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Treatment of Ischemia Reperfusion Injury with RNA Interference

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Graduate Program in Surgery
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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Monograph Format

by

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Abstract

Ischemia reperfusion injury (IRI) occurs during transplantation and causes apoptosis and inflammation. The purpose of this research was to determine the effect of caspase-3, complement 3, and RelB gene silencing in the reduction of IRI using an *in vitro* model.

LLC-PK1 cells were used along with antimycin A for the *in vitro* IRI model. Prior to exposure to antimycin A, cells were transfected with caspase-3, C3, and RelB small interfering RNA (siRNA) alone or in combination and then analyzed.

The relative risk reduction of apoptosis in antimycin A treated cells with caspase-3 siRNA was 46.6% (p=0.019), RelB siRNA 42.8% (p=0.038), and complement 3 siRNA 13.9% (p=0.968). Combinations caspase-3 and RelB siRNAs showed significant changes, but were similar to transfection with caspase-3 and RelB alone.

Caspase-3 and RelB siRNA are effective at reducing apoptosis in an *in vitro* model of IRI and will be used in future large animal studies.

Keywords

RNA Interference, siRNA, Ischemia Reperfusion Injury, Transplantation, Apoptosis, Complement Pathway, RelB, Hypoxia.

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Table of Contents

List of Tables

List of Figures

List of Appendices

Chapter 1

1 Introduction

1.1 Overview

End stage renal disease (ESRD) affects many patients in Canada and the number continues to increase. ESRD is generally defined as chronic kidney disease that is being treated by dialysis or renal transplantation (1). In 2012, there were 41,252 people living with ESRD, and 5,431 patients were newly diagnosed with ESRD. This is nearly two fold that which were diagnosed in 1993 (2). In Canada and the United States, the most common causes of ESRD include diabetes, hypertension, glomerulonephritis, and cystic kidney disease (2, 3). Other less common causes include drug induced ESRD and pyelonephritis (2). Patients with ESRD often have significant co-morbidities including coronary artery disease, hypertension, and diabetes which leads to increased morbidity and mortality as well as increased health care costs (1). ESRD leads to a significant amount of health care spending, and in the United States in 2011, ESRD accounted for $34.3 billion in Medicare costs (3).

Renal replacement therapy (RRT) is the mainstay of treatment for ESRD. ESRD may be treated using different types of RRT which include hemodialysis, peritoneal dialysis, and renal transplantation. The type of RRT that is initiated will depend on many factors such as age, co-morbidities, availability of the various options, and supports at home. Hemodialysis requires vascular access and this can be achieved in the form of primary arteriovenous (AV) fistulas, synthetic AV fistulas, and double lumen catheters (1). Hemodialysis can be performed at home or in an outpatient setting. Peritoneal dialysis requires an intra-abdominal peritoneal dialysis catheter and is performed at home by patients. For patients who are candidates, dialysis often provides a bridge to renal transplantation. Renal transplantation provides the best treatment for ESRD. However, not all patients are candidates for transplantation and significantly, there are a limited number of donor organs available.

1.2 Renal Transplantation

1.2.1 Overview

The first successful renal transplant was performed in 1954 and involved two identical twins (4). Since that time, the field of transplantation has expanded rapidly and many important milestones have been reached. In 1961, azathioprine was first used to prevent rejection in an unrelated kidney transplant patient (5). This patient and the next ultimately died shortly after transplantation due to the toxicity of azathioprine. However, the third patient was treated successfully and azathioprine eventually became well established in the prevention of rejection (5). Prednisone, tissue typing, and the development of antilymphocyte globulin also improved outcomes through the 1970s (5). Unfortunately, large doses of prednisone leading to steroid dependence contributed to increased morbidity and mortality. The introduction of cyclosporine in 1979, and then tacrolimus in 1989, allowed for significant improvements in the prevention of rejection (6). Refinements in the timing of the administration of anti-rejection medications have lead to an improvement in the morbidity associated with immunosuppresion. Since 2001, pre-transplant treatment with an antilymphoid antibody followed by tacrolimus post-transplant has been shown to be effective and reduces the complications associated with long-term immunosuppresion (6).

Renal transplantation continues to be the best form of RRT in the treatment of ESRD. There have been numerous studies which demonstrate a survival benefit in patients who have undergone renal transplantation compared to those who continue with dialysis for RRT (7-10). Renal transplantation is also more cost effective in the long-term, but has higher initial costs. The per person per year costs for Medicare in the United States in 2011 were $87,945 for hemodialysis, $71,630 for peritoneal dialysis, and $32,922 for renal transplantation (3). Renal transplantation is also more convenient for patients as they do not need to undergo dialysis multiple times per week. Although there are significant benefits with renal transplantation, there continues to be a worldwide shortage of available organs for transplantation. In Canada in 2012, there were 1054 renal transplants performed, and 2469 patients on the waiting list (2). Organs may be retrieved from living donors or deceased donors. In the past, deceased donors were those who were

determined to have a neurologically determined death (NDD). In order to expand the amount of available organs, efforts have been made in recent years to procure organs from patients who have undergone a cardiac arrest. This has been termed donation after cardiac death (DCD). There has also been a move to increase the number of living and deceased donors by accepting organs from those who do not meet the usual criteria. This has been termed expanded criteria donor (ECD). These new categories have also created the need to define a standard criteria donor (SCD) which is a donor that has undergone brain death and does not meet the criteria for an ECD (11).

1.2.2 DCD and ECD in Transplantation

DCD donors are deceased donors in whom cardiac arrest or cessation of cardiac function occurs prior to procurement of organs (11-13). This results in a situation in which the organs which are procured are either not perfused or under perfused for a period of time and remain in the donor's body until death can be confirmed and the patient taken to the operating room (OR). This is commonly referred to as the warm ischemic time (WIT). The functional WIT more specifically refers to the period of time during which the donor's systolic blood pressure drops below 50mmHg or the arterial oxygen saturation drops below 70 percent, and lasts until the organs are perfused with preservation solution. Once procured and placed on ice, this signifies the start of the cold ischemic time (CIT) (12). The WIT in organs from DCD donors causes an additional insult to the organs that is not seen in organs from NDD donors as the WIT is very short and lasts for only a few minutes. This leads to fewer organs being accepted for transplantation from DCD donors and a higher rate of delayed graft function (DGF) in transplanted kidneys. The discard rate for organs which have been procured but are not transplanted is around 20 percent for DCD donors (14). DGF is usually defined as the need for dialysis within the first week of transplantation and this leads to increased costs and morbidity (15). The rate of DGF in kidneys from DCD donors is twice that of kidneys from SCD donors, however, although the rate of DGF is significantly higher, the five-year overall graft survival rate is similar between the two groups (14). The use of DCD donors in Canada continues to increase while the NDD donor rate has remained relatively stable over the last ten years (Figure 1) (2).

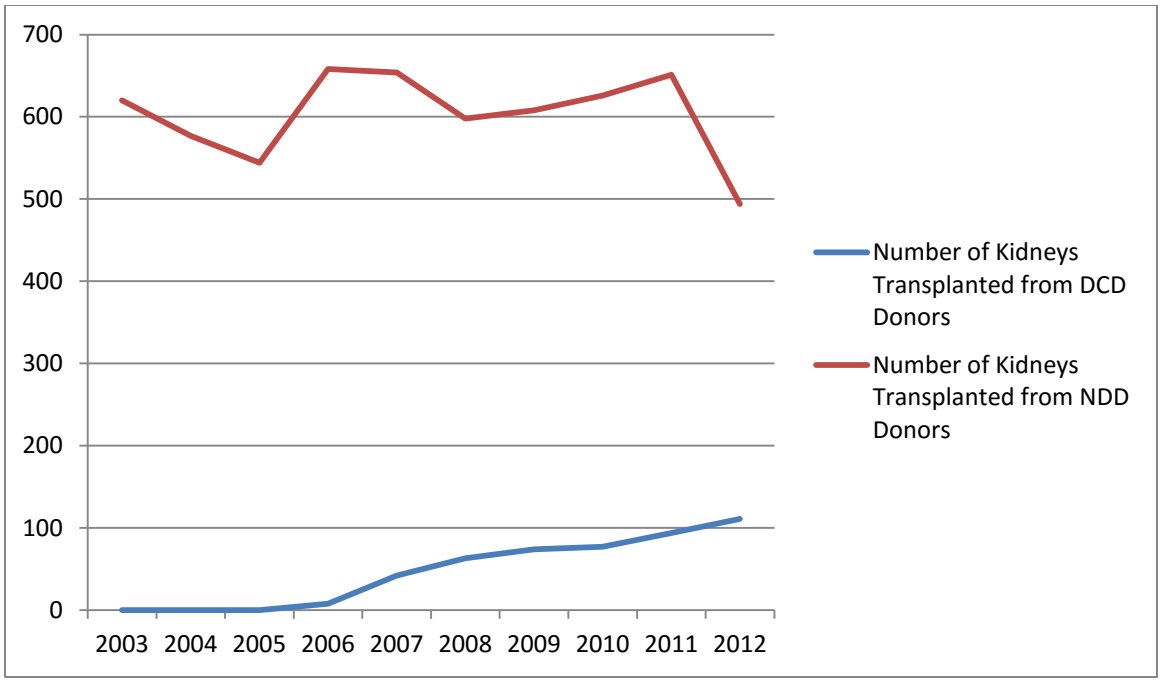


Figure 1 - Kidneys transplanted from DCD and NDD donors in Canada from 2003 to 2012 (2).

There are five different categories of DCD donors according to the modified Maastricht classification (12, 13). These include the following:

1. Dead on arrival
2. Unsuccessful resuscitation
3. Cardiac arrest after withdrawal of life support in patients who are not brain dead
4. Cardiac arrest after brain death
5. Unexpected cardiac arrest in an intensive care unit (ICU) patient

The classes can also be more generally categorized into controlled and uncontrolled DCD donors (11). Controlled donors come from classes 3 and 4, and uncontrolled donors from classes 1, 2, and 5. A controlled DCD donor is a patient whose family has provided consent for organ donation, but as in class 3, is not brain dead, or in class 4, an unexpected cardiac arrest occurs in an already identified donor who is brain dead. Class 4 donors are very rare and most of the controlled DCD donors come from class 3. In this case, life support is withdrawn after the transplant team is ready, and the donor monitored

until there is no mechanical cardiac function or signs of life. This can be determined by an arterial catheter, loss of pulsatile blood flow on Doppler ultrasound, or loss of blood flow on echocardiography (16). Asystole on a cardiac monitor is also sufficient for this determination as there can be no mechanical cardiac function if there is no electrical activity. After this, the donor is further monitored for a set period of time to ensure that there is no return of cardiac function or auto-resuscitation. This period of time varies from hospital to hospital and is the focus of ongoing debate as the removal of organs cannot contribute to the death of the donor (12, 16). This time must not be prolonged so as not to increase the WIT, however. A time of 5 minutes is generally accepted as an appropriate amount of time in order to ensure that the cessation of cardiac function is permanent, as the longest reported time of auto-resuscitation after asystole is 65 seconds (16, 17). The determination of death must then be done by someone who is not associated with the transplant team to avoid any conflict of interest. After the determination of death, the donor is transferred to the OR, if they are not there already, for procurement of the organs. Withdrawal of life support can occur either in the OR or in the ICU. The OR allows for a decreased WIT, however the ICU allows for the family to remain by the bedside until the donor is deceased and, in general, is a better place for withdrawal of life support.

Uncontrolled DCD donors are significantly different from controlled DCD donors in that warm ischemia will already be established before the decision is made to consider donation. This type of donation is practiced in some countries (e.g. Spain) and usually only makes use of the kidneys (12). After the determination of death in potential uncontrolled DCD donors, the common femoral artery is canulated with a double-balloon, triple lumen catheter with the lower balloon lodged at the aortic bifurcation and the upper balloon above the renal arteries. The kidneys are then perfused with cold preservation solution. A catheter is also inserted into the femoral vein to allow outflow of the preservation solution. At this point consent is obtained from family members, if not already done, and the donor transferred for procurement of the kidneys (18). Alternatively, an external cardiac compression and ventilation machine is used until consent can be obtained by family and then the common femoral artery is canulated (19). Strict criteria are needed to ensure that the kidneys obtained from these donors are of a

high enough quality for transplantation. In a retrospective review from Belgium, Dupriez et al. found that over 14 years, 39 procedures were initiated on potential uncontrolled DCD donors. Of the 78 kidneys available, only 40 were procured, and of these, only 25 were transplanted. Overall, there was a 32 percent kidney conversion rate (percentage of kidneys transplanted from donors who had the procedure initiated). They also found that DGF occurred in 54 percent of kidneys and the one-year graft survival rate was 86 percent (18). Uncontrolled DCD occurs in a number of countries, and in some is the only form of DCD. Protocols vary between countries for both controlled and uncontrolled DCD including determination of death, preservation of organs while awaiting family consent, and an opt-in versus opt-out system of consent for organ donation (20). This leads to many ethical considerations for both types of DCD as it is important to ensure that procurement of the organs is not the cause for death.

ECD donors present another challenge in transplantation. An ECD donor is defined as a donor who is over the age of 60, or who is between the ages of 50-59 with two of the following: hypertension, cerebrovascular accident as the cause of death, or a creatinine above 120umol/L. The use of ECD donors is necessary to increase the amount of organs available for transplantation, however these organs are of a lower quality due to the donor characteristics. ECD donors may either be living or deceased donors and the overall rate of DGF is approximately 30% which is below the rate seen in DCD donors (14). The five year graft survival rate has been shown to be 72.1% and 86.9% for ECD and SCD deceased donor kidneys, respectively, in a recent study by Papachristou et al. (21). They also found that the overall survival rates were similar at 5 years between the groups and slightly favoured the SCD group at 10 years. Additionally, older patients were more likely to receive kidneys from ECD donors and this may have contributed to the decreased overall survival at 10 years.

Although many advances have been made in transplantation, the need to develop ways to protect the graft during procurement, transport, and implantation is still in need of novel methods. This is especially important in DCD and ECD organs which inherently are of a lower quality than those obtained from living or NDD donors who meet the criteria for a SCD. There is a significant amount of injury that can occur during this time and it is a

type of ischemia reperfusion injury (IRI). IRI occurs when there is a partial or complete cessation of blood flow to a tissue or organ followed by a reperfusion phase. Therapies that target IRI may have a significant benefit in increasing the quality and survival or grafts, and demand further investigation and research.

1.3 Ischemia Reperfusion Injury

1.3.1 Overview

IRI occurs in a number of different clinical settings including myocardial infarction, shock, acute limb ischemia, compartment syndrome, and transplantation (22). IRI occurs as a result of many complex molecular pathways and can be separated broadly into the ischemic and reperfusion phases. The duration of the ischemic phase is variable and related to the amount of injury observed. The amount of injury observed is also dependent on the type of organ or tissue affected with the brain and heart being most susceptible with irreversible damage seen in less than 20 minutes and at 20 minutes, respectively. The kidney can withstand normothermic ischemia for up to 30 minutes (23).

Therefore, in order to decrease the amount of IRI in renal transplantation, kidneys are stored at 4^0C after being flushed with a preservation solution to decrease the metabolic activity of the cells by 12 to 13 times (24). This allows for the identification of a recipient as well as transportation of the graft which can take many hours. These hypothermic conditions, however, have been shown to detrimental in experiments that have exposed renal tubular rat cells to hypothermia and subsequent rewarming without ischemia (25). Cell changes were seen which were similar to acute tubular necrosis in renal failure. A balance must therefore be achieved between protecting the grafts from IRI with hypothermic conditions and preventing cold-induced injury. IRI is a complex process and requires an understanding of the molecular and cellular changes which occur in order to identify the potential therapies.

1.3.2 Molecular Events in IRI

As has been stated, IRI can be broadly separated into the ischemic and reperfusion phases. During these ischemic phases, blood flow to the affected organ or tissue is

interrupted, either completely (as in transplantation) or incompletely, and leads to decreased oxygen delivery. This causes a switch from aerobic to anaerobic metabolism, reduced ATP production, a decrease in the pH due to lactic acidosis, and a buildup of toxic metabolites which cannot be cleared (22, 26, 27). Additionally, there is a buildup of sodium ions as the Na^+/K^+ ATPase is inhibited by the low pH and ATP levels and the Na^+/H^+ antiporter attempts to expel H^+ ions to increase the pH. The increased concentration of sodium in the cell also causes an influx of water which causes the cells to become edematous. Arrest of glycolysis with a buildup of the intermediates further increases the osmolarity in the cells and edema (22, 28).

A rise in the concentration of sodium in the cell also leads to a reversal of the Na^+/Ca^{2+} antiporter, and, along with a reduced uptake into the endoplasmic reticulum, there is a significant increase in calcium ions in the cell (22, 23, 28). High concentrations of calcium within the mitochondria lead to mitochondrial permeability transition pore (mPTP) opening and the release of cytochrome c. Cytochrome c is important in the initiation of apoptosis. mPTP opening does not happen immediately during the ischemic phase due to the acidotic environment and will occur during the reperfusion phase when the pH returns to a more physiologic level (22, 23). The increased calcium concentration will also lead to priming of caspases, phospholipases, and calcium dependent calpains which will also be activated when the pH is corrected (28). Calpains and caspases are proteases that are important in cell injury and death, and calpain inhibition has been shown to reduce the effects of IRI in an *in vivo* setting using rats (29). Caspase activation leads to apoptosis and will be expanded on in section 1.3.3: Cell Death in IRI.

Additionally, increased cytosolic calcium causes activation of Ca^{2+}/calmodulin-dependent protein kinases (CaMKs). There are a number of different types of CaMKs, and some lead to cell death and adverse outcomes following IRI, while some have been shown to have a protective effect by inducing autophagy and decreased organ injury (30, 31). Taken together, all of these changes set the ischemic cells up for an additional and more severe injury seen when the organ or tissue is reperfused and oxygen is delivered to the cells.

Reperfusion occurs in transplantation when the graft is once again perfused with blood from the recipient. This is obviously necessary for the function of the graft, but in itself, this event sets off an additional round of cellular injury that builds on the cellular processes during the ischemic phase. Two important events that take place during this time include the return of oxygen to the cell and the return to a physiologic pH. As the extracellular pH rises, the cells rapidly expel H^+ ions via the Na^+/H^+ antiporter. This further increases the sodium concentration in the cell and once again reverses the Na^+/Ca^{2+} antiporter, increasing the calcium concentration in the cell (22). This further exacerbates the effects of calcium overload which occur during ischemia including mPTP opening and protease activation. Reperfusion also allows for the production of reactive oxygen species (ROS) which cause a significant amount of injury to the organ or tissue.

ROS are generated during the ischemic and reperfusion phases, but mostly occur during the reperfusion phase. The principle ROS produced is superoxide ($O_2^{-}\cdot$), as was shown in research that used superoxide dismutase (SOD) to effectively treat IRI, and is formed by the electron transport chain and by a number of different enzymes including cytochrome P450 oxidases, NADPH oxidase, nitric oxide synthase and xanthine oxidase (23, 32).

In the mitochondria, the electron transport chain involves a number of redox reactions which lead to the reduction of molecular oxygen to water and drives the production of ATP. Normally, most of the oxygen is completely reduced, however, there is a baseline leak of superoxide that accounts for about 1-2% of the molecular oxygen used. This superoxide is rapidly scavenged by SOD and cytochrome c and does not cause much damage (23, 32). In IRI, the mitochondria accounts for a significant amount of ROS via increased production as well as a decreased capability to scavenge the ROS which are produced (23).

Xanthine oxidase has been known for some time to be an important source of ROS and produces superoxide and hydrogen peroxide from molecular oxygen, water, and hypoxanthine (33, 34). Superoxide produced by xanthine oxidase and other enzymes may interact with iron-sulphide containing enzymes and inactivate them, but it is usually rapidly converted by SOD into molecular oxygen and hydrogen peroxide and is not

thought to contribute very much to the overall injury. However, superoxide may also be converted to the more reactive hydroperoxyl radical or react with nitric oxide (NO) to form peroxynitrous acid (ONOOH). Hydrogen peroxide can also generate the hydroxyl free radical (OH·) (23, 32). These free radicals may then go on to create oxidative cellular stress. This occurs through the oxidation of macromolecules and the entire process can be separated into the initiation, propagation, and termination steps (32). The initiation step involves creation of the free radical as mentioned above. The propagation step in cells involves the production of alkyl radicals from alkene groups followed by the addition of molecular oxygen to the alkyl radical to produce a peroxyl radical. The termination step involves a single electron redox reaction to a non radical. This step often produces a molecule which is unstable within the cell structure. For example, lipid peroxidation can affect the cell and mitochondrial membrane, DNA oxidation can create mutations within the genetic code, and amino acid oxidation or nitration can damage proteins and affect their function (32). Whether or not a cell dies depends on the extent of damage caused by the ROS and whether the cell is able to repair the affected macromolecules.

This release of ROS is followed by the migration of leukocytes to the affected tissue or organ which in turn produce a large amount of ROS and proteases, leading to further injury and cell death (34). The significant changes in cellular ion concentrations and the massive release of ROS in IRI leads to inflammation and activation of the immune system and can induce cellular death in a number of different ways.

