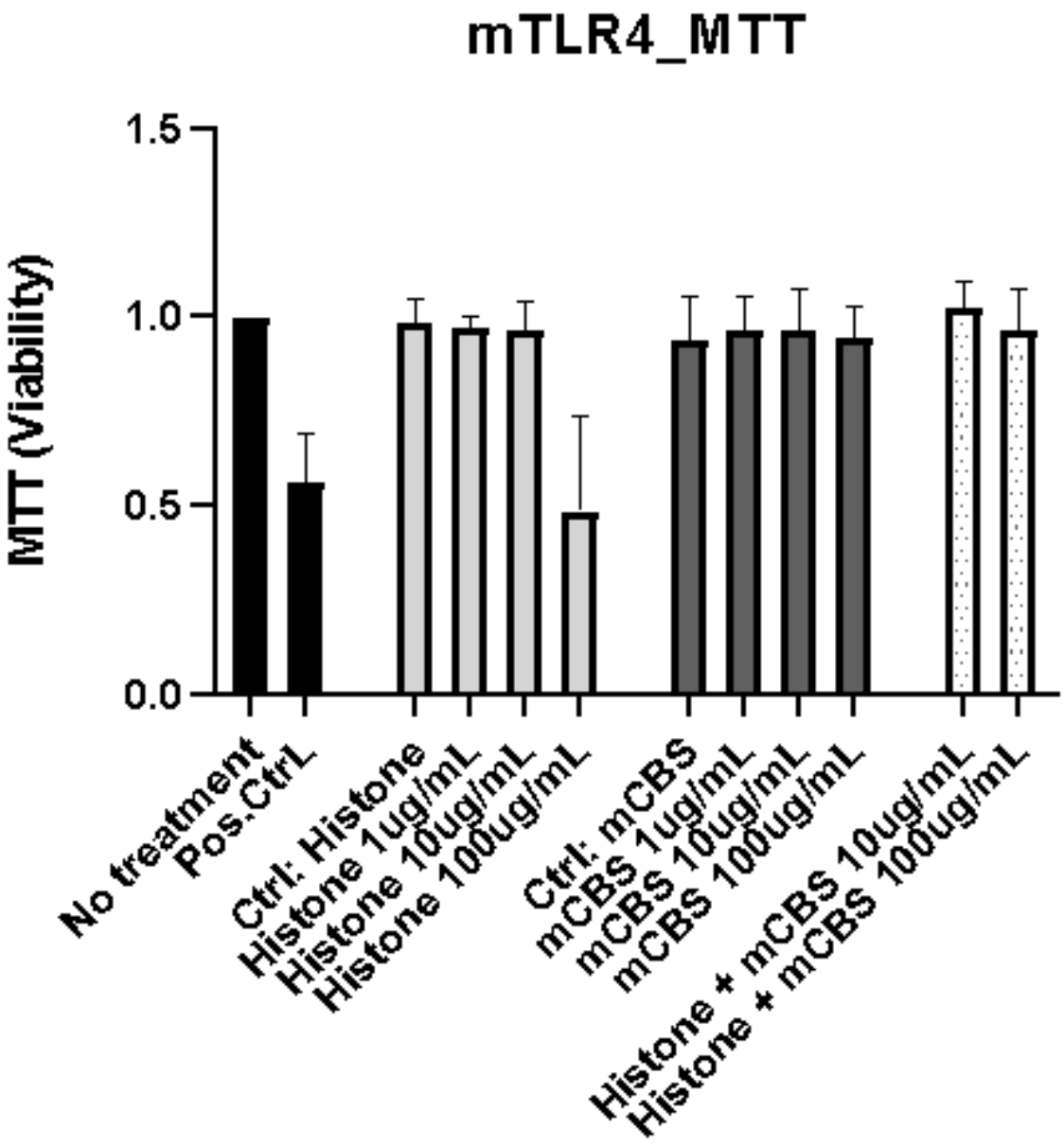
using a BioTek PowerWave XS Microplate Spectrophotometer purchased from BioTek, Vermont, USA.

Detection of NF-kB-induced SEAP production was determined with the use of HEK-Blue Detection medium and carried out according to the manufacturers protocol. In short 4x104 cells/well were seeded in a 96-well plate. Cells were treated over-night with mCBS and NF-kB activity was indirectly measured with a BioTek PowerWave XS Microplate Spectrophotometer purchased from BioTek, Vermont, USA at 630 nm, based on the accumulation of hydrolyzed SEAP colour substrate in the medium.

Results

To further investigate whether histones are capable of directly stimulating TLR4, a HEK293 reporter cell line expressing the TLR4, MD-2 and CD14 co-receptor genes was used. Stimulation with a TLR4 ligand induces the expression of the reporter gene as measured by the levels of secreted alkaline phosphatase. First, the toxicity of histones to these cells was determined using an MTT assay. Histone concentrations up to 10 mg/mL did not affect reporter cell viability but 100 lg/mL resulted in a significantly increased cell death (Figure 12.1) Co-incubation with mCBS prevented the cytotoxic effects of histones added to the HEK293 reporter cells. Next, TLR4 activity was measured after treatment overnight. The positive control of LPS caused a large increase in TLR4-mediated NF-KB activation, as expected (Figure 12.2). A small increase in NF-KB activity was seen at the highest concentration of 100ug/mL of histones, but a similar effect was seen in control (null) cells lacking TLR4, indicating it was not related to TLR4 stimulation (Figure 12.3). The inability of histones to stimulate TLR4-mediated NF-KB activation.