1.3.3 Cell Death in IRI

Cellular death in IRI occurs through apoptosis, autophagy, necrosis, and a recently discovered process known as necroptosis or regulated necrosis (23). Apoptosis is the term given to programmed cell death and leads to cell shrinkage, blebbing, nuclear fragmentation, degradation of DNA, exteriorization of phosphatidylserine, and phagocytosis of the cell fragments in the absence of inflammation (35, 36). This process is essential to the normal growth and development of all organisms and all cells contain the enzymes which are required for the initiation and progression of apoptosis. These enzymes are the caspases and can be activated through intrinsic or extrinsic pathways

when the cell is exposed to conditions which necessitate its death in a programmed, non-inflammatory fashion (35).

The intrinsic pathway can by activated by different types of cellular stress including DNA damage, oxidative stress, hypoxia, UV radiation, toxic compounds, and reduced growth factors (23, 36). This causes the integration of the pro-apoptotic proteins in the Bcl-2 protein superfamily into the outer mitochondrial membrane leading to the release of cytochrome c and other apoposis initiating proteins. Following its release into the cytosol, cytochrome c binds to apoptotic protein activating factor-1 (Apaf-1), forming an apoptosome which activates caspase-9. Caspase-9 then activates caspase-3 which leads to apoptosis through the degradation of many cellular proteins (23, 35, 36). Other proteins released from the mitochondria include endonuclease G (endoG) which is responsible for DNA fragmentation, as well as Smac/DIABLO and Omi-HtrA2 which bind and cleave inhibitors of apoptotic proteins (IAPs). IAPs are required for the inhibition of caspases (23, 35). The extrinsic pathway in apoptosis is activated by the binding of ligands to death receptors. These ligands include TNF superfamily proteins. Important death receptors include Fas, TNFα, and TRAIL which undergo trimerization when activated. Activation leads to the recruitment of the death domain proteins FADD and TRADD which activate caspase-8. Activated caspase-8 can then activate caspase-3, caspase-6, and caspase-7 leading to apoptosis (36). Apoptosis is an important form of cell death that occurs in IRI through both the intrinsic and extrinsic pathways.

Another important form of cell death in IRI is autophagy which does have some overlap with apoptosis. Autophagic death involves the development of autophagic vacuoles or autophagosomes and does not involve inflammation, DNA degradation, or caspase activation (35, 36). Autophagosomes engulf damaged organelles or protein complexes and then fuse with lysosomes which contain enzymes that are able to degrade these structures. Autophagy is a normal pro-survival cellular process that leads to the turnover of macromolecules and can help during episodes of cellular stress such as infection, by providing amino acids and fatty acids. However, in times of severe stress, such as in IRI, autophagy can actually lead to cell death through ongoing degradation of cellular components (23). Many of the proteins involved in signaling in autophagy are also

involved in apoptosis, especially the Bcl-2 superfamily, and as such, there is significant overlap between the two processes (36). Both of these forms of cell death do not produce an inflammatory reaction as is seen in necrosis.

Necrosis occurs in IRI as well and involves cell and organelle swelling, mitochondrial dysfunction, and eventual rupture of the plasma membrane and spilling of intracellular contents (23). This involves an inflammatory response as mast cells are attracted to the necrotic cells. Originally, this was thought to be an accidental type of cell death without cellular regulation and involving cell death that did not appear to be autophagic or apoptotic (35). Recently, however, the emerging concept of regulated necrosis has been developed in which the cell death is genetically controlled (23, 37). There are different forms of regulated necrosis such as necroptosis, ETosis, ferroptosis, NETosis, parthanatos, pyronecrosis, and pyroptosis. Necroptosis is the most studied and best understood currently. The receptor interacting protein kinase 1 (RIPK1) was first shown to be important in programmed cell death that was caspase independent and produced a necrotic morphology by Holler et al. (38). This represented an important discovery that lead to many further advancements in research into regulated necrosis. RIPK3 was subsequently identified and these two kinases have been shown to be essential in regulated necrosis. Interestingly, RIPK1 and RIPK3 signaling can lead to apoptosis as well, but when caspase-8 is inhibited, they activate mixed lineage kinase domain like (MLKL) leading to regulated necrosis. RIPK1 can also induce up-regulation of the non canonical NF-κβ pathway and increased cell death (23, 37). Regulated necrosis has been shown to be an important form of cell death in IRI, and is thought to be possibly more prevalent than apoptosis (39, 40). This was shown in a renal IRI model using two separate inhibitors. The first was necrostatin-1 which inhibits RIPK1, and the second was zVAD, an inhibitor of the caspases. It was shown that necrostatin-1 reduced organ damage, while zVAD did not produce similar results (39).

Cells exposed to IRI can die through any of the mechanism described above and much research has focused on the inhibition of one or more of these mechanisms. It has been difficult to determine which mechanism predominates and is responsible for the most cell death. This is likely due to the fact that there are many different cellular pathways

controlling these processes and a lot of redundancy to ensure that cells which can no longer be salvaged are degraded. As a result of the significant injury caused by IRI and the reduced function of the graft following transplantation, it would be extremely beneficial to target one or more of the mechanisms to protect the cells and prevent both programmed and accidental cell death. Targeting the cells of the effected tissue or organ may not be enough however, as there is significant involvement of both the innate and adaptive immune systems in IRI.

1.3.4 Innate Immunity in IRI

Immune responses have been well studied in the context of graft rejection and effective therapies to deal with this have been developed which has lead to many advancements in transplantation. The innate and adaptive immune systems also respond to the injury caused by IRI, can lead to further cell death and damage and may be an important area to target in the prevention and treatment of IRI. Multiple pathways have been shown to be involved in the initiation and propogation of IRI.

The innate immune system acts as a first line of defense against invading pathogens as well as tissue injury caused by conditions such as hypoxia. This is possible through the recognition of danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). DAMPs are produced during IRI and include high mobility group box-1 (HMGB-1), heparin sulphate, ROS, and heat shock proteins (41). Toll-like receptors (TLRs) are one class of PRR present on innate immune cells and TLR signaling is known to be activated in IRI. For example, TLR-4 is upregulated in renal IRI and leads to increased injury (42, 43). TLR signaling can occur through MyD88 dependent and independent pathways and leads to the activation of Nf-κβ. NF-κβ is an important transcription factor controlling cell survival, apoptosis, inflammation, and cell proliferation and can activate pro-survival or apoptotic processes depending on the situation and degree of cellular injury (44). NF-κβ is important in the upregulation of inflammatory cytokines and the innate and adaptive immune response following injury or infection. NF-κβ can lead to both the upregulation and downregulation of inhibitors of apoptosis, such as those in the Bcl-2 family. It may also upregulate pro-apoptotic genes, such as Bax and PUMA. It is very important in the

inflammatory response following IRI and subsequent cellular damage and death. NF-κβ factors contain Rel homology domains such as RelA, RelB, and c-Rel which are responsible for DNA binding. Different NF-κβ pathways exist including the canonical, non-canonical, and atypical pathways. RelB is part of the non-canonical pathway and its activation has been linked to apoptosis (45). Silencing of RelB with RelB siRNA in a mouse model of renal IRI showed a significant benefit in terms of renal function, histology, and overall survival (46). NF-κβ signaling is complex and has significant cross-talk with other cellular signaling pathways as well creating a potential for increased cell death or survival (44). Targeting the NF-κβ pathway may be challenging given its complexity and involvement in the regulation of many different genes, but may also offer some benefit in reducing apoptosis and the immune response early in the process of IRI.

The complement system is also an important part of innate immunity and is activated in IRI. The complement system contains three pathways which converge with the activation of complement 3 (C3) which subsequently leads to formation of the membrane attack complex (MAC) which is composed of C5b-C9. The MAC is responsible for inducing direct cell lysis and the C3a and C5a fragments, known as anaphylatoxins, act as powerful mediators of inflammation. The complement pathways include the classical, alternative, and lectin pathways. The classical pathway is activated by the binding of antigens to immunoglobulins (IgG, IgM) or C-reactive protein (CRP), which then bind complement 1q (C1q). This activates C1s and C1r which cleave C4 and lead to the formation of C4b and C2b (from C2). This constitutes the C3 convertase which cleaves C3 into C3a and C3b and leads to activation of the rest of the complement cascade. The alternative pathway begins with the spontaneous hydrolysis of C3 into C3b and C3a. C3b is able to bind directly to the surface of pathogens and activate the complement cascade leading to the MAC. The lectin pathway involves mannose binding lectin (MBL) which binds to carbohydrate moieties on bacterial cell membranes. When activated, MBL, in association with the MBL-associated proteins (MASPs), cleaves C4 and activates the complement cascade (47). Although initially thought to only occur in the presence of infection, all of these pathways have been found to be involved in sterile inflammation such as IRI due to the release of DAMPs (48, 49).

Complement activation is known to induce significant damage following transplantation from formation of the MAC as well as stimulation of cytokine and chemokine formation and through progression to fibrosis (47). However, this has mostly been observed in animal models. C3 production by kidney endothelial cells has been shown to be induced by immune stimulation and can possibly modulate the adaptive immune system (50). Tubular epithelial cells are also known to express C3a and C5a receptors and signaling by these receptors leads to harmful effects (47). Furthermore, silencing of C3 and C5a receptor in mouse models of IRI has been shown to be effective at improving the function of the affected kidneys (51-53). Currently, a clinical trial involving eculizumab, a C5 monoclonal antibody, is ongoing with the goal of reducing DGF in renal transplant recipients who have received a kidney from an ECD or SCD donor with a CIT of 18-40 hours (ClinicalTrials.gov identifier: NCT 01919346). C3 and C5 are both important components of the complement cascade in all three pathways and may represent potential targets in the amelioration of IRI.

Other factors involved in innate immunity include natural killer (NK) cells, macrophages, neutrophils, and T cells. Dendritic cells (DCs) provide an important link between the innate and adaptive immune system by acting as professional antigen presenting cells (APCs). DCs arrive at the site of injury and then travel to lymph nodes to present antigens to T cells (41). The adaptive immune response during IRI at the time of transplantation may lead to episodes of acute rejection as well as long-term graft failure (54).

1.3.5 Effect of IRI on Graft Function in Renal Transplantation

In renal transplantation, IRI is a type of acute kidney injury (AKI) which can lead to primary non-function, slow graft function, and DGF (15). In transplantation, there is a WIT, CIT, and a reperfusion phase. The WIT is considerably longer in DCD donors as there is time during which the organs are not perfused and the donor remains at a normothermic temperature. This time generally lasts anywhere from 20 minutes to 1 hour. A recent study by Salmeron-Rodriguez et al. found that the mean WIT was 24.88 minutes for Maastricht class III donors (55). In NDD donors, however, this time is very short and lasts for only a few minutes. The WIT and CIT create a hypoxic environment

where many cellular changes occur leading to cell injury and death. Both phases contribute to the damage that occurs during transplantation, and decreasing these times leads to lower rates of cellular injury and DGF (28, 56). Although the overall graft survival rates are similar between DCD and NDD donor kidneys, the rate of DGF and discarded organs are higher in DCD donor kidneys (14, 56). Discard rates of up to 34 percent have been observed with DCD donors and, therefore, is an important area to target to attempt the salvage and protection of these organs (56).

The need to protect the graft during procurement, transportation, and implantation is paramount in order to improve graft function and survival after transplantation. Treatment of the graft during these phases as well may lead to lower discard rates, higher quality grafts, and lower rates of DGF. The current therapies used around the time of transplantation will be further discussed below.

1.4 Current Treatment of IRI in Renal Transplantation

Currently, there are a limited number of effective therapies for IRI in transplantation and there is a need to develop treatments for IRI in order to improve the organs available for transplant and expand the number of organs which can be used. The selection of appropriate donors is an essential first step in order to obtain high quality organs, however, with the expansion to ECD and DCD donors, more and more marginal grafts are being used to increase the supply of organs. A recent FDA workshop on IRI in kidney transplantation sought to discuss the current state of knowledge of IRI and its effects on kidney transplantation (15). Past and current therapies were discussed at this workshop and the need for more research was highlighted. Some of the therapies which have been tested clinically include those listed in Table 1.

Table 1 Summary of Therapies for the Treatment of IRI in Clinical Kidney Transplantation (Continued on page 18)

Donor Type	Treatment	Outcomes	Reference
NDD	Furosemide vs. no treatment prior to reperfusion	No difference in DGF	(57)
Deceased donors	Omnibonia (vit. A, B complex, C, and E) vs. no treatment prior to reperfusion	Lower serum Cr up to 6 days, but no difference thereafter	(58)
DCD	Pentoxifylline at induction of anesthesia in recipients followed by once a day for 48h vs placebo	No difference in DGF	(59)
DCD	Enlimomab (anti-ICAM1 mAb) 3h pre-transplant followed by once a day for 5 days vs placebo	No difference in DGF	(60)
DCD	Epoetin-β before transplant and at 12h, 7 days, and 14 days vs no treatment	No difference in DGF or graft survival	(61)
Deceased donors	EPO-α at time of transplant vs placebo	No difference in DGF or SGF	(62)
NDD	IGF-1 at transplant and then twice a day for 6 days vs placebo	No difference in DGF, serum Cr, or inulin clearance	(63)
DCD	Dopamine vs dopamine-1 receptor antagonist infusion for 48h	No difference in urine output or serum Cr	(64)

Donor Type	Treatment	Outcomes	Reference
DCD	3 5min cycles of remote ischemic conditioning in unilateral lower limb (clamping of external iliac artery) at time of transplantation vs sham procedure	Reduced serum creatinine and urinary NGAL, increased GFR	(65)
DCD	YSPSL (blocks leukocyte adhesion) vs placebo	Higher rates of DGF in treated group	(66)
Living donor	Spironolactone 1 day prior and 3 days post transplant vs placebo	No difference in renal function, but decreased urinary hydrogen peroxide	(67)
Cochrane Review of RCTs	Calcium channel blockers (CCBs) in the peri-transplant period	Significant reduction of ATN and DGF in the treated groups	(68)
NDD	N-acetylcysteine 1h prior to and 2h following angiography to confirm brain death vs placebo	No difference in DGF	(69)
NDD	Methylprednisolone 3h prior to procurement vs placebo	No difference in DGF	(70)
Deceased donors	Vitamin C added to perfusion solution	No difference in DGF	(71)
Deceased donors	Human rh-SOD prior to reperfusion followed by a 1h infusion afterwards vs placebo	No difference in GFR or serum creatinine	(72)

Most of the trials mentioned above showed no difference in outcomes or only short term effects. Currently, machine perfusion has garnered a lot of interest in clinical trials. Machine perfusion involves cannulating the renal artery and vein and perfusing the kidney with preservation solution during transport. Previously, kidneys would be placed in static cold storage at 4^0C in preservation solution. The theory behind using machine perfusion is that continuous perfusion of the organ will allow for oxygen delivery, washout of toxic metabolites, better assessment of the kidney, and maintain the microvasculature (73, 74). Despite these potential benefits, machine perfusion, like many of the other therapies, has been shown in randomized controlled trials to have no overall effect on graft survival (75, 76). However, in two meta-analyses, the rate of DGF has been shown to be significantly decreased in the machine perfusion groups (OR 0.64, p=0.03, and OR 0.56, p=0.008) (77, 78). These meta-analyses also showed no difference in 1 year graft survival between the two groups. Notwithstanding these studies, many groups have adopted machine perfusion as the standard of care for DCD and ECD kidneys as well as some groups advocating its use for all SCD kidneys. Although machine perfusion has not improved graft survival rates, it can still be a valuable tool in the reduction of DGF, which leads to increased costs and morbidity, as well as allowing for the assessment of organs prior to implantation, and the delivery of novel therapies in the *ex vivo* setting. The variable parameters of machine perfusion are also the subject of ongoing study including temperature, pressure, and pulsatility as well as the use of oxygen and substrate carrying preservation solutions (79).

1.5 RNA Interference

1.5.1 Overview

RNA interference (RNAi) was described in 1998 by Fire et al. (80). This marked a significant discovery for which the Nobel Prize for Physiology or Medicine was awarded in 2006. In their landmark paper, it was shown that double stranded RNA containing a sense and antisense sequence for a specific gene is much more effective at gene silencing than single stranded RNA molecule (either sense or antisense) in *C. elegans*. They hypothesized that the double stranded RNA molecule was required to be incorporated into a complex that included a catalytic component as a few molecules lead to significant

gene silencing. They also believed that it was likely that the silencing occurred at the post-transcriptional level as targeting sequences containing introns did not lead to the same amount of gene silencing. Later on, they did indeed show that in RNAi the primary DNA sequence was not altered, targeting the upstream operon had no effect on the activity of the downstream gene, and that there was a significant decrease in the targeted gene transcripts in the nucleus (81). With ongoing research, it was further identified that RNAi is important in the normal regulation of many genes with the endogenous production of small RNAs. These small RNAs actually account for a significant portion of coding regions of the genome for which no function was originally identified and may regulate up to 60% of the entire genome by over 1000 different RNA molecules (82, 83). In addition, the production of small RNAs by cells was shown to lead to the control of viral infection by leading to degradation of viral RNA (82). From this point, it was discovered that there are different mechanisms of RNAi which make use of different types of RNA molecules including micro RNAs (miRNA), small interfering RNAs (siRNA), short hairpin RNAs (shRNA), and PIWI-interacting RNA (piRNA) (83). These different mechanisms of RNAi rely on RNA molecules from different sources and different cellular machinery, but generally produce similar effects with all relying on an Argonaute family protein to help direct it to the correct gene (82, 83).

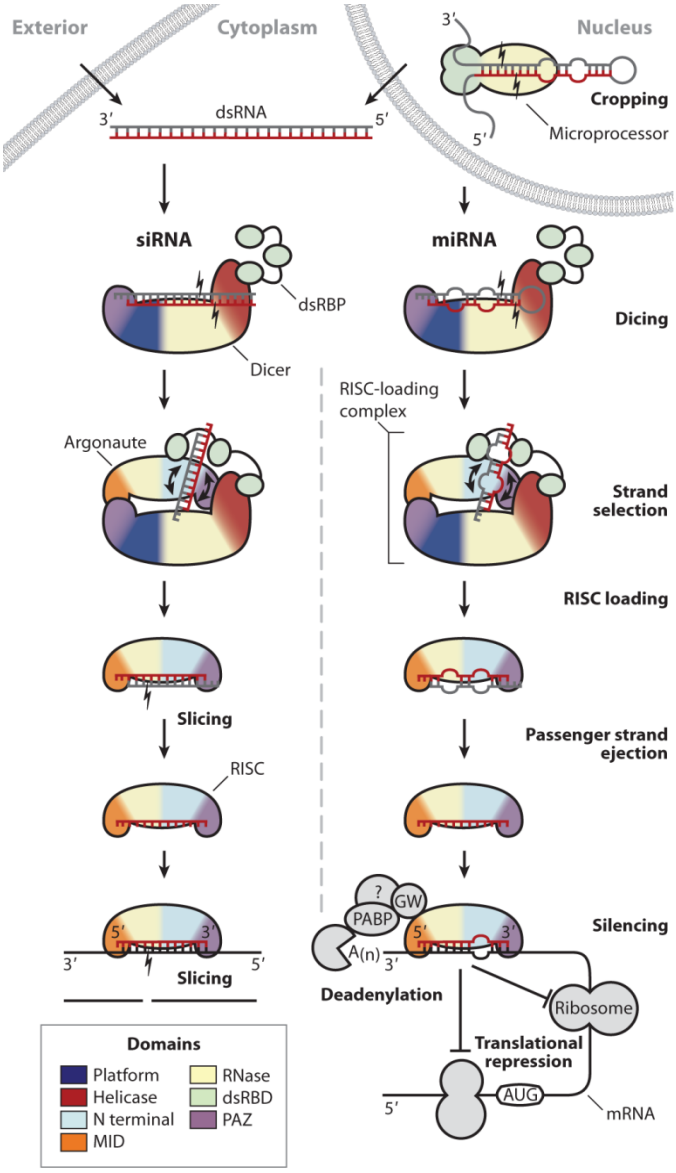
1.5.2 RNAi Mechanisms

The different RNAi mechanisms rely on miRNA, siRNA, and piRNA. miRNAs are endogenously produced by the cell. These RNA molecules are initially transcribed as pri-miRNAs that are at least 1000 nt long and contain single or multiple hairpins where the sequences are complimentary to each other. The pri-miRNA is processed in the nucleus by Drosha and DGCR8. DGCR8 binds to double stranded RNA and correctly positions the RNase Drosha 11 bp from the stem of the hairpin so that it can cleave the pri-miRNA into 65-70 bp segments known as pre-miRNA (84). The pre-miRNA is then exported to the cytoplasm by Exportin-5 and RanGTP (85). In the cytoplasm, the pre-miRNA is further processed by the endoribonuclease Dicer and loaded onto an Argonaute protein as a 21 to 25 bp double stranded sequence (82, 83). A third protein, a double-stranded RNA binding protein (dsRBP) helps with the activity of Dicer and the loading of the Argonaute

protein and together with Dicer and Argonaute, forms the RNA induced silencing complex (RISC) (82, 83, 86). Once the RISC is loaded with the double stranded miRNA, one strand, the guide strand, is retained while the other strand, the passenger strand, is discarded (82, 86). Once all of this has occurred, the RISC can bind to single stranded RNA sequences, such as mRNA, which are complimentary to the guide strand which it has loaded.

There are four different Argonaute proteins in humans, and only one, Ago2, has the ability to cleave single stranded RNAs when there is perfect complementarity with the guide strand. This is known as slicing and does not often occur in miRNA induced gene silencing. miRNA are usually complementary to the target over their seed sequence (bp 2-8) but then bind imperfectly along the remainder of their sequence to the 3' untranslated region (UTR) of mRNAs. Once the RISC is bound to the mRNA, another protein, GW182, is recruited to complex and is needed to activate additional cell machinery and localization to P bodies (83, 87). In the P bodies the complexes repress translation and cause deadenylation and destabilization of the target mRNA rather than slicing, as occurs with perfect complementarity (82, 83). As a result of the imperfect binding, one miRNA molecule can affect a number of different genes and regulate their expression.

In contrast to miRNAs, siRNAs are completely complementary to the target mRNA sequence. siRNAs can be produced endogenously by the cell or may be supplied exogenously by viral infection or intentional treatment with synthetic RNA molecules. siRNA molecules may occur as 21-25 bp double stranded RNA molecules and be directly loaded onto an Argonaute protein, or as shRNA which is first processed by Dicer and then loaded onto an Argonaute protein. Once the siRNA molecule is taken up into the cytoplasm, it follows the same process as a miRNA molecule would. Due to its perfect base pairing with the target mRNA, however, it allows for slicing of the mRNA and gene silencing, rather than requiring recruitment of GW182 and translational repression or modification of the mRNA (83). Both siRNA and miRNA lead to post transcriptional gene silencing.



Wilson RC, Doudna JA. 2013.
Annu. Rev. Biophys. 42:217–39

Figure 2 – RNA interference mechanisms involving siRNA and miRNA.

This figure displays the exogenous administration of siRNA which enters the cell and is loaded into the RISC which allows for binding to and slicing of its complementary mRNA. Production and processing of miRNA is also shown which leads to silencing through destabilization of the target mRNA. Reproduced with permission and copyright © from Annual Reviews, Wilson R.C. and Doudna J.A. *Annu. Rev. Biophys.* 2013, 42: 217-39.

The third type of RNAi involves the use of piRNA. The exact mechanism of piRNA induced RNAi is still being studied and is not as well known as that of miRNA or siRNA induced RNAi. piRNAs function without the use of Dicer and are initially produced as long, single stranded RNAs. These precursors are then exported to an area in the cytoplasm known as the nuage which is associated with the nucleus and mitochondria. Here they are processed by the endonuclease Zucchini and possibly other endonucleases. After processing, the piRNAs are loaded into the RISC complex as 26 – 31 nt molecules which are longer than miRNAs or siRNAs. Following formation of the RISC complex, the piRNAs can lead to gene silencing in two different ways. First, they can bind the complementary mRNA and slice it. In this mechanism, they can enter a ping-pong amplification cycle where the sense strand is loaded onto the RISC which cleaves the antisense strand. The antisense fragment is then loaded onto the RISC to cleave the sense strand. Second, they can translocate to the nucleus and cause transcriptional gene silencing by identifying transposable transcripts and recruiting chromatin and DNA modifiers. This is thought to be important in the prevention of germ line mutations (83).

The field of RNAi is constantly expanding and is the subject of intense research. RNAi has been applied in many different research settings and the use of siRNA can help to identify potential gene targets for the treatment of many different conditions including IRI.

1.5.3 RNAi in Ischemia Reperfusion Injury

As outlined above, IRI involves many complex cellular events which lead to cell death and damage. Therapies targeting IRI in the transplant setting are limited and require further investigation and development. RNAi is an exciting new field that may have an application in the treatment of IRI. A significant amount of *in vitro* and small animal work has already been performed, as well as some large animal work. Much of the work has focused on targeting pathways involved in cell death, inflammation, and immunity.

Identifying optimal gene targets for silencing in IRI is imperative if a clinically significant response is expected. IRI involves many cellular processes and it is difficult to recognize which genes may have the greatest effect on not only cell damage and death,

but longer term outcomes such as DGF in renal transplantation. The use of RNA micro-arrays may help to identify which genes are up regulated and during which phase in the IRI process. Recent work by Damman et al., has shown that hypoxia, coagulation, and complement pathways were enriched in human DCD donors after the first warm ischemic time, and that pathways related to endoplasmic reticulum (ER) and mitochondrial stress, RNA degradation, and DNA repair were associated with DGF (88). This study was unique in that biopsies of the kidneys were taken prior to donation so that they could be compared to biopsies taken 45 to 60 minutes following reperfusion. A proposed study design involves biopsies taken at 30 minutes and 30 days after transplantation in living and deceased donor kidneys to identify potential siRNA targets through microarray technology (89). Microarrays have the potential to return a large amount of data and it will be important to sort through all of the information so that genes with the most effect on IRI can be targeted.

Table 2 lists genes which have been targeted by siRNA in the research setting in order to improve IRI.

Table 2 - List of *in vivo* Studies Using siRNA in the Treatment of Renal IRI
(Continued on page 26)

Target	Animal	Outcome in Treatment Group	Reference
p53	Mice, Rats	Attenuation of apoptosis	(90, 91)
IKKβ	Rats	Decreased BUN, Cr, and cell injury on histology	(92)
ETaR	Rats	Decreased BUN, Cr, cell injury on histology, and cytokine production, and increased NO production	(93)
S1P(2)R	Mice	Reduced BUN, Cr, and cell injury on histology	(94)
RelB	Mice	Reduced BUN, Cr, cell injury on histology, TNFα, and improved survival	(46)
Fas	Mice	Reduced apoptosis and cell injury on histology, and improved survival	(95)
C3	Mice	Reduced BUN, Cr, cell injury on histology, TNFα, and improved survival	(52)
C5a	Mice	Reduced BUN, Cr, cytokines, and chemokines	(51)
Fas and Caspase-8	Mice	Reduced BUN, Cr, apoptosis, and necrosis	(96)

Target	Animal	Outcome in Treatment Group	Reference
Caspase-3 and C3	Mice	Reduced BUN, Cr, cell injury on histology, and improved survival	(53)
Caspase-3 and Caspase-8	Mice	Reduced BUN, Cr, cell injury on histology, and improved survival	(97)
Caspase-3	Pigs	Reduced apoptosis, inflammation, HMGB-1, and cell injury on histology, and improved renal function	(98-101)

As can be seen, most of these studies involve the use of small animal models. Different outcomes were assessed but often included biomarkers of renal function and histological examination after sacrifice. This limits the applicability to humans, but avoids the increased costs associated with large animal models. These studies have repeatedly shown a benefit with the use of siRNA and various gene targets. This research has set the stage for a transition to using siRNA in large animal IRI models and eventually, clinical trials in humans.

Caspase-3 siRNA has been used extensively from the *in vitro* setting to a porcine auto-transplantation DCD model (98-101). In the auto-transplantation model, Yang et al., showed that caspase-3 siRNA protected porcine kidneys up to two weeks following transplantation. This DCD model involved injecting caspase-3 siRNA into the kidney prior to static cold storage for 24 hours and then again systemically following auto-transplantation (101). The results of this study are encouraging as progress is being made towards human trials. Recently, one human phase I trial (ClinicalTrials.gov identifier: NCT00802347) was completed using I5NP (a p53 siRNA) in the prophylaxis of DGF following transplantation of DCD kidneys. Results of this trial are not yet available.

The translation to a clinical setting has been difficult, however, and is the focus of ongoing research. There are challenges associated with delivery of the RNA including uptake and degradation, as well as off-target and systemic effects.

1.5.4 Challenges with Translation to a Clinical Setting

There are many challenges that must be overcome prior to using siRNA in the clinical setting. These include the design and delivery of the siRNA as well as efficacy, off-target consequences and systemic effects. The design of siRNA needs to take into account thermodynamic stability, immunostimulatory motifs, secondary structure, and internal repeats in order to create a stable molecule that does not result in degradation nor incite an immune response (89, 102). Computer algorithms and known mRNA sequences are relied on to identify potential sequences that will be effective at gene silencing. Naked, or unmodified siRNA, is often used in research settings. The siRNA can also be chemically modified, however, to improve its stability. Chemical modifications include changes at

the 2’ position of the ribose by incorporating 2’-O-methylpurines or 2’-O-fluropyrimidines enhances the stability and effectiveness of the siRNA (89, 103). Another modification known as locked nucleic acid (LNA), has been used as well to improve stability of the siRNA by linking the 2’ oxygen with the 4’ carbon in the ribose through a methylene bridge. This allows for improved binding to its complementary sequence (104).

The delivery of siRNA presents another challenge. While numerous methods of *in vitro* delivery have been developed, it is difficult or impractical to use these methods in an *in vivo* setting. The most common methods for siRNA transfection in the *in vitro* setting make use of lentiviruses, liposomes, nanoparticles, antibodies, and electroporation. These delivery methods are employed as naked siRNA is easily degraded and it is difficult to get the siRNA across the cell membrane to where it will have its effect. All of these methods have the potential to harm the cells and, in the case of viral transfection, create an immunologic response (105). Research continues to be done to improve translation of *in vitro* delivery methods to the *in vivo* setting.

In the *in vivo* setting, delivery of naked siRNA has been successful with hydrodynamic injection into the tail vein in mice (106). This method involves the injection of a relatively large amount of fluid over a short period of time, leading to a sudden increase in the intra-vascular volume and pressure. Although the mechanism is unclear, this is then thought to facilitate delivery of RNA or DNA to cells by making them more permeable as the liver, which has the largest number of fenestrae in its vessel walls, has the highest uptake of the delivered genetic material (106). This method has been employed in renal warm ischemia models involving mice in which an improvement in renal function and 8-day survival were seen with complement 3 and casapse-3 siRNA (53). This approach may not be applicable to the clinical setting, however, given the large volume injections. Currently, a number of phase I/II clinical trials in areas other than transplantation and IRI are employing delivery methods such as liposomes or using naked siRNA in the treatment of diseases such as cancer, hypercholesterolemia, various ophthalmologic conditions, and familial adenomatis polyposis (FAP) (105). The only

clinical trial involving IRI, as mentioned previously, makes use of naked siRNA delivered intravenously following graft implantation.

After the design and delivery of the siRNA has been considered, the efficacy and safety of the molecule must also be determined. The design algorithms used to create the siRNA also take into account how effective the molecule should be at silencing. The siRNA should be targeted to a sequence that is at least 50 to 75 nt downstream from the start codon and should avoid any introns to improve silencing (103). After the siRNA is made, however, it must be tested, as the silencing effect which is predicted does not always occur. Often, three or more sequences will be designed in order to increase the probability of developing an effective molecule that can provide the highest silencing efficiency. Testing of the siRNA molecules usually occurs in cell cultures prior to *in vivo* studies as these are less expensive. Use of siRNA *in vivo* may not show the same results however, as the siRNA may be not be taken up by the targeted cells, degraded by RNases, cleared by the kidneys, and cause immunologic reactions leading to a reduced silencing efficiency (89).

The safety of siRNAs must be determined before they can be used regularly in clinical settings. Off-target effects can result from the siRNA binding to an mRNA other than the target mRNA leading to its degradation by the RISC complex, an immune response to the siRNA, or toxicity from the delivery vehicle (89). In some cases of non-specific binding, the siRNA can act like a miRNA sequence. As stated previously, miRNAs require complementarity over their seed sequences, but then bind imperfectly over the rest of the sequence and can lead to silencing of a number of different genes. If an siRNA has the same bases in the seed sequence location of a miRNA it will bind to the 3` UTR of mRNAs other than its target. This effect can be reduced with proper design of the siRNA (107). The delivery of siRNAs can also lead to complete occupation of the RISC complexes with the exogenous siRNA and not allow normal RNAi through miRNA silencing. This may have a wide range of effects that are difficult to predict and avoid (89, 107). Certain siRNA molecules can also be immunostimulating and lead to activation of the innate immune system which is thought to occur via activation of TLRs. This usually occurs with double stranded RNAs that are longer than 30 bp, but can occur

with 19 bp sequences as well (108). This effect can either be beneficial if the object is to stimulate an immune response, or detrimental, as in the case of IRI, where it is necessary to avoid further injury caused by the treatment. The delivery vehicle may also be toxic to the cells and this toxicity must be balanced with the ability to achieve adequate uptake and gene silencing. Transplantation, however, offers the unique ability to deliver a treatment such as siRNA in an *ex vivo* setting and avoid off-target or systemic effects.

1.6 Incorporating siRNA Delivery with Machine Perfusion

Due to the issues with delivery of siRNA, it is proposed that machine perfusion may be the ideal way to delivery siRNA during the *ex vivo* phase of transplantation. Machine perfusion allows for the intravascular delivery of siRNA to the target organ without affecting the entire organism. This also allows for control of the perfusion parameters such as pressure and temperature. Clinically, the temperature is set at 4^0C in order to take advantage of the reduced metabolism and better preservation. The hypothermic conditions create a cold ischemic insult, however, and it is known that increasing times of cold ischemia are associated with an increased risk of DGF(109). Currently, there is ongoing investigation into whether or not normothermic or subnormothermic conditions may lead to better outcomes (110-112). The advantages of using higher temperatures include avoiding the cold ischemic insult and maintaining the normal metabolic function of the organ as long as oxygen and nutrients are supplied appropriately. Artificial preservation solutions have been developed which include oxygen carriers and recently, an acellular hemoglobin named Hemoxycarrier® has been used in a porcine autotransplantation model to deliver oxygen and has shown promising results (113). In the case of siRNA transfection, it may also be beneficial to use normothermic temperatures as opposed to the standard hypothermic conditions to ensure optimal uptake by the cells. It has been shown by Brasile et al., that normothermic perfusion lead to adequate transfection after 24 hours (114). This was using a viral vector to deliver genetic material rather than siRNA. Some work has been done with siRNA delivery to porcine kidneys in an *ex vivo* setting, but only during static cold storage (101).

The pressure with which the kidneys are perfused and whether or not it is pulsatile are also the subject of ongoing debate. Patel et al., showed that for continuous perfusion, a pressure of 75mmHg is better than a pressure of 55mmHg (115). It also appears that pulsatility may improve creatinine clearance even if it is preceded by static cold storage (79). Integrating the delivery of siRNA with machine perfusion will require the determination of optimal temperature and pressure settings to both ensure that adequate gene silencing occurs and the organ experiences the most benefit from the machine perfusion. This has yet to be studied in depth.

1.7 Study Rationale

This project was designed with the purpose of developing porcine siRNAs which are effective at silencing their target genes and can be used in the future in a porcine DCD model while taking advantage of the benefits of machine perfusion. As the cell death pathways are quite redundant, we believe that it is important to target more than one pathway in order to achieve a clinically significant effect. We have therefore decided to target the intrinsic and extrinsic apoptotic pathways with caspase-3 siRNA as both pathways require its activation. We have also targeted the complement pathways with complement 3 siRNA as all three pathways converge with its activation. Finally, we have targeted the Nfκβ transcription factor pathway with RelB siRNA. In order to prove the effectiveness of the siRNA prior to using it in large animal studies, *in vitro* testing with an ischemia reperfusion model was used. A commercially available pig kidney tubular epithelial cell line was used throughout the project. The tubular epithelial cells are very susceptible to hypoxia and represent an appropriate cell type to treat in an IRI model (116). Two *in vitro* models of IRI were used and included a hypoxia chamber and antimycin A. The hypoxia chamber allows for complete control over the oxygen, carbon dioxide, and nitrogen concentrations as well as the temperature. Antimycin A is produced by *Streptomyces* sp. and inhibits complex III of the electron transport chain (ETC) (117-119). Upon inhibition of the ETC, the cell switches to anaerobic metabolism and a hypoxic environment is mimicked. These IRI models allowed us to assess the normal gene expression in response to hypoxia and re-oxygenation as well as that following siRNA transfection.

Chapter 2

2 Materials and Methods

Before translation to a clinical setting, it is important to prove that novel interventions are effective in a laboratory setting. This project was designed to demonstrate that silencing genes involved in the apoptotic, inflammatory, and complement pathways is an effective method to reduce IRI in an *in vitro* setting. This was accomplished to provide evidence for further studies involving large animals with eventual translation to a clinical setting.

2.1 Cell Culture

LLC – PK1 cells (ATCC© CL-101™) were used for all experiments. This is a commercially available cell line which has been cultured from pig kidney tubular epithelial cells. Cells were cultured in 15mL of medium 199 containing 3% fetal bovine serum (FBS) as well as penicillin and streptomycin in 75mL flasks. Cells were used between passage 2 and 25 and recovered using 0.05% trypsin for 5 minutes at 37^0C after washing with phosphate buffered saline (PBS).

2.2 Transfection with siRNA in Untreated Cells

RNA interference was achieved through the use of siRNA. Three different siRNA molecules were obtained (Sigma-Aldrich) for each of Caspase-3, Complement 3, and RelB. These sequences contain 3’ dT overhangs and are shown in Table 3.

Table 3 - siRNA Sequences

Gene	siRNA sequence (5' to 3' sense strand)
Caspase-3 #1	GUGUUUCUAAAGAAGACCA
Caspase-3 #2	CUUCUUGUAUGCAUAUUCU
Caspase-3 #3	CCUUCACAAACUUGAAAUA
Complement 3 #1	GCAUUGACAUCAAACGGGA
Complement 3 #2	CAGAUGACUUUGACGAUUA
Complement 3 #3	GACAAACUGGGUCAGUACA
RelB #1	CGGCCUUCCUGGAUCACUU
RelB #2	CGCUGACGCGGGACUCGUA
RelB #3	GCUACGGAGUGGACAAGAA

Transfections in normal, untreated PK1 cells were carried out to determine which of the three sequences induced the best gene silencing. Cells were plated in 12 well plates ($1.5x10^5$) and incubated overnight at 37^0C to allow them to reach a confluency of 90%. PK1 cells were then transfected using Lipofectamine® 2000 (Life Technologies). This was accomplished by adding 0.5µL to 100µL of OptiMEM® (Life Technologies) and incubating at room temperature for 5 minutes. Following this, 1µg of siRNA was added along with 100µL of OptiMEM and incubated at room temperature for 20 minutes. This was then added to a single well. A ratio of 2µL to 1µg (Lipofectamine® 2000 to siRNA) was used as this allowed for adequate gene silencing and did not induce cell death. At the time of transfection, an additional 300µL of OptiMEM was added, and then after 4 hours, 500µL of medium 199 containing FBS, penicillin, and streptomycin was added. Cells were incubated at 37^0C overnight.

2.3 Hypoxia Chamber Conditions

The H85 HypOxystation® (HypOxygen) was used to simulate ischemic conditions and $1.5x10^5$ cells, plated in 12 well plates, were placed in the chamber for 0.5-24h at 1% O_2, 5% CO_2, and 94% N_2, at 37^oC in 1mL glucose free Dulbeco's Modified Eagle Medium (DMEM). Experiments were carried out using media which had not be de-oxygenated, as well as that which had oxygen removed by bubbling a mixture of nitrogen, carbon dioxide, and hydrogen gas through the media for 3 minutes. The hypoxia phase was then followed by a re-oxygenation phase for 24h at 21% O_2, 5% CO_2, and 37^oC, which included changing the media to medium 199 containing FBS, penicillin, and streptomycin. A negative control was kept at 21% O_2, 5% CO_2, and 37^oC with glucose free DMEM during the hypoxic phase with a change in the media to medium 199 containing FBS, penicillin, and streptomycin during the re-oxygenation phase.

2.4 Treatment with Antimycin A

Antimycin A (Sigma-Aldrich®) was purchased and re-suspended in 95% ethanol according to the manufacturer's instructions. PK1 cells ($1.5x10^5$) were plated in 12 well plates and time and dose response experiments were performed to determine the conditions which would produce a significant amount of injury. Varying concentrations (5 – 200 μM) of antimycin A as well as varying times of exposure (1 – 4 hours) and recovery (3 – 24 hours) were used. It was determined that treating the cells with 10μM antimycin A in PBS containing Mg^{2+} and Ca^{2+} for 3 hours followed by 3 hours of recovery induced a significant amount of apoptosis and cell death while still maintaining approximately 60 percent viable cells. The recovery period involved changing the media to Medium 199 containing 3% FBS, penicillin, and streptomycin. Negative control cells were exposed to 3 hours of PBS containing Mg^{2+} and Ca^{2+}, followed by the recovery phase in normal media.

2.5 Treatment with Antimycin A Following Transfection with siRNA

In order to determine if the different siRNA molecules were effective at protecting the cells from injury and death, PK1 cells were transfected 24 hours prior to treatment with antimycin A. Once again, PK1 cells ($1.5x10^5$) were plated in 12 well plates and incubated overnight at 37^0C. Once at a confluency of approximately 90 percent, the cells were transfected with caspase-3, complement 3, and RelB siRNA according to the methods outlined above in 2.2. The following combinations of siRNA were used:

1. Caspase-3 alone
2. Complement 3 alone
3. RelB alone
4. Caspase-3 and complement 3
5. Caspase-3 and RelB
6. Complement 3 and RelB
7. Caspase-3, complement 3 and RelB

Following transfection, the cells were incubated overnight at 37^0C and then washed with PBS and treated with antimycin A according to the methods outlined in 2.4 using 10μM of antimycin A for 3 hours followed by 3 hours of recovery. Following this recovery phase, cells were analyzed by flow cytometry, quantitative real-time polymerase chain reaction (qPCR), and Western blotting.