Figure 12.1 and 12.2. HEK293 cells transfected with TLR4, MD-2, and CD14 co-receptor genes versus control



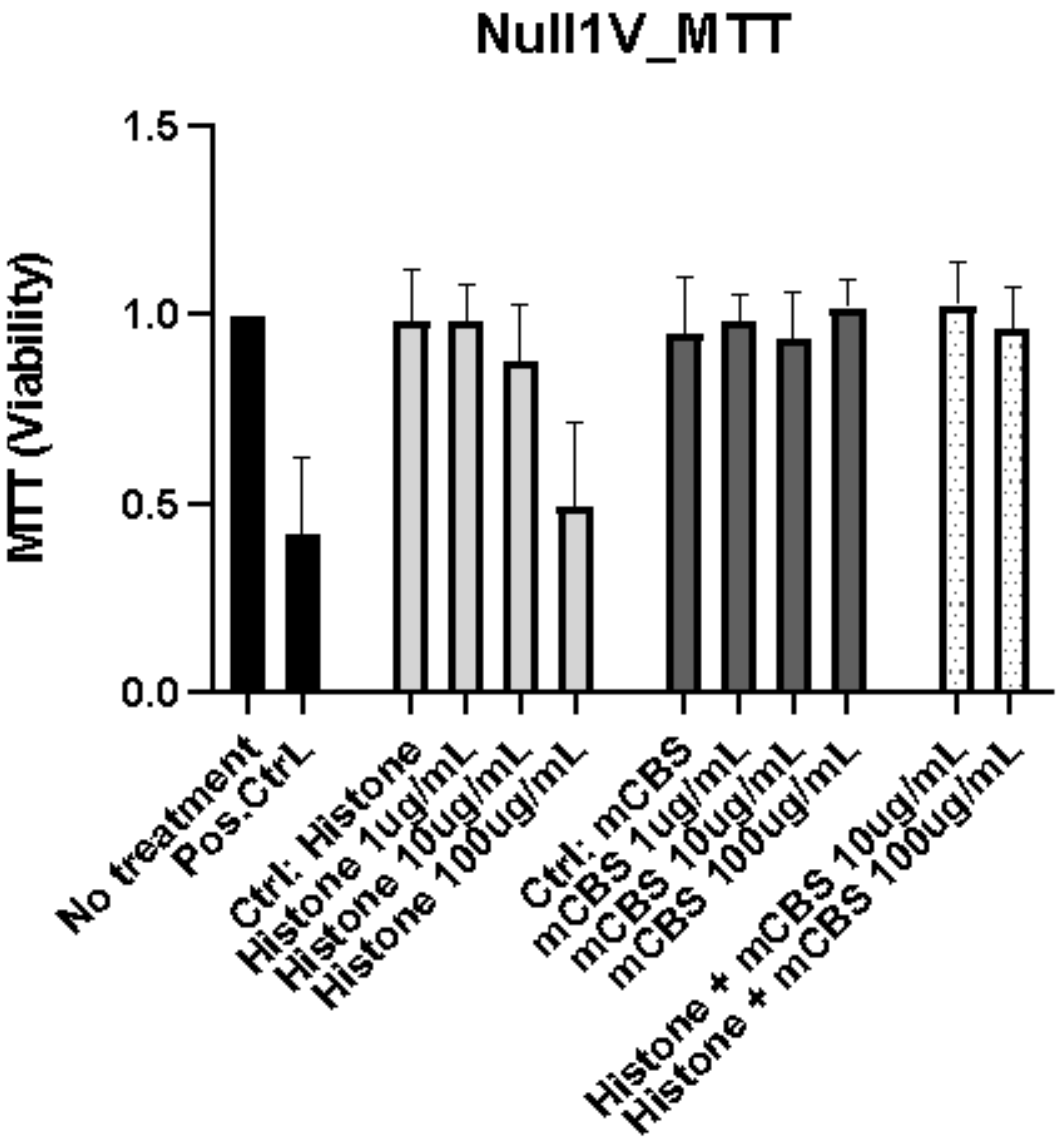


Figure 12.1 and 12.2. Survival of HEK293 cells treated as indicated, as measured by MTT assay. The positive control of LPS and 100 µg/ml histones decreased cell viability, equally in cells expressing the TLR4 receptor 12.1 or null vector 12.2. There was no significant difference (ns) between groups by 1 way ANOVA (N=6 independent experiments).

Figure 12.3 **HEK293 reporter cells expressing a TLR4 reporter**

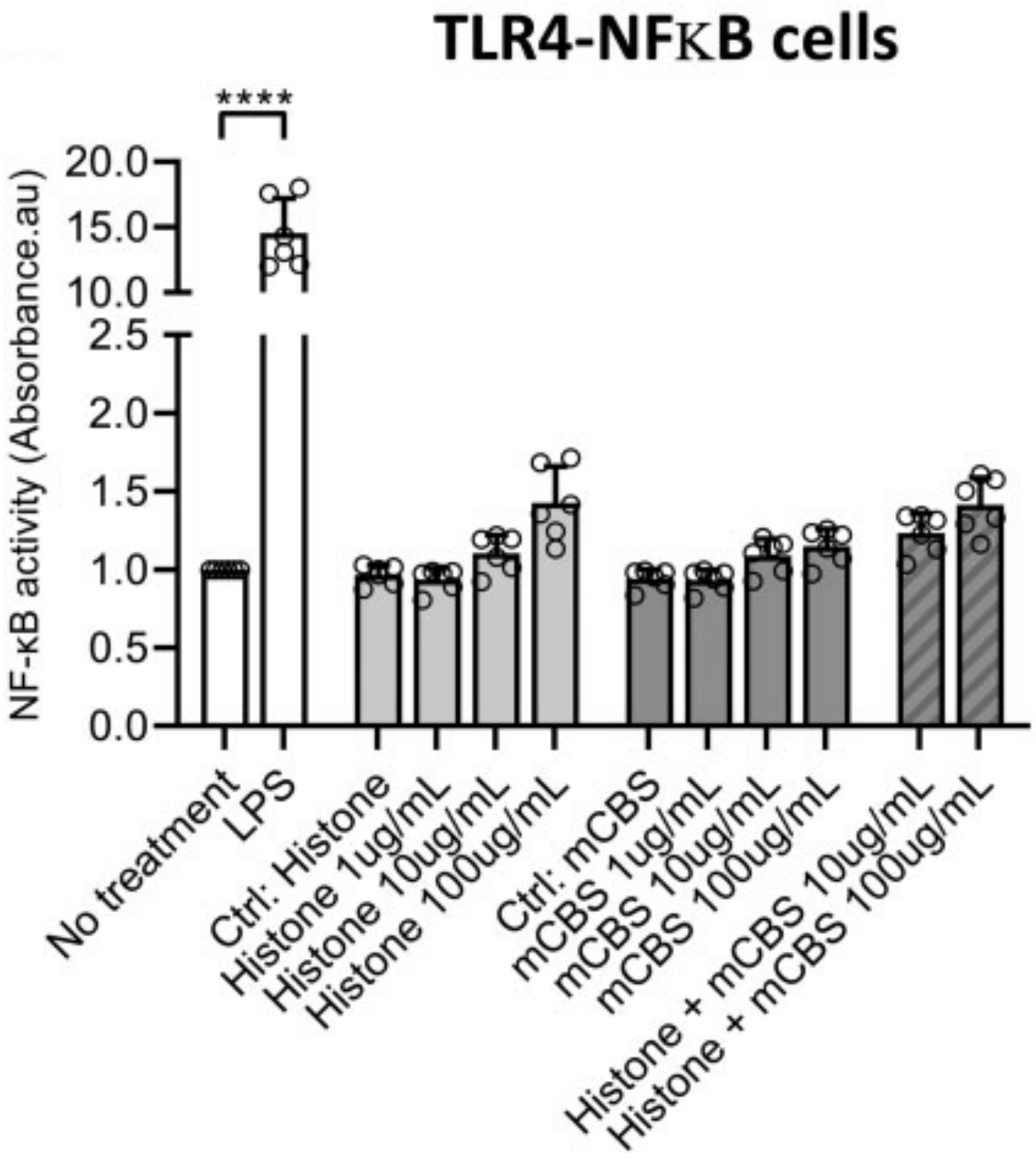


Figure 12.4 **HEK293 reporter cells expressing a null control construct**

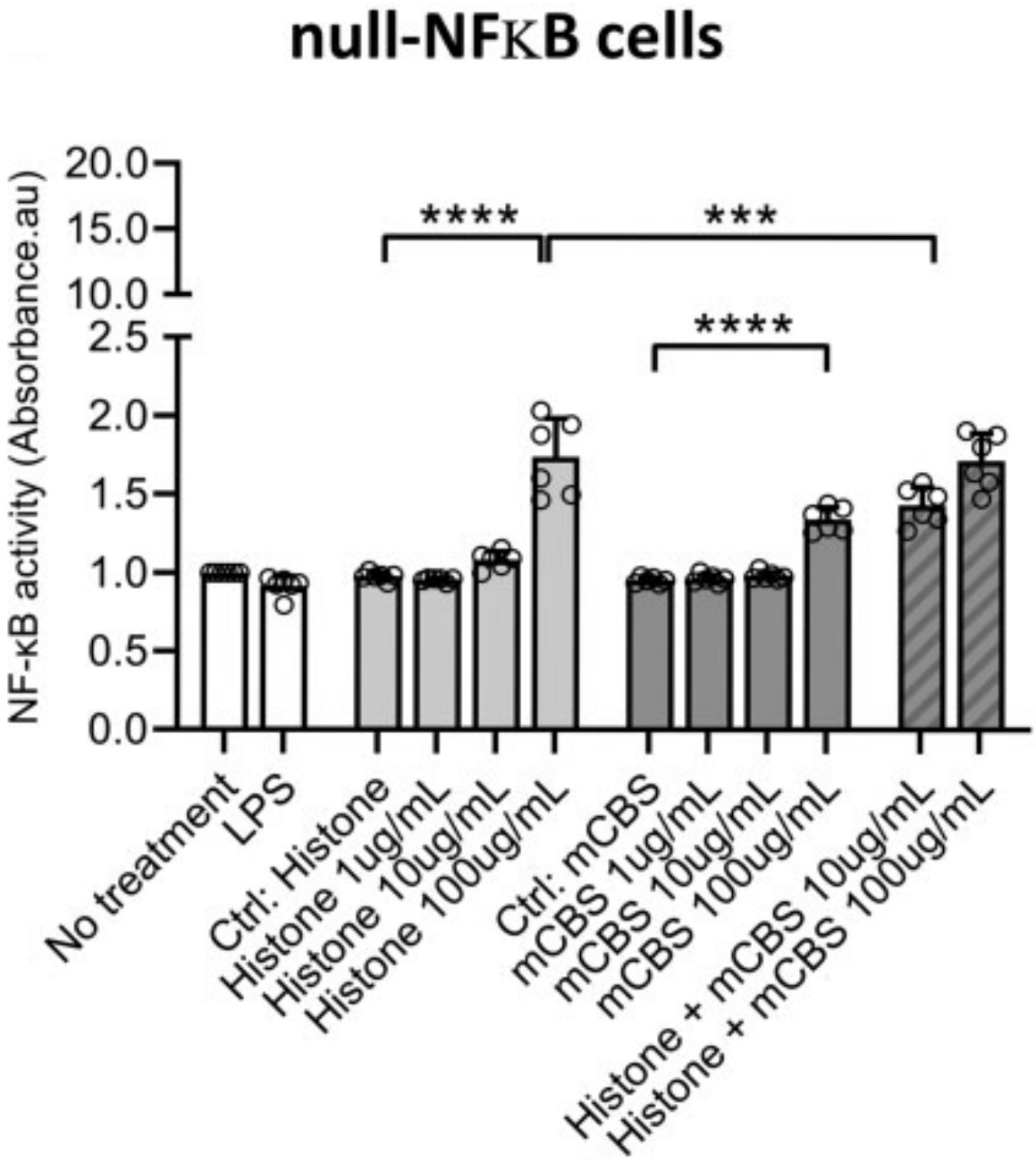


Figure 12.3 and 12.4. HEK293 reporter cells expressing a TLR4 reporter (fig 12.3) or null control construct (fig 12.4) were exposed to histones, mCBS or LPS overnight, then secreted embryonic alkaline phosphatase was measured as an index of

reporter activity. The positive control of LPS caused a huge increase of NFjB activity as expected (N = 6 independent biological experiments) ($P < 0.01$) analysed by one-way ANOVA and Tukey post-test. A small increase in NFjB activity was seen at the highest concentration of 100lg/mL histones, but as this occurred similarly in control cells, it was not related to TLR4 stimulation.