2.6 Flow Cytometry Analysis

Cells were analyzed following all experiments for apoptosis and viability by flow cytometry using the Beckman Coulter FC 500 flow cytometer. Following the recovery phase, cells were trypsinized and collected, along with any floating cells in the media, by centrifugation at 1500rpm for 5 minutes. Cells were then washed with 1mL of PBS and centrifuged at 1500rpm for 5 minutes. Cells were then stained with Annexin V and propidium iodide by resuspension in 100μL of Annexin V buffer according to the manufacturer's instructions (BD Biosciences). Cells were then incubated at room temperature in the dark for 15 minutes. Following this, 300μL of Annexin V buffer was

added and the cells were analyzed by flow cytometry. In order to compensate correctly between the channels, two extra samples were included; one stained only with Annexin V and one stained only with propidium iodide. An unstained control was also included to account for any baseline fluorescence of the cells. No gates were used on the cells as all of the cells, regardless of size, were important for the analysis.

2.7 Quantitative Real-time PCR

RNA was isolated immediately following the recovery phase using TRIzol® (Life Technologies). According to the manufacturer's instructions, 500µL of TRIzol® was added to the adherent cells and gently pipetted up and down a few times and then collected into a 1.5mL Eppendorf tube. At this point the solution was either stored at -20^{0}C for future use or used immediately by adding 100µL of chloroform, inverting by hand for 15-30 seconds and incubated at room temperature for 2-3 minutes. Samples were then centrifuged at 12,000g for 15 minutes at 4^{0}C and the supernatant transferred to a new 1.5mL Eppendorf tube to which 250µL of isopropanol was added. The samples were inverted gently and then incubated at room temperature for 10 minutes. This was followed by centrifugation at 12,000g for 10 minutes at 4^{0}C and the supernatant was discarded. The pellet was washed with 500µL of 75% ethanol, resuspended, and centrifuged at 7,500g for 5 minutes at 4^{0}C. The supernatant was discarded and the pellet air dried. Once dried, an appropriate amount (depending on pellet size) of nuclease free water was added to the RNA pellet and incubated at 55-60^{0}C for 10 minutes. At this point the RNA was either used immediately or stored at -80^{0}C for future use. In order to ensure good quality RNA, 1µL of the RNA sample was analyzed by gel electrophoresis using a 1% agarose gel containing ethidium bromide.

Once isolated, the RNA was used to create complimentary DNA (cDNA). The RNA concentration was measured by diluting 2µL of the RNA sample in 98µL of nuclease free water and detecting the absorbance at 260 and 280 nm with a spectrophotometer. The A260/A280 ratio was calculated to identify issues with RNA quality. cDNA was then created by using 3µg of RNA. The RNA was combined with 0.5µg of Oligo dT primers, and nuclease free water (to a volume of 13µL) and incubated at 70^{0}C for 10 minutes followed by a quick chill on ice. Following this, 3.5µL of nuclease free water, 2µL of

10X reverse transcriptase buffer, 1µL of 10nM dNTPs, and 0.5µL of M-MuLV reverse transcriptase (New England Biolabs) were added. The samples were then incubated at 42^0C for 50 minutes and then 70^0C for 15 minutes to inactivate the reverse transcriptase. The cDNA was then stored at 4^0C for future use.

Following the formation of cDNA, quantitative real-time PCR (qPCR) was used to determine the relative expression of the genes of interest. cDNA was diluted 10 times with nuclease free water and used in the qPCR reactions. This was combined with 5µL of SensiFAST™ SYBR® No-ROX reagent (Bioline), 200nmol of forward and reverse primers, and 2.3µL of nuclease free water for a complete reaction volume of 10µL. Primer and cDNA concentrations were optimized using varying concentrations of both. The reactions proceeded as outline below:

1. 95^0C for 2 minutes
2. 95^0C for 10 seconds
3. 60^0C for 10 seconds } repeat 39x
4. 72^0C for 20 seconds
5. 95^0C for 30 seconds
6. 60^0C for 10 seconds → melt curve
7. 95^0C end

Primers were designed using Primer-BLAST (NCBI) with a targeted annealing temperature of 60^0C (120). The primer pairs are shown in Table 4.

Table 4 - Primer Sequences for qPCR

Gene	Primers (5' to 3')
β-actin	Forward TCCACGAAACTACCTTCAACTC
	Reverse GATCTCCTTCTGCATCCTGTC
Caspase-3	Forward TCTAAGCCATGGTGAAGAAGGAAAAA
	Reverse GGGTTTGCCAGTTAGAGTTCTACAG
Complement 3	Forward GCGCAGCATCAATAAGGCAAGAGA
	Reverse CAGCGCACAATAGGAGGGACAGAG
RelB	Forward ACGTACCTGCCTCGGGACCAC
	Reverse CCAGGCCAGGGAGGGATACAGT

qPCR products were analyzed using gel electrophoresis with a 1% agarose gel containing ethidium bromide to ensure the presence of only one band at the expected length. Bio-Rad CFX Manager™ 3.1 software was used to analyze qPCR results using the ΔΔCt method with β-actin as the housekeeping gene. The following formula was used:

$$\Delta C_{T\,(test)} = C_{T\,(target,\,test)} - C_{T\,(reference,\,test)}$$

$$\Delta C_{T\,(control)} = C_{T\,(target,\,control)} - C_{T\,(reference,\,control)}$$

$$\Delta\Delta C_T = \Delta C_{T\,(test)} - \Delta C_{T\,(control)}$$

$$\text{Normalized expression ratio} = 2^{-\Delta\Delta C_T}$$

Results are displayed as relative expression with the control group arbitrarily set at 1.

2.8 Western Blotting

Western blotting was performed to assess protein expression following injury by antimycin A and gene silencing using siRNA. Protein was isolated from PK1 cells using radioimmunoprecipitation assay (RIPA) buffer containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) by pipetting up and down a number of times and then incubating on ice for 15 minutes. Samples were then centrifuged at 12,000g for 10 minutes at 4^0C. The supernatant was then transferred to a new Eppendorf tube and either used immediately or stored at -80^0C for future use.

Protein concentration was then measured using the Bradford assay by adding 2µL of the sample to 98µL of Bradford buffer and vortexing. Concentration was then measured using a spectrophotometer and measuring the absorbance at 595nm and subtracting the absorbance of the blank. The protein samples were then combined with an appropriate amount of 5x protein binding buffer and boiled for 10 minutes.

SDS-PAGE was then used to separate proteins. The components of the gels are shown in Table 5.

Table 5 - SDS Polyacrylamide gels used for Western blots

	12% gel	**Stacking gel**
ddH_2O (mL)	1.7	3.4
30% Bis-acrylamide mix (mL)	2.0	0.83
1.5M Tris (pH8.8) (mL)	1.3	1.0M Tris (pH 6.8) 0.63
10% SDS (mL)	0.05	0.05
10% APS (mL)	0.05	0.05
TEMED (mL)	0.002	0.005

After the gels had been made, 30µg of protein was loaded into the stacking gel and run at 80V until it was through the stacking gel and then run at 120V through the rest of the gel in running buffer. Once the bands had reached the end of the gel, the electrophoresis was stopped and the stacking gel removed from the running gel. The running gel was then placed next to a nitrocellulose membrane. This was then placed in a tank of transfer buffer and run at 100V for 1 hour. Buffer composition is listed in Table 6.

Table 6 - Western blot buffer composition

10X Transfer Buffer	
Tris	30.3g
Glycine	144.1g
ddH_2O	1L
5X Running Buffer	
Tris	15g
Glycine	72g
SDS	5g
ddH_2O	1L

1X running buffer was prepared using 200mL of 5X running buffer and 800mL of ddH_2O. 1X transfer buffer was prepared using 100mL of 10X transfer buffer, 200mL of methanol, and 700mL of ddH_2O.

Following protein transfer, the membrane was blocked with 5% milk for 1 hour at room temperature. The primary antibodies were then diluted in TBS-T (Table 6), added to the membrane, and incubated overnight at 4^0C on a shaker.

Table 7 - Primary antibodies used in Western blots.

Primary antibody	Dilution in TBS-T
β-actin (mouse monoclonal) Santa Cruz Biotechnology® Inc.	1:5000
Caspase-3 (rabbit polyclonal) Santa Cruz Biotechnology® Inc.	1:2000

Following incubation overnight, the primary antibodies were removed and the membrane washed with TBS-T three times for 10 minutes each. The secondary antibodies (Table 7) were then added to the membrane and incubated at room temperature for 1 hour on a shaker. The secondary antibodies were then removed and the membrane washed with TBS-T three times for 10 minutes each.

Table 8 - Secondary antibodies used in Western blots.

Secondary antibody	Dilution in TBS-T
Goat anti-mouse Santa Cruz Biotechnology® Inc.	1:5000
Goat anti-rabbit Santa Cruz Biotechnology® Inc.	1:5000

The membranes were then analyzed by enhanced chemiluminescence using Clarity™ Western ECL blotting substrate (Bio-Rad). Membranes were exposed using the FluorChem™ M System (Protein Simple®) and densitometry was used to quantitatively analyze the blots. β-actin was used as a reference.

2.9 Statistical Analysis

All statistical analyses were completed using IBM® SPSS® for Windows, Version 24.0. One way ANOVA was used to compare means between more than two groups with Tukey post hoc analysis. The Kruskal-Wallis test was used to compare means between more than two groups if the data was non-parametric. Student's t test was used to compare means between two groups. Results were considered significant with a p value of < 0.05. All experiments were completed in triplicate.

Chapter 3

3 Results

3.1 Validation of Gene Silencing by siRNA

Three separate siRNA sequences were obtained from Sigma-Aldrich for each target gene. Preliminary experiments were performed to identify the sequences which produced the most silencing. This included both qPCR to assess the effect on the mRNA as well as flow cytometry to assess uptake of the siRNA into the cell.

3.1.1 qPCR siRNA Silencing Results

qPCR results for caspase-3, complement 3, and RelB siRNA in normal PK1 cells are shown in Figures 3, 4, and 5.

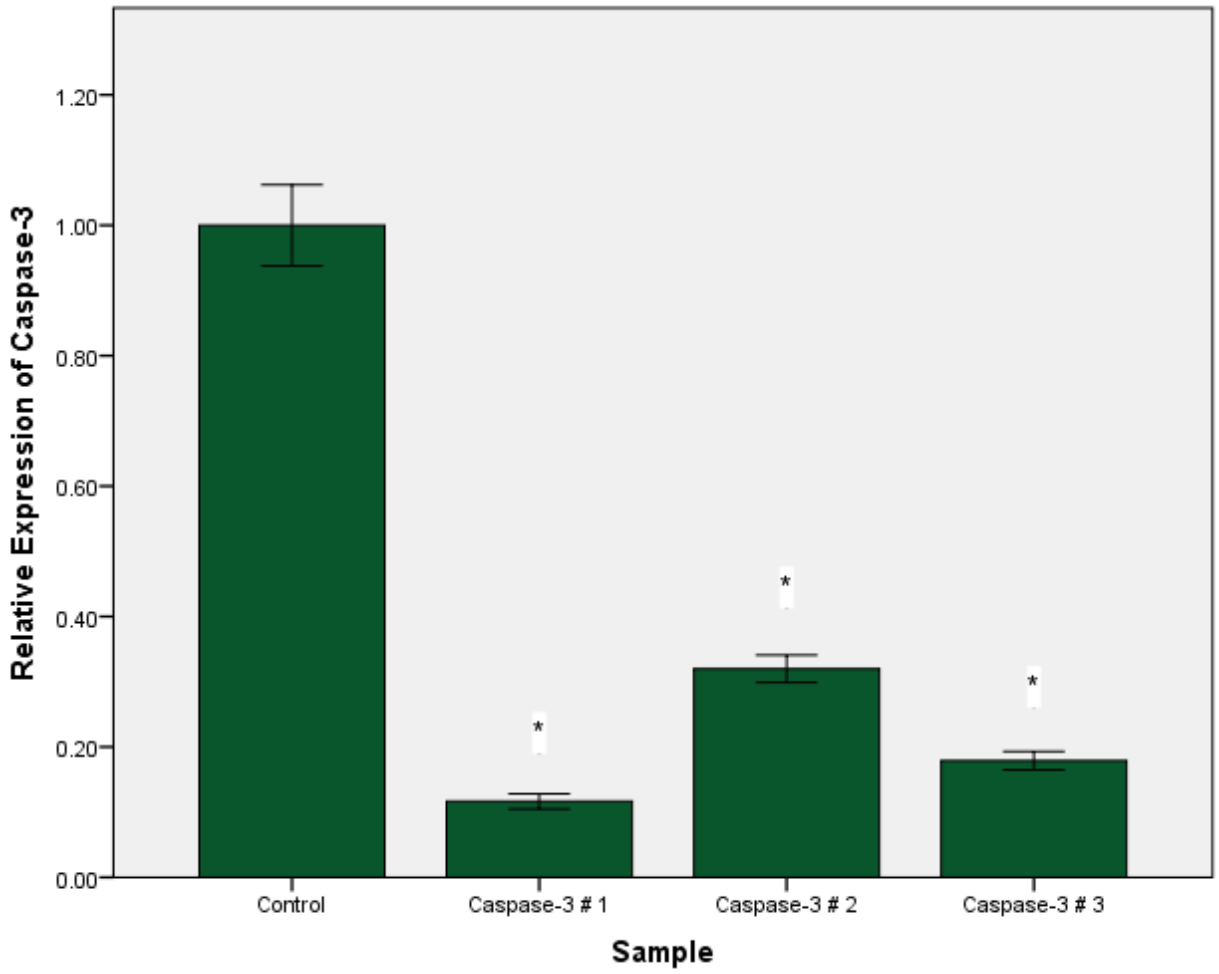


Figure 3 - Silencing of caspase-3 with siRNA.

Three sequences were obtained in order to identify which was most effective. Relative expression for the control sample was arbitrarily set at 1. Caspase-3 # 1 provided the most silencing and was used in further experiments. * $p < 0.05$ compared to control, n = 3, error bars: SEM +/-1.

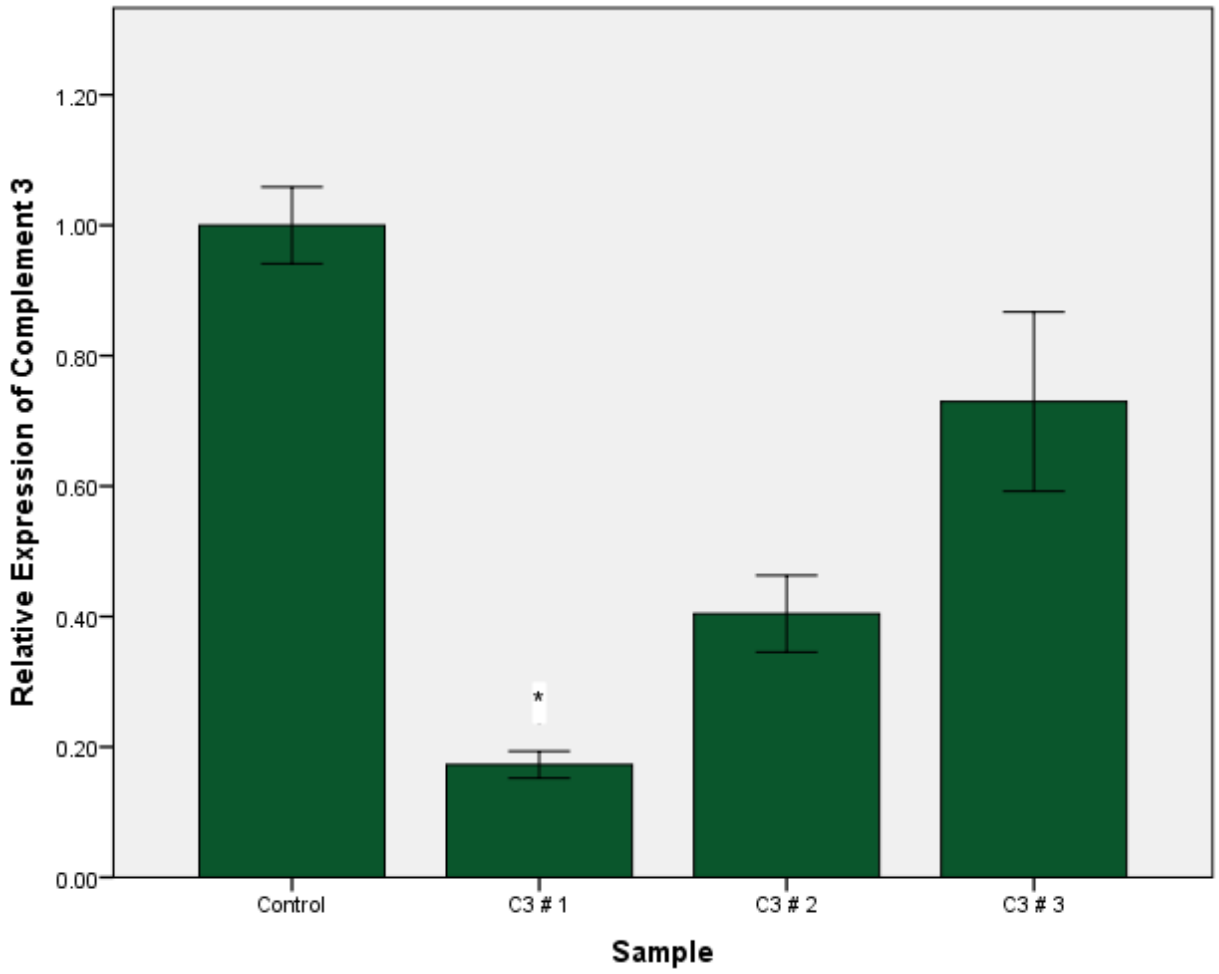


Figure 4 - Silencing of complement 3 with siRNA.

Three sequences were obtained in order to identify which was most effective. Relative expression for the control sample was arbitrarily set at 1. C3 # 1 provided the most silencing and was used in further experiments. * $p < 0.05$ compared to control, n=3, error bars: SEM +/- 1.

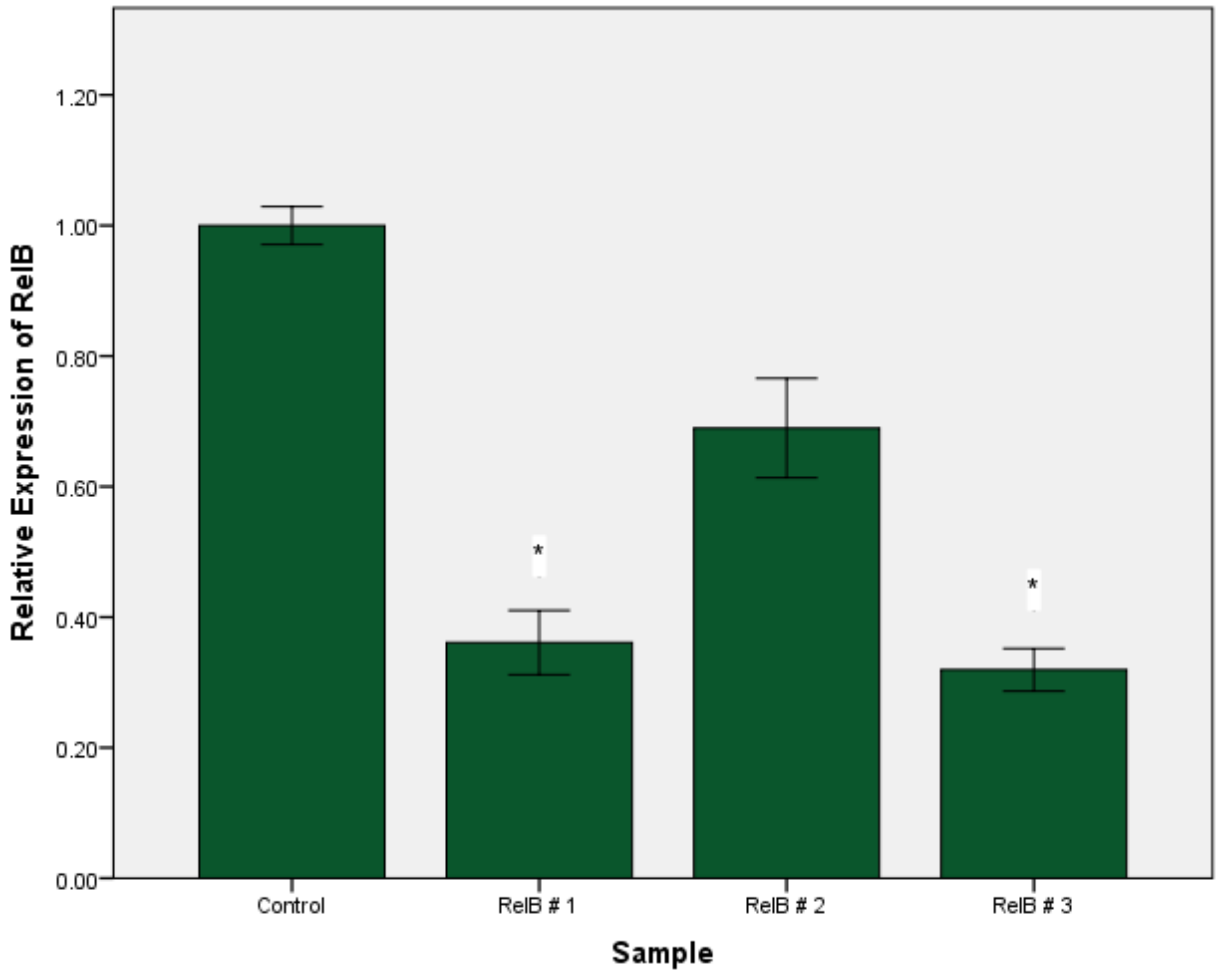


Figure 5 - Silencing of RelB with siRNA.

Three sequences were obtained in order to identify which was most effective. Relative expression for the control sample was arbitrarily set at 1. RelB # 3 provided the most silencing and was used in further experiments. * $p < 0.05$ compared to control, n=3, error bars: SEM +/- 1.

3.1.2 Western Blot Results Following siRNA Silencing

Silencing of caspase-3 using siRNA was confirmed using Western blots. Figure 6 shows a clear decrease in caspase-3 following transfection with all of the caspase-3 siRNA sequences. This proves that the siRNA is effective at reducing both the mRNA levels as well as the protein levels of caspase-3.

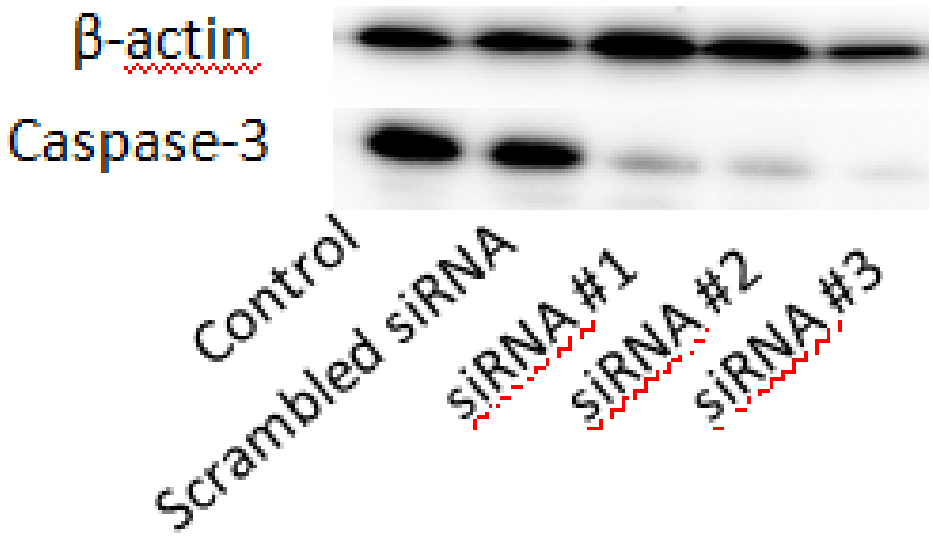


Figure 6 - Western blot using protein isolated from PK1 cells following transfection with caspase-3 siRNA.

3.1.3 Flow Cytometry Results of Transfection Efficiency

One of the caspase-3 siRNA molecules was labeled with Dy547 fluorescent dye in order to assess cellular uptake following transfection. A ratio of 2μL of Lipofectamine® 2000 to 1μg of Dy547 labeled Caspase-3 siRNA was used in these experiments. Flow cytometry was used to determine the percentage of cells containing the labeled siRNA (Figure 7). Transfected cells were also stained with Annexin V and propidium iodide to ensure that the transfection with siRNA did not cause an increase in cell death (Figure 8).

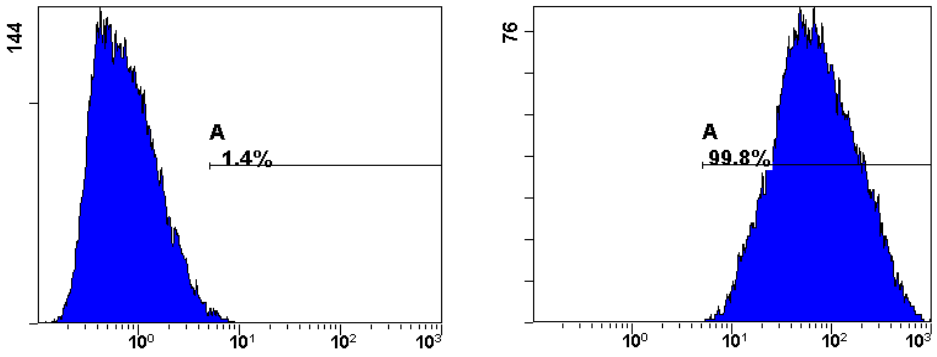


Figure 7 - Flow cytometry histogram with transfected PK1 cells.

Cells which have not been transfected, left, and is used as a control, and PK1 cells which have been transfected with Dy547 labeled caspase-3 siRNA, right. Uptake is excellent at close to 100 percent using the ratio of 2µL Lipofectamine® 2000 to 1µg of siRNA. This allowed us to be confident in the fact that we were able to have good cellular uptake of the siRNA molecules.

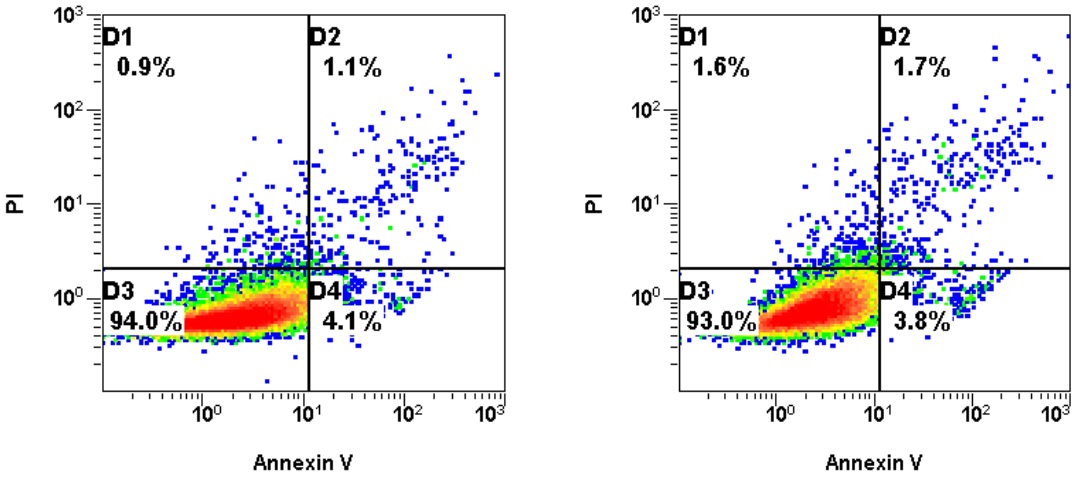


Figure 8 – Viability of PK1 cells following transfection.

PK1 cells which have not been transfected, left, and PK1 cells transfected with caspase-3 siRNA using Lipofectamine® 2000, right. Cells were stained with Annexin V and propidium iodide. This result shows that the number of viable cells (93% vs 94%) were very similar between the non-transfected and transfected cells.

3.2 Hypoxia Chamber Experiments

The H85 HypOxystation® (HypOxygen) was used to induce hypoxia in order to simulate IRI. This hypoxia chamber was thought to be the ideal IRI model for *in vitro* experiments as it can be used to closely replicate the conditions encountered in IRI. This chamber allowed for reliable monitoring and control of temperature and of the concentrations of oxygen, carbon dioxide, and nitrogen. Following exposure to the hypoxic environment, cells were allowed to recover in normal conditions to simulate a reperfusion phase.

3.2.1 Flow Cytometry

Cells were collected for analysis by flow cytometry following re-oxygenation. Annexin V and propidium iodide were used to assess for apoptosis and necrosis respectively. Experiments were carried out using different hypoxia times and are shown below (Figures 9 and 10). A hypoxic time of 24 hours was repeated three times to confirm the data (Figure 11).

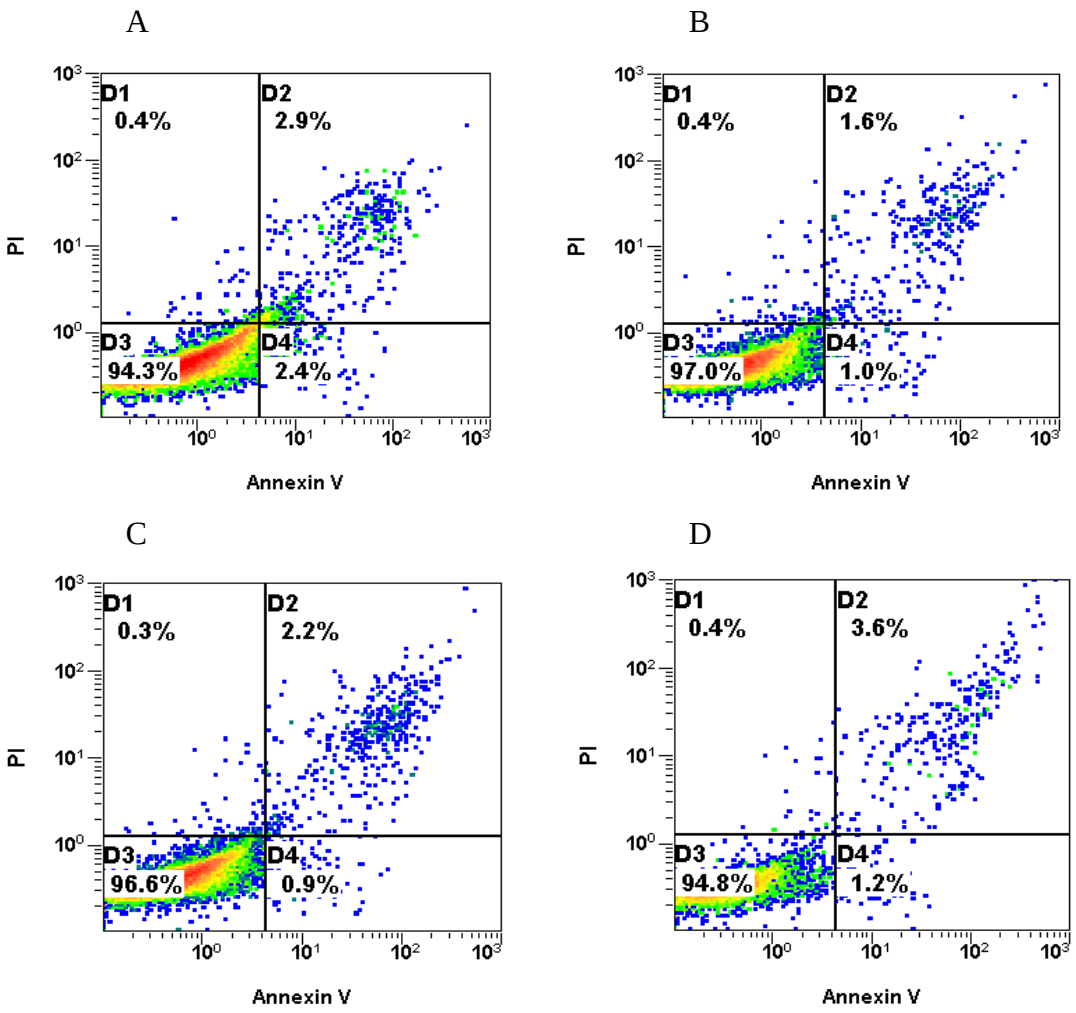


Figure 9 - Flow cytometry results from the hypoxia chamber.

Normal PK1 cells which were used as a negative control (A), and PK1 cells which have been exposed to hypoxia for 15 hours (B), 18 hours (C), and 24 hours (D). This was followed by a re-oxygenation phase of 24 hours. Cells were stained with Annexin V and propidium iodide. Even with long hypoxic times (24h), there is no difference in the number of viable cells between the negative control and the cells which were exposed to the hypoxic environment.

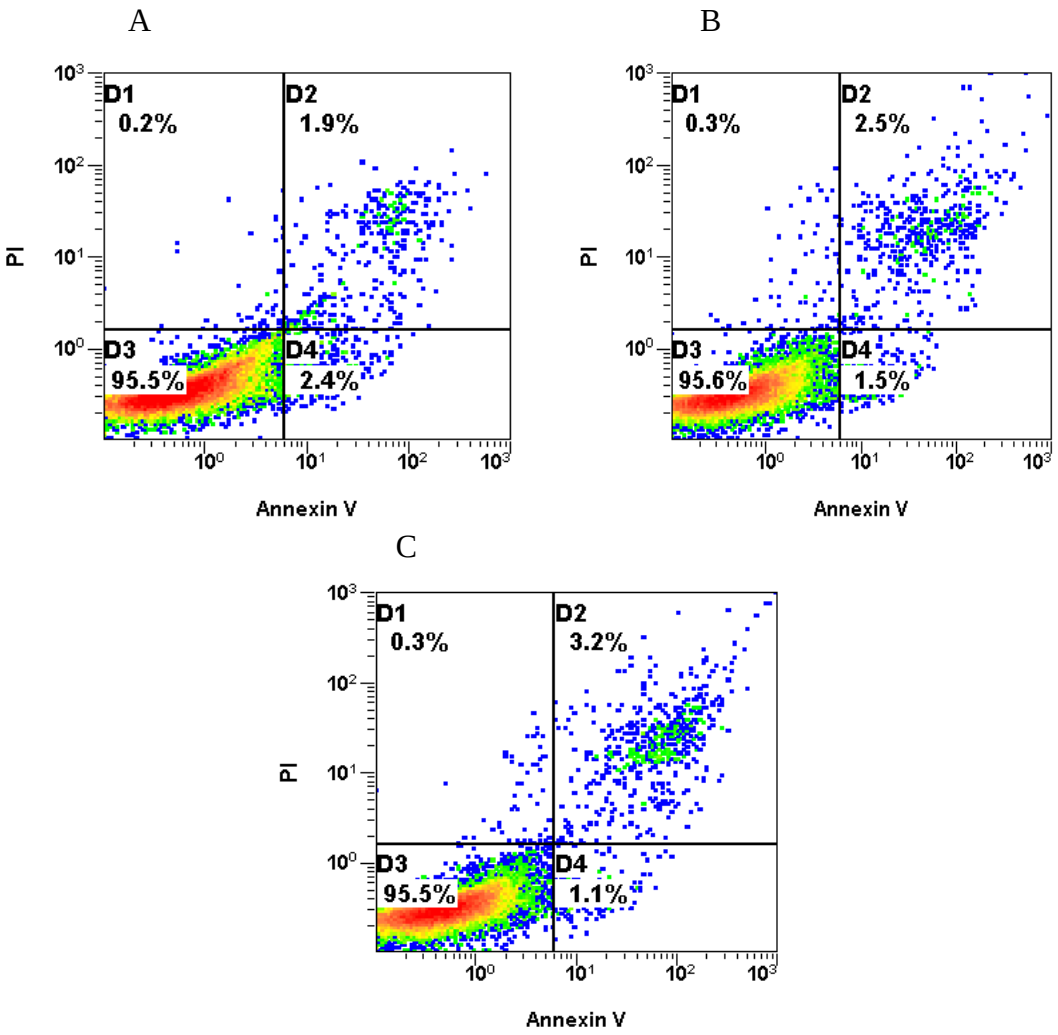


Figure 10 - Flow cytometry results from the hypoxia chamber.

Normal PK1 cells which were used as a negative control (A) and PK1 cells which have been exposed to hypoxia for 16 hours (B), and 24 hours (C) followed by a re-oxygenation phase of 24 hours. Cells were stained with Annexin V and propidium iodide. Again, these results show that there is no difference in the cells which were exposed to the hypoxic environment and the negative control cells which were kept at normal conditions.

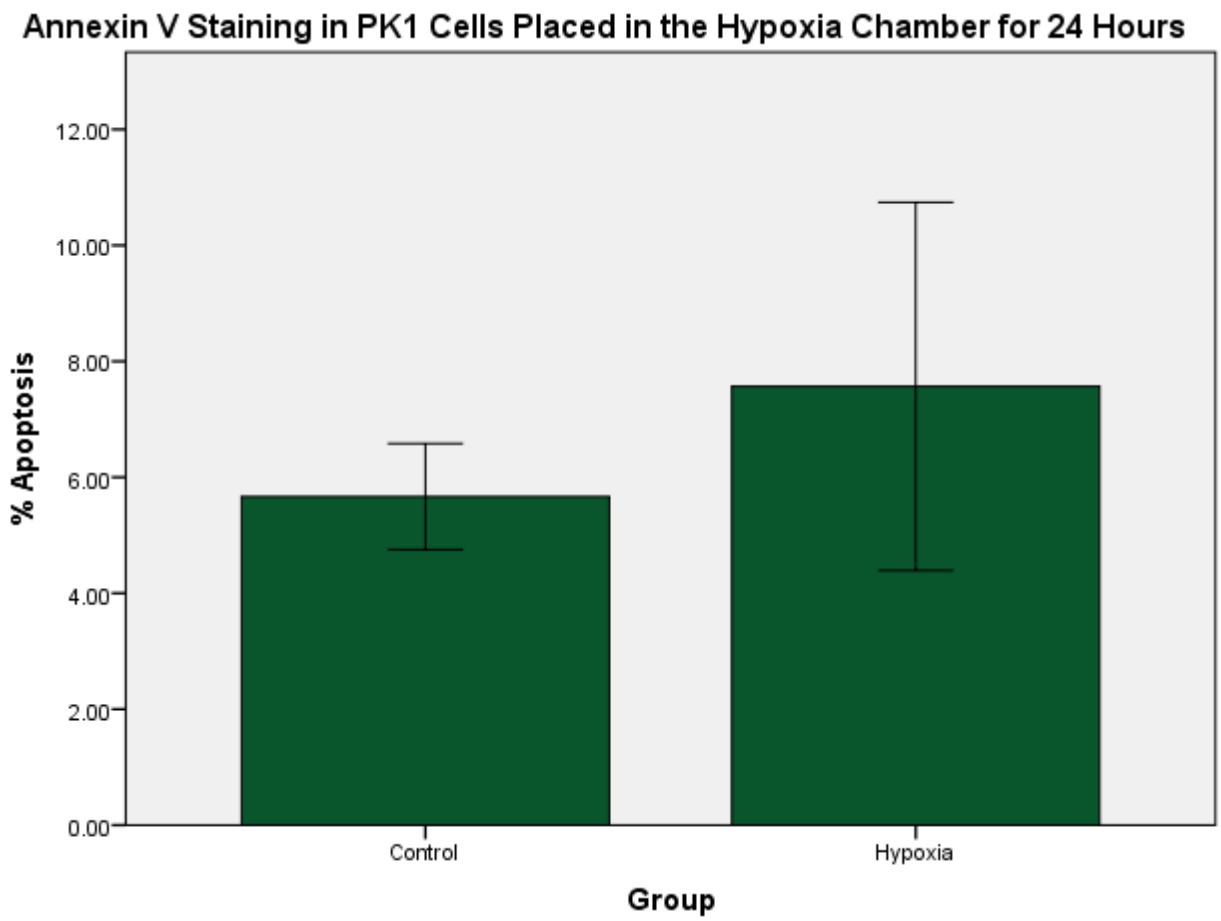


Figure 11 – Combined flow cytometry results from PK1 cells placed in the hypoxia chamber for 24 hours.

Following the hypoxic insult, cells were allowed to recover in normal media in a re-oxygenation phase that lasted 24 hours in order to simulate reperfusion. There was no significant difference found between the groups. p=0.596, n=3, error bars: +/- 1 SE. The hypoxia chamber experiments were repeated multiple times and the combined 24h results are shown here to show that even with long hypoxic times, the number of apopotic cells are not significantly different between the groups. Shorter times were used, as indicated above, and repeated, but again no difference was seen. This lead to a change in the IRI model as this model was not producing cell death in this cell line.

3.2.2 qPCR

As seen in figure 12, there was lower expression of caspase-3 seen in PK1 cells which had been placed in the hypoxia chamber. This was contrary to what was expected based on the length of time the cells were present in the hypoxia chamber. Various hypoxic (30 minutes – 48 hours) and re-oxygenation (3 hours – 24 hours) times were used over the course of these experiments to identify the optimal conditions required to induce cell injury and death, however, very inconsistent results were obtained. Due to the unexpected flow cytometry and qPCR results, a different IRI model was required.