Chapter 13 – HIPe but not mCBS reduces infarct size in a rat coronary artery reperfusion model

Rats (280–360 g) were anaesthetised with an initial dose of 100–120 mg/ kg pentobarbital. Tracheostomy was performed, and artificial ventilation was achieved by connecting to a Small Animal Ventilator purchased through Harvard Apparatus. 1-lead electrocardiogram (ECG) was recorded using PowerLab 4/30 system purchased through AD Instruments and LabChart 7 software.

Drug or vehicle (PBS) were injected into the vein using a 25 G needle attached to a syringe. Treatments were administered 30 min before ligation of the coronary artery. Thoracotomy was performed and a silk suture was placed underneath the left anterior descending (LAD) artery and coronary artery occlusion was achieved by ligation of the suture. The heart was subjected to 30 min of ischaemia followed by 2 hr reperfusion. HIPe (2 mg/kg), mCBS (100 mg/kg), or vehicle (PBS) was given via i.v. injection 10 min before reperfusion. mCBS was alternatively given by bolus (100mg/kg) followed by infusion throughout I/R at a rate of 0.83 mg/kg/min via jugular vein. Once the protocol was completed, the coronary artery was ligated permanently, the animal was euthanized by severing the aorta and the heart was removed. Evans Blue dye [0.5 in high-Kþ (30 mM) PBS] was injected to demarcate the non-at-risk area. The heart was then stained using methods previously described.

Results

To determine whether histone antagonists are effective in vivo, anaesthetised rats were subjected to 30 min coronary occlusion via suture ligation of the left anterior descending artery followed by 2 h reperfusion. 10 min prior to the onset of ischaemia the rats were administered vehicle (PBS) or mCBS (100 mg/kg) as a bolus via i.v. injection, or mCBS (200 mg/kg) divided equally as an initial i.v. bolus then followed by infusion throughout I/R. The ischaemic area at risk (AAR) was similar in all groups (figure 13.1 and 13.2). mCBS did not significantly affect the infarct size in vivo in comparison to the vehicle (Figure 13.3). In contrast to mCBS, HIPe (2 mg/kg), which is selective for histone H4, significantly reduced myocardial infarct size from 68.6 ± 6.8% to 39.6 ± 6.0% (P = 0.008, Figure 5B).

Figure 13.1 **The area at risk (AAR) of the left ventricle (LV), in an *in-vivo* rat coronary artery occlusion model of I/R**

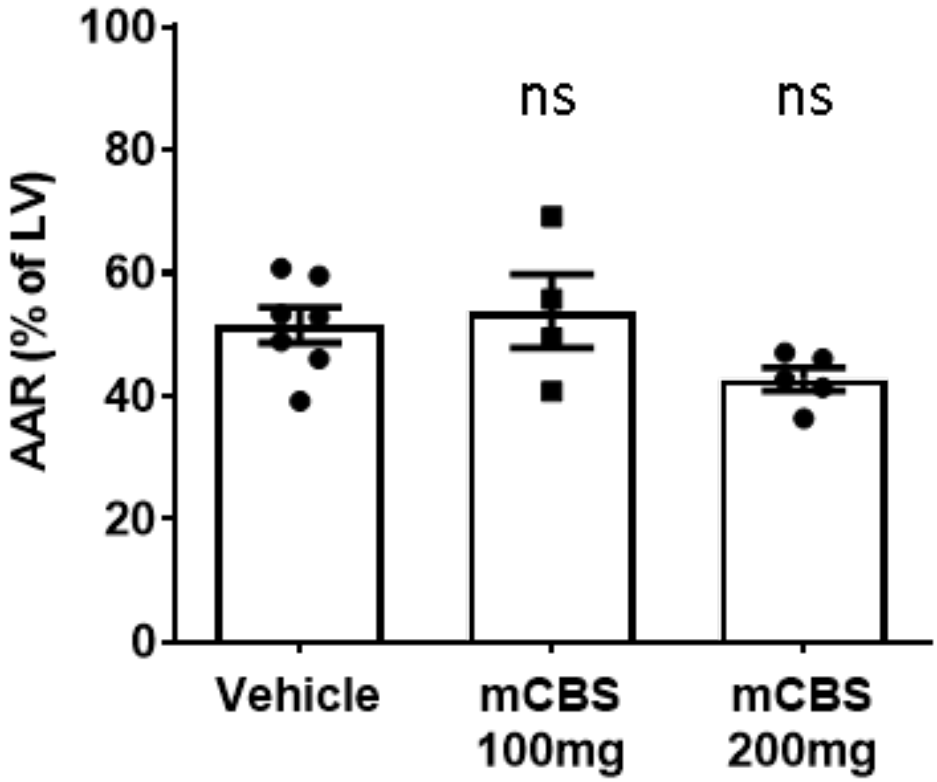


Figure 13.2 **The area at risk (AAR) of the left ventricle (LV), in an *in-vivo* rat coronary artery occlusion model of I/R**