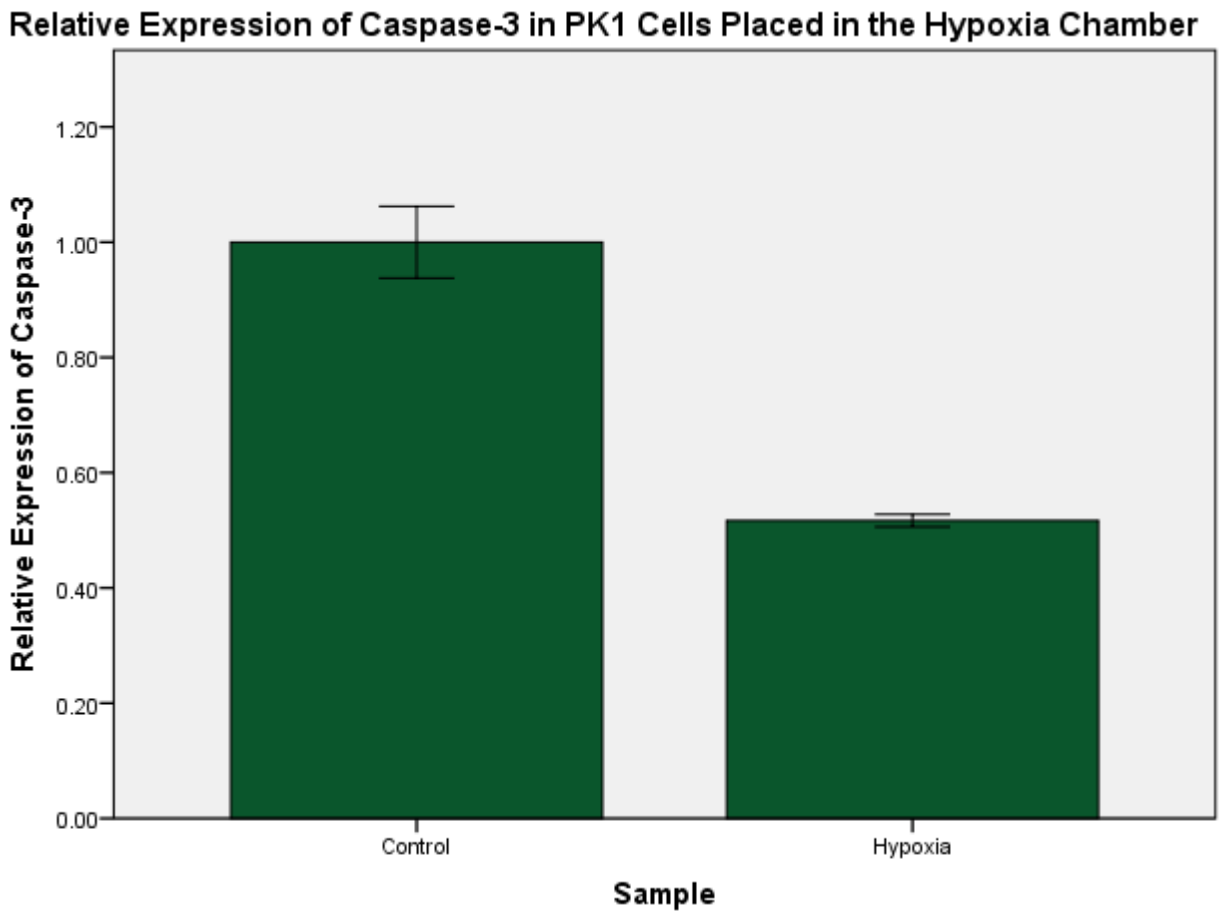


Figure 12 - qPCR results from PK1 cells placed in the hypoxia chamber for 24 hours.

Following the hypoxic insult, cells were allowed to recover in normal media in a re-oxygenation phase that lasted 24 hours in order to simulate reperfusion. p=0.333, n=2, error bars: SEM +/- 1.

3.3 Antimycin A Model of Ischemia Reperfusion Injury

Due to the difficulties with using the hypoxia chamber to induce IRI in an *in vitro* setting, a second IRI model had to be used. Antimycin A inhibits complex III of the electron transport chain and effectively induces a hypoxic environment. Prior to transfection with siRNA, optimal conditions had to be established for its use.

3.3.1 Flow Cytometry and Dose Response Experiments

Various exposure times and concentrations of antimycin A were used in order to obtain a significant injury from which a difference could be observed following treatment with siRNA. Initially exposure times were maintained between 1 and 4 hours and followed by a recovery phase that lasted up to 24 hours. This produced either almost no injury at lower concentrations, or too much cell death at higher concentrations (Figure 10). As antimycin A must be dissolved in 95 percent ethanol, a control exposing cells to an equivalent volume of 95 percent ethanol was included. Due to the small volume, no difference from the negative control was observed (Figure 13). The model was then adjusted to include a shorter recovery time and improved results were obtained with an increased number of viable cells (Figure 14).

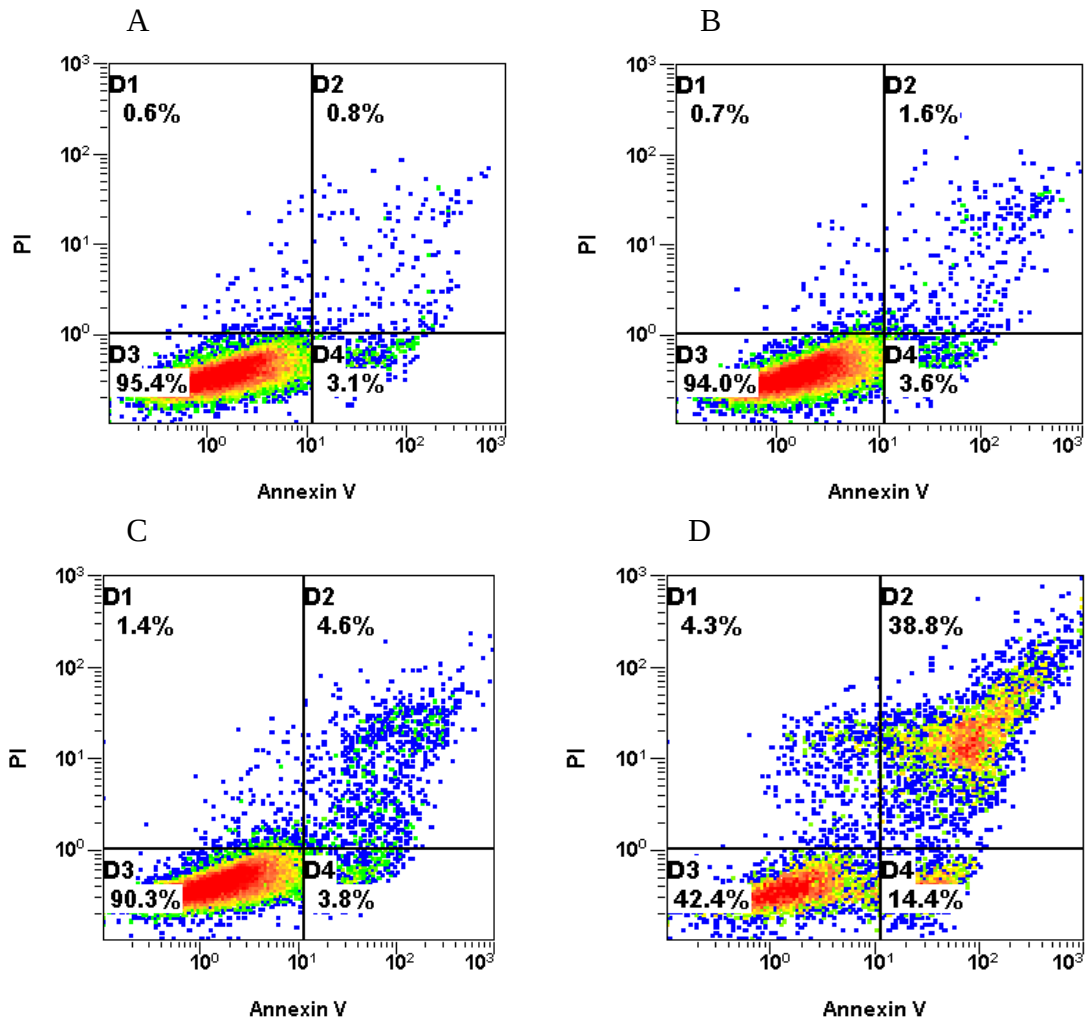


Figure 13 – Antimycin A experiments.

Normal PK1 cells which served as a negative control (A). PK1 cells exposed to 2µL of 95 percent ethanol (B). Media was replaced with PBS which did not contain antimycin A during the treatment phase in both of these controls. PK1 cells treated with 2.5µM antimycin A for 3 hours followed by 24 hours of recovery (C) and PK1 cells treated with 10µM antimycin A for 3 hours followed by 24 hours of recovery (D). Cells were stained with Annexin V and propidium iodide.

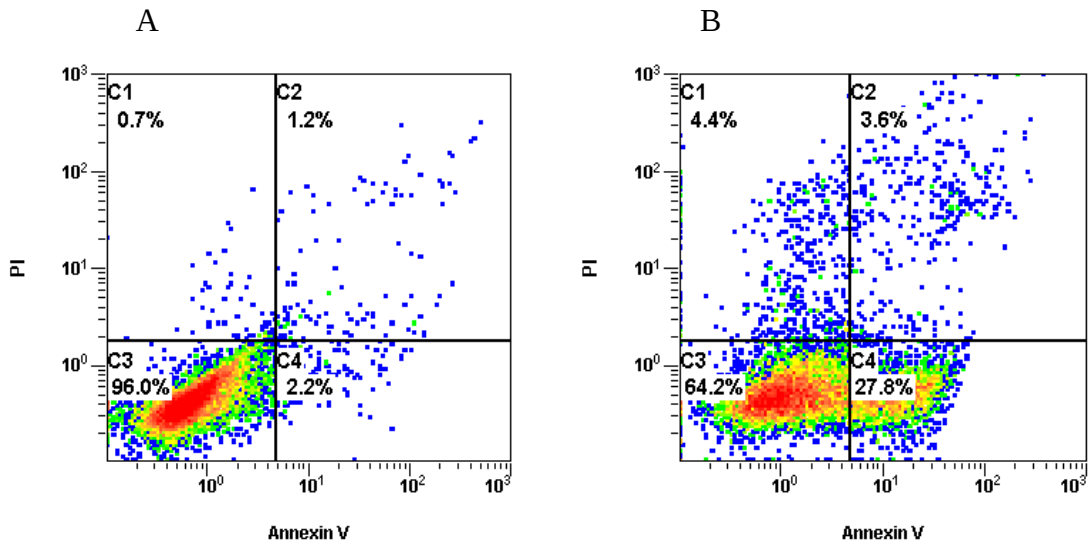


Figure 14 – Antimycin A experiments.

Normal PK1 cells (A) and PK1 cells treated with 10µM antimycin A for 3 hours followed by 3 hours of recovery (B). Cells were stained with Annexin V and propidium iodide.

From these experiments, an exposure time to antimycin A of 3 hours followed by a 3 hour recovery phase in normal media was used. Due to the small volume of ethanol used (which was necessary to dissolve the antimycin A), it did not have an effect on cell death. Also, the cells treated with antimycin A had the media changed to PBS during the exposure time to ensure that there was no available glucose, and the negative control confirmed that this did not have an effect on cell death.

3.3.2 Flow Cytometry and Adjustment of siRNA:Lipofectamine Transfection Ratios

As shown above, using a ratio of 2μL of Lipofectamine® 2000 to 1μg of siRNA allowed us to obtain a transfection efficiency close to 100 percent without causing obvious cell injury or death (Figures 7 and 8). However, when PK1 cells were transfected with siRNA 24 hours prior to treatment with antimycin A, an increase in apoptosis was observed compared to cells which were only treated with antimycin A and not transfected (Figure 15). This lead to a need to re-adjust the Lipofectamine® 2000 to siRNA ratio as the liposome is known to be toxic to cells. Caspase-3 Dy547 labeled siRNA was again used to obtain an optimal transfection ratio for use with antimycin A and results are shown in Figure 16. Transfected cells were also stained with Annexin V and propidium iodide and analyzed using flow cytometry to ensure that cells survived following transfection and treatment with antimycin A. PK1 cells were transfected with caspase-3 siRNA and then treated with 10μM antimycin A for 3 hours followed by 3 hours of recovery. Results are shown in Figure 17.

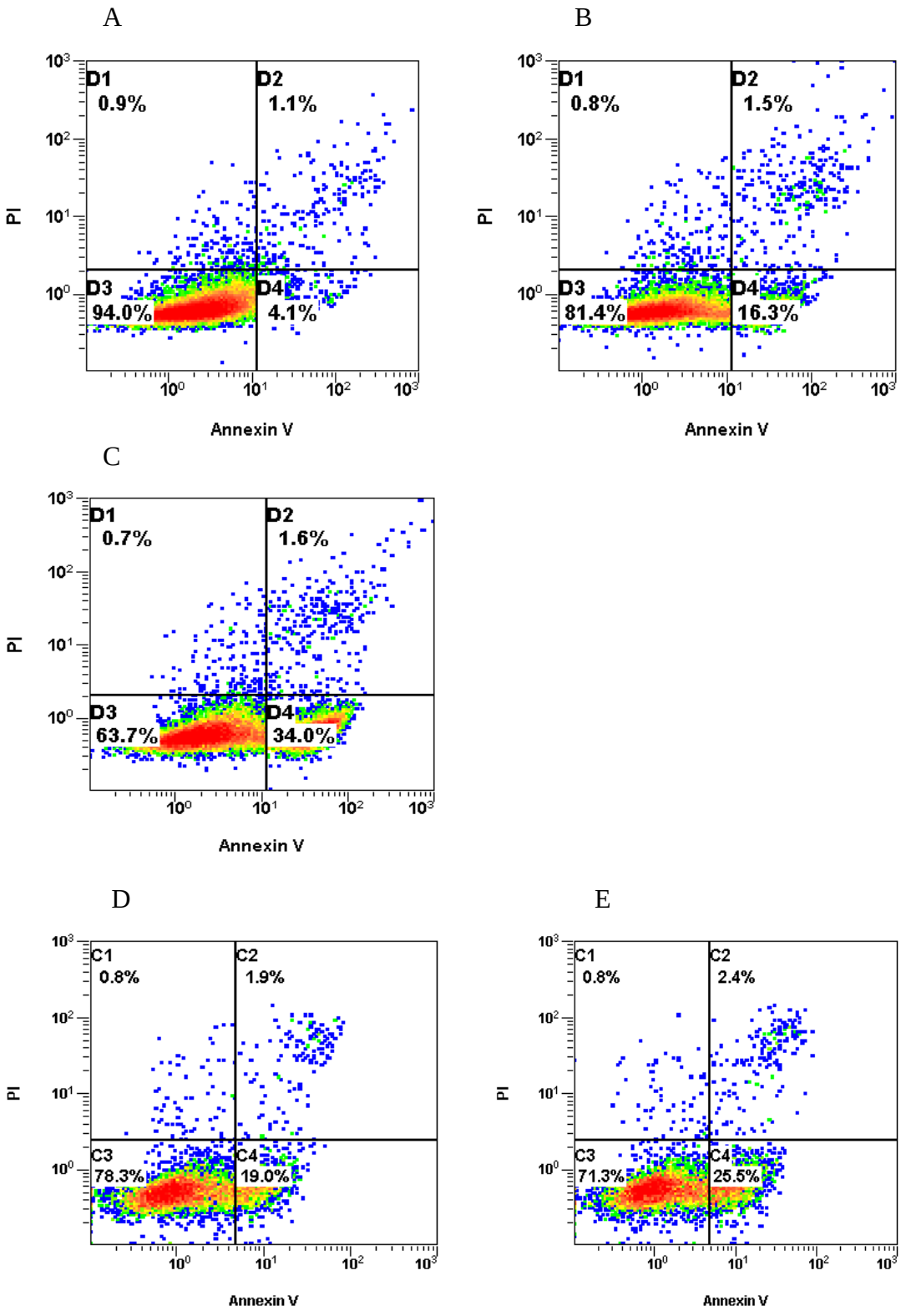


Figure 15 – Antimycin A treated PK1 cells following transfection with siRNA.

Normal PK1 cells (A). PK1 cells treated with 10μM antimycin A for 3 hours followed by 3 hours of recovery (B) and PK1 cells treated with antimycin A in the same way after transfection with caspase-3 siRNA (C). PK1 cells treated with 10μM antimycin A for 3 hours followed by 3 hours of recovery from a seperate experiment (D) and PK1 cells treated with antimycin A after transfection with scrambled siRNA (E). Cells were stained with Annexin V and propidium iodide.

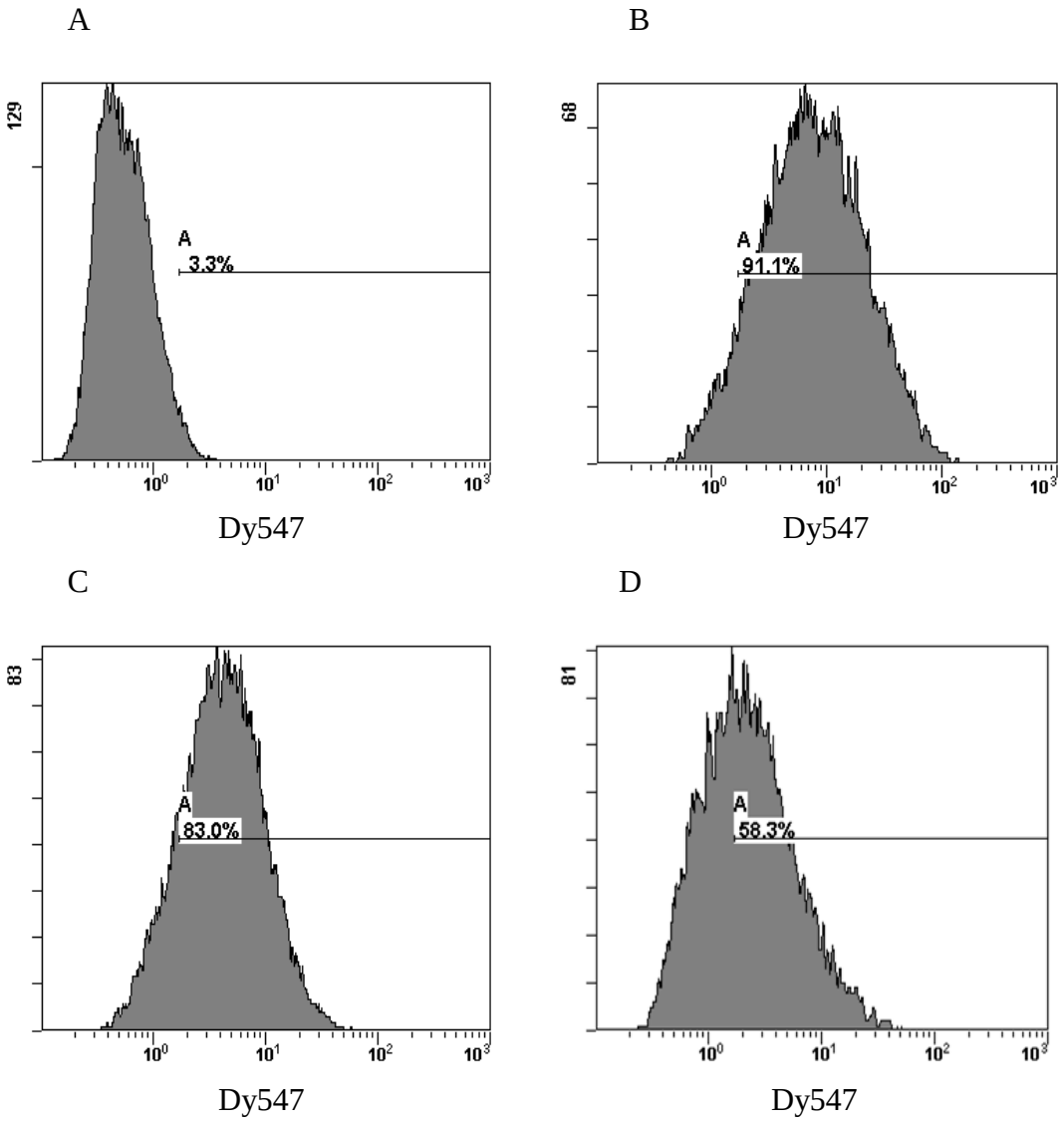


Figure 16 – Adjusted siRNA to Lipofectamine® 2000 ratios.

Varying ratios of Dy547 labeled caspase-3 siRNA and Lipofectamine® 2000. Non-transfected control (A), 1µL Lipofectamine® 2000 to 1µg siRNA (B), 0.5µL Lipofectamine® 2000 to 1µg siRNA (C), and 0.25µL Lipofectamine® 2000 to 1µg siRNA (D). As the ratio decreased, there was less uptake, but this need to be weighed against the issues with toxicity of the liposome.