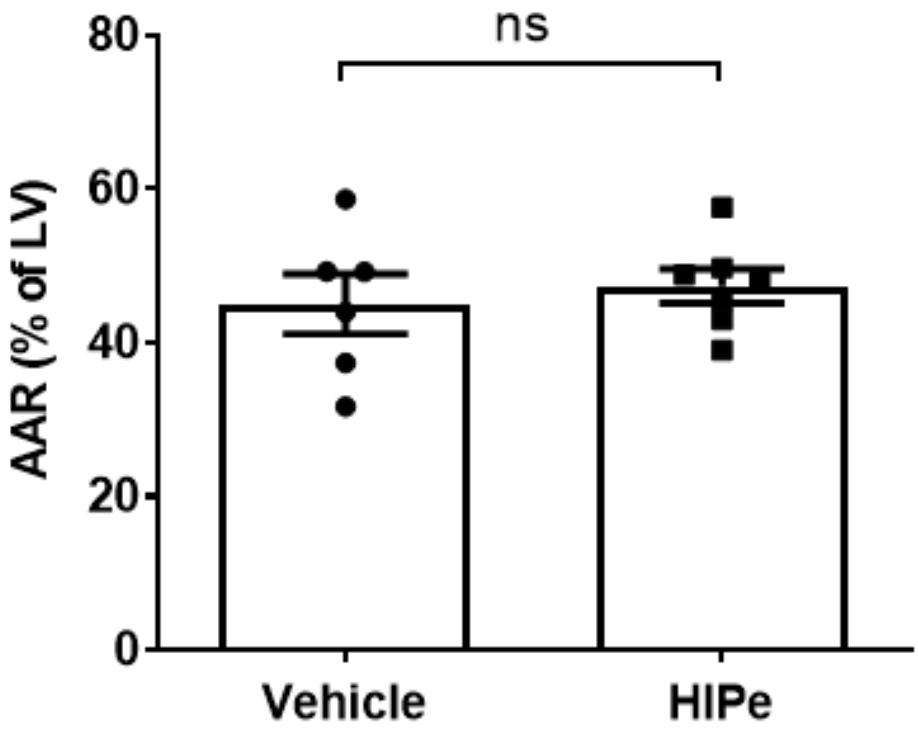


Figure 13.1 and 13.2. The area at risk (AAR) of the left ventricle (LV), in an *in-vivo* rat coronary artery occlusion model of I/R, after administration of vehicle or drugs as indicated. There was no significant difference (ns) between groups by 1 way ANOVA (N=7,4 and 5 in A; N=6 and 7 in B).

Figure 13.3 In an in vivo rat model of ischaemic and reperfusion injury

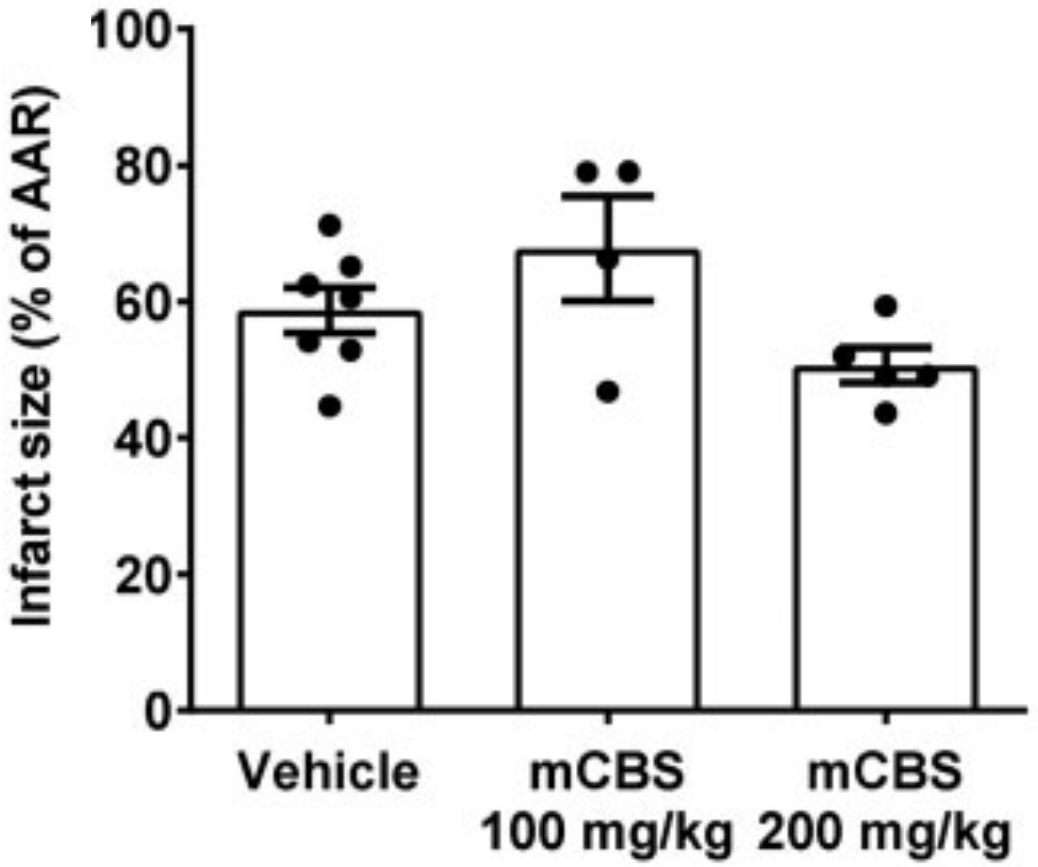


Figure 13.3. In an in vivo rat model of ischaemic and reperfusion injury, the histone-H4 specific neutralizing peptide HIPe, but not mCBS, reduced infarct size. (A) Infarct size as a percentage of area at risk (AAR) in an in-vivo rat coronary artery occlusion model of I/R, after administration of vehicle or mCBS. N = 4–7 hearts per group.

Chapter 14 Discussion -

Extracellular histones have been detected in several different pathological processes ranging from sepsis to atherosclerosis. Histones in conjugation with NETs have also been detected in the thrombus burden of patients suffering from ST elevation MI. During the process of necrosis or pyroptosis, DNA fragmentation will occur, and theoretically, DNA fragments, including histones, will be released into the extracellular matrix. The first step in identifying the role of extracellular histones in ischaemia- reperfusion injury is to prove their presence during the pathological process. Free extracellular histones have not been previously identified to be present in the penumbra of the infarct zone. However, both cell necrosis and pyroptosis have been identified in ischaemic tissue, which raises the possibility that during the process of ischaemia, intracellular debris, which includes DNA and histones, would be released into the area that is the extracellular matrix. DAMPs can originate from different sources and include extracellular proteins, such as biglycan and tenascin C, and intracellular proteins, such as high-mobility group box 1 (HMGB1), histones, S100 proteins, heat-shock proteins (HSPs), and plasma proteins, like fibrinogen, Gc-globulin, and serum amyloid A (SAA).

Quantifying the histones and nucleosomes that are detectable in the perfusate of hearts undergoing Ischaemia/reperfusion injury

Using a protein detection kit or ELISA assay, we were able to demonstrate the presence of nucleosomes and histones in the perfusate immediately after ischaemia and during the early stages of reperfusion. This correlates with previous studies,

which suggest that up to 50% of cardiomyocyte death occurs at reperfusion. However, histones bound to nucleosomes are not cytotoxic; it is free unbound histones, specifically H_2 and H4, which have demonstrated cytotoxic properties in earlier experiments(68). The next step in protein detection must be to identify the presence and quantify the specific amount of H4 histone.

Second, despite our best attempts with the current ELISA, we were unable to produce a concentration dose curve on the ELISA plate reader for histones. Without the concentration dose curve, we were unable to estimate the physical amount of histones present in the perfusate, nor could we quantify whether there is any correlation between infarct size and the amount of histone released. Instead, we were only able to calculate the percentage of histone in comparison to the control. These are both shortcomings of the ELISA plate we used. The ELISA assay specific to H4 is extremely expensive, and the control sample of histone that is given with the current kit we used had no concentration estimate. To overcome this problem, we quantified proteins using the DELPHI technique. The advantage of this technique is that we were able to use our own histone as the control at the concentrations we predetermined. Using the information provided in the manufacturer's leaflet, we were able to ascertain a concentration curve through which we calculated the concentration of histone H4 in each Langendorff perfusate. This technique allowed us to quantify the specific amount of free H4, rather than quantifying all free histones, including those bound to nucleosomes.

Histones are cytotoxic to isolated cardiomyocytes

Isolated histones (Sigma-Aldrich) when incubated in the presence of rat cardiomyocytes are cytotoxic towards the cells in a dose-dependent fashion. At concentrations above 40 ug/ml, they resulted in close to 100% cell death. These findings are corroborated by similar experiments within the literature(136). Histones are also implicated in the pathogenesis of sepsis-induced cardiomyopathy(96). However, the mechanism of how histones achieve their cytotoxic effects is disputed, with some researchers showing evidence for a Toll-like receptor-dependent mechanism(136) and others disputing the role of TLR, and instead demonstrating a direct effect of the histone on the cell membrane(68). The next step in my work was to delineate a mechanism of action. There is some evidence within the literature to suggest that histones can activate TLRs and exert their effects through the innate immune system. Our cell culture experiments also allowed us to shed some light on the mechanism of histone cytotoxicity by inhibiting TLRs by culturing cardiomyocytes exposed to histones in the presence of a specific TLR4 inhibitor. However, this method had some shortcomings. It has been shown that histones can activate multiple TLRs including TLR2 and TLR4, therefore using a specific TLR antagonist may not be enough to prove that histones are not using a secondary TLR. These shortcomings can be overcome by targeting the TLR downstream intracellular signalling cascade using a cell line with a knockout motif. Alternatively, we could use antibodies that bind to TLRs, or by isolating cardiomyocytes from TLR knockout rats. Another possible mechanism is culturing histones with cells that can fluoresce when TLR-dependent mechanisms are activated. This would allow us to quantify how much of the histone effect is via intracellular signalling pathways that utilise TLR.

TLR activation is also believed to play a crucial role in activating the intracellular

inflammasome, a protein structure that causes cell death via pyroptosis and allows the release of IL1β into the surrounding cellular matrix. There is a growing body of interest surrounding the role of pyroptosis in ischaemia -reperfusion injury, the mechanism of intracellular inflammasome formation, and subsequent IL-1β pro-inflammatory cytokine release. It is believed that pyroptosis may contribute to the excessive cell death that occurs after perfusion is restored to the ischaemic environment. Caspase inhibitors have been shown to inhibit the tissue death seen in ischaemia -reperfusion injury of the heart. (137)

Figure 14.1. Mechanisms of inflammasome activation.

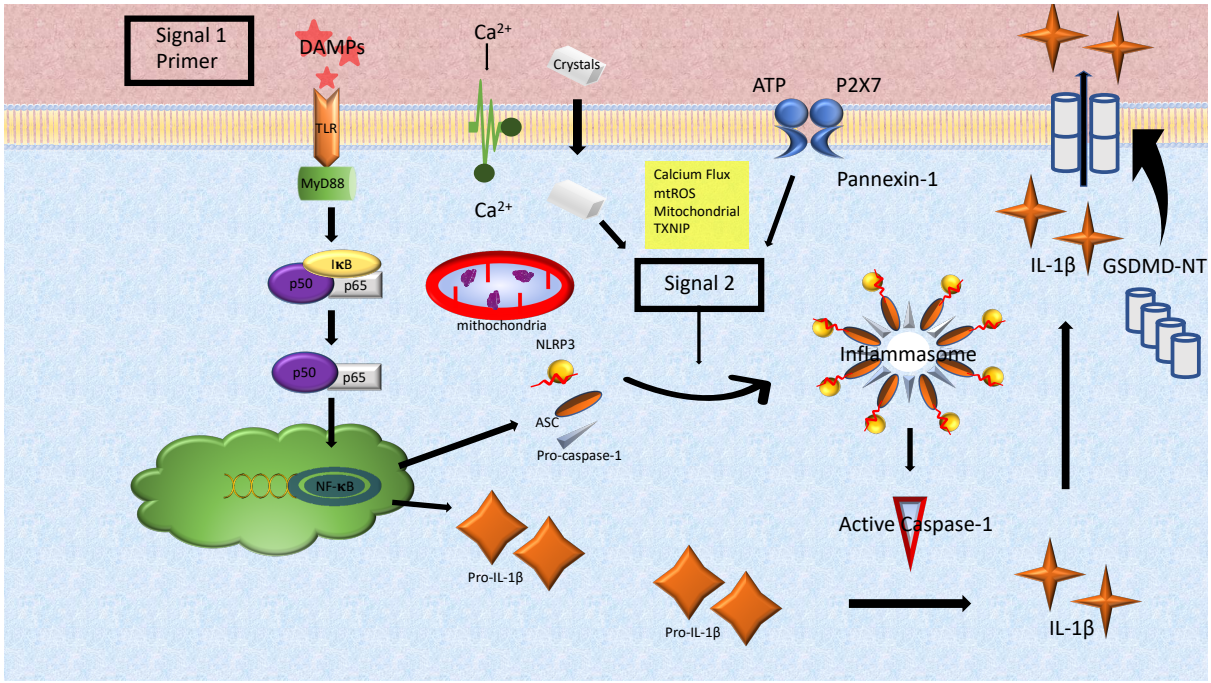


Figure 14.1 The activation of the inflammasome requires a danger-associated molecular pattern (DAMP) to stimulate TLR. This in turn activates NF-κβ mediated protein synthesis and generation of the components of the inflammasome (ASC, NLRP3, and pro-caspase 1), which accumulate within the cellular matrix. This step is called priming and requires an external pro-inflammatory signal. The second signal occurs independent of DAMP and is thought to be activated by a cell death-associated change in the cellular environment. Examples include calcium flux, mitochondrial ROS, and crystals. This signal stimulates the formation of the inflammasome protein; the inflammasome acts via caspase-1 and cleaves pro-ILβ into its active form IL-1β. This pro-inflammatory cytokine leaves the intracellular environment via the protein GSDRMN, a pore-forming molecule that ultimately kills the cell.