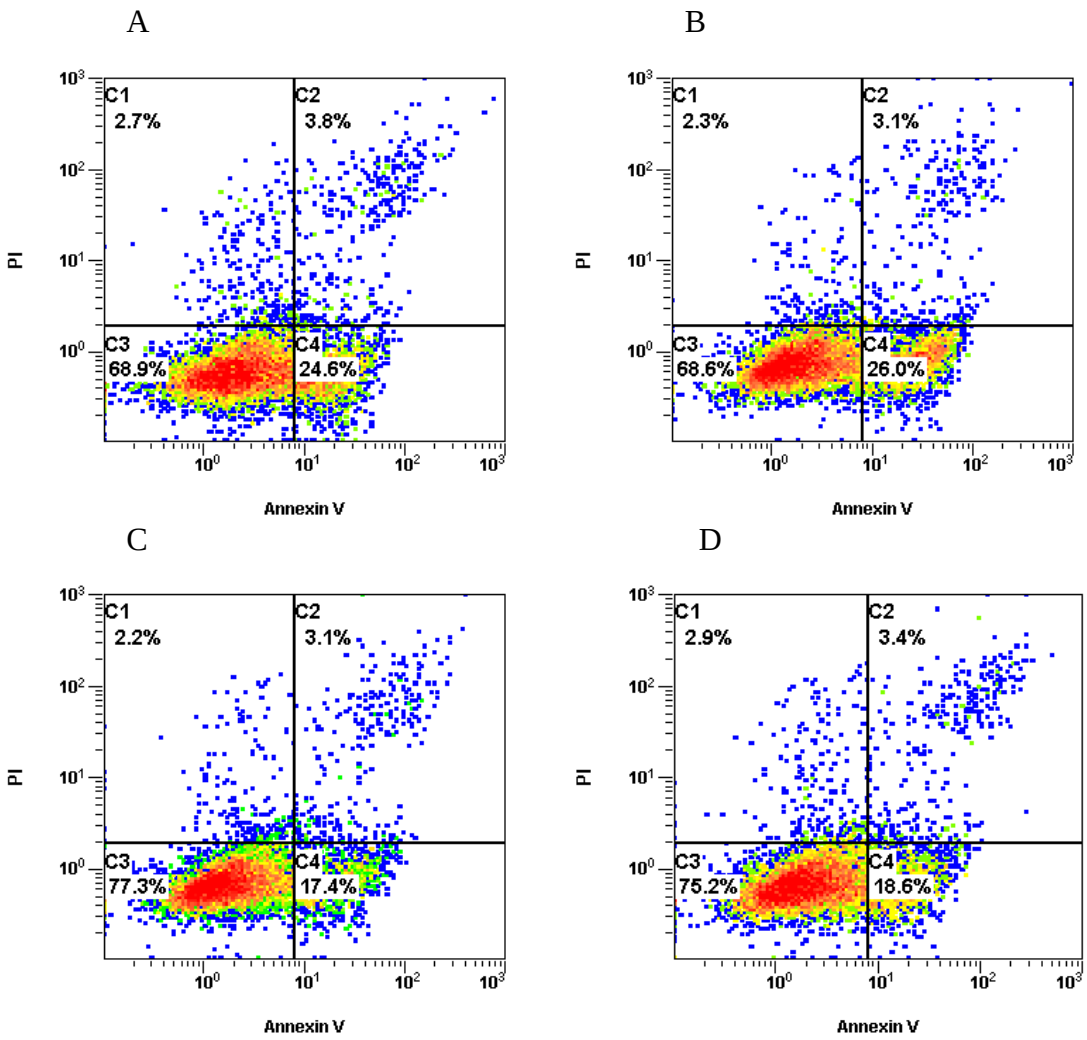


Figure 17 - Varying ratios of caspase-3 siRNA and Lipofectamine® 2000 in antimycin A treated PK1 cells.

Non-transfected control (A), 1µL Lipofectamine® 2000 to 1µg siRNA (B), 0.5µL Lipofectamine® 2000 to 1µg siRNA (C), and 0.25µL Lipofectamine® 2000 to 1µg siRNA (D). Cells were stained with Annexin V and propidium iodide. This shows that the ratio of Lipofectamine® 2000 to siRNA had an effect on apoptosis in cells that were both transfected and exposed to antimycin A and this needed to be taken into account.

Following these experiments, it was decided to proceed with a 0.5µL Lipofectamine® 2000 to 1µg siRNA ratio as this provided us with an adequate transfection efficiency (83%) and showed a benefit in terms of cell survival when cells were treated with antimycin A. This ratio was used for all future experiments.

3.4 Antimycin A and siRNA Experiments

Following the development of an adequate IRI model using antimycin A, PK1 cells were transfected with caspase-3, complement 3, and RelB siRNA alone or in combination 24 hours prior to treatment with antimycin A. The cells were treated with Antimycin A for 3 hours followed by 3 hours of recovery in normal medium and then collected for flow cytometry analysis as well as RNA isolation.

3.4.1 Gene Expression of Caspase-3, C3, and RelB

RNA was isolated to assess the effect on gene expression during treatment with antimycin A and transfection with siRNA. RNA was reverse transcribed to cDNA and qPCR performed to identify relative expression of caspase-3, complement 3, and RelB. Results are shown in Figures 18, 19, and 20 respectively. All of the siRNAs showed a reduction in relative expression of their respective genes compared to their negative controls. Only caspase-3 expression was significantly elevated in the positive control relative to the negative control following treatment with antimycin A, however. Complement 3 expression was actually significantly decreased after cells were treated with antimycin A.

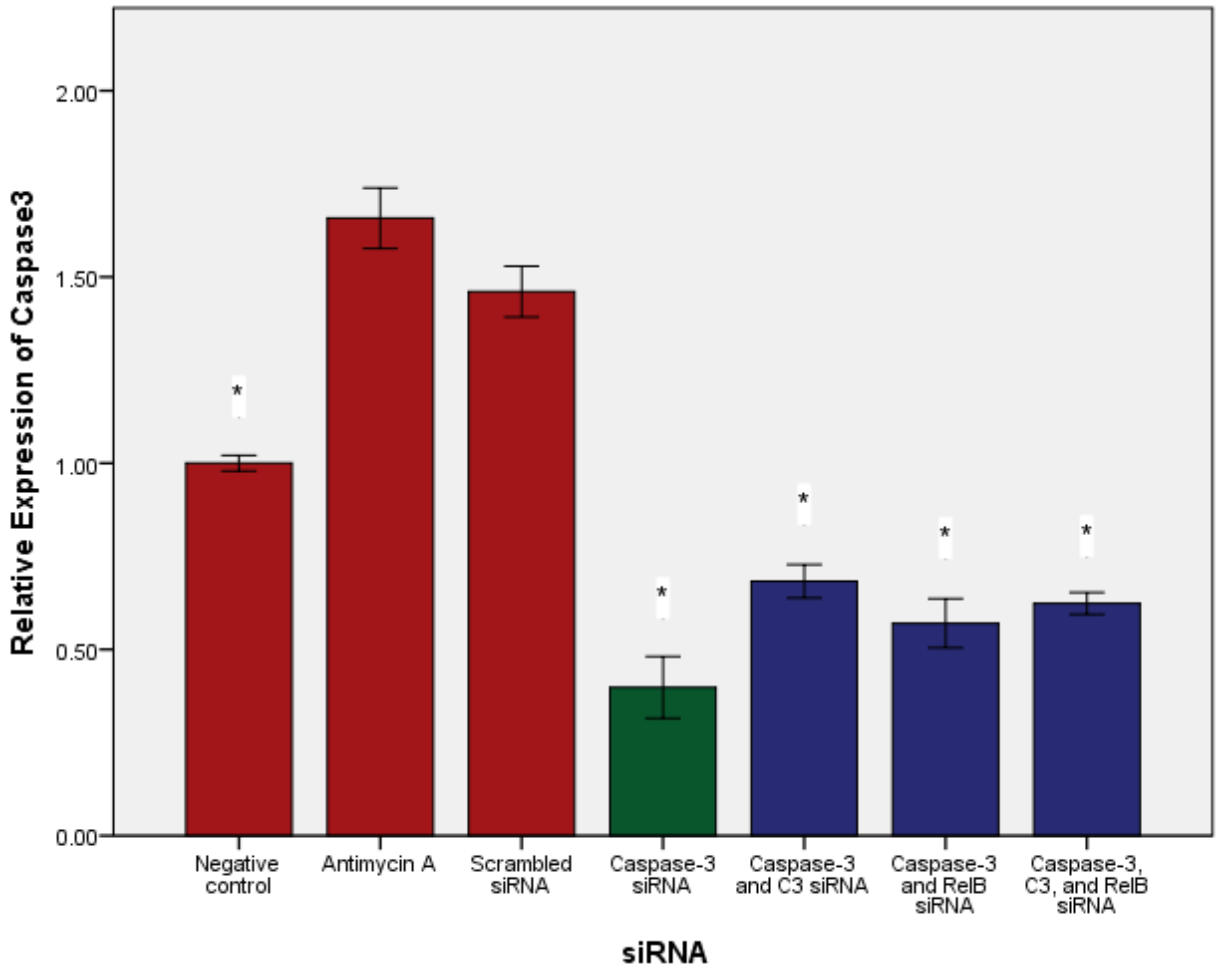


Figure 18 - Caspase-3 expression in PK1 cells following treatment with antimycin A after transfection with siRNAs listed.

* $p < 0.05$ compared to positive control (antimycin A), n=3, error bars: SEM +/- 1.

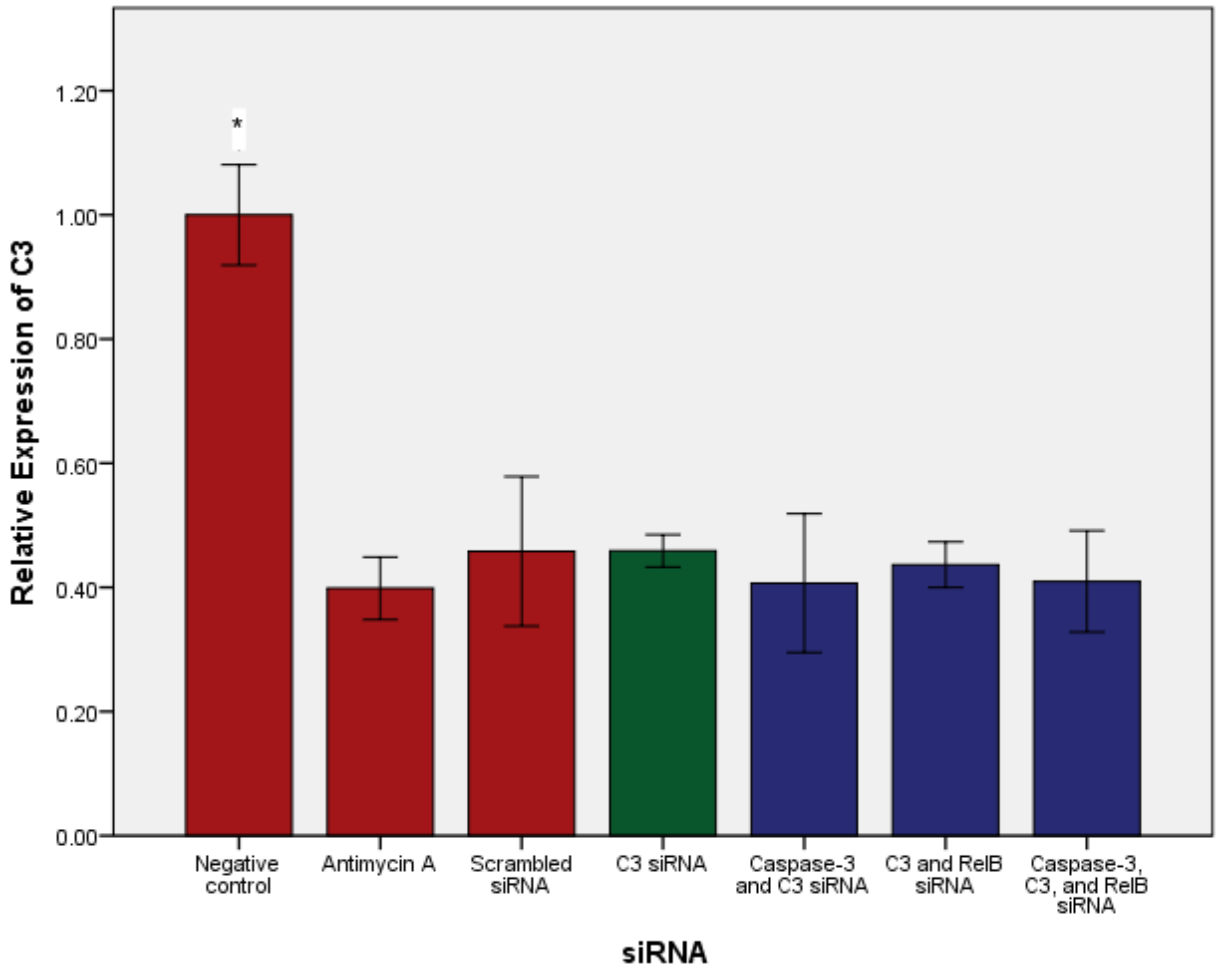


Figure 19 - Complement 3 expression in PK1 cells following treatment with antimycin A after transfection with siRNAs listed.

* $p < 0.05$ compared to positive control, n=3, error bars: SEM +/- 1.

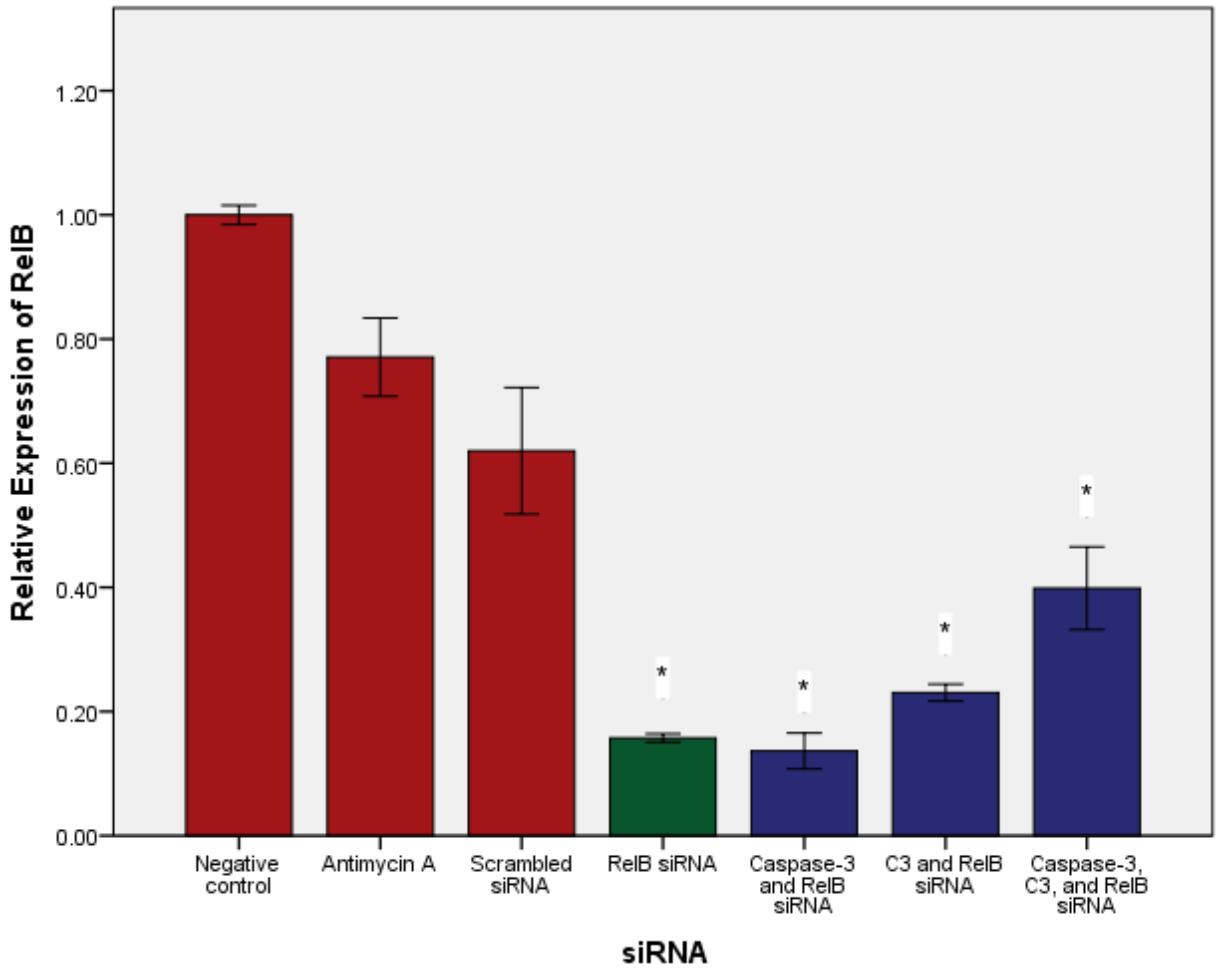


Figure 20 - RelB expression in PK1 cells following treatment with antimycin A after transfection with siRNAs listed.

* $p < 0.05$ compared to positive control, n=3, error bars: SEM +/- 1.

3.4.2 Flow Cytometry Results Following Treatment with Antimycin A and siRNA Transfection

Flow cytometry was used to assess for apoptosis and cell viability using Annexin V and propidium iodide staining. Results are shown in Figures 21 and 22 and Table 9. A significant reduction in apoptosis was seen when caspase-3 and RelB siRNA was used (either alone or in combination). The relative risk reduction was quite pronounced as shown in Table 9. A synergistic effect was not observed when two or three different siRNAs were used in combination. Complement 3 siRNA did not provide any benefit in terms of reduction of apoptosis or increased cell viability.

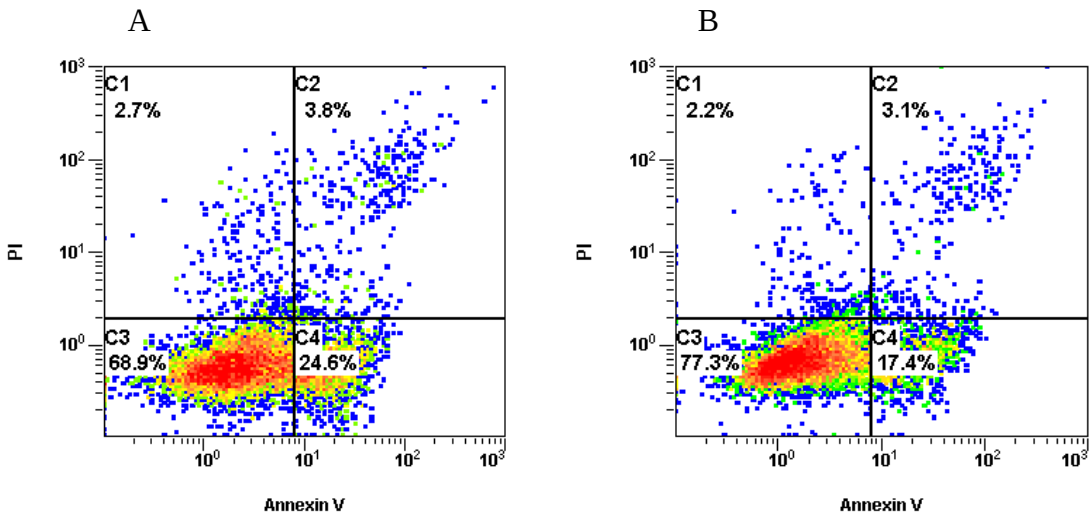


Figure 21 - A representative flow cytometry result of PK1 cells following treatment with antimycin A and transfection with caspase-3 siRNA.

PK1 cells treated with antimycin A for 3 hours followed by a recovery phase of 3 hours (A) and PK1 cells following transfection with Caspase-3 siRNA followed by treatment with antimycin A (B). Cells were stained with Annexin V and propidium iodide. This shows a large decrease in the percentage of apoptotic cells in the siRNA treated group.

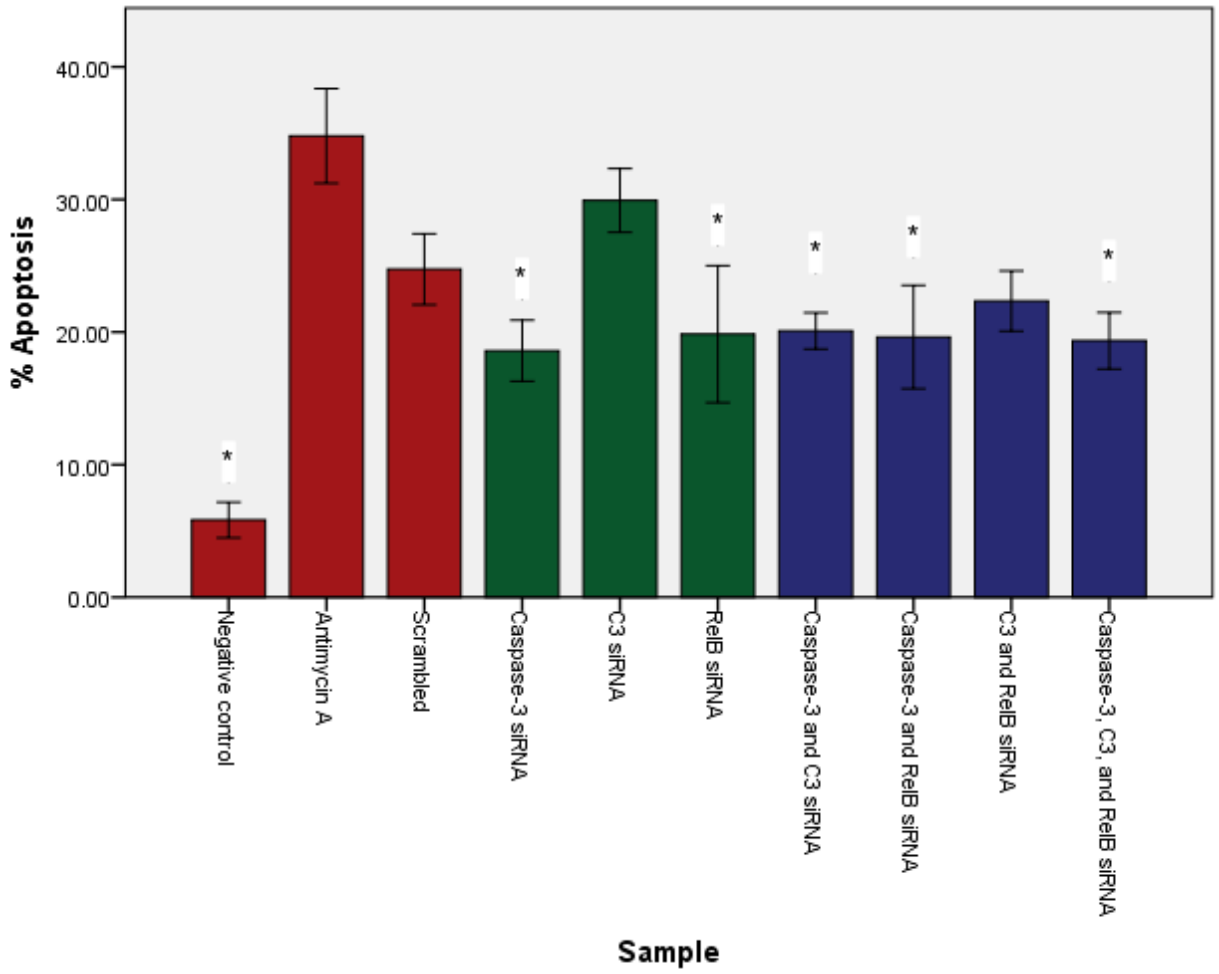


Figure 22 - Percentage of apoptotic cells as measured by flow cytometry using Annexin V staining.