mCBS protects cardiomyocytes from the cytotoxic effects of histones

High dose mCBS (100 ug/ml) in vivo significantly reduces the cytotoxicity of histones (40 mg/ml) on isolated rat cardiomyocytes. A dose of 25 ug/ml mCBS was ineffective at protecting cardiomyocytes. This correlates with confidential data from Sirtex, the manufacturers of the compound, who demonstrated that a dose of 100 ug/ml mCBS was able to protect human endothelial cells from the cytotoxic effects of histones in vitro. The structure of mCBS gives it properties as an anion scavenger that can interact with positively charged histones. Heparin also exhibits a similar effect at a lower potency level. In vitro and in vivo heparin also demonstrated an ability to protect endothelial cells and cardiomyocytes from the cytotoxic effects of histones (106, 107, 138). It is felt that heparins exert this effect by binding to histones(105) and preventing them from interacting with the cell membranes of bystander cells. However, the use of heparin-based compounds in this way is limited by the procoagulant effect of heparin at high doses. mCBS has the important advantage of lacking the procoagulant effect of heparin and can be given at much higher doses, making it far more effective than heparin at neutralising histones. The next stage was to see if the effects of mCBS can be reproduced in an ex vivo model of ischaemia-reperfusion injury.

In a regional ischaemia model, mCBS improved the coronary flow rate after ischaemia -reperfusion

This is not an unexpected finding, as the Sirtex confidential data demonstrate that

mCBS protects endothelial cells from the cytotoxic effects of histones. Coronary flow rate (CFR) is also a marker of endothelial function, as ischaemia causes endothelial cell death, disordered NO production, and ultimately microvascular obstruction (MVO). It may be that by protecting endothelial cells from the deleterious effects of histones, there may be a reduction in the amount of MVO. This is an incidental finding within the experiment and may require further investigation at a later stage. To further investigate this, we could test mCBS in a hypoxic/normoxic rat aortic ring model to ascertain the effect of mCBS on endothelial function.

Why was there so much difference in the results between regional ischaemia and global ischaemia?

mCBS did not reduce the size of the infarct in the regional ischaemia model of Langendorff but did reduce the size of the infarct in the global ischaemia model. This is an unexpected finding and, in many ways, weakens our theory that histone antagonism may be cardioprotective. However, there are several technical differences between the two experimental models that may partly explain the different findings.

First, the infarct size volume in the regional ischaemia model was relatively small, with a mean size of 37.6 and a large standard deviation of 10.23. In the global ischaemia model, there was a mean size of 55.4 with a standard deviation of 6.8. The larger spread in infarct size with the regional ischaemia model alters the power calculations so that a larger sample size is required and there is a greater chance of a type 2 error. Conversely, the global ischaemia model gives a much larger infarct

size with a smaller spread, which reduces the likelihood of a sampling error.

Second, regional ischaemia only disrupts the flow of perfusate through a small section of the heart; a significant part of the myocardial substrate still has perfusate washing through the tissue. As a result, through ischaemia, the drug is not actually in contact with the infarction zone and necrotic tissue. At the point of reperfusion, the suture across the heart is loosened to allow the drug to reach the ischaemic zone; however, the period of local ischaemia will inevitably disrupt the microvasculature of the heart, and there may still be inadequate drug delivery to the infarct zone during reperfusion. In comparison, during global ischaemia, there is no flow of the perfusate drug through the whole of the heart during ischaemia, and to keep the heart warm, it is bathed in a bath of perfusate and drug. As the heart is also immersed in the drug during ischaemia, the drug delivery may be much better in comparison to the regional model, where the drug only perfuses through the heart. Global ischaemia results in an equal amount of microvascular obstruction in both the healthy tissue and the ischaemic tissue, which would mean this model allows for a better distribution of the active drug throughout the heart during reperfusion.
These differences may also partly explain why we were unable to detect histones in the perfusate of hearts within the regional ischaemia, but were able to detect them in the hearts undergoing global ischaemia.

mCBS did not cause a reduction in the amount of circulating histones

There was no difference in the amount of detectable histone in the perfusate from hearts that we subjected to global ischaemia in the presence of mCBS or vehicle. Initially, this seems like a surprising finding; however, the method of detection of histones is not accurate. There is no ELISA assay that is specific for free histones; instead, the cell death detection ELISA assay is a tool used to detect histones present in oligonucleotide samples. This kit has been used by other researchers attempting to quantify histone amount(68); however, there is an important distinction to be made between histones in oligonucleotide samples and free histones. Nucleosomes containing DNA and histones are part of the protein that is quantified when looking for histone-containing oligonucleotide samples. However, nucleosomes do not possess the pro-inflammatory effects of free histones; this is felt to be because the DNA chains within the nucleosomes change the structure of the histone(139). Nucleosomes, when injected into mice, do not cause the catastrophic response that is exhibited when free histones are injected into mice(140). Nucleosomes have also demonstrated little or no affinity for interacting with TLR(141, 142). In the absence of a specific ELISA for histones and the shortcomings within the literature for methods of histone detection that other researchers have used, our new technique of quantifying histones is an important finding. We overcame this hurdle by switching to using DELFIA immunoassays, which are a good alternative to ELISA assays as described previously. This technique allows you to use your own antibodies to bind to histones within the perfusate. As such, we were able to quantify a more exact amount of histone H4 release from infarcted rat hearts. This also allowed us to use more accurate concentrations of histone H4 in our in vitro isolated rat cardiomyocyte model. Also, the concentration of histone H4 was comparable to concentrations that have been shown to cause cardiomyopathy

in septic human patients, lending more weight to our theory of histone-induced cardiotoxicity.