All groups, except for the negative control, were treated with antimycin A for 3h followed by 3h of recovery. Cells were transfected with the indicated siRNA 24h prior to treatment with antimycin A. * $p < 0.05$ compared to positive control (antimycin A), n=3, error bars: SEM +/- 1. Caspase-3 and RelB siRNA were effective alone and in combination in reducing apoptosis in the antimycin A treated cells. No synergistic effect was seen, however.

Table 9 - Relative risk reduction of apoptosis after RNAi in PK1 cells treated with antimycin A.

n=3 for all samples, one way ANOVA with Tukey post hoc analysis used for statistical analysis.

Sample	Relative Risk Reduction (%)	P value
Caspase-3 siRNA	**46.6**	**0.019**
C3 siRNA	13.9	0.968
RelB siRNA	**42.8**	**0.038**
Caspase-3 and C3 siRNA	**42.2**	**0.043**
Caspase-3 and RelB siRNA	**43.7**	**0.034**
C3 and RelB siRNA	**35.6**	**0.129**
Caspase-3, C3, and RelB siRNA	**44.3**	**0.029**

Chapter 4

4 Discussion

There is a significant need for more donor kidneys in order to address the large number of patients who are currently waiting for a kidney transplant. In Canada in 2012, there were 2469 patients on the waiting list for a renal transplant with only 1054 transplants performed (2). The donor pool was traditionally limited to SCD donation in order to ensure high quality grafts. Recently, however, the donor pool is being expanded to include DCD and ECD donors in order to address the need for more available donor kidneys. These organs are of lower quality given the co-morbidities present in ECD donors and the prolonged WIT in DCD donors. Much advancement has been made in transplantation since the first successful kidney transplant which has made transplantation possible by overcoming issues with rejection. There is a lack of effective therapies targeted at improving the quality of the graft during procurement, transport, and implantation, however. This is an important area of research, especially in kidneys from DCD donors where there is a prolonged period of decreased perfusion followed by no perfusion.

IRI is unavoidable during renal transplantation and can cause significant cell damage and death. This is especially prominent in DCD donation due to the lengthened WIT and leads to an increased rate of DGF. IRI is a complex process that involves the activation of many different molecular pathways. Most therapies targeted at IRI have not proceeded past the experimental stage, unfortunately. Machine perfusion is being used clinically and involves perfusing the kidney with perfusion solution prior to implantation. This has been shown to decrease the rate of DGF, but has not increased overall graft survival (77, 78). Novel therapies for IRI are needed to increase the amount and quality of available organs for transplantation in order to adequately treat the many patients who are on the waiting list.

A lot of work has been done in the *in vitro* setting as well as small animals to develop therapies for IRI. One area of research has focused on the use of RNAi to target specific

genes involved in the cellular pathways which are up-regulated in IRI. RNAi has proven to be very useful in these models; however, the translation to a clinical setting has been difficult due to issues around the stability and delivery of these small RNA molecules as well as potential off-target effects. Transplantation does offer the possibility of treating the organ in an *ex vivo* setting where off target effects can be minimized and conditions can be closely monitored. More research needs to be performed in large animals before this can be fully implemented in a clinical setting.

This project was designed with the intent of developing effective porcine siRNAs in an *in vitro* setting which could be used in future large animal *ex vivo* and *in vivo* experiments. The use of RNAi in large animal transplantation models has been limited to the targeting of apoptosis. Given the complex and redundant pathways involved in cell damage during IRI, however, we identified a need to target multiple pathways in order to enhance the treatment effect. Previous work using mice IRI models has shown a benefit to combining caspase-3 and complement 3 siRNA (53), as well as caspase-3 and caspase-8 siRNA (97). For this reason we have targeted caspase-3 (apoptosis), complement 3 (innate immunity), and RelB (transcription factor involved in the inflammatory response). Prior to use of these combinations in a large animal model, it was important to ensure the effectiveness of the siRNAs in an *in vitro* IRI model. With the future use of a porcine model in mind, it was decided to use PK1 cells which are pig kidney tubular epithelial cells. The tubular epithelial cells are susceptible to ischemia and the PK1 cell line is commercially available.

4.1 siRNA Development and Testing

Caspase-3, complement 3, and RelB siRNA sequences were obtained from Sigma-Aldrich® and were tested in normal PK1 cells. All of these genes showed constitutive expression. Most of these sequences provided some form of gene silencing and the best one from each group was chosen to be used in further experiments. The level of silencing was excellent for the sequences chosen as it was around 80 percent for caspase-3 and complement 3, and 70 percent for RelB in these normal cells. There was no obvious effect on the cellular morphology following transfection and silencing and flow cytometry was performed to ensure that the transfection did not induce any cell death

(which was confirmed). A Western blot was performed following caspase-3 transfection to ensure that the silencing effect extended to the protein level and was not only limited to mRNA expression. This was confirmed as well. The development of effective siRNA molecules was essential for the success of the rest of the experiments undertaken.

4.2 Hypoxia Chamber

In the past homemade chambers have been used to create hypoxic environments by continuously flushing the chambers with nitrogen or a combination of gases such as nitrogen, carbon dioxide, and hydrogen. These chambers may be unreliable and cannot always be monitored closely. The hypoxia chamber used in these experiments was a specialized chamber from HypOxygen and provided a controlled environment where the temperature, humidity, and concentration of oxygen, carbon dioxide, and nitrogen could be controlled and closely monitored. This hypoxia chamber was thought to be able to provide the optimal *in vitro* model for IRI as published studies that have made use of *in vitro* IRI models often use either a homemade version of an anaerobic chamber or antimycin A to induce hypoxia (121). Unfortunately, results obtained from the hypoxia chamber experiments did not align with what was expected. The PK1 cells which were used were very resistant to hypoxia and showed no obvious morphological changes even after 24 hours at 1 percent O_2. The experiments were adjusted many times in order to determine which variables were important in developing a reproducible amount of cellular injury and death. This was accomplished by varying times in the hypoxia chamber as well as re-oxygenation times after the cells were exposed to hypoxia. The type of medium was also changed to either include glucose or not, as well as to de-oxygenate or not prior to use in the hypoxia chamber. Additionally, cell confluence from 60 to 90 percent was used. In each case, the PK1 cells remained quite resistant to hypoxia and there was not a significant amount of cell death induced by using the chamber. After a number of experiments it was decided that a more reliable model of IRI was needed for the *in vitro* experiments.

4.3 Antimycin A

This prompted the switch to using antimycin A which is molecule produced by *Streptomyces* sp. and inhibits complex III of the electron transport chain and induces a hypoxic state (117-119). Antimycin A has been used effectively as a model for IRI in other studies (43, 121). Experiments were performed to produce an optimal model that induced cell death while still ensuring that a significant percentage of cells remained alive. The medium was changed to PBS during the treatment with antimycin A to try and produce an environment that mimicked that which occurs in transplantation when there is no perfusion and a lack of glucose. The PK1 cells were found to be quite susceptible to the antimycin A and the cell injury appeared to worsen with the recovery phase in normal, glucose containing, medium. If the cells were allowed to recover for 24 hours, cell death was much more pronounced than if only 3 hours were allowed for recovery. The recovery phase was used to simulate reperfusion. After treatment with antimycin A, most dead or dying cells were either in early apoptosis (Annexin V positive, propidium iodide negative) or early necrosis/late apoptosis (both Annexin V and propidium iodide positive). There was not a significant amount of necrosis seen (Annexin V negative, propidium iodide positive).

Once this model had been developed, transfection with siRNA was performed to identify the best time for transfection and examine how the cells reacted to both transfection and treatment with antimycin A. It was found that the best time for transfection was the day prior to treatment with antimycin A. This ensured that the transfection time was adequate and that the effect would be seen in the mRNA and protein expression. It was important to have the transfection as close to the treatment with antimycin A as possible to better mimic the clinical scenario and to ensure that the non-transfected negative control did not become overgrown and experience cell death due to overcrowding.

Although the transfection protocol which had been developed to test the siRNA did not produce any significant amount of cell death, it was noted that when combined with antimycin A treatment, more cell death occurred than with antimycin A treatment alone. This was thought to be due to the use of the liposome Lipofectamine® 2000 which can be toxic to cells and induce cell death. The amount of liposome needed to be decreased

therefore in order to ensure that additional cell death was not caused by its use. Different ratios were then used in order to identify a ratio which would allow for an adequate transfection efficiency and gene silencing, but not be harmful to the cells. This was achieved and a ratio of 1μL of Lipofectamine® 2000 to 2μg siRNA was used.

4.4 Antimycin A and siRNA Transfection

Once the IRI model had been developed and reliably induced injury and cell death in the PK1 cells, experiments were performed to assess the efficacy of the three siRNAs which had been chosen (caspase-3, complement 3, and RelB). It was observed that all of the genes were silenced compared to the positive and negative controls. Only caspase-3 and RelB showed any benefit in terms of reducing apoptosis and increasing the percentage of viable cells (Figure 19 and 20). Caspase-3 silencing produced a benefit when silenced alone or in combination with complement 3 and RelB. There was a benefit seen when all three target genes were silenced as well. Caspase-3 is important to the intrinsic and extrinsic apoptotic pathways and it makes sense that silencing of this gene will lead to improved cell survival. Caspase-3 mRNA expression and activated caspase-3 protein levels were elevated following treatment with antimycin A. Although caspase-3 mRNA was significantly decreased following transfection in the antimycin A treated groups, the same was not found with regards to activated caspase-3 protein levels. There was a difference in the transfected groups from the scrambled siRNA group, but not from the positive control. Given the fact that procaspase-3 is present in normal cells, silencing may prevent more caspase-3 protein from being produced during IRI, but will not have an effect on that which has already been produced and can be activated.

RelB silencing produced a benefit alone and in combination with caspase-3 silencing in terms of cell viability and apoptosis. There was a benefit seen when all three target genes were silenced as well. RelB is a transcription factor which is part of the NF-κβ signaling pathway and is responsible for the activation of many different genes. Attempting to silence part of this pathway could produce varying results as it can affect both survival and death. The mRNA expression of RelB was not significantly elevated when the cells were treated with antimycin A, however a benefit in terms of cell viability and reduced apoptosis was noted when RelB was silenced.

Complement 3 silencing did not produce a benefit in terms of reducing apoptosis or increasing the number of viable cells. Additionally, the mRNA expression of complement 3 was significantly lower in the antimycin A treated cells. The RNA was only isolated following the recovery phase so it is possible that complement 3 is up-regulated later on in the process of IRI or only in the presence of other cell types. Complement 3 is at the convergence of all of the complement pathways and is constitutively produced by tubular epithelial cells. The complement system is known to be important in the propagation of cell injury following IRI and targeting this system was thought to useful in treating IRI (47, 88, 122). This project was limited to *in vitro* studies using tubular epithelial cells. The complement system requires many other cell types to function include leukocytes such as macrophages. The results which were obtained showing that complement 3 silencing did not have any effect on cell death may be a result of this limitation. Complement 3 silencing in mouse models of IRI has been shown to be effective (52). It was still important to develop a porcine complement 3 siRNA that is effective at gene silencing and can be used in future *ex vivo* or *in vivo* experiments.

Interestingly, there was no synergistic effect observed when multiple genes were silenced. This may have occurred for a number of reasons. First, the RISC complexes may have been completely saturated leading to competition between the sequences and a reduced silencing as some samples showed higher expression of mRNA when genes were targeted as opposed to a single gene. Second, IRI is a complex process and many different pathways are involved in cell damage and death and this redundancy may have lead to the results which were obtained. Third, only one cell type (tubular epithelial cells) was used in this study and this does not fully represent what occurs in an *in vivo* setting. The inflammatory response which is affected by RelB and complement 3 did not occur due to the lack of immune cells.

4.5 Conclusions

In conclusion, caspase-3 and RelB siRNA were effective at gene silencing, increasing cell viability, and reducing apoptosis in an *in vitro* IRI model using antimycin A. Complement 3 siRNA was also tested, but did not show any significant difference in these outcomes. Interestingly, Complement 3 mRNA expression in this model was actually reduced. This project has also highlighted the need for large animal testing of therapies targeted at IRI and the limitations associated with *in vitro* and ischemia only models.

4.6 Future Directions

This project has lead to the development and confirmation of efficacy of porcine siRNA molecules. Future directions for this project involve the use of pigs as a large animal model to further study how RNA interference can be used effectively in the reduction of IRI. A DCD model will be used in which the kidneys will be subjected to warm ischemia prior to being perfused with preservation solutions. The delivery of siRNA molecules will be accomplished through the use of machine perfusion of isolated porcine kidneys. Optimal parameters for siRNA delivery will be determined and include temperature and perfusion pressure.

This project will make use of an ischemia-only model of IRI in that the kidneys will not be reperfused with blood. Although this has some limitations, the process of IRI in the transplant setting has discrete ischemic and reperfusion phases. This allows for the opportunity to intervene in each of these phases and targeting events in the ischemic phase may be the most important in order to prevent subsequent events from occurring during the reperfusion phase. Preliminary results have been obtained with porcine kidneys which were procured after 1 hour of warm ischemia followed by either 24 or 42 hours of perfusion at 37^0C. Apoptosis was increased with increasing time on pump. These results are shown in Appendix B. The use of a large animal model is an important step in the translation to a clinical setting and will allow us to confirm our results which we have obtained from this *in vitro* model.

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Appendix A: List of Abbreviations

AKI	Acute kidney injury
ANOVA	Analysis of variance
C3	Complement 3
DCD	Donation after cardiac death
ECD	Expanded criteria donor
miRNA	Micro RNA
NDD	Neurologically determined death
PCR	Polymerase chain reaction
piRNA	PIWI-interacting RNA
qPCR	Quantitative real time polymerase chain reaction
RNAi	RNA interference
RRT	Renal replacement therapy
SCD	Standard criteria donor
shRNA	Short hairpin RNA
siRNA	Small interfering RNA

Appendix B – Preliminary Results from Pig Studies

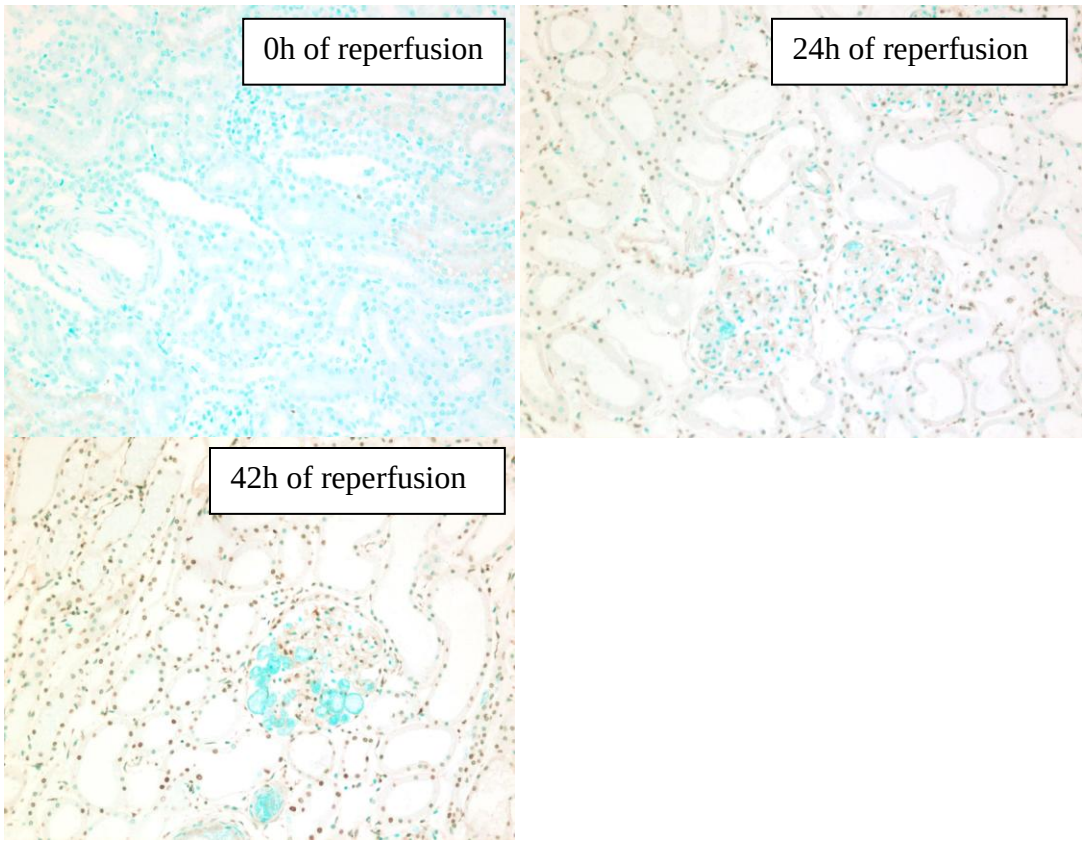

Figure 23 - TUNEL staining of porcine kidney tissue following 1h of warm ischemia and 0, 24, and 42h (as indicated) of perfusion at 37^0C. Graphical representation showing percentage of cells staining positive for apoptosis.

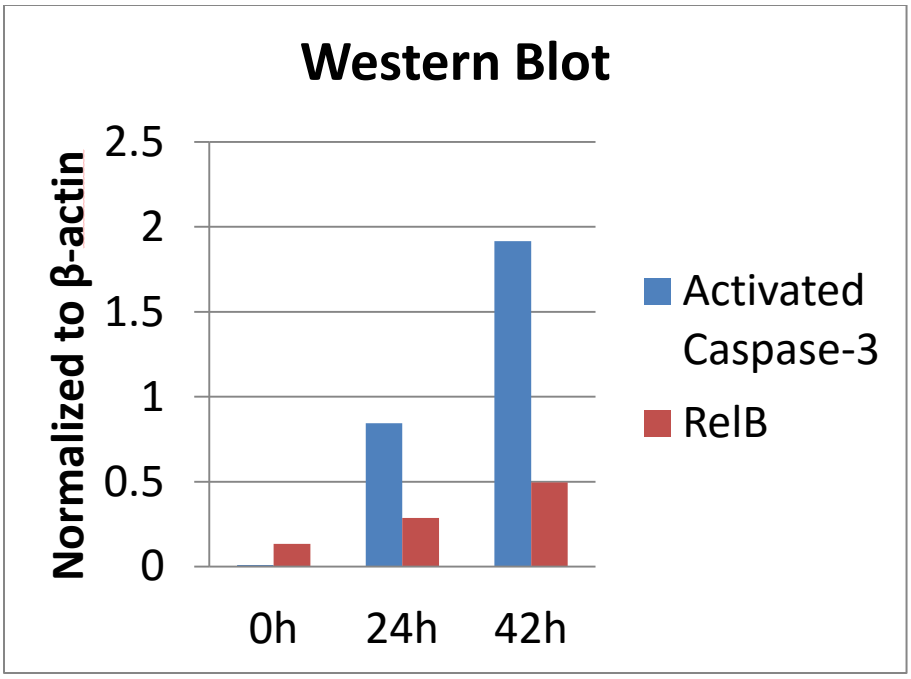

Figure 24 - Quantitative Western Blot results showing expression of activated Caspase-3 in pig kidney tissue. n=1.

As can be seen in Figures 23 and 24, apoptosis is increased in tissue obtained from pig kidneys which have undergone 1h of warm ischemia followed by perfusion at 37^0C for 24 and 42 hours. Activated caspase-3 is also increased in the tissue and supports the use of caspase-3 siRNA as a potential treatment for IRI in an ischemia only model.

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8.5 The licensing transaction described in the Order Confirmation document shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such licensing transaction shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.If you have any comments or questions about the Service or Copyright Clearance Center, please contact us at 978-750-8400 or send an e-mail to info@copyright.com.

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