Histone cytotoxicity occurs via a TLR4 independent process.

We found that resatorvid (TAK-242), a selective TLR4 inhibitor that binds the intracellular domain of TLR4 and suppresses its signalling, did not affect cell death caused by histones. Further experiements by collaboraters that I have included in this thesis showed further evidence for a TLR4 independent process. Furthermore, histone H4 addition to a TLR4-reporter cell line did not cause any activation beyond a small amount of non-specific activation that was also seen in the control (null) cells lacking TLR4. These results were supported by experiments in HL-1 cardiomyocytes in our group in which histones failed to cause any nuclear translocation of NF-κB. Together, these results suggest that extracellular histones are not, in fact, DAMPs, as they do not cause the TLR4-mediated activation of NF-κB. These data, coupled with my findings, have shown that histone-induced cardiomyocyte cell cytotoxicity occurs independently of TLR4 activation. In this regard, atomic force microscopy was used by Silvestre-Roig et al. to demonstrate that recombinant histone H4 is capable of causing cell membranes to bend and directly causes pore formation. Small-angle X-ray scattering was used to show that the N-terminal domain of histone H4 causes a similar degree of membrane remodelling of small unilamellar vesicles as other known membrane-remodelling proteins. Importantly, the HIPe peptide was shown to bind to the N terminus of histone H4 and prevent histone H4 from interacting with and altering cell membranes. Taken together, the experiments using TAK-242 and HIPe, and our previous experiments, demonstrate that HIPe strongly nullifies the

pore-forming effect of histones. My work has provided some basis for the use of potential novel anti-inflammatory therapies that target the deleterious effects of ischaemia- reperfusion injury after coronary artery occlusion. This has potential uses in a number of cardiovascular pathologies, including atherosclerosis, coronary artery thrombosis, and sepsis-induced cardiomyopathy. The widespread potential applications for compounds like HIPe and other compounds that can target the toxic release of necrotic compounds from cells that undergo disorganised cell death in acute illness have we believe a significant future potential.

HIPe but not mCBS is cardioprotective in an in-vivo model of rat coronary artery occlusion

In contrast to the ex vivo Langendorff experiments, mCBS did not signicantly affect the infarct size following I/R in vivo. This indicates that mCBS may not be cardioprotective in the presence of blood at the doses used in the setting of I/R. We speculate that the reason for this is that the non-specific nature of the electrostatic binding means that mCBS may bind with greater affinity to other proteins in high abundance in blood, leaving insufficient amounts to sequester histones in an acute I/R model (143). However, in contrast, HIPe which is selective for histone H4, significantly reduced myocardial infarct size in vivo. It will be important in future experiments to address the question of whether HIPe is cardioprotective when administered at, or shortly after, reperfusion, which is the clinically relevant time-point in patients presenting with an acute MI. The innate immune system appears to play a role in cardiac I/R injury,(144) as TLR inhibitors reduce infarct size in animal models (145) TLR activation is also believed to play a crucial role in activating the

intracellular inflammasome, a protein structure that causes cell death via pyroptosis, thereby allowing the release of IL-1b into the surrounding cellular matrix(144). Evidence for pyroptosis in I/R injury comes from studies with caspase-1 inhibitors, which reduce cardiac I/R injury(137) and the IL-1b inhibitor, Canakinumab, which in humans has shown to result in a 15% reduction in mortality associated with all cause cardiovascular disease (146).

Both free extracellular histones and histones incorporated into NETs have demonstrated cell cytoxicity in vitro experiments. extracellular histones have shown to trigger inflammation and cell death, either by stimulating pro-inflammatory cytokines resulting in the activation of cell death pathways or through the process of NETs. In human observational studies, raised histone serum levels have been demonstrated in multiple trauma patients and correlate with the severity of coagulopathy, endothelial damage, and inflammation(63). Isolated histones are known to activate TLR2 and TLR4; furthermore, TLR knockout mice are protected from the lethal effects of histones administered intravenously(42). In an ischaemic stroke model, histone infusion increased the infarct size and, conversely, histone neutralisation via an antibody infusion resulted in a reduction in infarct size(64). Similar cytoxic effects of histones have been demonstrated in kidney injury(65), sepsis(41), and even hair follicle death(66)

Conversely the role of NETs in cytoxicity is more complex. Large amounts of circulating NETs in unwell and septic human patients are associated with septic shock and organ failure (147). This could be due to increased NETosis induced cell necrosis or decreased clearance of extruded products with studies. However Although NETs have been shown to be associated with cell cytoxicity, studies have shown that NET aggregates can inhbit inflammation in a murine model of gout

partially through degradation of cytokines (148). This demonstrates that more research is required to delineate the complex roles of NET associated cytoxicity before we can identify NETs as a target in preventing cell cytoxicity.

COVID-19 Limitations

Unfortunately, I feel the need to mention the severe disruptions I had during 2020 and my PhD research as a result of the COVID-19 pandemic. During the first wave, my research was deemed non-essential in the first national lockdown in March 2020. I opted to pause my research and take up a role as a medical registrar on a COVID-19 and general cardiology ward in East Kent University hospitals, looking after patients with COVID-19. I worked there for four months in total. Unfortunately, once the research world normalised, my time out of my clinical training programme came to an end in September 2020. As a result, the final year of my PhD research was severely disrupted, which limited the number of experiments I could do in total.

Chapter 14 - Publications that arose from my work

Rauf A*, **Shah MK***, Yellon D, Davidson DM. Role of Caspase 1 in Ischemia/Reperfusion Injury of the Myocardium. *Journal of Cardiovascular Pharmacology*. 2019 (* Joint first author)

Shah MK, Yellon D, Davidson DM. The role of Extracellular DNA and Histones in Ischaemia-Reperfusion Injury of the Myocardium. *Cardiovascular Drugs and Therapeutics*. 2020

Shah MK, He Z, Rauf A, SB Kalkhoran, Heiestad CM, He D, Stensløkken K, Parish CR, Soehnlein O, Arjun S, Davidson SM, Yellon D. Extracellular histones are a target in myocardial ischaemia reperfusion injury. *Cardiovascular Research.* 2021

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